Wheat pollen viability and physiological, biochemical and biophysical factors impacting pollen storability

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktorin der Naturwissenschaften

(Dr. rer. nat.)

genehmigte Dissertation

von

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2022

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Tag der Promotion:	18.11.2022

For my family

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Thesis abstract

Pollen has high potential to preserve and exchange nuclear genes of plant genetic resources. To facilitate breeding programs, short- and more importantly long-term pollen preservation protocols have been established for many species. Long-term pollen preservation, particularly in wheat breeding programs, and especially with spatially and temporally isolated parents, would be of great interest to expand genetic diversity and to facilitate hybrid seed production. Wheat sheds tricellular pollen at maturity which loses the ability to germinate under ambient conditions within one hour. So far, neither short- nor long-term storage protocols for wheat pollen have been established, and physiological, ultrastructural and biochemical processes in pollen after shedding are hardly understood. To gain a comprehensive overview of processes contributing to fast viability loss, in this thesis, three consecutive studies on the viability and storability of wheat pollen were conducted. First, a comparison of different viability tests and their reliability for use in wheat pollen was made (**Chapter 2, Manuscript 1**). Second, two different environmental factors (temperature and relative humidity, RH) were investigated for their influence on wheat pollen longevity, physiological properties, pollen ultrastructure and metabolism (**Chapter 3, Manuscript 2**). Finally, experiments were conducted to investigate the feasibility of cryopreservation for wheat pollen (**Chapter 4, Manuscript 3**).

In the first study a consistent semi-solid *in vitro* pollen germination medium containing raffinose, boric acid, calcium chloride and gelrite was formulated and compared against existing media (Jian et al. 2014; Jayaprakash et al. 2015; Cheng and McComb 1992) for wheat pollen. The medium formulation resulted in consistent germination percentages for fresh pollen of > 87%. The germination was correlated with pollen viability assessed by impedance flow cytometry (IFC viability, $r = 0.67$, $P < 0.001$) and fluorescein diacetate (FDA, $r = 0.54$, $P < 0.05$) staining (**Chapter 2, Manuscript 1, Figure 5**). However, when the medium was used with other *Poaceae* pollen species, germination was low and assumed to require further adaptation. Although, FDA and IFC viability can be easily applied both, FCR and IFC, seem to overestimate pollen viability (**Chapter 2, Manuscript 1, Figure 5**). Therefore, two viability tests, *in vitro* germination and IFC viability, were applied in consecutive studies.

The second work revealed that low temperature (~ 4 °C) and high RH (> 90%) could keep pollen viable with a maximum of > 70% pollen germination after 60 minutes storage. The metabolic changes were most pronounced in unfavourable conditions (low RH and room temperature) were pollen lost most of its viability (pollen germination reached only 10%) after short storage of only 20 to 30 minutes. Under these conditions, wheat pollen showed extensive and deleterious changes in the ultra-structure (intine and cytoplasmic organization), fluctuations in primary metabolite concentration, and changes in water content (WC) (**Chapter 3, Manuscript 2, Figures 1, 2, 6, 7**). Overall, metabolic status, ultrastructural and WC changes lead to irreversible damages and viability loss suggesting that wheat pollen is not equipped with sufficient protection mechanisms to survive longer periods.

Additionally, in these two studies we found differences in germination percentage (**Manuscript 1, Figure 6; Manuscript 2, Supplemental Figure S4**) and metabolite concentrations of specific compounds (**Manuscript 2, Figure 4**) between different genotypes tested. Thus, it is suggested that the genotype may have an important influence on pollen survival. Further

research with a wide range of genotypic implications could reveal marker genes that might influence wheat pollen viability under different conditions.

In the third study, it was tested if wheat pollen may be able to survive cryopreservation. Therefore, wheat pollen had been dried and cooled under different regimes. Rapidly dried wheat pollen to WC above the unfrozen water content (0.91 ± 0.11 mg H_2O mg^{-1} DW) for 5 min retained IFC viability of around $6.1 \pm 8.8\%$ after fast cooling and warming but were not able to germinate. Nevertheless, damages induced by dehydration and cryo-injury during ultra-low temperature exposure seemed to occur to a lesser extent in the rapidly dried pollen compared to fresh pollen and pollen dried at ambient conditions for both, slow and rapid cooling/warming. Thus, within a very small window of a specific pollen WC and further adjustment pollen may survive cryopreservation storage. Future research and amendment of fast-drying and an optimisation of the cooling/warming rate will reveal whether the survival rate of pollen can be increased after exposure to cryo-storage. The use of cryoprotection may have favourable effects on the survival. Further suggestions for possible improvements of cryopreservation will be discussed in one of the sections of discussion.

Zusammenfassung

In dieser Arbeit wurden drei aufeinanderfolgende Studien zur Lebensfähigkeit und Lagerfähigkeit von Weizenpollen durchgeführt. Zunächst wurde ein Vergleich verschiedener Lebensfähigkeitstests und ihrer Zuverlässigkeit für die Verwendung bei Weizenpollen durchgeführt (Kapitel 2, Manuskript 1). Zweitens wurden zwei wichtige Umweltfaktoren (Temperatur und relative Luftfeuchtigkeit) auf ihren Einfluss auf die Langlebigkeit von Weizenpollen, die Pollen-physiologischen Eigenschaften, Pollen-Ultrastruktur und ihren Stoffwechsel untersucht (Kapitel 3, Manuskript 2). Schließlich wurden Experimente durchgeführt, um die Machbarkeit der Kryokonservierung von Weizenpollen zu untersuchen (Kapitel 4, Manuskript 3). Die Ergebnisse der einzelnen Teile werden direkt in den entsprechenden Kapiteln diskutiert. In der abschließenden Diskussion in Kapitel 5 werden I) die wichtigsten Ergebnisse der Arbeit mit zusätzlichen Aspekten diskutiert, II) ein Modell entwickelt, um die Sensitivität der Weizenpollen nach der Ausschüttung aus den Antheren näher zu erörtern und III) Anregungen für weitere Untersuchungen gegeben. Außerdem soll ein Ausblick mögliche Zukunfts-Szenarien für die Anwendung gelagerter Weizenpollen für die Weizen-Hybridzüchtung aufzeigen.

Für die Hauptteile dieser Arbeit in den Kapiteln 2 bis 4 können die Ergebnisse wie folgt zusammengefasst werden. Als erstes wurde ein halbfestes Pollenkeimungsmedium mit Raffinose, Borsäure, Calciumchlorid und Gelrite formuliert, um die Pollenschlauchentwicklung in vitro zu testen, und mit bestehenden Medien (Jian et al. 2014; Jayaprakash et al. 2015; Cheng und McComb 1992) für Weizenpollen verglichen. Die *In vitro*-Keimung basiert auf der Fähigkeit des Pollens, seine natürliche Funktion der Bildung eines Pollenschlauchs zu erfüllen. Daher ist dies ein zuverlässiger Test, der routinemäßig für die Bewertung der Lebensfähigkeit von Weizenpollen verwendet werden kann. Die hier verwendete Mediumformulierung führte bei der Verwendung von frischem Pollen zu konsistenten Keimungsraten. Einige Proben zeigten ein Maximum von > 87% Pollenschlauchwachstum. Die *In vitro*-Keimung wurde weiterhin mit anderen biochemischen und – physikalischen Vitalitäts-Tests verglichen. Der *In-vitro*-Keimungstest zeigte eine gute Korrelation mit zwei weiteren Tests, in welchen die Pollenlebensfähigkeit mittels Impedanz-Durchflusszytometrie (IFC-Viabilität) und Fluoresceindiacetat (FDA)-Färbung ermittelt wurde (Kapitel 2, Manuskript 1, Abbildung 5). Allerdings muss das Medium bei der Verwendung mit anderen *Poaceae*-Pollenarten angepasst werden. Die FDA-Färbung und die Messungen für die IFC-Vitalität sind zwei Methoden, die einfach zu handhaben waren und ebenfalls zuverlässige Ergebnisse lieferten für die Verwendung mit Weizenpollen. Sie sind daher als gute Alternative anzusehen für die routinemäßig Testung der Pollenlebensfähigkeit. Die FDA-Färbung und IFC-Viabilität scheinen jedoch die Lebensfähigkeit der Pollen zu überschätzen, da die Prozentsätze viele Prozentpunkte über der Keimfähigkeit der *in vitro* und *in vivo* gewachsenen Pollenschläuchen lagen (Kapitel 2, Manuskript 1, Abbildung 5).

Zweitens wurde in dieser Arbeit festgestellt, dass Weizenpollen auf Umweltveränderungen bereits kurz nach Entlassung aus den Antheren umfangreiche Veränderungen und biochemische Reaktionen zeigen. So wurden schädliche Veränderungen in der Ultrastruktur (Intine und zytoplasmatische Organisation), Schwankungen in der Konzentration von Primärmetaboliten und Veränderungen im Wassergehalt festgestellt, die alle tiefgreifende Auswirkungen auf die Lebensfähigkeit und das Überleben von Pollen während der Lagerung haben können (Kapitel 3, Manuskript 2, Abbildungen 1, 2, 6, 7). Es wurde zudem festgestellt, dass Pollen bei niedriger Temperatur (~ 4 °C) und hoher relativer Luftfeuchtigkeit (> 90%) länger lebensfähig bleiben und nach 60 Minuten Lagerung noch maximal zu > 70% keimen können. Die metabolischen

Veränderungen waren unter ungünstigen Bedingungen (niedrige Luftfeuchtigkeit und Raumtemperatur) am stärksten ausgeprägt, wo der Pollen nach kurzer Lagerung von nur 20 bis 30 Minuten den größten Teil seiner Lebensfähigkeit verlor (die Pollenkeimung erreichte nur noch 10%).

Drittens können Weizenpollen die Kryokonservierung möglicherweise überleben, wenn die verwendete Methode sorgfältig angepasst und weiter optimiert wird. In den hier durchgeführten Experimenten behielten Weizenpollen, die 5 Minuten lang schnell auf einen Wassergehalt oberhalb des ungefrorenen Wassergehalts (0.91 ± 0.11 mg H_2O mg^{-1} DW) getrocknet wurden, nach schnellem Abkühlen und Erwärmen eine IFC-Lebensfähigkeit von etwa $6.1 \pm 8.8\%$, waren aber nicht mehr keimfähig. Dennoch schienen die Schäden, die durch Dehydrierung und Abkühlung induziert wurden, bei den schnell getrockneten Pollen in geringerem Maße aufzutreten als bei frischen und bei Umgebungsbedingungen getrockneten Pollen, sowohl bei langsamer als auch bei schneller Kühlung/Erwärmung. Somit ist möglich, dass Weizenpollen eine Kryokonservierung überleben könnte, wenn der Pollen innerhalb eines sehr kleinen Fensters eines bestimmten Pollenwassergehalts schnell getrocknet wird und die Temperaturen schnell abgesenkt werden durch direktes Eintauchen in flüssigen Stickstoff. Hierzu werden Anregungen für weiterführende Experimente in der abschließenden Diskussion in Kapitel 5 gegeben.

Keywords:

Wheat pollen, pollen viability, pollen conservation

Schlagwörter:

Weizenpollen, Pollenvitalität, Pollenkonservierung

1. Introduction

1.1. Wheat (*Triticum aestivum* L.)

1.1.1. Evolution, distribution and importance of wheat

Phylogenetic classification and use

Wheat (*Triticum aestivum* L.) belongs to the family *Poaceae* (formerly known as *Gramineae*) subfamily *Pooideae* and tribe *Triticeae* (Soreng et al. 2017; GBWG et al. 2001). The *Poaceae* is a large and variable family of monocotyledonous flowering plants collectively named as grasses with >12,000 species occurring in almost any terrestrial habitat (Soreng et al. 2015). The *Triticeae* tribe within the grass family includes 501 species including important crops like wheat, barley (*Hordeum vulgare*), and rye (*Secale cereale*) (Soreng et al. 2017). Wheat has been used for the production of numerous products, mainly bread, pasta and beer; nowadays, wheat is mostly associated with pasta manufacture (durum wheat) or the baking industry (bread wheat) in western countries. However, it has also been used for development of non-food products such as cosmetics, as an energy crop for bioethanol and biomass production (Bell et al. 1995), is used as feed and for fermentation into alcoholic beverages (Kaparaju et al. 2009; Glithero et al. 2013; Karlsson et al. 2016).

Wheat evolution

Bread wheat is an allohexaploid (2n=6x=42, AABBDD), monocotyledonous, annual plant. Gametes regularly contain 21 haploid chromosomes during meiosis. Chromosomes are organized in the A, B and D genomes with each genome normally containing seven pairs of chromosomes (Martín et al. 2018). It has a genome size of 16 Gbp and more than 85% repetitive sequences.

Hexaploid wheat may have evolved from two hybridization events. In the first, the A genome progenitor *T. urartu* combined with the B genome progenitor, a yet unknown species from the *Sitopsis* section closely related to *Aegilops speltoides*, to form a primitive tetraploid wheat (*T. turgidum*, 2n=4x=28, AABB) (Feuillet et al. 2008). Domestication of tetraploid wheat most likely occurred in the Fertile Crescent of the Middle East >10,000 years ago from which later domesticated emmer (*T. dicoccoides*) and durum or pasta wheat (*T. durum*) evolved (Luo et al.

2007). Hexaploid wheat occurred more than 7,000 years ago after the second hybridization event between domesticated emmer and *A. tauschii* (Feuillet et al. 2008; Matsuoka 2011).

An annotated reference sequence of bread wheat variety Chinese Spring has been released in 2018, giving access to 107,891 high-confidence genes (IWGSC et al. 2018). Recently, multiple reference-quality genome assemblies of 10 bread wheat lines were generated (Walkowiak et al. 2020).

Distribution and global production

Wheat is grown across a wide range of environments on 218 million hectares annually (http://www.fao.org/faostat/en/#data/QC, accessed January 20th, 2021). It has a broad adaptation and is cultivated different climatic zones ranging from Scandinavia to South America and across Asia (https://wheat.org/wheat-in-the-world/, accessed January 20th, 2021). For growth, wheat requires a minimum temperature of 3 °C to 4 °C and tolerates maximum temperatures of about 32 °C. Optimal growth occurs around 25 °C. Therefore, the production concentrates between latitudes 30°N and 60°N and 27°S and 40°S and wheat grows best on well drained soils up to heights of about 4,500 m above sea level. Most wheat production occurs in areas with 375 to 875 mm annual precipitation with a tolerance from 250 to 1,750 mm annual precipitation (Kimber and Sears 1987; Briggle and Curtis 1987). Largest wheat producer was China with almost 137 million tonnes in 2021, followed by India, Russia and USA (http://www.worldagriculturalproduction.com/crops/wheat.aspx, accessed March 2nd, 2022). The current forecast of the global wheat production in 2022/2023 is > 770 million tonnes. It is the second most produced crop in the world and ranges between maize (forecast 2022/2023 > 1.18 billion tonnes) and rice (forecast 2022/2023 > 520 million tonnes).

World nutrition

The wheat grain, in some cases and for simplicity termed seed, consists mainly of 60-75% carbohydrates, while proteins account for up to 18%, and lipids up to 2%. In addition, wheat grains also contain valuable other components such as certain vitamins and minerals (Alvarez and Guzmán 2018). Wheat is one of the most important crops for human nutrition consumed by more than 2.5 billion people in over 89 countries. About 536 million tonnes are forecasted to be used for human nutrition in 2022/2023, an expected rise by 0.9% over 2020/21 (https://www.fao.org/3/cb9427en/cb9427en.pdf, accessed July 7th, 2022). In many low- and middle-income nations it exceeds maize or rice as a source of protein (https://archive.wheat.org/wheat-in-the-world/, accessed April 13th, 2022).

1.1.2. Wheat breeding and hybridization

The beginning of wheat breeding and today's yield stagnation

Species of the tribe *Triticeae* hold a huge capacity for inter- and intraspecific hybridization (Rey et al. 2021). As early as 9,000 BC, man was cultivating emmer in an area with natural occurrence of *A. tauschii* (Bell 1987). Natural hybridization of these wheat progenitors and the associated emergence of *T. aestivum* in the Fertile Crescent led to its cultivation and establishment of an important food source around the center of its origin. Human selection, initially unconscious during cultivation, led to the worldwide spread of wheat (Charmet 2011). Breeding by intentional crossing did not begin until the end of the 18th century. Motivated by Darwinian theories of adaptation to a habitat through variation and natural selection (Darwin 1859; Darwin and Murray 1868), Rimpau (1891) and Vilmorin (1880) independently achieved notable successes in their first hybrid wheat crosses, resulting in the development of superior wheat varieties through artificial hybridization and selection of the best offspring (Lupton 1987). Wheat breeding based on scientific knowledge took off when the Mendelian insights were rediscovered at the beginning of the last century (Biffen 1905). A great leap was made with the introduction of dwarf genes, which led to the modern short-grown and high-yielding varieties and gave rise to the so-called 'Green Revolution' around the 1960s (Hedden 2003; Venske et al. 2019). Since then, wheat breeding has continually improved wheat yields, grain quality, disease resistance and other valuable plant traits. Nowadays (2018), high-yielding wheat varieties produce around 3.42 t ha^{-1}. Before Green Revolution, commercially grown wheat varieties achieved about 1.05 t ha^{-1} (Ritchie et al. 2022).

Today's agriculture faces a multitude of challenges with an increasing global population, climate change-induced droughts, floods and other severe weather, shrinkage of fertile arable land, and emerging plant diseases. Beside meeting the food demand through yield increases, food quality is also of high importance, especially in developing countries with low nutrient density food. Moreover, breeders appear to have exhausted most of useful genetic variability of wheat and modern plant breeding of wheat has led to an elite crop gene pool narrowing the genetic diversity. Thus, the improvements of breeding and yield increases have stagnated in the recent years (Ray et al. 2013; Ray et al. 2012a). This is concerning as future projections indicate that yields will need to more than double by 2050 to meet the rising food demand and offset stagnating yields in a world with ongoing climate change (Tilman et al. 2011; McCouch et al. 2013; Singh et al. 2021). Continuous efforts are therefore needed to develop and implement improved strategies and methods in wheat breeding programmes.

Wheat hybrid breeding

Motivated by the high yields and resistance of hybrid lines in allogamous (cross-pollinating) maize at the beginning of the 20th century (Duvick and Cassman 1999), scientist and breeders started to exploit the potential of heterosis in autogamous (self-pollinating) wheat. Hybrid wheat results from crosses between two genetically different and carefully selected pure wheat lines and heterosis describes "*the phenomenon in which hybrids [...] are more robust or vigorous than their parents*" (Timberlake 2013).

The first heterosis effects in wheat were reported for plant height in 1919 (Freeman 1919). From 1960s onwards, many scientists have reported significant heterosis in wheat (Merfert et al. 1988; Pickett 1993; Singh et al. 2010; Longin et al. 2012) and the first economically successful hybrids of wheat have entered the wheat seed market in the 1980s (Pickett 1993). The high costs of seed production, the lack of cost-efficient and completely satisfactory hybridization mechanisms, the self-pollinating floral biology in wheat, and the competition to high-yielding and cost-efficient line varieties have hampered hybrid breeding success and paused the progress of hybrid wheat breeding in the early 1990s. Wheat breeding efforts in the last decades thus have focused on increasing resistance to diseases and abiotic stresses, and were based on a limited population of elite genotypes (Schneider et al. 2021; Reynolds and Borlaug 2006; Braun et al. 2010).

Plant fitness and yields in wheat can greatly benefit from the exploitation of heterosis. Numerous reports have shown that wheat hybrid lines are able to outperform inbred lines in the field in terms of higher total yields (Longin et al. 2013; Gowda et al. 2012) and yield stability (Mühleisen et al. 2014; Jordaan et al. 1999; Gowda et al. 2012), better abiotic stress tolerance (Longin et al. 2013; Sutka 1994, 1981), less susceptibility for biotic stress (Beukert et al. 2020; Longin et al. 2013; Gowda et al. 2014), and increased root capacity (Macholdt and Honermeier 2017). Results of a survey of over 600 German farmers and agricultural advisors on their experiences with wheat hybrid lines in the field have confirmed scientific research data in practice. In their experience, hybrid plants were more yield stable particularly in dry weather (Macholdt and Honermeier 2017).

Revival of hybrid wheat breeding and remaining challenges

The stagnations in yield gain in recent years, the growing demand for food and the higher requirements for abiotic and biotic stress tolerance increased the interest in hybrid breeding once again. Most hybrid programs have focused on the establishment of a male sterile female

inbred line to use it in crosses with a male fertile inbred line (Whitford et al. 2013). Some of the most common fertility control systems involve the use of chemical hybridization agents (CHA), a genetic male sterility control like cytoplasmic male sterility (CMS) or nuclear-encoded male sterility (NMS), or introducing biological sterility through self-incompatibility (SI) and environment-sensitive genetic male sterility (EGMS) (Selva et al. 2020). However, several limitations accompany wheat hybrid seed production. The production is costly, time-consuming, and there is a limited yield stability and seed quality. Additionally, restrictions to specific environments, or difficulties in introgression of desired genes hamper the use of hybrid lines in large commercial production (Selva et al. 2020). The integration of genomic selection tools can help to obtain high-performing hybrid wheat lines whereby a large number of possible inbred combinations can be predicted using statistical models that link genotypes to phenotypes (Kumar et al. 2021). Advances in marker-assisted breeding strategies can help in the selection process to find favorable alleles for desired traits in early generations (Bonnett et al. 2005). In addition, the availability of the fully annotated wheat genome reference sequence, RefSeq v.1.0 (IWGSC et al. 2018), the improved RefSeq v.2.0 (IWGSC, 2019, https://wheat-urgi.versailles.inra.fr/Seq-Repository/Assemblies), and recent advances in high-throughput genotyping platforms support the discovery of quantitative trait loci (QTLs), and prediction of marker effects on phenotypes become more accurate (Kumar et al. 2021).

However, one main problem in hybrid production of wheat remains on the side of pollen production, viability and spread (Longin et al. 2012). For instance, the amount of shed pollen is much lower in wheat (1,000 to 3,800 pollen per anther) compared to maize (more than 7,000 pollen grains per anther) and rye (19,000) (De Vries 1971); the viability of wheat pollen is limited to about one hour (D´Souza 1970); and the pollen sink time is rather quick because the pollen is big and heavy (De Vries 1974). Additionally, the proportion of pollen that are released outside the flower varies greatly between 3 to 80% and is dependent on the variety (Beri and Anand 1971). These floral characteristics are all factors contributing to a low cross-fertilization ratio. Thus, till now only 1% of the global wheat growing area is planted to hybrid lines (Longin et al. 2012). Moreover, most hybrid breeding programs so far have not focused on pollen biology and improvements in viability and storability after pollen release. Programs towards establishing an efficient hybrid seed production system, however, can benefit greatly from investigations of wheat pollen biology and improvements in pollen viability and longevity.

1.2. Wheat flower morphology, wheat pollen development and growth

1.2.1. Wheat flower structures

The flowers in wheat are grouped together in spikelets. In most of the grass species, flowers are small and the spikelets sit loosely on branches forming an open panicle. However, wheat flowers are tightly arranged forming a characteristic compact head which is called spike or ear (**Figure 1A, D**). Wheat spikes bear two rows of spikelets, which are alternately arranged on the central axis, the rachis. The number of spikelets per unit length of the rachis, their arrangement and appearance can vary greatly among different wheat varieties (Würschum et al. 2018). For example, some varieties have very crowded spikelets with short internodes at the rachis while others are widely separated, and the rachis becomes visible between the spikelets. Each spikelet is sessile and has a secondary axis, the rachilla, on which the single flowers (florets) are attached on either side of the rachilla node in alternating pattern (**Figure 1B, E**). Spikelets can contain up to nine florets (Goss 1968), but usually only up to four grains are developed (De Vries 1971). At the base of each spikelet are two empty bracts called glumes (Perry and Belford 2000). Another two bracts, lemma and palea, surround each individual floret. The lemma may have an awn at its tip, whereas the palea is awnless (Goss 1968). The floret itself bears both, the male and female flower structures. The male organs comprise three stamen consisting of elongated anthers which are situated on a filament. The anthers are the organs in which male spores (microspores) are formed and develop into pollen grains (**Figure 1C, F**). The female organs comprise a single pistil with an ovary and two short styles each carrying a feathery stigma (Goss 1968; De Vries 1971; Perry and Belford 2000). Two lodicules are located at the base of the ovary, the function of which is to push the lemma and palea apart during flowering to allow an opening of the floret (Goss 1968).

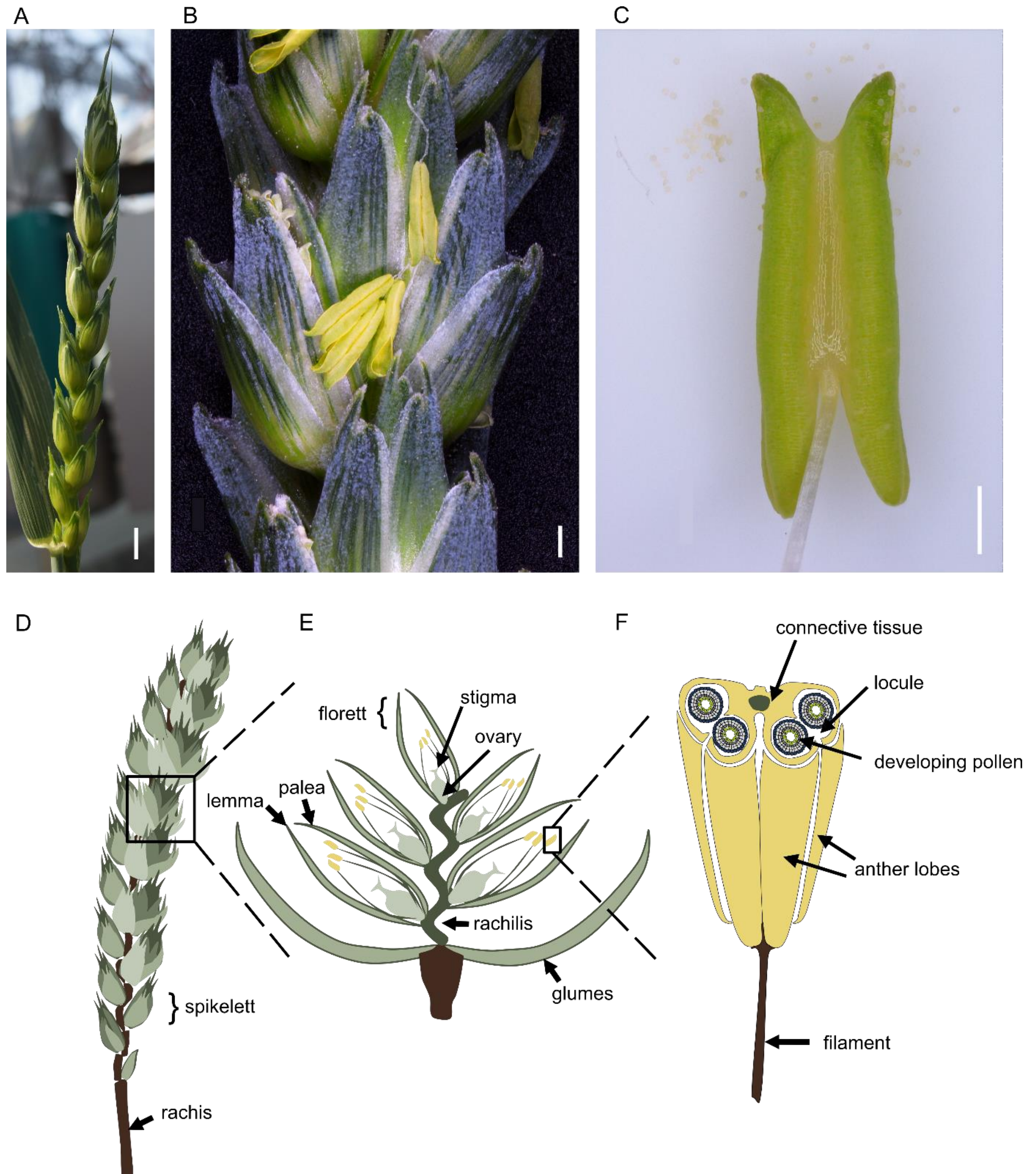

Figure 1 Photographs (A – C) and schematic representation (D – E) of the inflorescence of wheat. (A) Wheat spike shortly before flowering. Individual florets are still closed. Scale bar 1 cm. (B) Close-up of an individual spikelet shortly after anthesis of first and second floret. Anthers have shed the pollen and are opened at the tips. Scale bar 1 mm. (C) Microscopic picture of a single anther after pollen shedding. Scale bar 500 µm. (D) Schematic representation of a spike with spikelets attached to the nodes of the central rachis in an alternating way. (E) Enlarged structure of a spikelet with 5 florets and two sterile basal glumes. Each floret consists of three anthers and one ovary with two faethery stigmas (not shown in detail). (F) Enlarged view on the anther structure consisting of four anther lobes connected by the connective tissue. Pollen development takes place within the locule (compare with **Figure 2**).

1.2.2. Wheat anthers and pollen

Wheat anthers are ~3.3 to 6.6 mm and the number of pollen produced per anther varies between 1000 to 3,800 pollen grains depending on wheat line. Larger anthers tend to produce also more pollen (Lersten 1987; Athwal and Kimber 1970; D´Souza 1970; Beri and Anand 1971; De Vries 1971). Mature wheat pollen are spheroidal or ovoid in shape, have one side where the pollen tube emerges (monoporate) (De Vries 1971), and are tricellular, meaning that they have completed second pollen mitosis at anthesis (Brewbaker 1967). The size of wheat pollen varies between 40 and 60 µm (Forlani 1953; Nathawat and Gandhi 1966). Although wheat pollen is relatively large, pollen shed outside the floret may be able to travel up to 50 m (D´Souza 1970).

1.2.3. Plant development and induction of flowering

Dependent on the vernalization response and variety, wheat development can take until full grain maturation between 15 and 37.5 weeks (Acevedo et al. 2002). Within few days quiescent wheat grains germinate after imbibition at a minimum of 35 to 45% water content based on dry weight (DW) (Evans et al. 1975) and at temperatures between 4 to 37 °C with 12 to 25 °C being optimum (Austin and Jones 1975). The wheat development can be divided into 10 stages from 1 water uptake and germination to 10 full maturation (Bruns and Croy 1983). After water uptake (Stage 1), the radicle (primary root) emerges first followed by the primary shoot including coleoptile (Stage 1), and both continue to grow quickly (Kirby 2002; Bruns and Croy 1983). At first, leaves of the main stem appear and later, side tillers grow from the main axis, but many will abort before anthesis (Acevedo et al. 2002; Gallagher and Biscoe 1978). In the tillering and leave erection phase (Stage 2 and 3), the lateral meristems expand and side leaves grow. The plants are usually vernalized by cold temperatures in this stages (Bruns and Croy 1983). Winter-type wheats require a vernalization period for flower induction. Spring-type wheats respond to vernalization only mildly or not at all (Flood and Halloran 1986). From germination onwards, internal and environmental signals activate the shoot tip (apex) of the main axis and of some of the side tillers that progressively changes in form and complexity through the initiation of primordia (Stage 4, Node Formation) (Acevedo et al. 2002; Liu et al. 2009; Kirby 2002). Here, Waddington et al. (1983) developed a more detailed description of the changes in the shoot apex ranging from '*Transition apex*' (Stage 1) to '*Pollination*' (Stage 10). The next noticeable stage in Waddington's scale is called double ridge stage (Stage 2.5). Each of the primordia that develop on the later spike consists of two parts (the double ridge). The lower, smaller ridge is a leaf primordium whose further development is repressed. The upper, larger ridge differentiates into the spikelet (Kirby 2002). From this stage onwards, differentiation of

various floral structures is in progression (see next sections). After the initiation of 20 to 30 individual flower units, a final apical flower primordium is formed which determines the end of further primordia initiation (Kirby 2002; Acevedo et al. 2002).

1.2.4. Anther development and role of the tapetum

Male and female gametes develop within the sexual organs of the flower. The formation of male sex organs, collectively known as androecium, starts when progenitor cells divide and differentiate to produce primordia of the future stamen. The filament and the anther grow after primordial cells of the stamen had been developed (Goldberg et al. 1993). Within the young anther, three different primary germ layers named L1, L2 and L3 evolve (Goldberg et al. 1993; Gómez et al. 2015). Cells of L1 and L3 layer show only a limited degree of differentiation (**Figure 2A**). Progenitors of L1 layer will differentiate to the epidermis cells, whereas cells of the L3 layer develop into the connective tissue, the vascular bundle and the inner tapetum. L2 layer cells, also called archesporial cells, will undergo several differentiation steps. The first differentiation will produce parietal and sporogenous cells. The parietal cells further develop into the anther wall, consisting of an outer (endothecium) and inner (middle wall) layer, and the outer tapetum (Shivanna 2003; Greyson 1994). The cells of the endothecium build up the mechanical layer and, therefore, are responsible to disperse the male gametes during anthesis (Greyson 1994; Pacini 2000). The sporogenous cells are located within a cavity of the anther (locule) will develop into microsporocytes or pollen mother cells (PMC) (**Figure 2B**) (Stanley and Linskens 1974).

The tapetum is a nutritive, apoptotic tissue located as the innermost cell layer of the anther. Depending on plant species, its cellular structure, and the relative relationship with microspores, two types of tapeta are recognized; the most commonly secretory and the amoeboid cell type (Vasil 1967; Pacini 1997; Pacini 2010). Wheat anthers belong to the secretory-type (Mizelle et al. 1989) which provide essential substances and energy for the developing microspores (Pacini et al. 1985; Scott et al. 2004) including cell wall components for the construction of the pollen exine layer, such as sporopollenin (Shivanna and Johri 1985; Ariizumi and Toriyama 2011), and enzymes for the release of microspores out of the meiotic tetrad (Goldberg et al. 1993). Furthermore, secretory tapetal cells produce the locular fluid (Pacini 2009; Furness 2008), which is released via the plasma membrane or by vesicles and connects all cell types including the microspores (Clément et al. 1998 and references therein; Owen and Makaroff 1995; Pacini and Dolferus 2016). Depending on species, the locular fluid can be detected between microsporocyte stage up until the early bicellular microspore stage (Clément et al. 1998;

Quilichini et al. 2014). Different substances are secreted and may fluctuate throughout coordinated anther and pollen development (Clément et al. 1998) including pectin (Clément et al. 1998; Aouali et al. 2001), proteins (Huang et al. 2013; Papini et al. 1999), lipid bodies (Parish and Li 2010; Dickinson and Lewis 1973; Wang et al. 2003), carbohydrates (Hesse and Hess 1993; Pacini and Franchi 1983; Santos de Oliveira et al. 2015; Clément and Audran 1999). Sugars are especially important to nourish the developing microspores which build up their own storage compounds (Engelke et al. 2010). The composition of the locular fluid may also vary depending on environmental conditions (Castro and Clément 2007; Clément et al. 1998; Pressman et al. 2012; Quilichini et al. 2014). The development of tapetum and PMC are closely linked and share some regulatory factors (Yang et al. 1999). During the development of the microspores from vacuolated state to first microspore mitosis, tapetal cells undergo a programmed cell death (PCD) (Papini et al. 1999; Carrizo García et al. 2017; Pacini 2010) and disintegration leading to pollen maturation and fertility. Thus, incorrect development of the tapetum or untimely ablation (premature or delayed degradation of the tapetum) disrupts the development of pollen and, hence, results in reduced pollen fertility or even male sterility (Lei and Liu 2020).

1.2.5. Development of pollen

The microsporocytes develop into the pollen grains through both, meiotic and mitotic cell divisions in processes known as microsporogenesis and microgametogenesis, respectively (Ma 2005; Gómez et al. 2015). Before the onset of meiosis, sporogenous cells divide asynchronically to form the PMC within the anther (**Figure 2C**). After a certain number of PMC have been produced, mitosis stops, and a pre-meiotic interphase is initiated accompanied by synchronization of subsequent meiotic events (Sauter 1971; McCormick 1993; Shivanna 2003). Once meiosis is triggered, various synthetic activities such as DNA, RNA and protein synthesis as well as organelle and cytoplasmic reorganization take place (Sauter 1971). PMC form a syncytium by callose deposits around each cell (Heslop-Harrison 1966). Neighbor PMC are connected by cytoplasmic channels (plasmodesmata) which allow efficient exchange of signals and nutrients between PMC, and maintain synchrony between the meiocytes (Shivanna 2003; Heslop-Harrison 1971a). Subsequently, individual PMC become isolated and meiotic cell division takes place. During meiosis, typically four haploid microspores are formed (Bedinger 1992). They are arranged in tetrads and surrounded by callose. In the late tetrad state, microspores become separated by degradation of the callose wall (**Figure 2D**) (Mizelle et al.

1989; El-Ghazaly and Jensen 1986). Thereafter, microspore development continues in microgametogenesis to form mature pollen grains.

After meiosis, microspores are released into a free stage marked by the dissolution of callose through secretion of a callase enzyme mixture (**Figure 2E**) (Lu et al. 2014). Before the microspores undergo mitotic cell divisions, a rearrangement of cytoplasm and organelles takes place involving active participation of microtubules (**Figure 2F**) (Twell 2011; Hafidh et al. 2016). Commonly, the nucleus migrates to the periphery of the microspore due to the formation of a single large vacuole by the coalescence of smaller vacuoles (**Figure 2G**) (Yamamoto et al. 2003). Thereafter, during the first mitotic division, the nucleus of the microspore separates asymmetrically to form two daughter nuclei (**Figure 2H and I**). A cell wall is built and separates the vegetative pollen cell from the smaller generative cell (**Figure 2J**). The former receives most of the organelles, whereas the latter will further mitotically divide to form the two sperm cells (**Figure 2K**). The vegetative cells together with the two sperm cells form the complete structure of the final multicellular male gametophyte, commonly named pollen or pollen grain which is ready to be shed and germinate after landing on a receptive stigma (**Figure 2L**).

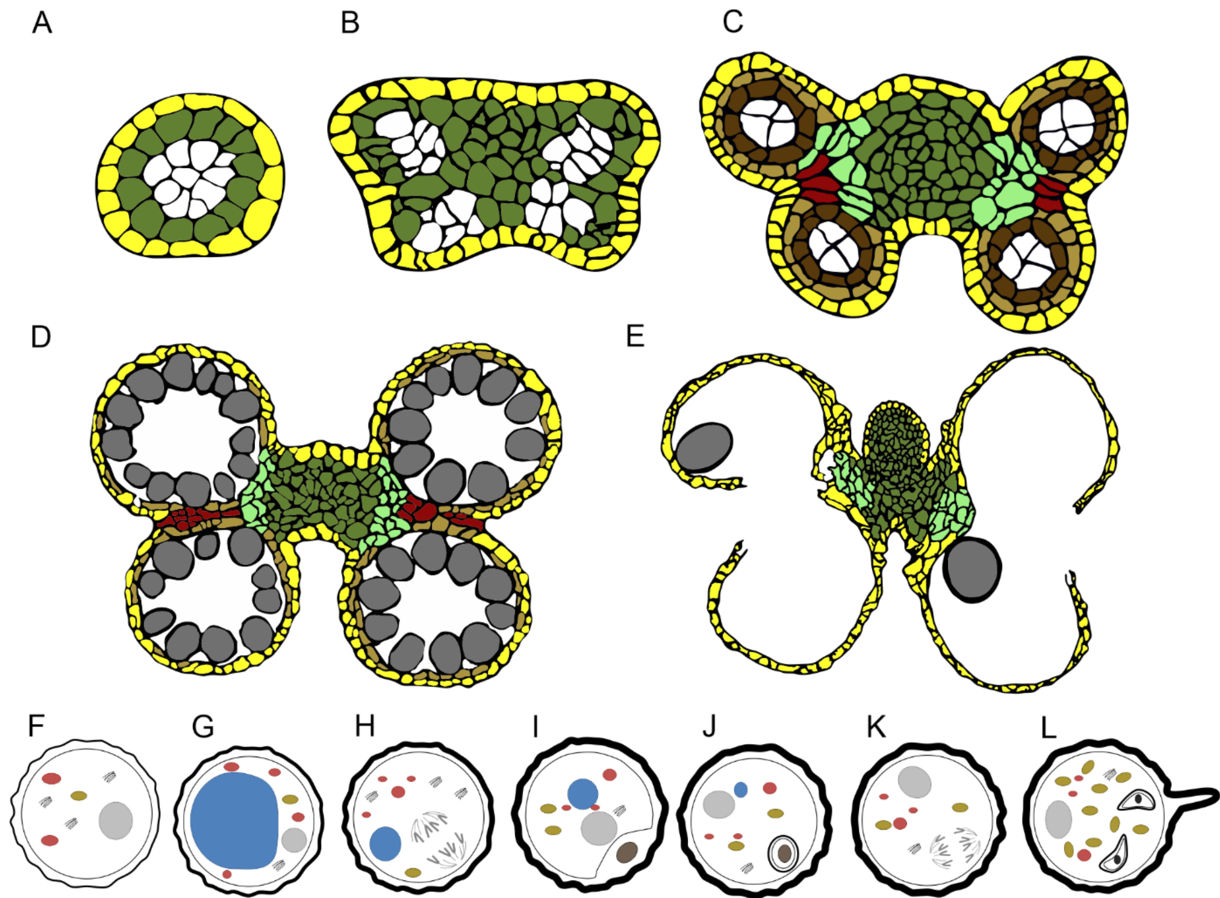

Figure 2 Schematic illustration of (A-E) anther and (F-L) pollen development. (A) In the anther primordium stage, cells of the layers L1 (yellow), L2 (green), and L3 (white) will initiate further differentiation. (B) Four independent lobes form during the sporogenous tissue stage, where sporogenous cells (white) start to differentiate from L1 and L2 layer. (C) During lobe formation and meiotic stage, the anther wall develops an outer epidermis (yellow), endothecium (light brown), middle layer (not shown) and tapetum (dark brown). At the stomium region (red), the thecae of the anther start to separate into the anther lobes. A connective tissue (light green) is built between them and the vascular tissue (green), which is more highly developed. Cells of the sporogenous tissue (white) divide to build PMC which will undergo meiosis (not shown). (D) After meiosis, microspores are formed and the middle layer (not shown) starts to degenerate as well as tapetal cells (dark brown) which leads to deposition of wall material onto the surface of the microspores. The stomium region (red) thins out which results in combination of the two thecae from each side of the anther lobe. (E) Finally, during dehiscence of the anther, the anther opens and mature pollen grains are released. (F) When meiosis is finished (**Figure 2C**), individual microspores are released from tetrads by dissolution of callose. Pollen wall formation (exine) starts through the deposition of sporopollenin onto the microspores. Different cell organelles like nucleus (grey), mitochondria (red), endoplasmic reticulum (ER, black lines), and plastids (light brown) can be distinguished. (G) The vacuole (blue) enlarges which leads to cytoplasmic rearrangement and migration of the nucleus to the periphery of the cell. (H) During pollen mitosis I, the nucleus divides asymmetrically. (I) Within the vegetative cell a generative cell (dark brown) is formed. The vacuole is resorbed, and the cytoplasm begin to fill up with storage reserves. (J) A cell wall is formed inside the vegetative cell to separate the newly formed generative cell. (K) Pollen mitosis II takes place in the anther and forms the tricellular pollen including the two sperm cells. (L) The mature pollen grain is filled up with storage reserves like starch (olive brown granules) and after release from the anther starts to grow pollen tubes upon landing on receptive stigmas. Simplified illustration after (Browne et al. 2018) and (Shivanna 2003).

1.2.6. Pollen wall formation, morphology and function

Among plant cell walls, pollen walls are the most complex ones differing vastly in their chemical composition and morphology (Blackmore et al. 2007). The pollen wall consists of three main domains, exine, intine, and pollen coat (Shivanna 2003; Wiermann and Gubatz

1992). The exine is the outer wall, which can have intricate and species-specific surface architectures that has led scientist to numerous discussions in terms of taxonomic classification and even to a new discipline, palynology (Blackmore et al. 2007). Two main descriptive sets are popularly used to further divide the exine layer (Shivanna 2003; Punt et al. 2007). The first descriptive set divides the exine into endexine and ektexine, with the ektexine further divided into tectum, columella and foot layer (Faegri and Iversen 1964). The other set divides the exine into sexine (with tectum and columella), and nexine with nexine 1 and 2, where nexine 1 is the pendant of the foot layer (Erdtman 1952). The columella is also known as bacula (Punt et al. 2007). The exine is mainly composed of a very durable compound, namely sporopollenin (Heslop-Harrison 1968a), which itself is made up of polymers derived from long-chain fatty acids and phenylpropanoids both synthesized from tapetal cells of the anther (Piffanelli et al. 1998; Wiermann and Gubatz 1992). The sculptures on the outer wall may have evolved as adaptations to promote pollen dispersal or to facilitate the attachment to the stigma (Quilichini et al. 2015). In wheat, intricate structures of the exine and pollen coat are absent (Pacini and Hesse 2005).

The intine is the inner wall that maintains the structural integrity of the pollen protoplast and has many of the chemical and structural characteristics of the primary wall of a somatic cell (Quilichini et al. 2015; Sitte 1953; Shivanna 2003). It is composed of hydrolytic enzymes, hydrophobic proteins, pectic polymers, cellulose and hemicellulose (Heslop-Harrison 1968a; Ariizumi and Toriyama 2011). The intine encompasses the grain more or less uniformly with only slight variation in thickness occurring usually at the apertural sites (Heslop-Harrison 1979c). During pollen germination, the intine serves as the only cell wall encasing the growing pollen tube, and is rapidly remodeled to assist growth while preventing premature rupture (Chebli et al. 2012).

Pollen wall development is a heterogenic process whereby the compounds of the exine are produced by tapetal cells of the sporophyte and the compounds for the intine are produced by the developing pollen grain (Knox 1984; Wiermann and Gubatz 1992; Dickinson and Heslop-Harrison 1968; Heslop-Harrison 1968a). The pollen wall development is initiated after telophase II has finished and microspores are in the tetrad stage (**Figure 2C, F**), and mainly is completed by the time of the first pollen mitosis (**Figure 2D, I**) (Knox 1984; Heslop-Harrison 1963; Scott 1994; Wiermann and Gubatz 1992).

Microspores in the tetrads are physically separated by a special callose wall (Skvarla and Larson 1966). At that time, the plasma membrane commonly starts to separate from the callose wall,

and the precursor of exine, primexine, is formed. It accumulates between the plasma membrane and the callose wall and consists of a microfibrillar cellulosic material (Heslop-Harrison 1968b, 1971b). The primexine was considered to be synthesized by the microspore (Fitzgerald and Knox 1995; Piffanelli et al. 1998; Southworth and Jernstedt 1995; Wilson and Zhang 2009). However, different genetic analysis provided evidence, that the sporophytic tapetal cells may be involved in the formation of primexine (Ariizumi et al. 2004; 2005; 2008; Hu et al. 2014; Guan et al. 2008; Ariizumi and Toriyama 2011). Shortly, after the beginning of primexine formation, future aperture regions are defined. It has been proposed that the endoplasmic reticulum (ER) blocks the deposition of primexine at specific sites (Heslop-Harrison 1971b, 1968a, 1963; Munoz et al. 1995; Scott 1994). Also, radially oriented rods, the probacula, develop at regular intervals, presumably opposite to tubules and ER and on the raised sites of the undulated plasmalemma (Heslop-Harrison 1971b; Quilichini et al. 2015; Paxson-Sowders et al. 1997). Probacula will become connected at their bases and, together with the arising lamellae at the plasmalemma, they form the foot layer (nexine 1). New material of primexine is deposited between the rods, and the heads of the probacula expand and eventually fuse to form a continuous roof, the protectum, which will further develop into the tectum of the sexine (Dickinson and Heslop-Harrison 1968). The basic exine pattern is laid down at that time (Paxson-Sowders et al. 1997; Heslop-Harrison 1971b). It is likely that primexine has also some selective binding capacities provided by special enzymes or receptors promoting polymerization of sporopollenin (Ariizumi and Toriyama 2011; Gabarayeva et al. 2010; Scott 1994). Thus, primexine is considered as ‘exine template’, ‘scaffold’ or ‘anchor’ (Skvarla and Larson 1966; Quilichini et al. 2015; Ariizumi et al. 2004; Scott 1994).

In the second phase of pollen wall formation, the callose wall is dissolved by callase and microspores are released from the tetrads into the tapetal fluid filling the anther locule (**Figure 2D**). Changes in pollen size and shape occur and the primexine wall may get stretched and thinned out (Heslop-Harrison 1971b). However, the primexine thinning may not be visible due to a rapid accumulation of wall material (Stanley and Linskens 1974). Sporopollenin is quickly deposited on the microspore surface, first in irregular form around the probacula. Then, it continues to build up until the complete surface is covered with exception for the apertural regions (Heslop-Harrison 1971b; Stanley and Linskens 1974). The final sporopollenin in the exine thereby is the product of polymerizations from precursors made up of fatty acid derivatives and phenolic compounds that have been synthesized by tapetal cells. The precursors are secreted from the tapetal cell to the apoplast via ATP binding cassette (ABC) transporter proteins and then perhaps is transported to the microspores via osmophilic orbicular structures,

also termed Ubisch bodies, located in the anther locule or other transport proteins (Ariizumi and Toriyama 2011; Zhu et al. 2013).

At the same time of accumulation of the sporopollenin, the probacula and protectum elongate and form the mature bacula and tecta of the sexine. The plasmalemma returns back to a smooth surface (Lou et al. 2014). The mature exine is visually completed by the time of the bicellular pollen stage (**Figure 2J**) (Ariizumi and Toriyama 2011). A number of different genes are involved in exine formation and some are specifically expressed in the tapetal cells during that stage, indicating sporophytic control (Quilichini et al. 2015; Jiang et al. 2013). Underneath the foot layer, intine is secreted by the microspore (Lou et al. 2014). As the tapetum gradually starts to undergo PCD during microgametogenesis, final changes of the exine wall and usually deposition of the pollen coat filling up cavities occur as a results from the breakdown of tapetal cells (Wilson and Zhang 2009).

1.2.7. Pollen composition

Gross mineral composition of wheat pollen was determined by Knight et al. (1972). It contains 5.3% ash, 250 mM mg^{-1} DW potassium, 20 mM mg^{-1} DW sodium, 100 mM mg^{-1} DW calcium, 220 mM mg^{-1} DW magnesium, 2640 mM mg^{-1} DW nitrogen, 160 mM mg^{-1} DW phosphorous, 180 mM mg^{-1} DW sulfur (units converted from milliequivalents 100 g^{-1} DW), 586 ppm ferrum (Knight et al. 1972). Petrovskaya and Tsinger (1961 cited by Goss 1968) found glucose and fructose but not raffinose or xylose in mature wheat pollen. Joppa et al. (1966) reported the accumulation of starch granules in the developing wheat pollen in the anther, however, starch is unevenly distributed with higher appearance towards the pore region compared to its opposite pole (Watanabe 1961 cited by Goss 1968).

To address the high energy consuming process of pollen germination and tube growth the vegetative pollen cell produces storage reserves (Pacini 1996). Sperm cells do not contain reserves due to their reduced cytoplasm and the general absence of plastids (Pacini et al. 1992; Clément and Pacini 2001). The two main types of reserves in pollen are carbohydrates and lipids (Rodríguez-García et al. 2003). Insoluble carbohydrates are mainly stored in form of starch in amyloplasts or occur as part of the pollen wall, while soluble poly-, di- and/or monosaccharides are mainly present in the cytosol (Clément and Audran 1999). Lipids are congregated in spherosomes (Pacini and Hesse 2004). All pollen grains probably also accumulate storage proteins, but they are less abundant (Piffanelli et al. 1998; Pacini 1996).

During pollen development, plastids differentiate to amyloplast (amylogenesis) once or twice depending on species (Clément and Pacini 2001; Franchi et al. 1996). In monocots, amylogenesis occurs once during pollen development (Pacini and Viegi 1995). Amyloplasts are always present after microspores are released from tetrads and the first haploid mitosis had occurred. However, starch reserves may be emptied before anthesis by translocation and/or hydrolyzed to sugars and lipids in case of the starchless pollen (Clément et al. 1994), or stored and utilized in dispersed pollen and during pollen tube growth in case of the starchy pollen (Franchi et al. 1996). Grass pollen mainly produce starch and contain little amount of other carbohydrates, thus, belong to the latter category (Shi and Yang 2010; Speranza et al. 1997).

1.2.8. The flowering process in wheat - anther dehiscence, pollen presentation and dispersal

The female egg cell inside the ovule is immobile and develops completely encased inside the ovary (Gifford and Foster 1989). Thus, pollen grains need to be dispersed from the anther and reach to the stigma in order to fertilize the egg cell (Pacini and Dolferus 2016). In wheat, flowering begins in one spikelet located in the middle third of the spike and progresses upwards and downwards along the spike. Within a spikelet, the primary floret flowers first followed by secondary and so on (De Vries 1971). Anther opening involves multiple anther cell types and is initiated when pollen development is fully completed. The opening of tetrasporangiate anthers is preceded by fusion of two neighboring loculi leading to a sudden increase of space for the maturing pollen grains (Pacini 2000). Two main steps mark the opening of anthers: first, locular fluid disappears and second, the anther opens (Pacini and Hesse 2004). The locular fluid, which provides substances from the mother plant and surrounds the developing pollen, disappears by evaporation through the anther epidermis and/or by resorption towards the stamen filament or other floral parts (Ge et al. 2001; Pacini and Hesse 2004; Heslop-Harrison et al. 1987). In wind-pollinated (anemophilous) pollen species like grasses, the mechanical layer consists only of endothecial cells (Pacini 2000). Cells of the mechanical layer are often already dead and may have lignified thickenings. Mechanical forces due to the thickening and water withdrawal causes stretching and folding of the cells leading to opening of the anther and pollen dispersal (Manning 1996). In chasmogamously flowering wheats (referred to as open-pollination wheats) florets open during anthesis exposing the pollen for wind-pollination (De Vries 1971). Floret opening is facilitated by water uptake of the lodicules from the ovary. Thereby, lodicules become very turgid, swell and push the lemma and palea apart. Maximum opening of the floret is reached within five minutes. During the same time, the filaments

suddenly elongate strongly making the anthers leave the floret and taking a pendent position. In cleistogamous wheats (closed flowering), filament elongation still takes place but is hidden within the closed floret (De Vries 1971). However, the opening of florets within the same plant may also depend on spikelet position and environmental condition (Kandaurov and Belkovskaja 1966; Leighty and Sando 1924; Früwirth 1905; Obermayer 1916; Rajki 1962). Simultaneously, the stigmas expand to expose a larger surface area for the shed pollen. When flowering finishes, the water withdraws from the lodicules, they collapse and the palea and lemma resume their position before flowering which leads to the closure of the floret (De Vries 1971). The duration of the flowering process of a single floret varies within a range of 5 to 60 minutes, and takes about 30 minutes in the mean depending on wheat line and environmental conditions (De Vries 1971; Früwirth 1905; Obermayer 1916; Percival 1921; Leighty and Sando 1924). Flowering of all fertile florets within a spikelet can last between 3 to 4 days, and the whole spike may have finished flowering after ~5 to 7 days (De Vries 1971; Früwirth 1905).

1.2.9. Pollen-stigma interactions and pollen tube growth

Upon landing on the stigma, pollen needs to stick on it and rehydrate. To support rehydration and initiation of pollen tube growth, stigmas provide water and nutrients in form of exudates. There are two categories of stigmas, the wet stigmas with generous amounts of exudates, and the dry stigmas with limited surface secretion (Heslop-Harrison and Shivanna 1977; Heslop-Harrison 1979c). It was shown, that plants with tricellular pollen like in wheat tend to have the dry stigma type (Van Went and Willemse 1984). The feathery stigma in wheat has two main branches and each has many stigmatic side branches on which outward curved papillae cells support adhesion of the pollen (De Vries 1971). After adhesion, water from the stigma will pass into the pollen grain most likely at the side of the aperture (Heslop-Harrison 1987). Within the pollen, extremely rapid changes in the cytoplasm and pollen wall occur involving reorganization of membranes, dissolution of lipid aggregates, vacuolation, repositioning of cell wall material, and protoplasmic streaming may be seen in the grains (Heslop-Harrison 1979b). The pollen rapidly starts with the formation of a pollen tube within 1 to 15 minutes (D´Souza 1970; Cheng and McComb 1992; Heslop-Harrison 1979b). Tube growth is guided by complex signaling and cell-to-cell communications between the tube and female gametophyte (Ge 2020; Higashiyama and Yang 2017; Dresselhaus et al. 2016). When the pollen tube emerges, the operculum in front of the aperture gets lifted allowing the tube to penetrate the cuticle of the papilla cells (Heslop-Harrison 1979b). Within the pollen tube, a characteristic zonation is established. Immediately at the tip there is a “clear zone” where secretory vesicles transport and

deposit polysaccharide-containing material onto the expanding cell wall (Taylor and Hepler 1997; Heslop-Harrison 1979b). The proximal region of the pollen shaft behind the tip contains many organelles, the two sperm cells and the vegetative nucleus which are transported alongside microtubules that maintain a constant distance to and support organization of the tip (Chebli et al. 2013). In the distal region actin microfilaments and microtubules form dense matrices to direct long-distance transport of organelles, vesicle and other “cargo” (Taylor and Hepler 1997; Chebli et al. 2013). Zones are also established in the cell wall with the pollen tube tip being flexible and becoming firmer and more stable towards the pollen tube shaft (Scholz et al. 2020). As the tube continues to grow between the cuticle and the underlying pectocellulosic wall of the stigma branches (Heslop-Harrison 1979b), many factors are involved in the guidance of the pollen tube towards the female gametophyte, including ion gradients, small GTPases, reactive oxygen species (ROS), and signaling phospholipids (Heilmann and Ischebeck 2016; Guo and Yang 2020; Ischebeck et al. 2010; Michard et al. 2017; Scheible and McCubbin 2019; Steinhorst and Kudla 2013; Vogler et al. 2019; Zhang et al. 2020). In the initial period of pollen tube growth, pollen uses the reserves stored in the pollen grain and the tube grows mainly autotrophically (Stephenson et al. 2003). In the later growth phase of the tube additional supplies like sugars, glycoproteins, free amino acids and phenolic compounds are provided by cells of the transmitting tract and other maternal tissues (Goetz et al. 2017). Finally, the pollen tube content with its two sperm cells is discharged into the degenerated synergid of the egg cell (Gao et al. 1992).

1.2.10. Wheat pollen viability and longevity

The assessment of the pollens’ biological function and fitness is crucial for plant breeders and for the purpose of plant genetic resource conservation. Early developed viability assays were based on one of the four following basic principles: intactness of the pollen grain, activity of pollen enzymes, the process of pollen germination, and the result of a successful fertilization event as the number of seeds or fruit set. One of the first observations of viable pollen was made already in the 1830s and was based on pollen tube growth (Amici 1830). Since then, numerous different pollen viability tests have been developed, and, along with them, different descriptions of pollen viability have evolved with varying degrees of significance. Dafni and Firmage (2000), summarized the most widely used viability tests and gave an overview about their usage, advantages and disadvantages (Dafni and Firmage 2000). Recently, new and refined techniques evolved including the use of high throughput chip-based measurements (Heidmann

et al. 2016), and new staining methods with the possibility of automated counting (Biswas et al. 2020).

Tricellular pollen such as wheat pollen have a short lifespan (Brewbaker 1967). The longevity of wheat pollen depends on environmental conditions such as temperature, relative humidity (RH), light and wind (Hoekstra 2002; De Vries 1971). Further, it was shown that the position of the floret within the spike can influence pollen viability. Primary and secondary florets within a spikelet are larger and show higher viability (Obermayer 1916; Ovcinnikov 1952). Mature wheat pollen loses the ability to fertilize a stigma after the first 15 minutes from anther opening under field conditions (20 °C, 60% RH). Storage under cold conditions (5 °C, 60% RH) prolong the survival time to 2h (D´Souza 1970). **Table 1** summarizes some published papers in which wheat pollen longevity has been studied. However, most of these works have shown that only short-term storage was possible. Among these studies, the maximal pollen storage time of 45 days was achieved in ultra-low temperature storage in liquid nitrogen (Andreica et al. 1988). However, none of these works showed in dept which effects different storage conditions may have on pollen viability, and whether pollen have an altered metabolism and ultrastructure.

Table 1 Overview of wheat pollen viability after different storage periods.

Storage temperature [numbers in °C]	Storage conditions	Survival time	Viability	Viability assessment	References
undefined	field conditions	15-20 min	initial viability unknown	unknown	Kul´bij (1959a); Kulbij (1959b) cited by Kison and Franke (1980), Barnabás and Kovács (1992) and De Vries (1971)
2-4	pollen stored in desiccator over calcium chloride	5 h	initial viability unknown	unknown	Kul´bij (1959b); Kulbij (1959b, 1959a) cited by Goss (1968); De Vries (1971) and D´Souza (1970)
undefined	undefined	60 min	decline of fertility to 5%	unknown	Kovacik and Holienka (1962) cited by De Vries (1971)
0-4	pollen stored within the anther in ambient atmosphere	15 days	initial viability unknown	unknown	Abramova (1966) cited by De Vries (1971)
4	unknown	1 day	unknown	unknown	Barnabás (1982) cited by Gill (2014).
undefined	pollen stored in the sun	3 h 40min	decrease from 71.5% to 50.3%	peroxide reductase staining*	Lelley (1966) cited by De Vries (1971)
undefined	pollen stored in the shade	3 h 40min	decrease from 71.5% to 56.1%	peroxide reductase staining*	Lelley (1966) cited by De Vries (1971)
cool temperatures	high RH	‘Prolonged’	initial viability unknown	unknown	Watkins and Curtis (1967) cited by De Vries (1971)
30	40% RH	30 min	~85% in fresh pollen	*in vivo* germination on male-sterile pistils	D´Souza (1970)
20	40% RH	45 min	~85% in fresh pollen		
5	40% RH	30 min	~85% in fresh pollen		
30	60% RH	60 min	~85% in fresh pollen		
20	60% RH	75 min	~85% in fresh pollen		
5	60% RH	120 min	~85% in fresh pollen		
30	95% RH	15 min	~85% in fresh pollen		

Storage temperature [numbers in °C]	Storage conditions	Survival time	Viability	Viability assessment	References
20	95% RH	45 min	~85% in fresh pollen		
5	95% RH	75 min	~85% in fresh pollen		
at RT in green house	ambient RH	< 5 min	0% seed set	seed set on emasculated spikes	Athwal and Kimber (1970)
4	75-90	4 days		benzidine hydrogen peroxide test* (detects catalase activity) compared with seed set	Khan et al. (1971)
at RT in green house	ambient air	55-65 min		seed set	Fritz and Lukaszewski (1989)
at RT	pollen stored in desiccator	35-40 min			
at RT in green house	ambient air	2-4 min		fluorescein diacetate staining after Heslop-Harrison and Heslop-Harrison (1970)	
at RT	pollen stored in desiccator	2-4 min			
-196 °C (liquid nitrogen)		45 days	17,2% after storage	*in vitro* germination	Andreica et al. (1988)

*Petrochenko (1964 cited by Goss, 1968) claimed that the benzidine hydrogen peroxide test is questionable. He showed that catalase remains active for several month, even when the pollen starts to lose its viability. Similarly, cytochrome oxidases and peroxidases remain active in dead pollen (Diakonu 1968 cited by Kison and Franke, 1980). However, in the study of Khan et al. (1971), their results of the benzidine hydrogen peroxide test were in close agreement with their results of the seed set.

1.3. Pollen storage and challenges

1.3.1. Characteristics and differences of bi- and tricellular Pollen

The majority of angiosperm plant species (70%) produce bicellular pollen which is incompletely developed at anthesis. These pollen grains contain a vegetative cell and a generative cell at the time of dispersal, the generative cell divides after rehydration during pollen germination. In contrast, in about 30% of species, pollen are shed in a fully developed, tricellular state containing already both sperm cells when disseminated (Brewbaker 1967). These pollen are also considered phylogenetically more advanced (Mulcahy and Ottaviano 1983). Before dispersal, pollen water content is reduced by evaporation or reabsorption from the surrounding anther tissues (Pacini 1994). Bicellular pollen are capable to tolerate desiccation to extremely low water contents (Franchi et al. 2002; Nepi et al. 2001). After dehydration bicellular pollen can reach very low water contents (11.1% DW) (Towill 1985), which helps the pollen to withstand adverse conditions and fluctuations in temperature and RH (Firon et al. 2012). However, bicellular pollen tend to be slow in recovering respiratory competence upon rehydration and the ultimate respiration rate achieved is low (Hoekstra and Bruinsma 1975). In contrast, tricellular pollen are desiccation-sensitive and are only slightly dehydrated at dispersal (Franchi et al. 2002; Williams and Brown 2018). The water contents in tricellular pollen at anthesis can be as high as 60% (e.g. maize pollen) and, thus, these pollen are referred to as ‘partially hydrated’ (Nepi et al. 2001). During anthesis, pollen are highly metabolically active and initiate quickly pollen tube growth after landing on the stigma (Hoekstra and Bruinsma 1975).

1.3.2. Pollen preservation approaches

Pollen is a valuable genetic resource for conservation and exchange of nuclear genes and can be considered as an additional propagule for breeders and researchers. The main objective of pollen preservation is the retention of its normal function, i.e. its ability to germinate *in vivo* and fertilize the female egg cell (Hanna and Towill 1995). Ideal pollen grains suitable for storage are small, desiccation tolerant and relatively simple to sample. Thus, many pollen species can survive storage for a few weeks in a dried state, e.g. at room temperature, over dry silica gel or over saturated salt solutions in a desiccator (Shivanna and Rangaswamy 1992). Depending on species, pollen grains can be stored for short duration under cold conditions with low RH (0–10 °C, 10–30% RH) or made amenable for long-term storage by cold storage at -20 °C or lower (Nair et al. 2017). Alternatively, freeze-drying was used to preserve pollen of fruit

trees, e.g. peach (*Prunus persica*), apple (*Malus pumila*), and Japanese pear (*Pyrus serotina*) for many years (Akihama and Omura 1986). Cryopreservation is a widely used method for long-term storage of pollen and involves storage at ultra-low temperatures (< -150 °C), in the liquid phase (-196 °C) of liquid nitrogen (LN) or its vapour phase (> -196 °C). Under these conditions, the molecular mobility is considered very low or absent and the stored pollen material can be maintained for long periods of time (Benson 2004; Sparks and Yates 2002). Cryopreservation is a highly promising and effective method to conserve pollen materials for various fields of application in research and agriculture (Naidoo et al. 2011), e.g. to increase efficiency of breeding programs through higher accessibility of germplasm around the world (Connor and Towill 1993). Especially when applied to pollen species with ineffective or absent pollinators, asynchronous or short flowering the use of cryopreserved pollen can enable crosses for hybridization (Dinato et al. 2020).

Although numerous protocols exist for various pollen species, there is no universal protocol. Successful cryopreservation has been achieved for pollen of at least 170 species, including fruit and forest trees, staple crops, vegetables, forage grasses, and ornamental economic and medicinal plants (Ganeshan et al. 2008; Zhang et al. 2009; Chaudhury et al. 2010; Karun et al. 2014; Wang et al. 2015; Popova et al. 2016). To date, there are only a few published sources in which attempts have been made to make wheat pollen storable (see **Table 1**).

1.3.3. Pollen banks

A pollen bank is a collection of pollen collected from different species of important fruit and forest trees as well as agricultural crops. Pollen banks have been established in several countries (**Table 2**). Stored pollen in a cryobank is an important plant genetic resource that is often established to complement field, seed and in vitro gene banks (Towill and Walters 2000). Each sample of pollen in a cryobank is collected and placed in an individual storage container, e.g. in so-called cryotubes of 1.5 to 2 ml. These tubes are then placed in storage boxes in racks inside LN tanks (**Figure 3**). Cryobank management systems often use barcodes to keep individual samples organized and to store additional information of the sample in a database, e.g. species, date of collection, plant state, treatments.

Table 2 Overview of pollen cryobanks and their number of stored pollen species.

Institution with pollen bank	**Country**	**No. of preserved pollen species**	**Reference**
Indian Institute of Horticultural Research (IIHR), Division of Plant Genetic Resources	India	572 accessions from 36 species	(Ganeshan and Rajasekharan 2000) https://www.iihr.res.in/technology-establishment-pollen-cryobank
National Bureau of Plant Genetic Resources (NBPGR)	India	65 accessions from 16 species; actual 616	(Mandal 2000); http://www.nbpgr.ernet.in/Research_Projects/Base_Collection_in_NGB.aspx (accessed 24th April 2022)
National Center for Genetic Resources Preservation (NCGRP)	USA	13 pear varieties, 24 Pyrus species	(Reed et al. 2000)
Fruit Tree Research Station	Japan	Unknown number of pollen species	(Akihama and Omura 1986)
La Direction de la Recherche Forestière	Canada	12 tree species in 83 seed orchards	(Mercier 1995)
Beijing Forestry University	China	71 species/varieties from 19 families	(Xu et al. 2014)
Beijing Laboratory of Urban and Rural Ecological Environment	China	102 species/varieties in 14 families and 32 genera	(Ren et al. 2019b)

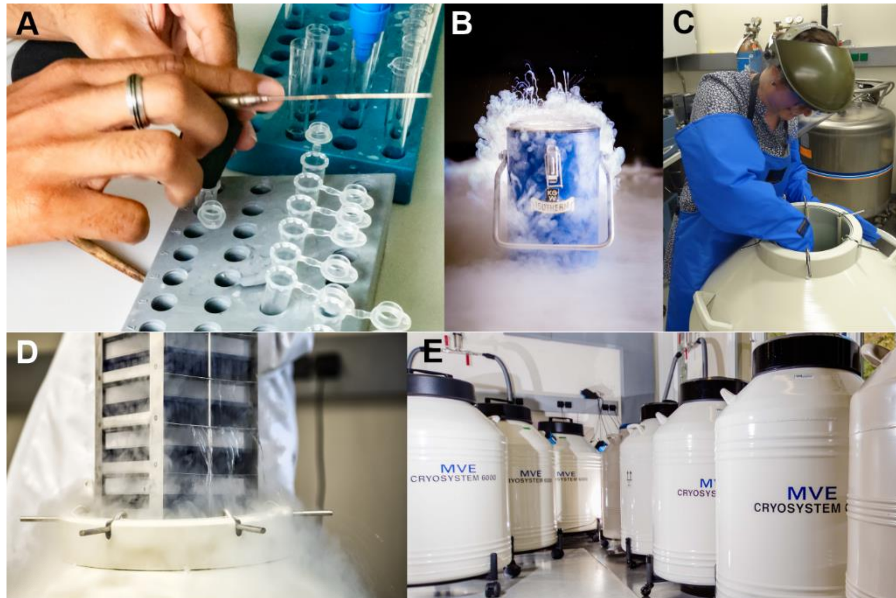

Figure 3: Pollen sampling for storage in liquid nitrogen. A) Wheat pollen was collected onto black carton paper. Part of the pollen can be transferred into 1.5-ml-tubes for analysis of viability, biochemical compounds, or for the use in other experiments. B) Pollen in cryo-sample tubes may be frozen in small Dewar containers before transfer to the LN storage tanks. C) Storage containers or tanks are used to store samples in the liquid (-196 °C) or vapour (>-196 °C) phase of LN. D) Individual samples are placed in boxes inserted a rack of a LN tank. E) LN storage tanks. Pictures from Cryo- and Stress Biology Group, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK).

Depending on the plant species, pollen from a pollen bank can be made available throughout the year. Thus, geographical, seasonal and physiological barriers can be overcome. For example, the use of stored pollen in plant breeding of woody plants can help to reduce or even eliminate the tedious, time- and resource-consuming care and maintenance of orchards and nurseries (Bajaj 1987). Environmental fluctuations in the release of pollen during pollination can be compensated for by pollen storage. Furthermore, stored pollen from field crops nearly eliminate the need to grow male lines for plant breeding. However, since pollen is an exhaustible resource, pollen banks cannot serve as the sole mode of conservation of nuclear genes. Seeds or clones must also be conserved *in vivo* or *in vitro* to ensure regular replenishment of the pollen bank (Towill and Walters 2000).

Also, for a large number of species, conservation in pollen banks is associated with biological problems like low pollen viability, limited pollen production, low regeneration potential of aged pollen, or technical problems such as labour-intensive steps in collection and further processing, lack of protocols for testing viability and conservation (Nair et al. 2017). There are no established pollen banks for wheat so far.

Thesis objectives

1) Assessment of wheat pollen viability

Wheat sheds tricellular pollen at maturity which loses rapidly the ability to germinate under ambient conditions. The aim of this study was to gain a comprehensive overview of processes and factors contributing to viability loss in wheat pollen. Reliable, quick and easy viability assessment tests are necessary to estimate the proportion of viable pollen after collection from mature flowers. So far publicly available information on viability testing for wheat pollen are scarce and wide-scale screenings and comparisons of suitable tests have not been undertaken. Thus, in the first study of this thesis we aimed to establish and compare quick and reliable viability assay for wheat pollen that can be used in future studies.

a. The first aim was to find a formulation of *in vitro* gemination medium where wheat pollen tube growth was present. Thus, media with different ingredients and concentrations were screened and the results compared with existing protocols from different authors.
b. Thereafter, the thesis aimed to optimize the own developed medium formulation to find optimal pollen tube growth. A stepwise improvement of the gemination medium by addition/omission of different plant growth regulators, osmotic substances, and other additives should help to find a suitable germination medium for wheat pollen.
c. To test also other viability tests, the best *in vitro* germination medium was compared with two staining methods (fluoresceine diacetate staining and Alexander staining), a chip-based high-throughput assay (impedance flow cytometry), and *in vivo* germination.
d. Finally, the suitability and performance of above-mentioned viability assays was investigated for pollen of other important cereals (maize, rye, barley).

2) Environmental factors affecting pollen viability

The pollen state and fitness at anthesis are crucial information that can help to improve protocols for long-term pollen storage. The second part of this work focused on elucidating factors that affect wheat pollen viability and physiological, structural and biochemical changes after the pollen is shed. Within the second part, the following objectives were investigated:

a. Changes of wheat pollen water content and viability in four wheat lines in response to the exposure to four different environmental conditions were studied. The effects on viability were measured with two viability assays chosen from the first part of this study whereas changes in water content should help to identify possible correlations.
b. A structural analysis of pollen morphology using light and electron microscopy was used to show physiological changes and possible damages due to storage.
c. A comprehensive gas-chromatographic analysis of changes of metabolites in important pathways (glycolysis and citrate cycle) was carried out to develop a metabolic map of wheat pollen.
d. Possible changes of carbohydrate reserves (soluble sugars and starch) in wheat pollen were analyzed during storage using ion-exchange chromatography and spectrophotometry.

3) An attempt to establish a protocol for wheat pollen preservation

Pollen has high potential to preserve and exchange nuclear genes of plant genetic resources. To facilitate breeding programs, short- and more importantly long-term pollen preservation protocols have been established for many species. Long-term pollen preservation, particularly in wheat breeding programs, and especially with spatially and temporally isolated parents, would be of great interest to expand genetic diversity and to facilitate hybrid seed production. However, favorable storage conditions for wheat pollen have not been studied yet and physiological, ultrastructural and biochemical processes in pollen after shedding are hardly understood. Thus, in the third part of this thesis I focused on the effects of dehydration and ultra-low temperature storage on physiological and morphological parameters and changes in pollen viability. Finally, I investigated whether cryopreservation can be applied for pollen storage in wheat.

a. To analyse transitional changes of intracellular water during freezing, controlled freezing was used with two different cooling/warming rates and the changes in water content in pollen were measured with differential scanning calorimetry.
b. To study pollen survival after cooling, the effects of different rates of dehydration and cooling/warming on structural changes were compared and analysed with the help of cryomicroscopy. Pollen viability before and after

cooling was measured with *in vitro* pollen germination and impedance flow cytometry.

2. Assessment of Pollen Viability for Wheat

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Type of authorship:	First author
Type of article:	Research article
Contribution to article:	design and conduction of experiments, data analysis, manuscript preparation
Co-authors contribution:	Manuela Nagel conceived and provided the project, participated in interpretation of the data and in writing the manuscript.
	Angelika Senula participated in design of experiments.
	Janka Reitz, Claudia Köpnick, and Hardy Rolletschek conducted parts of the experiments.
	Andreas Börner provided substantial resources.
	All authors proofed and corrected the manuscript.
Journal:	Frontiers of Plant Science, Section Crop and Product Physiology
Impact Factor:	4.106
Date of Publication:	2020-01-22
DOI:	10.3389/fpls.2019.01588

2.1. Manuscript 1

ABSTRACT

Wheat sheds tricellular short-lived pollen at maturity. The identification of viable pollen required for high seed set is important for breeders and conservators. The present study aims to evaluate and improve pollen viability tests and to identify factors influencing viability of pollen. In fresh wheat pollen, sucrose was the most abundant soluble sugar (90%). Raffinose was present in minor amounts. However, the analyses of pollen tube growth on 112 liquid and 45 solid media revealed that solid medium with 594 mM raffinose, 0.81 mM H_3BO_3, 2.04 mM $CaCl_2$ at pH5.8 showed highest pollen germination. Partly or complete substitution of raffinose by sucrose, maltose, or sorbitol reduced *in vitro* germination of the pollen assuming a higher metabolic efficiency or antioxidant activity of raffinose. *In vitro* pollen germination varied between 26 lines ($P < 0.001$); between winter (15.3 ± 8.5%) and spring types (30.2 ± 13.3%) and was highest for the spring wheat TRI 2443 (50.1 ± 20.0%). Alexander staining failed to discriminate between viable, fresh pollen, and non-viable pollen inactivated by ambient storage for >60 min. Viability of fresh wheat pollen assessed by fluorescein diacetate (FDA) staining and impedance flow (IF) cytometry was 79.2 ± 4.2% and 88.1 ± 2.7%, respectively; and, when non-viable, stored pollen was additionally tested, it correlated at $r = 0.54$ ($P < 0.05$) and $r = 0.67$ ($P < 0.001$) with *in vitro* germination, respectively. When fresh pollen was used to assess the pollen viability of 19 wheat, 25 rye, 11 barley, and 4 maize lines, correlations were absent and *in vitro* germination was lower for rye (11.7 ± 8.5%), barley (6.8 ± 4.3%), and maize (2.1 ± 1.8%) pollen compared to wheat. Concluding, FDA staining and IF cytometry are used for a range of pollen species, whereas media for *in vitro* pollen germination require specific adaptations; in wheat, a solid medium with raffinose was chosen. On adapted media, the pollen tube growth can be exactly analyzed whereas results achieved by FDA staining and IF cytometry are higher and may overestimate pollen tube growth. Hence, as the exact viability and fertilization potential of a larger pollen batch remains elusive, a combination of pollen viability tests may provide reasonable indications of the ability of pollen to germinate and grow.

Keywords: *in vitro* pollen germination, raffinose, recalcitrant pollen, impedance flow cytometry, fluorescein diacetate staining, stigmatic germination

Abbreviations: FDA, fluorescein diacetate; IF, impedance flow cytometry; IAA, indole acetic acid; GA_3, gibberellic acid; PEG, polyethylene glycol; Pep, peptone water; EACA, ε-aminocaproic acid.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is an allohexaploid (2n = 6x = 42, AABBDD), self-pollinating species adapted to a wide range of temperate environments (Shewry, 2009). Annually about 770 million tons of wheat seeds are produced for human diet and agricultural purposes (http://www.fao.org/faostat/en/). Although, there had been significant increases in the global wheat yield development until 1990ies, about 38.8% of the global wheat production area has experienced yield stagnation in the recent years (Ray et al., 2012). Here, hybrid breeding promises an increase in grain yield and stability through the recognition of heterotic pattern (Zhao et al., 2015). Among other factors, successful wheat hybrid breeding relies on the haploid male gametophyte that is shed after second pollen mitosis completed. The mature tricellular wheat pollen has relatively high moisture contents (McCormick, 2004) and is known to be short-lived. Therefore, pollination is necessary within 30 to 40 min after pollen shedding to achieve successful seed sets (Fritz and Lukaszewski, 1989).

Viable pollen is important for species dispersal, fitness, and survival of the next plant generation. It is also essential for directed plant breeding and, consequently, crop improvement. Pollen viability comprises different aspects of pollen performance such as fertilization ability, germinability, and stainability (Dafni and Firmage, 2000). Common techniques to elucidate pollen viability are staining techniques, *in vitro* germination, seed set as well as *in vivo*, and semi-*in situ* germination on the excised stigma, also termed stigmatic germination. In the last two, pollen tube growth toward or on stigmas is observed by contrasting dye and the results are assumed to give most accurate estimations of the seed set (Esser,1955; Dionne and Spicer, 1958). However, incompatibilities, postfertilization barriers and limited measurability may restrict the accuracy of these tests (Dafni and Firmage, 2000).

Staining of pollen aims to visualize specific compounds, contents, or cellular compartments related to pollen viability. Potassium iodide, aniline blue, and acetocarmine stain starch, callose, and chromatin, respectively, and the absence of colors indicate non-viable pollen (Stanley and Linskens, 1974). Comparable, the Alexander stain discriminates aborted pollen grains from non-aborted pollen grains. Here, the cytoplasm is colored red, whereas the cell walls are stained green. When the cytoplasm is absent, the green cell walls become visible and indicate a lack of function (Alexander, 1969). In the 1970ies, Heslop-Harrison and Heslop-Harrison (1970) developed a viability test based on the fluorochromatic reaction which included tests of the membrane integrity and enzyme activity. The non-fluorescent polar dye fluorescein diacetate (FDA) passes intact semi-permeable membranes. Intracellular nonspecific esterases hydrolyze

FDA and the fluorescein accumulates in the cytoplasma which shows a bright green fluorescence. When the plasmalemma is impaired the fluorescein can leave the pollen grains and a uniform background fluorescence can be observed which is an indication that the pollen grains are non-viable. Although staining methods offer the possibility to distinguish aborted and non-aborted fresh pollen, they often fail to discriminate different viability levels (Ge et al., 2011).

Refined viability estimations can be achieved by *in vitro* germination or the impedance flow (IF) cytometry. IF cytometry measures electrical cell properties of single cells using microfluidic chips and has been successfully applied to measure the physiological cell state of bacteria (David et al., 2012). Pollen grains are stimulated by radio frequencies from 0.1 to 30 MHz with alternating current and resulting data are related to cell size, membrane capacitance, cytoplasmic conductivity of single cells, and cell concentration. For mature cucumber, sweet pepper, and tomato pollen, high correlations were found between viability results of IF cytometry and FDA staining (Heidmann et al., 2016). Nevertheless, the potential of pollen to germinate and grow can be best analyzed by pollen tube growth in liquid or on solid media, also termed *in vitro* germination. Brewbaker and Kwack (1963) developed one of the first comprehensive pollen culture media suitable for 86 species. For wheat pollen, Cheng and McComb (1992) achieved maximum pollen tube length on medium containing raffinose. Due to high expenditures for raffinose, Jian et al. (2014) and Jayaprakash et al. (2015) replaced raffinose either by sucrose or maltose, respectively and achieved pollen germination up to 95% (Jayaprakash et al., 2015). However, except of these studies, wheat pollen germination has not been investigated in greater detail up to now. Therefore, the present study aims to compare pollen germination, staining assays, and IF cytometry across different wheat lines to establish a reproducible protocol to assess wheat pollen viability and to elucidate influencing factors. The term "pollen viability" will be used as an umbrella term describing the capacity of pollen to live, grow, germinate, or develop (Dafni and Firmage, 2000). By comparing 157 solid and liquid media, varying in main sugars, H_3BO_3, $CaCl_2 \cdot 2H_2O/Ca(NO_3)_2 \cdot 2H_2O$ concentration, pH, and other components, we selected a raffinose based medium triggering the highest *in vitro* germination. The results were compared with the pollen viability assessed by semi *in vivo* germination on the stigma, termed stigmatic germination, FDA staining, Alexander staining, and IF cytometry in various wheat lines. To test the suitability of the medium for other species of the *Poaceae* family, pollen of different rye, barley, and maize lines were germinated and results are discussed in relation to the required energy source for pollen germination, the effect of the line/species, and environment.

MATERIAL AND METHODS

Plant Material and Pollen Extraction

Seeds of 26 wheat, 25 rye (*Secale cereale* L.), 11 barley (*Hordeum vulgare* L.), and 4 maize (*Zea mays* L.) lines comprising listed varieties, breeding lines, and accessions were used for pollen viability assessments. Seeds were provided by the Federal Ex situ Gene Bank at IPK Gatersleben (https://doi.org/10.5447/ ipk/2019/9), Germany and from the Maize Genetics Stock Center (http://maizecoop.cropsci.uiuc.edu) (**Supplementary Table S1**) and germinated in a standard culture medium (Substrate1, Klasmann-Deilmann GmbH, Geeste, Germany) at 20 ± 2°C. One-week old seedlings of wheat, rye, and barley lines were subjected to 4 ± 1°C for four (spring types) and six weeks (winter types). Vernalized plants were transferred into pots containing a sand/soil mixture (70% compost soil, 20% white peat, 10% sand) and grown under optimum conditions (regular watering and fertilization, 16 h light) at 20 ± 2°C in the greenhouse. Maize seeds were germinated in 0.25 L pots at 22/20°C (day/night) and 13 h light. After 3 weeks, plantlets were transferred to 20 L pots and developed under optimum conditions (regular watering and fertilization) at 25/20°C (day/night) and 16 h light. In total, seeds of 19 wheat, 25 rye, and 11 barley lines were sown in two cultivation periods in August (1st set) and September (2nd set) in 2017 and mature pollen was available between November and January (1st set) and between December and March (2nd set). At the beginning of anthesis, spikes were cut between 8:00 and 10:00 a.m., kept in water and used within 6 h. Only mature pollen was used for all experiments. To stimulate pollen maturation, awns, glumes, and lemmas were carefully removed and pollen was sampled when lodicules swelled, the stigma fanned out, filaments elongated, and anthers enlarged and turned greenish to bright yellow (**Figure 1, Supplementary Video S1**). Before the tip of the anther opened, at minimum three anthers were taken and pollen shedding was supported by opening gently with a needle. For some experiments, non-viable pollen was required. According to Fritz and Lukaszewski (1989), wheat pollen loses germination at ambient conditions within 45 min. Therefore, to achieve non-viable pollen, we stored the wheat pollen at ambient conditions (50.0% RH and 23°C) for >60 min. For each replicate, anthers of different plants were used. Due to variations in pollen production, lines varied between some experiments.

Modification and Evaluation of Liquid Pollen Growth Media

To assess pollen tube growth, liquid media based on Cheng and McComb (1992); Jayaprakash et al. (2015), and Jian et al. (2014) were modified in contents of $CaCl_2 \cdot 2H_2O$/ $Ca(NO_3)_2 \cdot 2H_2O$, H_3BO_3 and the effect of different sugars and pH values was evaluated. Media were prepared using between 300 and 900 mM of sucrose, maltose, and raffinose and pH was adjusted to 5.8

and 7.3 (**Supplementary Table S2**). The media according to Jayaprakash et al. (2015) and Jian et al. (2014) contained 0.81 mM $MgSO_4 \cdot 7H_2O$ and 0.99 mM KNO_3 in addition. By using the sitting drop technique (Shivanna and Rangaswamy, 1992), 10 to 20 µl of the liquid media were placed in the wells of microscopic slides. Spikes of the lines ‘Ferrum’, ‘Piko’, and ‘Hermann’ were harvested and for each of the three replicates, pollen was pooled from mature anthers of one single spike. Pollen were shed on the droplets (without coverslip to avoid hypoxia) and further development was observed under the microscope immediately (Eclipse LV100, Nikon, Tokyo, Japan) (Stanley and Linskens, 1974). Due to rapid crystallization of the sugars, the detailed count of pollen tubes was not possible. Therefore, the frequency of pollen tubes and pollen bursting, and pollen tube lengths was estimated for a total of about 500 pollen grains located in ten microscopic fields (**Supplementary Figure S1**). Low, medium, and high frequency of pollen tubes refers to 1, up to 10 and more than 10 pollen grains, respectively, that developed pollen tubes. Low, medium, high frequency of pollen bursting refers to one, half of the pollen batch and most pollen that burst, respectively. Pollen tubes were defined as short, medium, and long when pollen tubes were shorter, between the one- and twofold or longer than the twofold diameter of the pollen, respectively.

Modification and Evaluation of Solid Pollen Growth Media

Solid media were assessed in three steps. 1st) Liquid media (**Supplementary Table S2**) according to Cheng and McComb (1992) and Jayaprakash et al. (2015) were solidified using 0.3% Gelrite and pH adjusted at 5.8 (**Supplementary Table S3**). Both media were further modified using ε-aminocaproic acid (EACA), peptone water (Pep), PEG 4000, PEG 8000, and different concentrations of H_3BO_3, $CaCl_2 \cdot 2H_2O/Ca(NO_3)_2 \cdot 2H_2O$, maltose, sucrose, and raffinose. All media were used to test pollen germination of the lines ‘Ferrum’, ‘Piko’, and ‘Hermann’. 2nd) A solid medium containing 0.81 mM H_3BO_3, 2.04 mM $CaCl_2 \cdot 2H_2O$, and 594 mM raffinose at 5.8 pH stimulated highest pollen germination in the 1st step and was termed “Basic” medium. To test the effect of different factors such as lines (‘Ferrum’, ‘Dialog’, ‘Einstein’, and TRI 9102), macronutrients (sucrose, maltose, mannitol, sorbitol), additives (H_3BO_3, EACA, IAA, GA_3) and concentrations, the “Basic” medium was further modified (**Table 1**) and the osmolality measured using a vapor pressure osmometer (Vapro 5520, Wescor, Langenfeld, Germany). 3rd) The “Basic” medium showed best results in the 2nd step and was consequently used for comparisons of pollen germination of 19 wheat, 25 rye, 11 barley, and 4 maize lines. All media were autoclaved for 20 min and stored at 5°C in sealed petri-dishes until usage.

For all solid media, three replicates were prepared and each replicate represented mature pollen from anthers of a single spike. The pollen was evenly dusted on the medium. To ensure an optimal pollen tube development, petri-dishes were placed without lid in a chamber and kept at 98.0 ± 0.5% relative humidity and 23°C for 30 min. Pollen germination was observed for each petri-dish by taking 10 non-overlapping pictures (Eclipse LV100, Nikon, Tokyo, Japan) at 100 x magnification (**Figure 2**). The total number of pollen, mostly between 500 and 1,000 pollen grains, burst pollen, and pollen tubes that exceeded the lengths of the pollen radius were counted manually using the software NIS elements v. 4.11 (Nikon Metrology, Brighton, USA) (Richter and Powles, 1993; Burke et al., 2007; Cerović et al., 2014). Shrunken or sterile pollen was not considered. After counting, the percentage of germinated and burst pollen was calculated.

Acetocarmine Staining to Identify Stigmatic Pollen Germination

In vitro pollen germination of the wheat line TRI 9102 was compared to stigmatic germination. Before anthesis, inner florets of wheat spikes were discarded and, in each spikelet, the two remaining immature florets were emasculated. After 2 days, wheat spikes were cut between 8:00 and 10:00 a.m., kept in water, and used within 6 h. Pollen was shed over fanned stigmas and germinated for about 30 min. For better visualization, stigmas were placed in a droplet of acetocarmine (Morphisto, Frankfurt, Germany) and kept in a chamber above filter paper soaked in 45% acetic acid for another 30 min. The stigmas were washed in 45% acetic acid and the ovaries carefully removed from the stigma. After washing, stigmas were placed into a drop of 45% acetic acid on a microscopic slide, covered using a coverslip, and germinated pollen was counted as previously described (**Figure 2**). Stigmatic germination was tested on 14 stigmas obtained from different plants (n = 14). In total, >70 fresh pollen grains and about 20 pollen grains stored at ambient conditions for >60 min were dusted on each stigma. Stigmatic pollen germination was compared to *in vitro* germination which was analyzed in 14 biological replicates (n = 14) using >300 fresh pollen and >300 stored pollen each.

Alexander Staining to Test Pollen Viability

In vitro pollen germination was compared to FDA staining (Heslop-Harrison and Heslop-Harrison, 1970) and Alexander staining (Alexander, 1969). Five biological replicates (n = 5) of about 300 to 600 pollen grains each were analyzed for the lines ‘Ferrum’, ‘Hermann’, and TRI 9102 each. FDA stain was prepared freshly before usage. Shortly, 2.4 mM FDA dissolved in acetone was mixed with 0.5 M sucrose solution until the solution turned milky (Pinillos and Cuevas, 2008). Fresh pollen was shed on a droplet containing FDA and pollen-FDA solution was mixed. Due to the bursting of the pollen, the stain fainted fast into the drop. Bright green

fluorescing pollen was observed using UV light and a fluorescence microscope (Axiolab, Zeiss, Jena, Germany) and counted as stainable (**Supplementary Figures S2A, B**). Alexander stain was prepared according to Alexander (1987). Again, fresh and stored wheat pollen grains were shed on a droplet of the Alexander stain placed on a microscopic slide and closed by a coverslip. Pollen stained magenta was considered intact, whereas blue-green pollen was considered to be nonviable or sterile (**Supplementary Figures S2C, D**).

Impedance Flow Cytometry

Pollen viability of the lines 'Ferrum', 'Hermann', and TRI 9102 was tested using IF cytometry and five replicates (n = 5) each. Briefly, for each replicate, between 500 and 1,500 of fresh pollen or non-viable, stored pollen grains were transferred into 1 mL IF cytometry measurement buffer (AF6, Amphasys, Lucerne, Switzerland), filtered using 100 μm pore size and loaded onto a channel chip (channel size 120 μm) inserted in the IF cytometer type Ampha Z32 (Amphasys, Lucerne, Switzerland). Measurements were carried out at 1 MHz at the default settings for wheat pollen (trigger level 0.1 V, frequency 1, modulation 3, amplifier 6, and demodulation 1). Data of $1.4 \times 10^3 \pm 640$ cells per sample using a concentration between 250 and 5.5 x 103 cells mL^{-1} were collected and analyzed using AmphaSoft v 2.0 version (Amphasys, Lucerne, Switzerland) (**Supplementary Figures S2E, F**).

Sugar Analysis

Soluble sugars were extracted from fresh and stored pollen samples (1.5 ± 0.8 mg) of the lines 'Ferrum', 'Hermann', and TRI 9102 using n = 4 biological replicates each. Pollen was mixed with 0.2 mL ethanol (60% v/v) and stored at −20°C until further analysis (Rolletschek et al., 2011). For efficient extraction, the samples were sonicated for 5 min. After centrifugation (5 min at 13,000 rpm) the supernatants were diluted with 10% methanol (1:50 v/v) and used directly for sugar analysis using ion-exchange chromatography coupled to pulsed amperometric detection (ICS-3000; Thermo Fisher). Chromatographic separation was carried out on a PA1 column (2 × 250 mm) and a PA1 guard column (2 × 50 mm) at 25°C by applying an isocratic run with 400 mM NaOH at a constant flow rate of 0.7 mL min^{-1} over 5 min. A mixture of glucose, fructose, sucrose, raffinose, stachyose, and verbascose was used as authentic standards and for external calibration. The identification of sugars was performed by comparing retention times of individual sugars in the reference vs. analyzed solution, and quantification was based on peak areas. Due to the low availability of pollen, the DW could not be determined. Therefore, the change in weight was measured on a second set of 'Ferrum' and TRI 9102 at a later time point. The fresh weight of fresh pollen was corrected by 0.39 and the fresh weight of stored

pollen was corrected by 0.91 to show the data on the content of individual sugars on a DW bases.

Statistical Analysis

Statistical analysis was conducted using SigmaStat 4.0 (Systat Software, 2016). All data were tested for normal distribution using the Shapiro-Wilk test. GenStat 18 (VSN International Ltd, 2016) was used to conduct Spearman correlation analysis at $P < 0.05$ and analysis of variance (ANOVA). Statistical differences were evaluated using the least significant difference at $P < 0.05$ (LSD5%). If the normality test failed, a balanced design was used for ANOVA and pairwise multiple comparisons using Holm-Sidak method was chosen.

RESULTS

Solid Medium Containing Raffinose Is Suitable for the Assessment of *In Vitro* Pollen Germination

Pollen tube growth was mostly stimulated when raffinose was available in liquid media. Of the 112 liquid media that were based on different protocols (Cheng and McComb, 1992; Jian et al., 2014; Jayaprakash et al., 2015) and differed in sugar composition (sucrose, maltose, raffinose) and in pH (pH 5.8, pH 7.3), pollen tube growth was visible in 25 liquid media. The highest number of germinated pollen in liquid media was found in media developed by Cheng and McComb (1992) containing raffinose (**Supplementary Table S2**). Here, the concentrations of H_3BO_3 and $CaCl_2·2H_2O$ affected significantly the pollen tube development. Reducing the H_3BO_3 concentration from 1.62 to 0.81 mM decreased the number of pollen showing pollen tubes, whereby the reduction of $CaCl_2·2H_2O$ from 2.04 to 1.02 mM resulted in a higher number of germinated pollen. When raffinose was partly substituted by sucrose or maltose a low frequency of pollen tube development was observed in two media with a pH adjusted to 5.8, each. However, liquid media were difficult to handle due to different focal planes, uneven distribution of pollen grains and rapid crystallization of sugars (**Supplementary Figure S1**). Therefore, in subsequent experiments selected liquid media, resulting in the highest pollen germination, were solidified and used for further germination tests.

Pollen germination could be quantified on solid media containing raffinose (**Figure 3, Supplementary Table S3**). Of in total 34 solid media, *in vitro* pollen germination was only observed on two media prepared according to Cheng and McComb (1992). On the average of four different lines, germination was 13.3 ± 10.4% in a media containing 1.62 mM H_3BO_3, 1.02 mM $CaCl_2·2H_2O$, and 594 mM raffinose and 32.7 ± 14.2% in a media containing 0.81 mM H_3BO_3, 2.04 mM $CaCl_2·2H_2O$, and 594 mM raffinose. However, in both media about 65% of

the pollen burst. In solidified media according to Jayaprakash et al. (2015), pollen tube growth was not detected (**Supplementary Table S3**). Therefore, the medium adapted from Cheng and McComb (1992) containing 0.81 mM H_3BO_3, 2.04 mM $CaCl_2 \cdot 2H_2O$, and 594 mM raffinose at 5.8 pH was further modified and the effect of different plant hormones (GA_3 and IAA), sugars (sucrose and maltose), sugar alcohols (mannitol and sorbitol), and additives (Pep, EACA) was investigated in detail (**Figure 3**). The osmolality of the different media ranged between 644 mmol kg^{-1} (sucrose) and 2,465 mmol kg^{-1} (sorbitol, **Table 1**). Among the four different lines, variations in pollen germination were observed, but this was not related to the osmolality of the solid media. Maltose, sorbitol, and sucrose inhibited most pronouncedly pollen tube development. The “Basic” medium showed highest pollen germination (39.7 ± 25.0%) and, therefore, was chosen for comparisons between different pollen viability tests, lines, and species.

Stigmatic Pollen Germination Was Higher Compared to *In Vitro* Germination

Wheat stigmas of emasculated florets stimulated pollen tube development of fresh pollen. In total, 52.4 ± 17.0% of the freshly harvested and mature pollen developed pollen tubes and grew toward or entered the stigma (**Figure 4**). A significantly ($P < 0.001$) lower germination (24.2 ± 17.2%) was observed for pollen grains on the “Basic” medium. In comparison, pollen stored at ambient conditions for 60 min revealed 1.8 ± 3.4 and 0.7. ± 2.4% for stigmatic and *in vitro* germination, respectively. However, the evaluation of stigmatic pollen germination was difficult due to chaotic pollen tube growth and the analysis of different focal layers in the microscope. Furthermore, the number of pollen grains for each stigma is limited and standardizations are hardly possible.

Analysis of *In Vitro* Germination, Fluorescein Diacetate Staining, Impedance Flow Cytometry, and Soluble Sugars to Assess Wheat Pollen Viability

Results of wheat pollen viability differed drastically in dependency of the assay (**Figure 5A**). In three wheat lines, percentage of *in vitro* pollen germination was compared with pollen stainability using FDA and Alexander staining and pollen viability after IF cytometry and were on the average 11.5 ± 14.5%, 40.2 ± 41.6%, 96.6 ± 3.4%, and 44.9 ± 44.4%, respectively. These results include the analysis of pollen stored under ambient conditions for >60 min, which was 0.3 ± 0.1%, 1.3 ± 4.2%, 97.7 ± 2.0%, and 1.6 ± 2.2%, respectively, and indicate that *in vitro* germination, FDA staining, and IF cytometry can discriminate between fresh and stored pollen (**Figure 5A, Supplementary Figure S2**). Alexander staining showed high viability results for all, fresh, and stored pollen in all lines and the results were significantly different ($P < 0.05$) compared to those obtained by other methods. Therefore, this approach was not appropriate for

wheat pollen viability assessment and was further excluded from comparisons. Significant correlations were observed between results of *in vitro* pollen germination and IF cytometry ($r = 0.67$, $P < 0.001$), and FDA staining ($r = 0.54$, $P < 0.05$) (**Figure 5C**). However, due to large differences between the results of *in vitro* germination, FDA staining, and IF cytometry, it can be hypothesized that the medium may still not sufficiently reflect the conditions which wheat pollen require for germination. Sucrose was the major sugar in fresh wheat pollen collected after pollen shedding (**Figure 5B**). Sucrose concentration was at 74.5 ± 16.7 µmol g^{-1} DW, 96.5 ± 26.4 µmol g^{-1} DW, and 79.3 ± 6.8 µmol g^{-1} DW and accounted for 84.5%, 88.7%, and 91.0% of the total amount of soluble sugars for 'Ferrum', 'Hermann', and TRI 9102, respectively. The content of sucrose was significantly higher ($P < 0.05$) in comparison to glucose, fructose, and raffinose representing 6.0%, 5.6%, and 0.4%, respectively, of the total amount of soluble sugars across the three lines. Raffinose was not detected in pollen of the line 'Ferrum', and stachyose, maltose, and verbascose in any line. Significant higher ($P < 0.001$) contents of sucrose, glucose, and fructose were found in stored wheat pollen resulting in significant negative correlations between results of *in vitro* pollen germination and sucrose ($r = -0.77$, $P < 0.001$), glucose ($r = -0.72$, $P < 0.001$), and fructose ($r = -0.73$, $P < 0.001$) (**Figure 5D**). The shift in major soluble sugars during viability loss might be a useful indicator for *in vitro* pollen germination.

Variations in the Response of Different Wheat Varieties to *In Vitro* Germination and Fluorescein Diacetate Staining

In vitro pollen germination on medium varied between lines ($P < 0.001$) and annuity (**Figure 6A**). There was a significant ($P < 0.001$) difference between winter and spring types showing on the average 15.3 ± 8.5% and 30.2 ± 13.3% pollen germination, respectively. Pollen germination of the spring wheat landrace TRI 13752 and the winter wheat landrace 'Lang-Dörflers Braunweizen' was lowest with 7.1 ± 6.0% and 3.2 ± 0.7%, respectively. In contrast, pollen of the spring wheat landrace TRI 2443 and the winter wheat variety 'Ferrum' germinated at 50.1 ± 20.0 % and 32.8 ± 18.1%, respectively. Across the 19 tested lines, higher percentages of pollen were stained by FDA staining (61.7 ± 20.3%) (**Figure 6**) in comparison to the lower percentage of pollen which germinated on the medium (23.1 ± 13.9%). Again, pollen stainability varied between lines ($P < 0.001$) and pollen of the variety 'Triso' showed the lowest germination at 15.0 ± 4.5%. The highest value was obtained for TRI 8891 at 98.3 ± 0.8%. For both, FDA staining and *in vitro* pollen germination, pollen viability was neither associated with biotype (landrace or variety) nor with the sets grown at different time points indicating that variability of pollen viability is influenced by other factors.

In Vitro Germination and Fluorescein Diacetate Staining of Pollen of Different Barley, Rye, and Maize Varieties

Pollen viability varied between lines, species of the *Poaceae* family and viability assays (**Supplementary Figure S3**). *In vitro* pollen germination ranged between 3.3% and 42.4% for 25 rye lines, between 0.4% and 13.6% for 11 barley lines and between 0.2% and 4.5% for four maize lines. These results were lower in comparison with *in vitro* germination of different wheat lines. Highest variations (< 0.001) for pollen stainability assessed by FDA staining were found between 25 rye lines which ranged between 5.4% and 90.8%. Barley and maize lines showed high pollen stainability for all lines and ranged between 81.1% and 97.1%, and 92.7% and 96.3%, respectively. However, correlations between *in vitro* pollen germination and pollen stainability assessed by FDA staining were absent. Comparable to wheat, pollen tube development was not associated with the biotype (landrace or variety) and sets grown at different time points. In summary, *in vitro* pollen germination varied between lines of rye, barley, and maize but was relatively low compared to wheat assuming that the medium for *in vitro* pollen germination may not provide optimal condition required for pollen germination of rye, barley, and maize pollen.

DISCUSSION

Successful pollination and double fertilization of gametophytes are essential processes for seed production and improvements in plant breeding. Viability assessment of pollen represents an important tool to estimate the physical, biochemical, and biological status and capacity of pollen to generate tubes, penetrate into the stigma, and elongate inside the style until two male gametes are released within female gametophyte. Aside from FDA staining and seed set, *in vitro* germination is seen as a reliable approach to indicate pollen viability but suffers on the development of specific media for different species.

Sugar Metabolism and Signaling May Determine the Efficiency for Wheat Pollen Germination

Pollen tube growth requires carbohydrates for respiration and elements and energy for cell wall synthesis and transportactivities. In wheat pollen, we showed that the soluble carbohydrate sucrose is most abundant and may serve as an important primary energy source in addition to the internal starch reservoirs (Speranza et al., 1997). Supplementary, external carbohydrates such as sucrose and hexoses are required and actively uptaken from the apoplast of the transmitting tissue (Goetz et al., 2017). However, in anthers, high levels of sucrose and the lack/low levels of glucose and fructose can indicate or result in the loss of pollen viability. The deficiency to metabolize sucrose to hexoses is caused by impairments of vacuolar and cell-wall

bound invertases due to the downregulation of the two genes, one encoding the vacuolar (Ivr5) and the other a cell-wall (Ivr1) isoform (Koonjul et al., 2004; Ba et al., 2019); and were found for wheat exposed to drought stress (Koonjul et al., 2004) or in cytoplasmic male sterile wheat plants (Ba et al., 2019). Therefore, we assume that invertases play important roles for accumulation of soluble sugars and, hence, wheat pollen germination.

Raffinose can serve as an important energy source for *in vitro* pollen germination. In wheat pollen, highest germination and longest pollen tube growth was stimulated on a medium containing raffinose as exclusive sugar. Raffinose is a trisaccharide composed of fructose, glucose, and galactose; and is cleaved into melibiose and fructose (Goetz and Roitsch, 1999). In wheat pollen, we found only minor amounts confirming the general knowledge that it is largely absent in cereals (McIntyre et al., 2012; Van den Ende, 2013). However, wheat invertases such as β-fructofuranosidase can hydrolyze both, sucrose and raffinose (Goetz and Roitsch, 1999). Due to an amino acid modification, cell-wall bound plant invertases have even a higher substrate specificity and a higher relative cleavage rate for raffinose compared to sucrose (Goetz and Roitsch, 1999). The subsequent transport of the different cleavage products may appear actively and in parallel (Goetz et al., 2017). Fructose can be transported by POLYOL/ MONOSACCHARIDE TRANSPORTER 1 (AtPMT1), 2 (AtPMT2) (Klepek et al., 2009), 5 (AtPMT5) proteins (Klepek et al., 2005), and a pollen-specific H+ -monosaccharide symporter STP6 (Scholz-Starke et al., 2003) across the pollen membrane. These transports are driven by a proton gradient (Slewinski, 2011; Rottmann et al., 2016). Their optimum is around pH 5.0 (AtPMT1 and AtPMT2) (Klepek et al., 2009) which may explain the higher efficiency in wheat pollen germination at pH 5.8 in comparison to 7.3. Summarizing, we assume that raffinose might be partly cleaved by cell-wall bound invertases and products are actively transported into wheat pollen to support pollen germination. Alternatively, the uptake of raffinose, fructose, and other cleavage products may occur simultaneously. However, the involvement of enzymes in sugar cleavage and uptake need to be explored further in wheat pollen in order to identify specific requirements for pollen germination.

Raffinose may support *in vitro* pollen germination in wheat by specific signaling and as a protecting agent. The substitution of raffinose or the supplement of maltose, sucrose, or sorbitol reduced dramatically wheat pollen germination (**Figure 3**). Also the raffinose components, such as fructose or galactose, failed to stimulate wheat pollen germination (Cheng and McComb, 1992). In Arabidopsis pollen, the hexokinase (HXK) enzyme, an important sensor for sugar metabolism and sugar signaling, has a lower sensitivity for fructose. Changes in the fructose to

glucose ratio (Hirsche et al., 2017) or deficiencies in the glucose signaling contribute to normal pollen tube growth (Rottmann et al., 2018) indicating that changes in the sugar composition may have substantial effects on pollen germination and the signaling pathways. Furthermore, raffinose is known as stress protective agent. In leaves, higher levels of galactinol and raffinose correlated with increased tolerance against drought, salinity, and cold stress and efficient protection against hydroxyl radicals (Taji et al., 2002; Nishizawa et al., 2008). It further provides protection to liposomes and is involved in the stabilization of cellular membranes under stress (Hincha et al., 2003). Nevertheless, pollen germination in the presence of a stigma was superior compared to *in vitro* pollen germination showing that the complex network of metabolic activities, signaling, and protection mechanism need to be further elucidated to improve the capability of pollen to germinate and grow on *in vitro* media.

Pollen Germination Depends on Species, Line, and Environmental Conditions

The *in vitro* pollen germination is assumed to be partly genetically controlled. On the raffinose-based medium "Basic", wheat pollen germination varied between 3% and 50% in 19 different lines and was often higher in spring-types compared to winter types. Also in rye, barley, and maize, pollen germinated between 3.3% and 42.4% (25 lines), between 0.4% and 13.6% (11 lines), and between 0.2% and 4.5% (4 lines), respectively. The composition of pollen wall, the exine, is presumably responsible, among other factors, for variations in *in vitro* germination and underlies various changes during pollen tube development. Therefore, a unique composition including the production of extraplastidial lipids and the storage of triacylglycerols in lipid droplets have evolved (Ischebeck, 2016). In wheat, the composition of extraplastidic phospholipids is dominated by 34:3 and 36:6 lipid molecular species which include linolenic acid (18:3) (Narayanan et al., 2018). The ratio between linoleic (18:2) and linolenic acid may vary as shown for oilseed rape pollen (Evans et al., 1990). Hoekstra et al. (1992) hypothesized that different degrees of poly-unsaturation may affect pollen viability and longevity. A higher degree of polyunsaturated fatty acids facilitates a greater membrane fluidity accelerating transport processes of cell wall precursors over the plasma membrane and, hence, stimulating fast pollen tube growth. Although lipids of minor quantities may also play important roles for pollen viability status (Jiang et al., 2015), the high degree of polyunsaturated fatty acids in wheat pollen might be prone to oxidative processes such as phenolic oxidation known for maize pollen (Žilić et al., 2014). Here, flavonoids, especially quercetin diglycoside, were found to protect pollen against oxidative stress. Pollen of sweet maize had the highest content of total phenolics and flavonoids, hence, the highest antioxidant capacity. Other protective compounds are cold, heat shock, and Fe-deficiency proteins (Jagadish et al., 2009). Therefore, we assume

that lipid composition and variations in protective compounds may cause genotypic variations in pollen viability.

Environmental conditions and the time point of pollen shedding can cause strong variations in pollen germination. Here, the right anther stage needs to be identified to determine the optimum time point of pollen harvest and to test mature pollen. Thereby, anther dehiscence depends on the rupture of the septa, expansion of locule walls, pollen swelling prior to anthesis, and rupturing of the stomium (Liu et al., 2006) (Supplementary Video S1). In wheat, anthers located in the middle of a spike and at the outer florets of the spikelets tend to open and to shed mature pollen first. Afterwards, anthers located at the inner florets dehisce and the anthers of the basal and apical spikelets follow (Lukac et al., 2012). Pollen maturation and shedding can be triggered by moderate temperature increases. However, high temperature stress during anthesis affects pollen viability and reduces seed set (Jiang et al., 2015; Bheemanahalli et al., 2019). Pollen of maize is highly sensitive to desiccation; and pollen is non-viable in more than 300 m distance of the mother plants (Luna et al., 2001). The viability of cotton pollen can be reduced when flowers are directly exposed to sunlight (Burke et al., 2004). In contrast, the halflife can be extended under cloudy atmospheric conditions (Ge et al., 2011). Here, the carbohydrate concentration plays again an important role. Sucrose and starch increase in pollen due to the reduction of the metabolism under heat stress (Aloni et al., 2001). In the current study, constant greenhouse conditions were applied to plants used for pollen production. However, variations in pollen germination might be triggered by diverse environmental stresses occurring across the year. Therefore, pollen germination of the wheat line TRI 9102 may have ranged between 7.4 and 64.2%. In summary, optimal environmental conditions during plant production and mature pollen are required to gain high pollen quality and germination.

Comparison of Viability Tests for Wheat Pollen

FDA staining, IF cytometry, *in vitro*, and stigmatic germination were able to distinguish fresh from stored wheat pollen. By comparing pollen viability of three lines (**Figure 5A**), results correlated highly between *in vitro* pollen germination and IF cytometry ($r = 0.67$, $P < 0.001$) and FDA staining ($r = 0.54$, $P < 0.05$). Higher pollen germination was found on stigmas for one line. At the stigma, nutritional supply and structural elements are assumed to be species specific and promote pollen germination. Fritz and Lukaszewski (1989) indicated that *in vivo* or *semi in vivo* assays were most accurate tests to assess pollen viability for wheat and triticale. However, difficulties in the identification of germinated pollen and high risks of incompatibilities (Dafni and Firmage, 2000) are unfavorable for quick reliable tests. A fast viability analysis is the

measurement of dielectric properties of pollen by IF cytometry (Heidmann et al., 2016). In wheat, it can discriminate between fresh and stored pollen and has shown high correlations between results of IF cytometry and FDA staining for pollen of tomato, cucumber, and sweet pepper (Heidmann et al., 2016). Furthermore, also FDA staining has been proven to indicate pollen viability for cherry (La Porta and Roselli, 1991), potato (Trognitz, 1991), and acacia pollen (Sedgley and Harbard, 1993). Nevertheless, when the analysis of stored, non-viable pollen is absent such as shown for wheat (**Figure 6**), rye (**Supplementary Figure S3A**), barley (**Supplementary Figure S3B**), and maize (**Supplementary Figure S3C**), correlation coefficients between FDA staining and *in vitro* germination are rather low. We conclude that FDA staining and IF cytometry tend to overestimate the ability of pollen to germinate and produce long pollen tubes whereas the *in vitro* germination test requires specific modification for different species and perhaps lines. When optimal growth conditions for pollen are absent on medium, the *in vitro* pollen germination might give an underestimation of the ability of pollen to germinate. Stored, non-viable wheat pollen was stained by the Alexander assay; hence, the test was excluded as viability test for pollen. The Alexander staining has been developed to identify the presence or absence of cytoplasm indicating sterility or maturity of pollen grains (Alexander, 1969). Therefore, Alexander staining is efficient when species produce a high number of aborted pollen (Atlagić et al., 2012; Silva et al., 2018). The use as viability test is disputed because stored and non-viable pollen having intact cytoplasm are stained and misinterpreted as viable (Heslop-Harrison et al., 1984). Comparable to the results in wheat, immature or incompletely formed pollen were stained in Johnson grass (Sorghum halepense) (Burke et al., 2007) and correlations between results of *in vitro* germination and Alexander staining were absent in asparagus pollen (Marcellán and Camadro, 1996). Therefore, we suggest that publications should clearly distinguish between germinability, stainability, and sterility/maturity if the Alexander staining is used.

CONCLUSIONS

Wheat is known for short-lived, recalcitrant pollen which cannot be stored so far. To work toward an efficient storage approach, in the present study, we assessed the viability of fresh and stored wheat pollen using 157 different solid and liquid media and identified a solid medium based on raffinose as most appropriate. Although raffinose is not the main soluble sugar in pollen, wheat invertases have a higher affinity to cleave raffinose compared to sucrose which is highly abundant in antheres. Therefore, raffinose might be transported in parallel with its cleavage product through the exine. When raffinose is accumulated, it may function as a

protective agent and stabilizes pollen membranes in addition. However, higher germination was achieved by stigmatic germination indicating complex structural and/or biochemical signals from the stigma that trigger higher percentages of pollen tube growth. *In vitro* germination of pollen was lower compared to pollen viability assessed by FDA and IF cytometry and varied between lines, species, and growth conditions. However, it is still elusive to which extent pollen classified as viable is able to germinate and grow. Therefore, it is recommendable to choose a combined approach of *in vitro* germination with FDA or IF cytometry in order to analyze the potential of pollen germination and viability correctly.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

FUNDING

This study was supported and publication fees provided by the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK).

ACKNOWLEDGMENTS

We like to acknowledge Stefanie Thumm, Doris Büchner, Jürgen Marlow for excellent technical support, Armin Meister for statistical advice, Traud Winkelmann, Hans-Peter Mock, Natalia Tikhenko, Myroslava Rubtsova for advice and critical comments on the manuscript. The work is part of a PhD project and was supported by Leibniz Institute of Plant Genetics and Crop Plant Research (IPK).

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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2.2. Tables and Figures

Table 1 Different modifications and osmolality of the solid media "Basic". ε-aminocaproic acid (EACA), $CaCl_2 \cdot 2H_2O$ ($CaCl_2$), peptone water (Pep), mannitol, sorbitol, gibberellic acid (GA_3) or indole-3-acetic acid (IAA) were added to the 'Basic' medium or raffinose was partly substituted by sucrose or maltose. Sugar concentrations of 292 mM, 396 mM and 594 mM correspond to 10%, 20% and 30%, respectively.

Media	**H_3BO_3**	**$CaCl_2 \cdot 2H_2O$**	**Sucrose**	**Maltose**	**Raffinose**	**EACA**	**Pep**	**Mannitol**	**Sorbitol**	**IAA**	**GA_3**	**Osmolality**
	mM	mM	mM	mM	mM	mg L^{-1}	mg L^{-1}	mM	mM	mM	mM	mmol kg^{-1}
Basic	0.81	2.04			594							700
EACA + Pep	0.81	2.04			594	500	100					865
EACA	0.81	2.04			594	500						864
Pep	0.81	2.04			594		100					767
high $CaCl_2$	0.81	3.04			594	500	100					887
high H_3BO_3	1.62	2.04			594	500	100					818
Sucrose	0.81	2.04	292		396	500	100					649
Maltose	0.81	2.04		292	396	500	100					644
Mannitol	0.81	2.04			594	500	100	300				970
Sorbitol	0.81	2.04			594	500	100		300			2465
IAA	0.81	2.04			594	500	100			0.01		934
GA_3	0.81	2.04			594	500	100				0.01	862

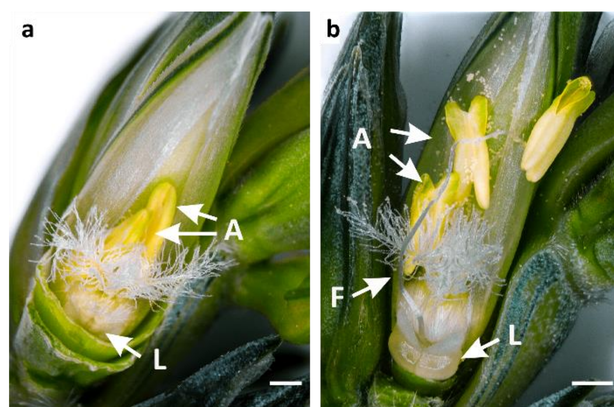


Figure 1 Structural changes in wheat florets before and after pollen shedding. (a) Before pollen shedding, filaments were short, anthers (**A**) closed, greenish yellow and showed fleshy anther lobes. To support the pollen extraction and maturation, awns, glumes and lemmas were removed. (b) Ten minutes after removing the glumes, lodicules (**L**) swelled, filaments (**F**) elongated, anther tips opened and mature pollen shed on the stigma (**S**). At this stage, the maximum pollen maturity was reached and pollen was used for all experiments. Scale bars = 1 mm.

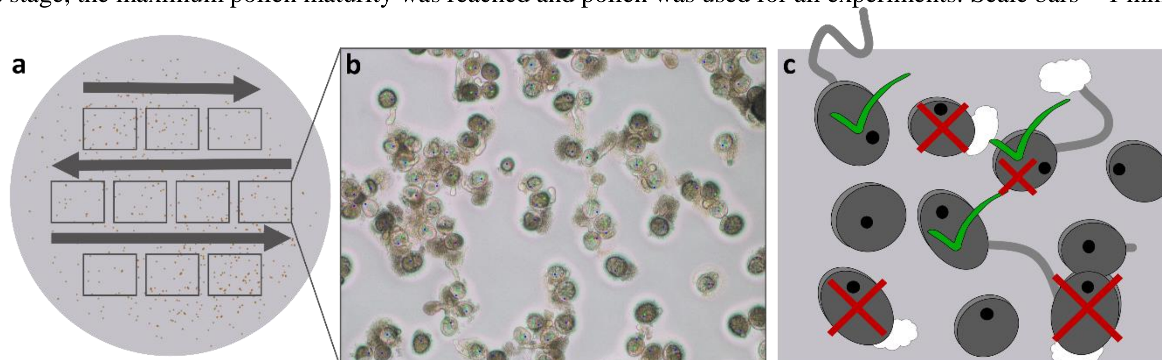


Figure 2 Evaluation of pollen germination on solid media. (a) Pollen was distributed on solid medium and 10 independent pictures (rectangles) were taken for further evaluation. (b) One of ten pictures taken for pollen germination analysis is shown. (c) Each picture was evaluated and the total number of pollen (black dots), germinated pollen (green ticks) and burst pollen (red crosses) counted.

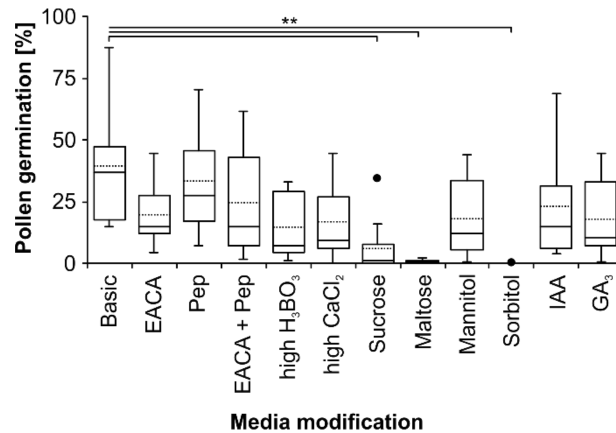


Figure 3 Wheat pollen germination depends on media composition. The "Basic" pollen medium was modified according to Table 1 and pollen of wheat lines 'Ferrum', 'Einstein', 'Dialog' and TRI 9102 (n = 3 for each line, >805 pollen for each replicate) were analyzed for pollen tube development (**Figure 2**). Box plots represent means (dashed line), medians (solid line), lower and upper quartiles; whiskers show the 98th percentile; and dots show outliers. Significant differences between the means of the media are indicated by the least significant differences at $P < 0.05$ (LSD5%). $CaCl_2$, calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$); EACA, ε-aminocaproic acid; GA_3, gibberellic acid; IAA, indole-3-acetic acid; Pep, peptone water; H_3BO_3, boric acid.

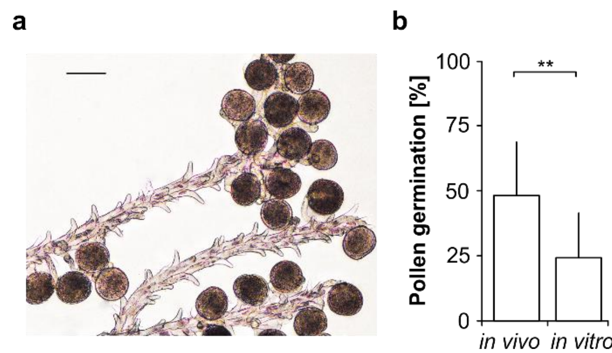


Figure 4 Improved wheat pollen tube growth in the presence of the stigma. (a) Pollen tubes grew towards stigma. (b) Comparison of stigmatic pollen germination and *in vitro* germination on medium using fresh pollen and pollen stored at ambient conditions for > 60 min. Mean and standard deviation of n = 14 replicates using >70 pollen each are shown for the line TRI 9102. The least significant differences at $P < 0.05$ (LSD5%) was estimated at 10.0%. Scale bar = 50 µm.

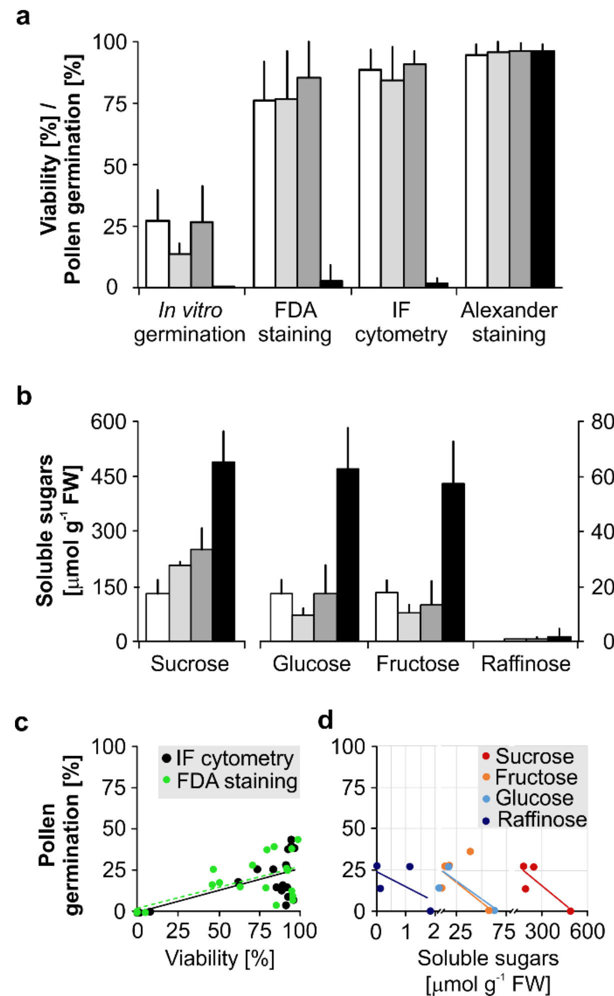


Figure 5 Assessment of wheat pollen viability using physiological, biochemical and physical tests. (a) Pollen viability was evaluated for fresh and stored pollen using *in vitro* germination, fluorescein diacetate (FDA) staining, impedance flow (IF) cytometry and Alexander staining (**Supplementary Figure S2**). Means and standard deviations of n = 5 replicates of fresh pollen are shown for the lines 'Ferrum' (white bar), 'Hermann' (light grey bar) and TRI 9102 (dark grey bar). The mean and standard deviation of the three lines is shown for stored pollen (black bars) kept under ambient condition for > 60 min. (b) Soluble sugars were measured in pollen collected immediately after pollen shedding and in pollen stored under ambient conditions for > 60 min. Mean and standard deviations of n = 5 replicates are shown for the lines mentioned above and for stored pollen (black bar). (c) Relationship between *in vitro* pollen germination and viability assessed by IF cytometry (r = 0.67, P < 0.001) and FDA staining (r = 0.54, P < 0.05) and (d) concentration of raffinose (r = -0.29, P = 0.175), glucose (r = -0.72, P < 0.001), fructose (r = -0.73, P < 0.001) and sucrose (r = -0.77, P < 0.001). Significant differences between the means of the viability assays, soluble sugars and lines are indicated by the least significant differences at P < 0.05 (LSD5%).

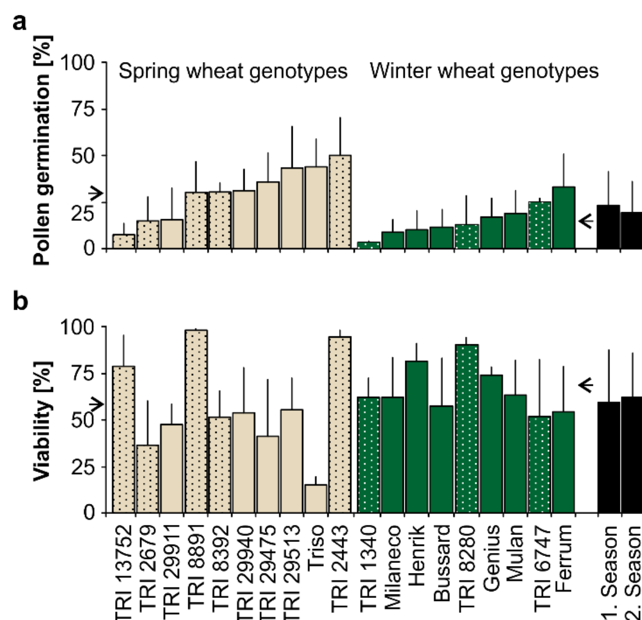


Figure 6 *In vitro* pollen germination and viability assessed by FDA staining varied among 19 different wheat lines. (a) *In vitro* pollen germination was analyzed for 10 spring and 9 winter wheat lines, among them 7 landraces (spotted bars) and 12 varieties (plain bars). Bars represent means and standard deviations of n = 3 (*) to 6 replicates of 100 to 300 pollen each. (b) Pollen stainability was analyzed by FDA staining. Bars represent means and standard deviations of n = 3 (*) to 6 replicates of 100 to 400 pollen each. Arrows indicate mean pollen germination/stainability for spring (left) and winter (right) wheat varieties, respectively. Significant differences between means of lines and spring and winter wheat (annuity) are indicated by the least significant differences at P < 0.05 (LSD5%). Seeds of all lines were grown in two intervals, in August (1st set) and September (2nd set) and mature pollen was available between November and January (1st set) and December and March (2nd set).

2.3.Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01588/full#supplementary-material

Supplementary Tables

Supplementary Table S1 Details of the tested genotypes. Wheat, rye and barley accessions were obtained from the Federal *Ex situ* Gene Bank for Agricultural and Horticultural Plants in Gatersleben, Germany (https://doi.org/10.5447/ipk/2019/9). Maize genotypes were obtained from the Maize Genetics Stock Center, USA (http://maizecoop.cropsci.uiuc.edu).

Accession name	Accession number	Annuity	Biotype	Botanical name	Country of donation/approval	Donor/ breeding company	Year of access/ approval
Ferrum		winter	variety	*Triticum aestivum* L.	Germany	KWS Lochow GmbH	2012
Hermann		winter	variety	*Triticum aestivum* L.	Germany	LIMAGRAIN GmbH	2004
Piko		winter	variety	*Triticum aestivum* L.	Germany	NORDSAAT Saatzucht GmbH	1994
Odyssee		winter	variety	*Triticum aestivum* L.	United Kingdom	Syngenta International AG	2011
Dialog		winter	variety	*Triticum aestivum* L.	France	KWS UK Ltd.	2008
Einstein		winter	variety	*Triticum aestivum* L.	United Kingdom	LIMAGRAIN GmbH	2001
Henrik		winter	variety	*Triticum aestivum* L.	Belgium	LIMAGRAIN GmbH	2009
Genius		winter	variety	*Triticum aestivum* L.	Germany	NORDSAAT Saatzucht GmbH	2010
Mulan		winter	variety	*Triticum aestivum* L.	Germany	NORDSAAT Saatzucht GmbH	2006
Bussard		winter	variety	*Triticum aestivum* L. var. *lutescens* (Alef.) Mansf.	Germany	KWS Lochow GmbH	1990
Milaneco		winter	variety	*Triticum aestivum* L.	Germany	KWS Lochow GmbH	2013
Lang-Dörflers Braunweizen Walthari	TRI 1340	winter	landrace	*Triticum aestivum* L. var. *milturum* (Alef.) Mansf.	Germany	Lang-Doerfler Niedertraubling/Ndb.	1947
Bezostaja 1	TRI 6747	winter	landrace	*Triticum aestivum* L. var. lutescens (Alef.) Mansf.	Soviet Union	N.I. Vavilov All-Russian Scientific Research Institute of Plant Genetic Resources	1963
Rode Ris	TRI 8280	winter	landrace	*Triticum aestivum* L. var. *lutescens* (Alef.) Mansf.	Netherland	Institute of Horticultural Plant Breeding Wageningen	1967
Triso		spring	variety	*Triticum aestivum* L.	Germany	Deutsche Saatveredelung AG	1996
Melissos	TRI 29940	spring	variety	*Triticum aestivum* L.	Germany	Fa. Strube Saatzucht KG	2003
Melon	TRI 29513	spring	variety	*Triticum aestivum* L. var. *lutescens* (Alef.) Mansf.	Germany	Fa. Strube Saatzucht KG	1997
Kalistos	TRI 29475	spring	variety	*Triticum aestivum* L. var. *lutescens* (Alef.) Mansf.	Germany	Fa. Strube Saatzucht KG	1999
Combi	TRI 29911	spring	variety	*Triticum aestivum* L.	Germany	Saatzucht Engelen Büchling e.K.	1990
	TRI 4399	spring	landrace	*Triticum aestivum* L. var. *icterinum* (Alef.) Mansf.	Europe	Agricultural Botanical Garden Szentes, Hungary	1956

	TRI 9102	spring	landrace	*Triticum aestivum* L. var. *ferrugineum* (Alef.) Mansf.	Austria	Mayr, alpine landrace collection	1922
	TRI 2679	spring	landrace	*Triticum aestivum* L. var. *aestivum*	India	Herrlich, Northwest-India-Nepal	1937
	TRI 2443	spring	landrace	*Triticum aestivum* L. var. *aestivum*	Nepal	Herrlich, Northwest-India-Nepal	1937
	TRI 8392	spring	landrace	*Triticum aestivum* L. var. *aestivum*	Mongolia	Mongolia collection mission	1964
	TRI 8891	spring	landrace	*Triticum aestivum* L. var. *aestivum*	Austria	Mayr, alpine landrace collection	1922
	TRI 13752	spring	landrace	*Triticum aestivum* L. var. *aestivum*	Lybia	Lybia collection mission	1982
	R 864	spring	landrace	*Secale cereale* L. subsp. *cereale*	Georgia	Georgia collection mission	1982
	R 687	spring	landrace	*Secale cereale* L. subsp. *cereale*	Poland	Poland collection mission	1976
Tiroler Sommerroggen	R 979	spring	landrace	*Secale cereale* L. subsp. *cereale*	Austria	Austria collection mission	1986
	R 594	spring	landrace	*Secale cereale* L. subsp. *cereale*	Czech Republik	Kühn, Brno	1975
	R 2034	spring	landrace	*Secale cereale* L. subsp. *cereale*	Russia	N.I. Vavilov All-Russian Scientific Research Institute of Plant Genetic Resources	2015
SOMRO	R 2480	spring	variety	*Secale cereale* L. subsp. *cereale*	Germany	Lochow-Petkus GmbH	1977
Polko	R 257	spring	variety	*Secale cereale* L. subsp. *cereale*	South Africa	Institute of Tropical and Subtropical Agriculture, Leipzig	1966
Beka	R 1470	spring	variety	*Secale cereale* L. subsp. *cereale*	Poland	IPK Genebank Gülzow	1967
Petkuser Sommerroggen	R 2319	spring	variety	*Secale cereale* L. subsp. *cereale*	Germany	IHAR Radzikow	1997
Sorom	R 2263	spring	variety	*Secale cereale* L. subsp. *cereale*	Germany	IPK Genbank Gülzow	1997

	R 1185	winter	landrace	*Secale cereale* L. subsp. *cereale*	Albania	Albania collection mission	1994
Anatolien	R 1874	winter	landrace	*Secale cereale* L. subsp. *cereale*	Turkey	IPK Genebank Gülzow	1997
	R 674	winter	landrace	*Secale cereale* L. subsp. *cereale*	Poland	Poland collection mission	1976
	R 708	winter	landrace	*Secale cereale* L. subsp. *cereale*	Slowakia	CSSR collection mission	1977
	R 973	winter	landrace	*Secale cereale* L. subsp. *cereale*	Georgia	Georgia collection mission	1986
Plato	R 2938	winter	variety	*Secale cereale* L. subsp. *cereale*	Germany	Hybro Saatzucht GmbH	2001
Warko	R 2937	winter	variety	*Secale cereale* L. subsp. *cereale*	Poland	DNKO Hodowla Roslin Sp. zo.o.	2000
Hacada	R 2936	winter	variety	*Secale cereale* L. subsp. *cereale*	Germany	KWS Lochow GmbH	1993
Motto	R 2935	winter	variety	*Secale cereale* L. subsp. *cereale*	Poland	DNKO Hodowla Roslin Sp. zo.o.	1991
Halo	R 2934	winter	variety	*Secale cereale* L. subsp. *cereale*	Germany	KWS Lochow GmbH	1977
Boruus		winter	variety	*Secale cereale* L. subsp. *cereale*			
Turbo		winter	variety	*Secale cereale* L. subsp. *cereale*	Poland	IPK Genebank Gülzow	2000
Borfuro		winter	variety	*Secale cereale* L. subsp. *cereale*	Germany	Saatzucht Steinach GmbH	1996
Visello		winter	variety	*Secale cereale* L. subsp. *cereale*	Germany	KWS Lochow GmbH	2006
Boresto		winter	variety	*Secale cereale* L. subsp. *cereale*	Germany	Saatzucht Steinach GmbH	2000
	HOR 4710	spring	landrace	*Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef.	Germany	VEG Saatzucht Boldebuck	1972

	HOR 4706	spring	landrace	*Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef.	Germany	VEG Saatzucht Boldebuck	1972
	HOR 2347	spring	landrace	*Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *erectum* (Rode) Alef	Germany	Schiemann Germany	1990
	HOR 2901	spring	landrace	*Hordeum vulgare* L. convar. *vulgare* var. *hybernum* Viborg	El Salvador	Schwatzi, Central America collection mission	1958
Belana		spring	variety	*Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef.	Germany	NORDSAAT Saatzucht GmbH	2003
Orthega		spring	variety	*Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef.	Germany	KWS Lochow GmbH	1996
	HOR 6974	spring	variety	*Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef.	Germany	VEG Saatzucht Derenburg	1974
Minerva	HOR 2492	spring	variety	*Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef.	The Netherlands	University of Agriculture, Wageningen	1990
Saale	HOR 2441	spring	variety	*Hordeum vulgare* L. convar. *distichon* (L.) Alef. var. *nutans* (Rode) Alef.	Germany	Institute for Plant Breeding, Halle	1990
Berendstets	HOR 3021	spring	variety	*Hordeum vulgare* L. convar. *vulgare* var. *hybernum* Viborg	Germany	Biologische Bundesanstalt Braunschweig	2000
Hiland	HOR 2754	spring	variety	*Hordeum vulgare* L. convar. *vulgare* var. *rikotense* Regel	USA	Barley collection, Beltsville	2000
PH 207		spring	Inbred line	*Zea mays* L.	USA		
A188		spring	Inbred line	*Zea mays* L.	USA		
B73		spring	Inbred line	*Zea mays* L.	USA		
A183		spring	Inbred line	*Zea mays* L.	USA		

Supplementary Table S2 Pollen tube growth, length and frequency of pollen bursting on liquid growth media. Liquid media are based on protocols following Cheng and McComb (1992) (Cheng), Jayaprakash, et al. (2015) (Jaya), Jian, et al. (2014) (Jian) and were modified in micro- and macronutrients and pH. Frequency of pollen tubes was estimated and assigned as low, medium and high when one, two to ten or more than ten pollen, respectively, developed pollen tubes. Pollen tube length was characterized based on the pollen diameter and characterized as short (smaller than the pollen diameter), medium (between the one- and twofold diameter) and long (more than the twofold diameter) (**Supplementary Figure S1**). Frequency of pollen bursting refers to a single pollen (low), half of the pollen batch (medium) or the complete pollen batch which bursted. PEG, polyethylene glycol.

Protocols	Sucrose [mM]	Maltose [mM]	Raffinose [mM]	pH	H_3BO_3 [mM]	$CaCl_2 \cdot 2H_2O$ [mM]	$Ca(NO_3)_2 \cdot 2H_2O$ [mM]	MgS [mM]	KNO_3 [mM]	PEG 4000 [g L^{-1}]	Frequency of pollen tubes	Pollen tube length	Frequency of pollen bursting
Cheng	300	-	-	5.8	1.62	2.04	-	-	-	-	-	-	-
Cheng	300	-	-	7.3	1.62	2.04	-	-	-	-	-	-	-
Cheng	600	-	-	5.8	1.62	2.04	-	-	-	-	-	-	-
Cheng	600	-	-	7.3	1.62	2.04	-	-	-	-	-	-	-
Cheng	750	-	-	5.8	1.62	2.04	-	-	-	-	-	-	-
Cheng	750	-	-	7.3	1.62	2.04	-	-	-	-	-	-	-
Cheng	900	-	-	5.8	1.62	2.04	-	-	-	-	-	-	-
Cheng	900	-	-	7.3	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	300	-	5.8	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	300	-	7.3	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	600	-	5.8	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	600	-	7.3	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	750	-	5.8	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	750	-	7.3	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	900	-	5.8	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	900	-	7.3	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	-	300	5.8	1.62	2.04	-	-	-	-	low	-	low
Cheng	-	-	300	7.3	1.62	2.04	-	-	-	-	-	-	-
Cheng	-	-	600	5.8	1.62	2.04	-	-	-	-	low	medium	medium

Cheng	-	-	600	7.3	1.62	2.04	-	-	-	-	high	long	low
Cheng	-	-	750	5.8	1.62	2.04	-	-	-	-	low	short	low
Cheng	-	-	750	7.3	1.62	2.04	-	-	-	-	medium	medium	medium
Cheng	300	-	-	5.8	0.81	2.04	-	-	-	-	-	-	-
Cheng	300	-	-	7.3	0.81	2.04	-	-	-	-	-	-	-
Cheng	600	-	-	5.8	0.81	2.04	-	-	-	-	-	-	-
Cheng	600	-	-	7.3	0.81	2.04	-	-	-	-	-	-	-
Cheng	750	-	-	5.8	0.81	2.04	-	-	-	-	low	-	low
Cheng	750	-	-	7.3	0.81	2.04	-	-	-	-	-	-	-
Cheng	900	-	-	5.8	0.81	2.04	-	-	-	-	-	-	-
Cheng	900	-	-	7.3	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	300	-	5.8	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	300	-	7.3	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	600	-	5.8	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	600	-	7.3	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	750	-	5.8	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	750	-	7.3	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	900	-	5.8	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	900	-	7.3	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	-	300	5.8	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	-	300	7.3	0.81	2.04	-	-	-	-	-	-	-
Cheng	-	-	600	5.8	0.81	2.04	-	-	-	-	low	-	low
Cheng	-	-	600	7.3	0.81	2.04	-	-	-	-	high	short	low
Cheng	-	-	750	5.8	0.81	2.04	-	-	-	-	low	-	medium
Cheng	-	-	750	7.3	0.81	2.04	-	-	-	-	high	medium	low
Cheng	300	-	-	5.8	1.62	1.02	-	-	-	-	-	-	-

Cheng	300	-	-	7.3	1.62	1.02	-	-	-	-	-	-	-
Cheng	600	-	-	5.8	1.62	1.02	-	-	-	-	-	-	-
Cheng	600	-	-	7.3	1.62	1.02	-	-	-	-	-	-	-
Cheng	750	-	-	5.8	1.62	1.02	-	-	-	-	-	-	-
Cheng	750	-	-	7.3	1.62	1.02	-	-	-	-	-	-	-
Cheng	900	-	-	5.8	1.62	1.02	-	-	-	-	-	-	-
Cheng	900	-	-	7.3	1.62	1.02	-	-	-	-	-	-	-
Cheng	-	300	-	5.8	1.62	1.02	-	-	-	-	-	-	-
Cheng	-	300	-	7.3	1.62	1.02	-	-	-	-	-	-	-
Cheng	-	600	-	5.8	1.62	1.02	-	-	-	-	-	-	-
Cheng	-	600	-	7.3	1.62	1.02	-	-	-	-	-	-	-
Cheng	-	750	-	5.8	1.62	1.02	-	-	-	-	-	-	-
Cheng	-	750	-	7.3	1.62	1.02	-	-	-	-	-	-	-
Cheng	-	900	-	5.8	1.62	1.02	-	-	-	-	-	-	-
Cheng	-	900	-	7.3	1.62	1.02	-	-	-	-	-	-	-
Cheng	-	-	300	5.8	1.62	1.02	-	-	-	-	low	-	low
Cheng	-	-	300	7.3	1.62	1.02	-	-	-	-	low	short	medium
Cheng	-	-	600	5.8	1.62	1.02	-	-	-	-	high	short	medium
Cheng	-	-	600	7.3	1.62	1.02	-	-	-	-	high	medium	low
Cheng	-	-	750	5.8	1.62	1.02	-	-	-	-	high	short	low
Cheng	-	-	750	7.3	1.62	1.02	-	-	-	-	high	short	low
Jaya	300	-	-	5.8	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	300	-	-	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	600	-	-	5.8	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	600	-	-	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	750	-	-	5.8	0.81	-	0.13	0.81	0.99	130	-	-	-

Jaya	750	-	-	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	900	-	-	5.8	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	900	-	-	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	300	-	5.8	0.81	-	0.13	0.81	0.99	130	low	long	medium
Jaya	-	300	-	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	600	-	5.8	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	600	-	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	750	-	5.8	0.81	-	0.13	0.81	0.99	130	low	-	low
Jaya	-	750	-	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	900	-	5.8	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	900	-	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	-	300	5.8	0.81	-	0.13	0.81	0.99	130	low	-	low
Jaya	-	-	300	7.3	0.81	-	0.13	0.81	0.99	130	medium	short	low
Jaya	-	-	600	5.8	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	-	600	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	-	750	5.8	0.81	-	0.13	0.81	0.99	130	-	-	-
Jaya	-	-	750	7.3	0.81	-	0.13	0.81	0.99	130	-	-	-
Jian	300	-	-	5.8	0.65	-	2.96	0.81	0.99	100	low	-	low
Jian	300	-	-	7.3	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	600	-	-	5.8	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	600	-	-	7.3	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	750	-	-	5.8	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	750	-	-	7.3	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	900	-	-	5.8	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	900	-	-	7.3	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	300	-	5.8	0.65	-	2.96	0.81	0.99	100	low	-	medium

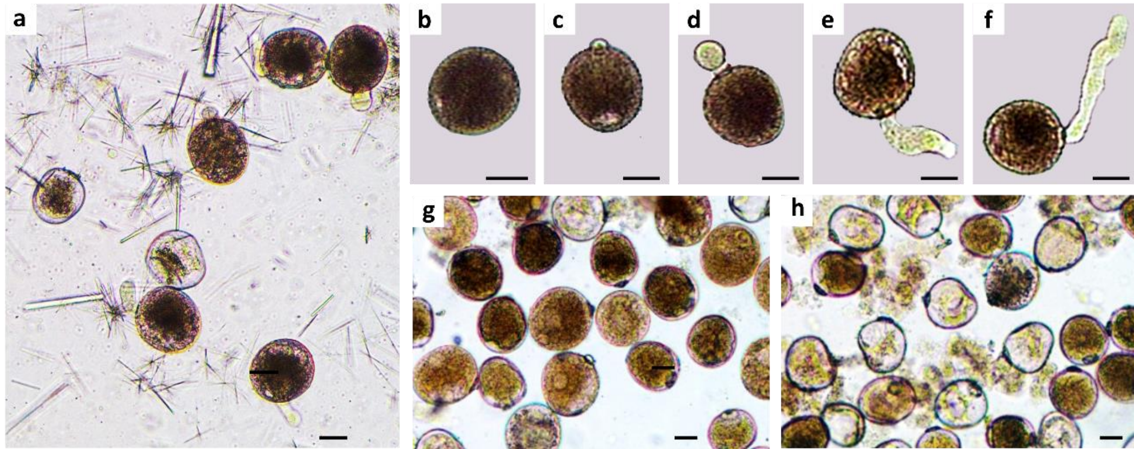
Jian	-	300	-	7.3	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	600	-	5.8	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	600	-	7.3	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	750	-	5.8	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	750	-	7.3	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	900	-	5.8	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	900	-	7.3	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	-	300	5.8	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	-	300	7.3	0.65	-	2.96	0.81	0.99	100	high	short	low
Jian	-	-	600	5.8	0.65	-	2.96	0.81	0.99	100	low	-	low
Jian	-	-	600	7.3	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	-	750	5.8	0.65	-	2.96	0.81	0.99	100	-	-	-
Jian	-	-	750	7.3	0.65	-	2.96	0.81	0.99	100	medium	short	low

Supplementary Table S3 Pollen germination and frequency of pollen bursting on solid media. Liquid media stimulating pollen tube growth were solidified and modified in micro- and macronutrients and pH. Mean and standard deviations of germinated and bursted pollen counted of the genotypes 'Ferrum', 'Piko', 'Hermann'are given in percentage. Cheng, Cheng and McOmb (1992); Jaya, Jayaprakash, et al. (2015); PEG, polyethylene glycol; EACA, ε-aminocaproic acid

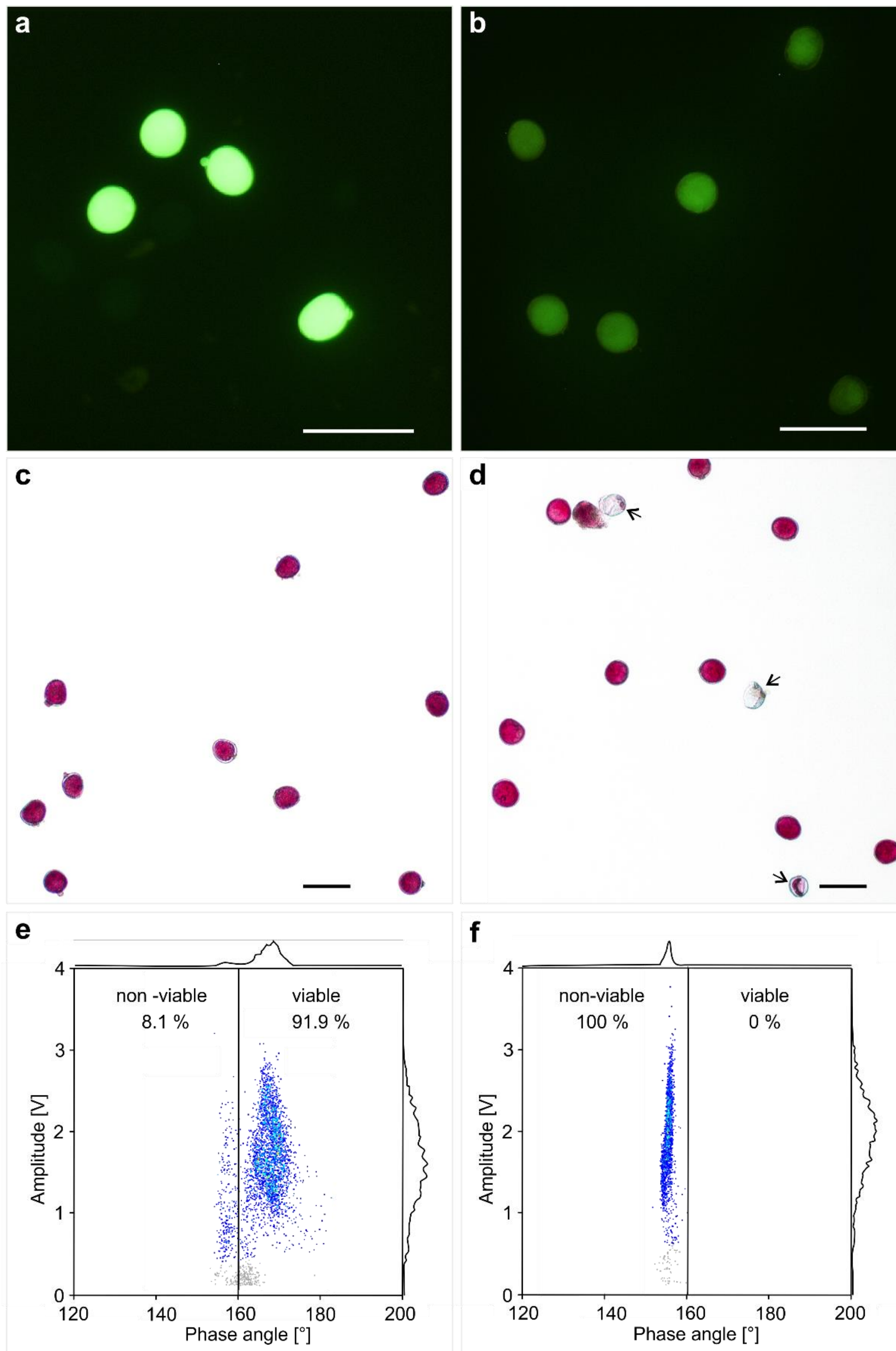
Protocols	Sucrose [mM]	Maltose [mM]	Raffinose [mM]	pH	H_3BO_3 [mM]	$CaCl_2 \cdot 2H_2O$ [mM]	$Ca(NO_3)_2 \cdot 2H_2O$ [mM]	EACA [mM]	Peptone water [mg L^{-1}]	PEG 4000 [g L^{-1}]	PEG 8000 [g L^{-1}]	Pollen germination [%]	Pollen bursting [%]
Jaya	-	584	-	5.8	0.81	-	**0.13**	**3.81**	**100**	**130**	-	-	-
Jaya	-	584	-	5.8	0.81	-	**0.13**	**3.81**	**-**	**130**	-	-	-
Jaya	-	584	-	5.8	0.81	-	**0.13**	**-**	**100**	**130**	-	-	-
Jaya	-	584	-	5.8	0.81	-	**0.13**	**3.81**	**100**	**-**	-	-	-
Jaya	-	584	-	5.8	0.81	**1.02**	**-**	**3.81**	**100**	**130**	-	-	-
Jaya	-	584	-	5.8	**1.62**	-	0.13	3.81	100	130	-	-	-
Jaya	-	584	-	5.8	**2.43**	-	0.13	3.81	100	130	-	-	-
Jaya	-	584	-	5.8	**4.85**	-	0.13	3.81	100	130	-	-	-

Jaya	-	584	-	5.8	0.81	-	**0.42**	3.81	100	130	-	-	-
Jaya	-	584	-	5.8	0.81	-	**0.64**	3.81	100	130	-	-	-
Jaya	-	584	-	5.8	0.81	-	**2.12**	3.81	100	130	-	-	-
Jaya	-	584	-	5.8	0.81	-	**4.23**	3.81	100	130	-	-	-
Jaya	-	584	-	5.8	0.81	-	0.13	3.81	100	**50**	-	-	-
Jaya	-	584	-	5.8	0.81	-	0.13	3.81	100	**100**	-	-	-
Jaya	-	584	-	5.8	0.81	-	0.13	3.81	100	-	**50**	-	-
Jaya	-	584	-	5.8	0.81	-	0.13	3.81	100	-	**100**	-	-
Jaya	-	**876**	-	5.8	0.81	-	0.13	3.81	100	130	-	-	-
Jaya	-	**1460**	-	5.8	0.81	-	0.13	3.81	100	130	-	-	-
Jaya	**584**	-	-	5.8	0.81	-	0.13	3.81	100	130	-	-	-
Jaya	**876**	-	-	5.8	0.81	-	0.13	3.81	100	130	-	-	-
Jaya	**1460**	-	-	5.8	0.81	-	0.13	3.81	100	130	-	-	-
Jaya	-	-	**198**	5.8	0.81	-	0.13	3.81	100	130	-	-	-
Jaya	-	-	**396**	5.8	0.81	-	0.13	3.81	100	130	-	-	-
Jaya	-	-	**505**	5.8	0.81	-	0.13	3.81	100	130	-	-	-
Cheng	-	-	252	**5.8**	**0.81**	**2.04**	-	-	-	-	-	-	-
Cheng	-	-	252	**7.3**	**0.81**	**2.04**	-	-	-	-	-	-	-
Cheng	-	-	252	**5.8**	**1.62**	**1.02**	-	-	-	-	-	-	-
Cheng	-	-	252	**7.3**	**1.62**	**1.02**	-	-	-	-	-	-	-
Cheng	**731**	**-**	**-**	5.8	**0.81**	**2.04**	-	3.81	100	-	-	-	-
Cheng	**-**	**833**	**-**	5.8	**0.81**	**2.04**	-	3.81	100	-	-	-	-
Cheng	**-**	**-**	**594**	5.8	**0.81**	**2.04**	-	3.81	100	-	-	32.7±14.2	61.7±18.3
Cheng	**731**	**-**	**-**	5.8	**1.62**	**1.02**	-	3.81	100	-	-	-	-
Cheng	**-**	**833**	**-**	5.8	**1.62**	**1.02**	-	3.81	100	-	-	-	-
Cheng	**-**	**-**	**594**	5.8	**1.62**	**1.02**	-	3.81	100	-	-	13.3±10.4	65.8±19.8

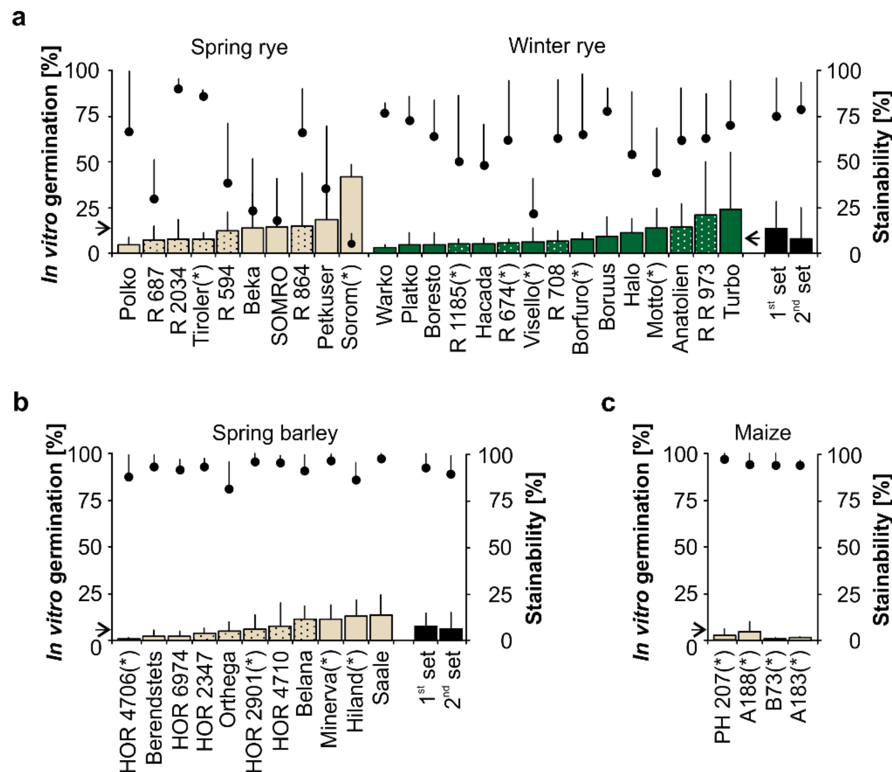
Supplementary Figures



Supplementary Figure S1 Pollen tube development and assessment in liquid media. (a) Raffinose, the main sugar in liquid media, crystallizes rapidly at room temperature. Therefore, exact evaluation was hampered and, pollen was categorized into (b) and (c) absent pollen tubes; (d) short pollen tubes (smaller than the pollen diameter); (e) medium pollen tubes (between the one- and twofold diameter); and (f) long pollen tubes (more than the twofold diameter) (Supplementary Table S2). (g) and (h) Frequency of pollen bursting was dependent on the genotype and ranged between low (g) and high (h). Scale bar = 30 µm.



Supplementary Figure S2. Results of different pollen viability assays. (a) Fluorescein diacetate (FDA) show different results for fresh and (b) stored pollen assessed at 95.0% and 0.1% pollen viability. Fresh pollen is shown in a bright green whereas stored pollen has a uniform background fluorescence. (c) Red pollen stained by Alexander solution indicate pollen viability whereas (d) the absence of staining show sterile pollen (arrows). The Alexander stain cannot distinguish between fresh and stored pollen. Therefore, pollen of 0% *in vitro* germination are also colored in red. (e) Histograms and dot plots are shown after impedance flow cytometry using the Ampha Z32 (Amphasys, Lucerne, Switzerland) at 1 MHz. Number of pollen were counted and viability of fresh pollen samples was estimated at 91.7%. (f) Stored pollen samples kept for several days at ambient conditions is used to estimate the threshold (vertical line) between fresh (right) and stored (left) pollen. Arrows indicate the site at which most dots were found. Scale bar = 100 µm.



Supplementary Figure S3. *In vitro* pollen germination and viability varied between species of the *Poaceae* family and their lines. (a) *In vitro* pollen germination (bars) was assessed on 'Basic' medium and compared to pollen stainability (points) assessed by FDA staining. Bars and points represent means and standard deviations of *in vitro* pollen germination of n = 3 (*) to 6 replicates of 100 to 300 pollen each and means and standard deviations of pollen stainability of n = 3 (*) to 6 replicates of 100 to 400 pollen, respectively. Arrows show the means of *in vitro* pollen germination for spring (left) and winter (right) lines. *In vitro* pollen germination (bars) and FDA staining was analyzed for a) 10 spring and 15 winter rye lines, among them 10 landraces (spotted bars) and 15 varieties (plain bars). Least significant difference at P < 0.05 (LSD5%) between lines was at 19.0% for *in vitro* germination and at 32.3% for pollen viability. Spring and winter rye differed significantly for pollen viability after FDA staining (LSD5% = 10.7%). (b) 11 spring barley lines, among them 6 landraces (spotted bars) and 5 varieties (plain bars) distinguish significantly for *in vitro* germination at LSD5% = 9.5% and for pollen stainability at LSD5% = 10.5%. (c) *In vitro* germination and pollen stainability were not significantly different among the four maize lines.

Supplementary Videos

Supplementary Video S1 Timelapse of anthesis shown within a wheat floret. Awns, glumes and lemmas were removed to observe swelling of the lodicules, elongation of the filaments, rupture of the anther tips and shedding of mature pollen on the stigma in a time interval of 8 minutes (161 images). At this stage, the maximum pollen maturity is reached and pollen was used for all experiments. The timelapse movie was created using the software NIS elements v. 4.11 (Nikon Metrology, Brighton, USA).

3. Metabolite and ultrastructural changes during wheat pollen storage

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Type of authorship:	First author
Type of article:	Research article
Contribution to article:	design and conduction of experiments, data analysis, manuscript preparation
Co-authors contribution:	Manuela Nagel conceived and provided the project, participated in interpretation of the data and in writing the manuscript.
	Till Ischebeck conducted the metabolite analysis with GCMS.
	Hardy Rolletschek was involved in the sugar analysis in experimental conduction and data analysis.
	Michael Melzer conducted the experimental part of transmission electron microscopy.
	All authors proofed and corrected the manuscript.
Journal:	intended submission: Plant, Cell & Environment
State of Manuscript:	in preparation

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ABSTRACT

Hybrid breeding may overcome yield stagnation in wheat but depends on successful fertilization of sterile female plants. At maturity, wheat sheds tricellular, short-lived pollen which may compromise fertilization. To understand factors and mechanisms influencing pollen viability and longevity, we aimed to investigate physiological, ultra-structural and metabolic changes during the exposition of pollen of four wheat lines to four environmental conditions. Here, we show that pollen germination ability decreased rapidly after mature pollen was shed. At 23°C and low relative humidity (RH) (R-L), pollen dropped to 10% germination after 18.9±0.1 min. At high RH (R-H) 10% germination was reached after 31.7±0.1 min. After 60 min under R-L pollen germination decreased to 1.1±2.2% and lost around 94% of the initial water content including visual changes in the pollen ultrastructure. At 9°C and low (C-L) and high RH (C-H), pollen water content was reduced by 68% and 39% and germination decreased by 51% and 25%, respectively, after 60 min, showing a close correlation ($r=0.8$, $P\leq0.001$) between pollen viability and water content. Over all conditions and lines, TRI 9102 was most resistant and pollen accumulated highest amounts of hexanedioic acid, pipecolate and lysine under R-L. When pollen of TRI 9102 was exposed to different environmental conditions (R-L, R-H, C-L, and C-H) for 60 min, proline, leucine, GABA, glycerol, sucrose and glucose showed strong increases, especially under R-L. In addition, also starch content increased significantly. The depletion of glucose-6-phosphate and fructose-6-phosphate indicates that starch may be actively synthesized in stored pollen. To speculate about possible protection mechanism and processes during pollen viability loss, we modelled the metabolic machinery. In conclusion, wheat pollen is prepared for immediate germination and the exposition of mature pollen to the environmental affects pollen viability. However, survival can be extended by environment and genotype-specific modulations and might be a selection criterion for future hybrid breeding.

Keywords: wheat pollen, pollen storage, metabolite analysis, sugar

Abbreviations: R-L, room temperature, low relative humidity; R-H, room temperature, high relative humidity; C-L, cold temperature, low relative humidity; C-H, cold temperature, high relative humidity

INTRODUCTION

The viability of pollen, the male gametophyte of higher plants, is crucial for fertilization and the survival of the species. About 30% of the angiosperm families (Williams et al. 2014), among them the *Poaceae*, shed tricellular pollen which has completed second pollen mitosis before shedding. (Franchi et al. 2002b; Brewbaker 1967). This pollen type is in a highly active metabolic state and germinates immediately after landing on the stigma (Carrizo García et al. 2015).

Pollen germination and tube growth is a highly dynamic and energy-demanding process. At this stage, transcripts for cell wall organization, and metabolism of lipids, proteins and major carbohydrates are highly abundant (Moon et al. 2018). For *Poaceae* species, starch is the main energy source after shedding (Speranza et al. 1997). They synthesize between 16.6 and 25.6% starch (Roulston and Buchmann 2000) during pollen development and store it in the amyloplasts of the vegetative pollen cell (Franchi, Bellani, Nepi, & Pacini, 1996) until usage. Thereby, the mature rice pollen is highly flexible and contains transcripts for amylases, catalyzing the hydrolysis of starch into oligosaccharides, transcripts for invertases participating in the hydrolysis of sucrose into glucose and fructose, and transcripts for starch synthesis, starch synthase (SS) and one ADP-glucose pyrophosphorylase (AGPase) (Moon et al. 2018). These enzymes are essential for decomposition, synthesis and translocation processes during pollen tube formation and make up, together with other proteins, between 20 to 30% of the pollen dry weight (DW) (Roulston et al. 2000). In combination with polysaccharides and glycolipids, proteins modulate the cytoskeleton and the pollen cell wall (Nägele et al. 2017) during tube growth and are further required to the final pollen-pistil interactions before the germ cells are released (Ischebeck 2016; Roulston and Cane 2000). For the pollen tube, majorly phospho- and glycerolipids are synthesized, most likely, from sugars and form large membrane lipids (Ischebeck 2016). Other lipids, such as neutral lipids, i.e. triacylglycerols, are present in oil bodies located in the cytoplasm of the vegetative pollen cell (Piffanelli et al. 1998). In maize pollen, lipid contents can be up to 17% of the pollen DW (Bianchi et al. 1990). However, changes in the composition of pollen phospholipids (Prasad et al. 2015; Wada et al. 2020) and starch synthesis (Lee et al. 2016) are closely associated with reductions in pollen germination and function.

Wheat (*Triticum aestivum* L.) is an important member of the *Poaceae* family; hence pollen is shed at a partially-hydrated state at > 30% water content (Pacini and Franchi 2020). The mature pollen germinates immediately on the style or on raffinose-containing media (Impe et al. 2020;

Cheng and McComb 1992). Wheat pollen is highly sensitive against environmental stress after shedding (Fritz and Lukaszewski 1989), and microspores between start of meiosis and the end of the tetrad stage (Koonjul et al. 2005). During pollen development, drought can induce pollen sterility including lower antioxidative activities, reduced carbohydrate accumulation in the spikes (Dong et al. 2017), and reduced invertase activities in the anthers with downstream effects on starch biosynthesis and changes in the carbohydrate profile. When wheat pollen is released from the anther, viability declines within 60 minutes at ambient conditions (Impe et al. 2020) and within 30 minutes under hot and dry field conditions (D´Souza 1970). A reduced pollen viability compromises wheat hybrid breeding programmes which rely on the fertilization of male-sterile female parents. Hybrid breeding promises an increase in grain yield through heterotic pattern (Zhao et al. 2015) and may overcome yield stagnation of the recent years (Ray et al. 2012a). However, for successful hybrid breeding programmes, the physiological and biochemical response of wheat pollen towards different environmental conditions needs to be understood to modulate the field design accordingly.

This study aims to examine the metabolite and ultrastructural changes in wheat pollen in response to different storage conditions and to decipher processes leading to the rapid viability loss in wheat pollen. In this work, we exposed mature pollen of four wheat lines to four storage conditions and studied in detail ultrastructural and metabolite changes during the viability decline. To elucidate the importance of the carbohydrate level and composition, we analysed in detail soluble sugars, and starch and tried to construct a hypothetic model of pollen viability loss in wheat.

MATERIALS AND METHODS

Plant material, pollen sampling and storage

Seeds of the spring wheat lines TRI 9102 (DOI:10.25642/IPK/GBIS/9074) and TRI 4399 (DOI:10.25642/IPK/GBIS/4399) were provided by the Federal *Ex situ* Gene Bank of Agricultural and Horticultural Plants at IPK Gatersleben. Seeds of the winter wheat lines ‘Ferrum’ (KWS, listed in Germany since 2012), ‘Hermann’ (Limagrain, listed in Germany since 2007) were commercially available. Seeds from all lines were germinated in a standard culture medium (Substrate1, Klasmann-Deilmann GmbH, Geeste, Germany) at 20 ± 2 °C. One-week old seedlings were subjected to 4 ± 1 °C for four (spring types) and six weeks (winter types). Vernalized plants were transferred into pots containing a sand/soil mixture (70% compost soil, 20% white peat, 10% sand) and grown under optimum conditions (regular watering and fertilization, 16 hours light) at 20 ± 2 °C in the greenhouse.

At the beginning of anthesis, spikes were cut between 8:00 and 10:00 a.m., kept in water and used within 6 hours. Only mature pollen was used for all experiments. To stimulate pollen maturation, awns, glumes and lemmas were carefully removed and pollen was sampled when lodicules swelled, the stigma fanned out, filaments elongated and anthers enlarged and turned greenish to bright yellow (Impe et al., 2020). Before the tip of the anther opened, at minimum three anthers were taken and pollen shedding was supported by opening gently with a needle.

Mature pollen was collected within a 5 min window and used immediately after anthesis (control) and termed 'fresh'. To cover a range of storage conditions, pollen was stored under room temperatures at low RH (R-L, 23.0 ± 0.4 °C, 60.0 ± 0.1% RH) and high RH (R-H, 23.0 ± 0.4 °C, 98.0 ± 0.8% RH) and under cold temperatures at low RH (C-L, 8.0 ± 1.2 °C, 72.0 ± 3.7% RH) and high RH (C-H, 8.0 ± 1.2 °C, 94.0 ± 1.9 %RH) for 10, 20, 30, 45, and 60 min (**Figure 1a**). Fluctuations in temperature and RH were monitored with a data logger (DL-120TH, Voltcraft, Hirschau, Germany).

Pollen viability determination

Pollen viability was assessed according to Impe et al. (2020) by *in vitro* germination, thereafter named germination, and impedance flow cytometry (IFC), termed IFC viability, using at minimum four biological replicates each if not otherwise stated. Briefly, pollen was germinated on a solid medium containing 594 mM raffinose, 0.81 mM H_3BO_3, 2.04 mM $CaCl_2$ at pH 5.8. Pollen tubes exceeding the lengths of the pollen radius were counted manually using the software NIS elements v. 4.11 (Nikon Metrology, Brighton, USA) and expressed as percentage of germination.

To determine IFC viability, at minimum 1000 pollen were transferred into 1 mL IFC buffer (AF6, Amphasys, Lucerne, Switzerland), filtered using 100 µm pore size and loaded onto a chip of 120 µm channel diameter. The chip was inserted in the IFC (Ampha Z32, Amphasys, Lucerne, Switzerland) and measurements were carried out at 1 MHz at the default settings for wheat pollen. IFC viability was analyzed using AmphaSoft v 2.0 version (Amphasys, Lucerne, Switzerland) and given as percentage.

Determination of pollen water content

Pollen of > 1 mg was collected in aluminum pans, hermetically closed and the fresh weight determined. Pans were perforated and exposed to 100 °C for 24 h. Afterwards, pans were reweighed and pollen water content (WC) calculated from the difference between fresh and dry mass and expressed as a percentage of the dry weight (DW).

Transmission electron microscopy

For comparative ultrastructural analysis, freshly collected pollen and pollen stored at room temperature at low RH (R-L) for 60 min was used for a combined high pressure freezing and freeze substitution sample preparation according to Daghma et al. (2011). Samples of stored pollen was directly dusted into a 1:1 v/v mixture of cyanobacteria and baker´s yeast. Mature pollen inside of opening anthers was used as fresh pollen to avoid desiccation during sample preparation. The cyanobacteria and yeast were maintained and the ready-to-use solution was provided by the Structural Cell Biology group, IPK Gatersleben. The mix of pollen/fresh anthers, cyanobacteria and yeast was inserted in a nitrocellulose membrane by capillary force and cut into 1 - 2 mm pieces. The filled nitrocellulose membranes were transferred onto 0.15 mm aluminium pans (Engineering Office M. Wohlwend GmbH, Switzerland) and covered with a 0.30 mm platelet. The samples were immediately shock-frozen under a pressure of ~2 kbar and transferred into an automated freeze substitution unit (Leica Microsystems, Bensheim, Germany). Further processing of the samples including freeze substitution, infiltration in Spurr’s resin (**Supplemental Table S1**), and sectioning of the fixed samples as well as ultrastructural analysis was performed as described by Daghma et al. (2011b).

Pollen metabolite analysis using gas chromatography - mass spectrometry (GC-MS)

Pollen metabolites were studied in two experimental set ups; the Genotype and Storage experiment. In the Genotype experiment, differences of metabolites detected in fresh pollen and pollen stored under R-L and R-H for 60 min were compared between the wheat lines ‘Ferrum’, ‘Hermann’, TRI 4399, TRI 9102. In the Storage experiment, metabolites analysed in fresh pollen of TRI 9102 were compared to metabolites of pollen stored under R-L, R-H, C-L, C-H for 10, 20, 30, 45 and 60 min.

In general, pollen was sampled immediately after anthesis (fresh) or storage as described above, transferred to glass vials and plunged into liquid nitrogen. Frozen samples were freeze-dried at -30 °C and 0.3 mbar (Freeze drier Alpha 1-4, Christ, Osterode am Harz, Germany) for 12 h and glass vials sealed under vacuum. Primary metabolites of freeze dried pollen were extracted in 0.5 mL methanol:chloroform:ddH_2O (v:v:v, 32,75:12,5:6,25). As internal standard 0.25 mL *allo*-inositol in ddH_2O (0.005 mg mL^{-1}) was added. The upper polar phase (100-200 µL) was used, evaporated under a stream of nitrogen and derivatized with 10 µL methoxamine hydrochloride in pyridine (30 mg ml^{-1}) and 20 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) to transform the metabolites into their methoxyimino (MEOX)- and trimethylsilyl (TMS)- derivatives (Bellaire et al. 2014). The derivatives were separated by

an Agilent 7890B gas chromatograph equipped with a capillary HP5-MS column (30 m x 0.25 mm; 0.25 µm coating thickness; J&W Scientific, Agilent) and mass spectra were recorded on an Agilent 5977N mass selective detector. Each sample was analysed twice; first using a longer gradient and a split of 1:10, and second with a shorter gradient and a split of 1:120 to analyse molecules of a high abundance. Helium was used as carrier gas with a flow velocity of 1 mL min^{-1}. The inlet temperature was set to 250 °C. A temperature gradient was applied and started at 70 °C for 2 min, increased by 5 °C min^{-1} (15 °C min^{-1} in the short gradient) to a final temperature of 325 °C hold for 2 min. The mass spectrometer was set at electron energy of 70 eV, an ion source temperature of 230 °C, and a transfer line temperature of 280 °C was used. Spectra were recorded in the range of 70-600 da e^{-1}.

Peaks were assigned with MSD ChemStation D.01.02.16 (Agilent Technologies, Waldbronn, Germany) by comparing the mass peaks to a list of collected standards. The final metabolites were identified by using the external standards or spectral libraries (Golm Metabolome Database Bioinformatics, 21, 1635-1638; NIST spectral library 2.0f). The intensity of each compound was divided by the internal standard *allo*-inositol. Due to the sylation process, amino acids can have two or three groups of trimethylsilyl, each having a different peak location. These values found were multiplied by a constant (**Supplemental Table S2**) and then combined. The final metabolite data were adjusted by the fresh weight of each sample. Due to low availability of pollen, the DW could not be determined directly. The change in weight was measured on a second set of samples at a later time point and the fresh weight corrected by a factor (**Supplemental Table S3**). For the further statistical analysis, corrected data were used and termed raw data (**Supplemental Table S4**, **S5**).

The raw metabolite data were normalized by dividing each metabolite value by the highest value found in the whole data set and statistically analyzed using MetaboAnalyst 4.0 (Chong et al. 2019) available at https://www.metaboanalyst.ca. The data were further pre-processed using Log-transformation and autoscaling (**Supplemental Figures S1-S3**). Univariate analysis by one-way ANOVA with Fisher's LSD post-hoc test was applied to assess statistically significant differences of single metabolites between samples stored under different conditions. Significance threshold was adjusted to $P \leq 0.05$.

Multivariate statistical analysis using unsupervised principal component analysis (PCA) was performed to identify intrinsic variation without considering sample-class membership. Thus, groups of samples are formed solely based on the structure of the metabolite data resulting in unbiased dimensionality reduction. Groups are separated when within-group variation is

substantially less than between-group variation (Young and Alfaro 2018). PCA was used to identify and exclude extreme outliers.

The supervised Partial Least Squares-Discriminant Analysis (PLS-DA) was used to maximise separation between pre-defined sample-classes and identify metabolites that significantly contributed to the classification. To avoid overfitting (Westerhuis et al. 2008), cross validation with 10-fold validation and 5 components and permutation tests with 2000 permutations were used. Cross validation determined the optimal number of components needed to build the PLS-DA model and defined by following performance measures: the sum of squares captured by the model (R^2), the cross-validated R^2 (termed Q^2), and the prediction accuracy (Xia and Wishart 2016). For biological data, at $R^2 > 0.7$ and $Q^2 > 0.4$, the model is regarded as good (Lundstedt et al. 1998). In the permutation test, the optimal number of components determined by previous cross validations and the original class assignment were considered and could be visually evaluated according to Bijlsma et al. (2006). For the prediction of class assignment, the ratio of the between sum of the squares and the within sum of squares (B/W-ratio) was calculated. If the B/W-ratio of the original class assignment was a part of the distribution based on the permuted class assignment, the difference between the two class assignments could not be considered as significant. If the B/W-ratio based on the original class assignment was higher compared to the ratios based on the permuted class assignments, the differences between the classes were statistically significant (Rubingh et al. 2006).

Variable importance in projection (VIP) score calculated by PLS-DA was used to select important features. The VIP score is a measure for weighted sum of squares of the PLS loadings; and the higher the VIP score, the higher the importance of the metabolite. Metabolites contributed significantly to the separation of the classes when the VIP scores were > 1.0 and P-value < 0.01. Further comparisons were conducted using heat maps constructed with ClustVis (Metsalu and Vilo 2015), available at https://biit.cs.ut.ee/clustvis/ using the average linkage of Euclidean distance between groups. Metabolites in the heat maps were ordered according to chemical class provided in **Supplemental Table S6.**

Sugar analysis in wheat pollen using ion chromatography

Soluble sugars were extracted from fresh pollen of wheat line TRI 9102 or stored pollen kept at R-L, R-H, C-L and C-H for 10, 20, 30, 45, and 60 minutes. Pollen was mixed with 0.2 mL ethanol (60% v/v) and stored at -20 °C until further analysis (Rolletschek et al. 2011). For extraction, the samples were sonicated for 5 min. After centrifugation (5 min at 13,000 rpm) the supernatants were diluted with 10% methanol (1:50 v/v) and used directly for sugar analysis

using ion-exchange chromatography coupled to pulsed amperometric detection (ICS-3000; Thermo Fisher). Chromatographic separation was carried out on a PA1 column (2×250 mm) and a PA1 guard column (2×50 mm) at 25 °C by applying an isocratic run with 400 mM NaOH at a constant flow rate of 0.7 mL min^{-1} over 5 min. A mixture of glucose, fructose, sucrose, raffinose, stachyose and verbascose was used as authentic standards and for external calibration. The identification of sugars was performed by comparing retention times of individual sugars in the reference vs. analyzed solution, and quantification was based on peak areas. The change in weight upon storage was measured on a second set of 'Ferrum' and TRI 9102 at a later time point. The fresh weight of fresh pollen was corrected by 0.39 and the fresh weight of stored pollen was corrected by 0.91 to show the data on the content of individual sugars on a DW basis.

Extraction of starch content

Total starch content in pollen was quantitatively determined using a kit from Megazyme (Total Starch Assay Kit, K-TSTA-50A, Megazyme, Wicklow, Ireland). Briefly, around 1 to 4 mg of pollen sample were immediately frozen in liquid nitrogen. Pollen starch measurements were modified to analyse pollen starch content in 96-microwell plates. Hence, all solution volumes of the reaction kit were reduced by one tenth, used to follow the manufacturer's instructions. The starch analysis involved a two-step hydrolysis; at first, the starch was partially hydrolysed to maltodextrins and totally solubilized by the action of α-amylase. At second, the maltodextrins were quantitatively hydrolyzed to glucose by amyloglucosidase. The quantity of glucose formed was measured spectrophotometrically with a glucose determination reagent comprising a buffered glucose oxidase – peroxidase solution containing 4-aminoantipyrine. The absorbance of the released quinoneimine dye was measured at 510 nm wavelength using the Cytation 5 multi detection reader (BioTek, Bad Friedrichshall, Germany). Starch content was calculated on DW basis.

Statistical analysis

Statistical analysis was conducted using SigmaStat 4.0 (Systat Software, 2016). Data were tested for normal distribution using the Shapiro-Wilk test. Accordingly, parametric or non-parametric tests were used to compare differences of the means. For the comparison of three or more samples one-way analysis of variance (ANOVA) was used. GenStat 18 (VSN International Ltd, 2016) was used to conduct Spearman correlation analysis at $P < 0.05$. Statistical differences were evaluated using the least significant difference at $P < 0.05$

(LSD5%). If the normality test failed, a balanced design was used for ANOVA and pairwise multiple comparisons using Holm-Sidak method were chosen.

RESULTS

Pollen water content and storage conditions affect pollen viability

Room temperature, low relative humidity and storage periods longer than 10 min cause a drop in wheat pollen viability **(Figure 1b)**. When pollen was exposed to R-L or R-H, pollen of four wheat lines reached 10% germination after 18.9 ± 0.1 and 31.7 ± 0.1 min, respectively, and decreased to 1.1 ± 2.2% and 3.4 ± 3.9%, respectively, after 60 minutes. Accordingly, IFC viability ($r_{germination\text{-}IFC\ viability} = 0.82$, $P = 0.09$) declined when pollen was stored at R-L for 60 min (3.1 ± 2.6%) but was higher at R-H (61.5 ± 9.3%) **(Figure 1c-d)**. In parallel, the pollen water content showed a significant decline and correlated positively with germination at $r_{germination\text{-}WC} = 0.77$, $P \leq 0.001$ and IFC viability at $r_{IFC\ viability\text{-}WC} = 0.88$, $P \leq 0.001$ (**Figure 1f**). After storage at R-L and R-H, pollen of the wheat lines lost around 94% and 60%, respectively, of their initial water content (1.65 ± 0.1 mg H_2O mg^{-1} DW) (**Figure 1e**). Under cold temperatures, pollen water content was less affected and reduced by 68% and 39% under C-L and C-H, respectively. Time to 10% germination was reached after 72.3 ± 0.5 and 205.1 ± 82.6 min, respectively **(Figure 1b).** Accordingly, germination reduced by 51% and 25% and IFC viability by 46% and 12%, respectively, when pollen was stored for 60 minutes **(Figure 1b-c**). Pollen germination of 'Ferrum', 'Hermann' and TRI 4399 showed comparable results ($P > 0.07$) after storage under different conditions. The pollen of the wheat line TRI 9102 showed higher resistance against R-H and C-L and had significantly higher germination percentages over longer storage intervals compared with 'Ferrum' and TRI 4399 (LSD5% = 9.98%, $P \leq 0.05$) **(Supplemental Figure S4).** Remarkably, all pollen samples with water contents above 0.7 mg H_2O mg^{-1} DW showed an IFC viability of > 50% whereas IFC viability decreased to less than 10% when pollen had water contents below 0.3 mg H_2O mg^{-1} DW. In conclusion, as soon as pollen was released from the anther, pollen water content ceased and contributed to a decline in pollen viability.

Pollen storage affects ultrastructure

Decrease in water content during R-L caused ultrastructural damages. When fresh pollen was dusted on pollen germination medium, pollen tubes developed within 5 minutes (**Figure 2a**). In fresh pollen, the intine was thin, filled with vesicles and was separated from the protoplast by a smooth plasma membrane (**Figure 2b, c**). The cytoplasm appeared homogenous and cell organelles such as dictyosomes, endoplasmic reticulum and mitochondria were clearly visible

(**Figure 2d**). In contrast, after 60 minutes storage under R-L, pollen tube development was absent on pollen germination medium (**Figure 2e**) and the pollen ultrastructure completely changed. The intine thickened and appeared to be more textured (**Figure 2f, g**). Vesicles in the intine clustered and moved to the plasma membrane which itself was invaginated and partially damaged (arrowheads in **Figure 2g, h**). The cytoplasm was highly disordered including more granular structures (asterisks in **Figure 2g, h**). Mitochondria and dictyosomes were shrivelled and less visible (**Figure 2h**). Under these conditions, the ultrastructure was irreversible damaged explaining low pollen viability after storage at R-L.

Wheat lines show minor differences in pollen metabolites

In total, 44 substances (40 annotated) comprising carbohydrates, amino acids, and intermediates of the glycolytic pathway and tricarboxylic acid (TCA) cycle (**Supplemental Table S6)** were found in pollen of the four wheat lines stored under various conditions. To analyse the variance of metabolites between genotypes of fresh pollen and pollen stored under R-L, the PLS-DA model was applied and was confirmed by cross validation with $Q^2 > 0.64$, $R^2 > 0.96$, accuracy = 75% (**Supplemental Table S7)** and permutation test **(Supplemental Figure S5).** The resulting first two PLS-DA components distinguished pollen samples between wheat lines and storage groups (**Figure 3a)**. The wheat lines ‘Ferrum’, and TRI 9102 were clearly separated whereas pollen samples of the lines ‘Hermann’ and TRI 4399 overlapped with either one or both groups. The storage condition R-L was a second factor and separated fresh pollen from pollen stored under R-L for 60 min (**Figure 3).** The storage groups but not wheat lines showed clear differentiation based on PCA with PC1 explaining 58.2%, PC2 10.3% and PC3 6.6% of the total variation **(Supplemental Figure S6).**

Out of the 44 analysed substances, the pollen of four wheat lines differed significantly in 12 compounds (VIP > 1.0) **(Figure 4, Supplemental Figure S7)**. Thereby, the VIP scores calculated by the PLS-DA model is an indicator for the importance of the differentially accumulated metabolites. The pollen of the wheat lines discriminated in hexanedioic acid (VIP = 2.51) and in the composition of seven amino acids, most pronounce the amino acid derivate pipecolate (VIP = 2.41). Based on the difference between fresh pollen and pollen stored under R-L for 60 min, most noticeable differences were found between pollen of ‘Ferrum’ and TRI 9102 which varied in the metabolites hexanedioic acid and the amino acids pipecolate, serine, valine and lysine. In conclusion, only few metabolites varied between wheat lines indicating that other factors than genotype-specific metabolite patterns are responsible for viability loss.

To investigate the storage conditions as source of variation, the more resistant wheat line TRI 9102 was chosen for further analysis.

Pollen metabolite levels gradually change during storage

Fresh pollen could be distinguished in their metabolite pattern from pollen stored for longer periods. To explore the extent of the change in metabolites over various conditions and storage intervals pollen of the wheat line TRI 9102 was exposed to R-L, R-H, C-L and C-H for up to 60 min. Cross validation (**Supplemental Table S7,** $Q^2 > 0.20$, $R^2 > 0.36$, accuracy = 41%) and permutation test **(Supplemental Figure S8)** of the PLS-DA model indicated that based on the metabolite levels, pollen samples of different storage conditions and intervals partly overlapped **(Figure 5, Supplemental Figure S9)**. Fresh pollen samples only overlapped with pollen samples stored under cold conditions which was confirmed by PCA (**Supplemental Figure S9**). Here, the first three components explained 64% (PC1, 40.8%; PC2, 16.7%; PC3, 6.5%) of total variance. To identify differences between storage intervals, each storage group (R-L, R-H, C-L, C-H) was analysed separately for the PLS-DA model **(Supplemental Figure S10, S11, S12)** and PCA **(Supplemental Figure S13, S14)**. Pollen metabolites of longer storage times (> 30 min) and most pronounced under R-L and R-H, clearly separated from metabolites of fresh pollen.

The pollen storage experiment revealed that most significant VIP scores were found in R-L (26 of 44 metabolites), followed by C-L (24), R-H (22) and C-H (21) (**Supplemental Figure S15).** During pollen storage under various conditions, the abundance of sucrose and associated myo-inositol rose significantly (VIP ≥ 1.0) in pollen stored under all conditions **(Figure 6)**. Fructose and glucose increased also under most conditions. Under R-L for 60 min, pollen reached total amounts of 12.5 ± 2.9 mg g^{-1} DW fructose, 13.2 ± 3.1 glucose mg g^{-1} DW and 146.3 ± 43.8 mg g^{-1} DW sucrose. In parallel, also starch content increased achieving under R-L, 142.6 ± 16.7 mg g^{-1} DW **(Figure 7).** Interestingly, the metabolite levels of glucose-6-phosphate and fructose-6-phosphate declined significantly (VIP ≥ 1.0, **Figure 6**) which may indicate a turn-over of the phosphate-reserves towards the soluble sugars and starch. Accordingly, we would also expect a lower abundance of glycerate-3-phosphate after various storage conditions. However, concentrations of glycerate-3-phosphate increased. Following the glycolytic pathway, pyruvate remained stable for pollen stored under cold conditions. The following increase of citrate, isocitrate and the significantly higher abundance of succinate and fumarate (VIP ≥ 1.0) over all different storage treatments indicate that the rise in concentration may derive from other sources then from hydrolysis of starch and soluble sugars. Protein and lipid degradation are

hypothesized to be a potential pool for the increase of metabolites in the TCA cycle and elsewhere.

From the 40 annotated compounds found, all 11 amino acids, except for aspartate, increased during pollen storage for 60 min **(Figure 6, Supplemental Figure S12)**. Particularly, GABA had, besides proline, the highest metabolite abundance, increased significantly (VIP = 1.1 to 1.5) during pollen storage and might be an additional source for the metabolite increase in the TCA cycle. In pollen stored under R-L, the concentrations of the amino acids glycine, isoleucine, leucine, proline, serine, threonine, valine and the amino acid derivates beta-alanine and pipecolate rose also significantly (VIP ≥1.0 to 1.3) (**Supplemental Figure S15**). Alanine and GABA increased significantly (VIP ≥ 1.3) in pollen stored under both room temperature conditions (R-L and R-H). Pollen kept under cold temperatures (C-L and C-H) for storage periods longer than 30 min showed significantly higher levels (VIP ≥ 1.2) of the amino acids glycine, leucine and valine. Simultaneously, under cold conditions, also glycerol levels increased as possible result of lipid degradation. Overall, the increase of amino acid concentrations and glycerol at specific storage intervals tends to be strongly dependent on storage temperature and humidity.

DISCUSSION

Ecological advantage of high pollen hydration

The partial-hydration of the wheat pollen (Heslop-Harrison and Heslop-Harrison 1992) contributes to immediate germination. Wheat pollen has an initial water content 1.65 ± 0.1 mg H_2O mg^{-1} DW (~ 62%) at maturity which is extremely high compared to partially-dehydrated pollen (< 10% WC) (Franchi et al. 2002b). Although wheat pollen is protected by sporopollenin, an extraordinary resistant and stable substance (Mackenzie et al. 2015), its exine is interrupted by a single aperture (Zhou and Dobritsa 2020) and small channels and cracks are visible in microscopic images. Other protective layers, such as pollenkitt that can impede rapid water transfer, are absent (Pacini and Hesse 2005). In rice pollen, which is also partially-hydrated, the gene expression is largely identical between mature and germinated pollen (Wei et al. 2010) indicating that required transcripts are available at shedding and enable immediate translation and rapid germination. Both, high water content and fast germination, might be interpreted as evolutionary response to the warm and humid climate at flowering time and offer advantages in a competition of male gametophytes after pollen release within a restricted area and a short time interval (Franchi et al. 2011). Monnet et al. (2020) estimated that 10% of the grass species

have established outside of their natural habitat, which is higher than the total proportion for vascular plants. Therefore, we assume that the partial-hydration of pollen may have contributed to efficient fertilization of single plants, and hence the widespread and global abundance of wheat and other grasses.

Wheat pollen is not equipped with sufficient protection mechanisms to survive longer periods

The open thermodynamic system of wheat pollen is highly prone to temperature fluctuations and water loss. When mature wheat pollen was released from the anther the water content was reduced by more than 95% under R-L but was less than halved under C-H (~39% water reduction) at 94% RH within one hour. Lower RH and higher temperatures reduce the time to 10% pollen germination from 205.9 ± 82.6 min under C-H to 72.3 ± 0.5 min under C-L to 31.7 ± 0.1 min under R-H and down to 18.9 ± 0.1 min under R-L. After 60 min under R-L, structures aggregated at the cytoplasm and within the intine, and the protoplast was severely damaged. In vegetative plant tissue, when the water potential decreases below -5 MPa, mechanical tension is imposed on the plasma membranes, the cytoskeleton is disrupted, and the distance between cell organelles and other compounds is reduced to a so-called juxtaposition facilitating novel interactions. A further decrease of the water potentials results in an unfolding and denaturation of proteins, a fusion of membranes and a loss of cellular compartmentalization leading to the loss of pollen viability (Oliver et al. 2020). The dehydration of wheat pollen was accompanied with massive increase of the most abundant metabolites, sucrose and proline, especially during extended R-L storage periods. Both metabolites are known to act as osmoregulators and slow water loss. Proline is also known as a molecular chaperone stabilizing proteins, membranes (Szabados and Savouré 2010) and the mitochondrial electron transport complex II (Hamilton and Heckathorn 2001). Sucrose molecules can replace water at hydrogen-bonding sites and maintain the native protein structure and spacing between phospholipids (Buitink and Leprince 2004). By this, increased levels of sucrose contribute to the transition of the cytoplasm from amorphous, gel-like matrix to a glassy state (Crowe et al. 1992). Although, there is a large variation in sucrose content in partially-dehydrated pollen of different species (Nepi et al. 2001b), a smaller amount tends to diminish the pollen longevity (Speranza et al. 1997) assuming that the increase of sucrose and proline in wheat pollen is not sufficient to protect pollen against water loss under different environmental conditions.

Starch and sugar accumulation

Starch is the main storage reserve supplying energy for pollen germination and final fertilization. However, also genes for starch synthesis, i.e. SS and AGPase were still enriched

in mature rice pollen (Moon et al. 2018). Lee et al. (2016) proposed that glucose-6-phosphate is translocated in the amyloplasts and the plastidic phosphoglucomutase (pPGM) converts glucose-6-phosphate to glucose-1-phosphat (Weigelt et al. 2009). The plastidial AGPase converts glucose-1-phosphate to ADP-glucose which is further synthesized, i.e. by SS, to starch. Impairments in the starch synthesis, such as mutations in the plastidic large subunit of AGPase, *OsAGPL4*, or the pPGM in rice, *OspPGM*, lead to significant reductions in the amount of starch and have dramatic consequences on successful fertilization (Lee et al. 2016). Wheat pollen stored under different conditions dropped in viability, water content and showed a massive increase of sucrose and glucose, among other metabolites (**Figure 6**) after 60 min storage. Comparably, sucrose levels increased in partially-hydrated pumpkin (*Curcubita pepo*) pollen stored for seven hours. However, in pumpkin starch levels reduced (Garcia et al. 2015) assuming that the starch was hydrolysed and used to maintain the high metabolic activity during quiescence. By contrast, in partially-dehydrated *Helleborus foetidus* pollen, increasing amounts of starch were paralleled with a depletion of sucrose, glucose and fructose during 0 °C storage (Vesprini et al. 2002). Surprisingly, in wheat pollen, starch increased in parallel with the content of soluble sugars indicating that the precursors, i.e. ADP glucose, glucose-1-phosphate and glucose-6-phosphate, were synthesized during storage but from a different source than starch or soluble sugars itself.

The increase of most metabolites may be caused by the initiation of massive degradation processes which trigger starch synthesis (**Figure 7**). In non-chlorophyllic tissues such as long-term stored wheat and barley seeds, viability loss was accompanied with a significant increase of glycerol, fatty acids, alanine, galactose, glucose and fructose. Especially, non-oxidized storage lipids such as triacylglycerols or structural lipids, i.e. phospho- and galactolipids, reduced; and mono-/diacylglycerols, lysophospholipids and fatty acids accumulated indicating that lipids were oxidized or enzymatically hydrolysed (Wiebach et al. 2019). In pollen, impairments in the lipid biosynthesis during pollen development have consequences on the concentration, composition and, hence, pollen structure and fertility. For example, the mutant *male sterile 33* (*ms33*) in maize (Zhang et al. 2018) or the knock-out of glycerol-3-phophate acyltransferase 3 (*osgpat3*) in rice lead to reduction of lipid molecules in the anthers, to a defective exine layer and, consequently a male sterility (Men et al. 2017). We speculate that during storage of wheat pollen, membrane and structural lipids were hydrolysed leading to a significant increase of glycerol, glycerol-3-phosphate and glycerate. The degradations of cell membranes and damages in the exine layer might be comparable to impairments of the glycerolipid biosynthesis. In parallel, the glycerol is assumed to be converted via glycerol-3-

phosphate and glycerate-3-phosphate to glucose-6-phosphate (Xue et al. 2017). The same might be true for conversion of proteins via pyruvate (Araújo et al. 2011). We speculated that enzymatic activity is triggered by the overall increase of soluble sugars and by a shift in pH and redox potential to a more reductive condition indicative for ageing processes during wheat seed storage (Nagel et al. 2019). Redox-sensitive enzymes of the carbohydrate pathway are AGPase, starch synthase (SS1) and glucan water dikinase (GWD1) and are activated during the shift of redox potential (Skryhan et al. 2018). In summary, during wheat pollen storage, products of lipid hydrolysis (**Figure 7**) may provide the source and required conditions for starch synthesis.

The ability of pollen to grow is inhibited during extended storage

Stable protein structures involving leucine and glycine are destabilized during dehydration and affect pollen cell wall and tube formation, and successful fertilization. Leucine is highly abundant in pollen (Ghosh et al. 2020) and plays an important role in the leucine-rich repeat (LRR) structural motif including 20 to 30 amino acids (Bella et al. 2008) that mediates specific protein-protein interactions (Kobe and Deisenhofer 1994). The LRR domain forms together with the extensin domain the so-called leucine-rich repeat extensins (LRXs), a major class of cell-wall proteins. The LRX protein *Pex1* was localized to the intine of maize pollen grains and callose-rich pollen tube cell wall (Rubinstein et al. 1995). Mutations of *LRXs* (*LRX9-11*) in Arabidopsis affect polysaccharide composition and integrity of the pollen tube cell wall leading to compromised pollen germination and tube growth (Wang et al. 2018). We assume during continuous water loss, the LRXs proteins are degraded, leucine released and the ability to form pollen tube walls is significantly inhibited the longer pollen is exposed to different environmental conditions. Comparably, glycine is a component of glycine-rich protein (GRP) and glycinebetaine. GRPs accumulate in the exine of microspores and mature pollen and play an important role in the differentiation (Takebe et al. 2020). Reduced levels of transcripts encoding for glycine-rich proteins, such as *LeGP92* or *OsGPR2*, correlate with altered and uneven exine formation in tomato (McNeil and Smith 2010) and deficiencies in the thickening of tapetal cell walls at the pollen mother cell stage in rice (Takebe et al. 2020), respectively. In addition, glycinebetaine is an important derivate of glycine and stabilize the quaternary structures of enzymes and complex proteins and maintains the highly ordered state of membranes at non-physiological temperatures and salt concentrations (Papageorgiou and Murata 1995). The decomposition of GRPs and glycinebetaine destabilizes the exine and protein structures but may stimulate the conversion to serine. Increased levels of D-serine activate glutamate receptor-like (*GLR*) family which encode functional Ca^{2+} channels. During pollen tube, Ca^{2+} diffusion is stimulated into the cytoplasm of the apical region of pollen tubes

(Michard et al. 2011) and, thus supporting and preparing pollen tube growth before pollen viability is lost.

At an early state of pollen viability loss, degradation products may stimulate further rescue programmes. GABA is known to inhibit the anion passage through Aluminium (Al^{3+})-activated malate transporter family of proteins (ALMT) (Ramesh et al. 2017). Therefore, it controls the apical growth in pollen tubes via tip-focused ion gradients and fluxes (Gutermuth et al. 2013). The pollen tube grows along a concentration gradient from a lower GABA concentration in the stigma to relatively high GABA levels in the septum surface and at the ovary walls. Therefore, slight increased GABA levels can stimulate tube elongation. However, higher concentrations prevent growth (Palanivelu et al. 2003; Renault et al. 2011). We speculate that under the proposed acidic conditions GABA accumulation is stimulated via activation of glutamate decarboxylase (Kinnersley and Turano 2000) to enhance the pollen tube performance. However, extended storage periods may facilitate GABA concentration which are detrimental for successful fertilisation.

Pollen survival may be enhanced by genotype-specific variations of hexanedioic acid, pipecolate and lysine. Especially, the line TRI 9102 showed elevated amounts of hexanedioic acid, pipecolate, lysine and unknown compounds. Lysine can be catabolized via different pathways to α-amino adipic acid, an hexanedioic acid bearing a single amino substituent, and pipecolatic acid, known as pipecolate (Návarová et al. 2012). The non-protein amino acid pipecolate seems to be critical for plant systemic acquired resistance and basal immunity to bacterial pathogen infection (Hartmann et al. 2017). Furthermore, the hexanedioic acid analogue isolated from an endophytic fungus associated with the traditional medicinal herb *Orchidantha chinensis* exhibited strong superoxide anion radical scavenging capacity (Luo et al. 2017). It showed also suppression to the mycelium growth of the fungus *Verticillium dahllae* and enhanced the resistance of eggplants (Liu et al. 2009b). Although, there has not been drawn any relationship to pollen viability yet, we speculate that some pollen metabolites may increase the protection against viability loss.

CONCLUSION

When mature wheat pollen was shed from the mother plants, pollen viability and water content dropped rapidly within minutes. Storage time could be extended by lowering temperatures and increasing RH. However, the depletion of pollen viability was accompanied by ultrastructural changes and an accumulation of metabolites, most pronounced proline, leucine, GABA,

glycerol, sucrose and glucose. In parallel, starch levels also increased leading to the assumption that the high metabolic activities facilitate starch synthesis at the expense of other compounds, most likely degradation of proteins and lipids. For successful hybrid breeding, pollen viability needs to be maintained under field conditions for a limited period. Therefore, genotype-specific characteristics need to be further evaluated to identify and delimit the source of the massive metabolite accumulation and to work towards the long-term storability of pollen.

AUTHOR CONTRIBUTION STATEMENT

DI, TI, MN designed the experiments. DI, TI, MM, HR conducted the experiments. DI analyzed the data. DI and MN wrote the manuscript. All authors proofed and corrected the manuscript.

ACKNOWLEDGMENT

We like to acknowledge Jürgen Marlow and Kerstin Wolf for excellent technical support. Traud Winkelmann, Hans-Peter Mock, Angelika Senula for advice and critical comments on the manuscript. The work is part of a PhD project and was supported by Leibniz Institute of Plant Genetics and Crop Plant Research (IPK).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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3.2. Tables and Figures

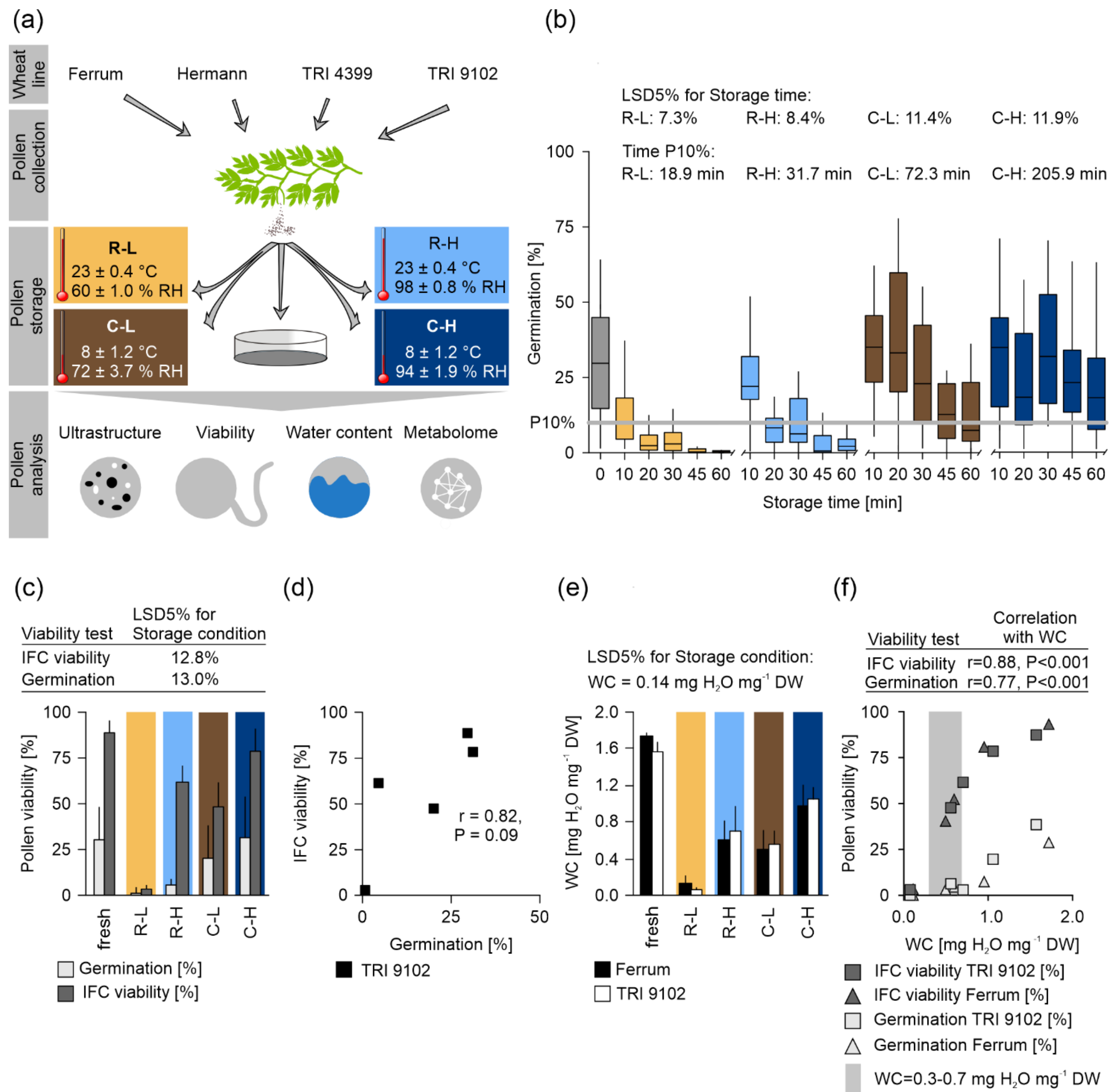


Figure 1 Decrease of pollen viability and pollen water content during storage under various conditions. (a) Experimental set-up to study pollen of four wheat lines stored under room temperatures at low RH (R-L, beige) and high RH (R-H, light blue) and under cold temperatures at low RH (C-L, brown) and high RH (C-H, dark blue) for 10, 20, 30, 45 and 60 minutes. After pollen storage, ultrastructure, viability, water content and metabolic changes were compared with fresh pollen (grey). (b) Whisker-box plots represent pollen germination of four wheat lines for fresh pollen and pollen stored under conditions described in (a). Time until pollen germination drops to 10% germination (grey line) and least significant differences at $P < 0.05$ (LSD5%) between storage times are provided for each condition above each plot. LSD5% between conditions is at 5.5%. Five biological replicates with 2700 to 7700 pollen each were tested for each line. (c) Comparison between pollen viabilities of wheat line TRI 9102 analysed by pollen germination (light grey) and impedance flow cytometry (IFC viability, dark grey) of fresh pollen and pollen stored as described in (a) for 60 min. LSD5% values between storage conditions and for each viability test are provided in the table above. (d) Relationship between pollen germination and IFC viability for pollen of the wheat line TRI 9102 as shown in (c). Pearson coefficient of correlation (r) is provided in the plot. (e) Comparison between pollen water contents (WC) of the wheat lines 'Ferrum' (black) and TRI 9102 (white) for fresh pollen and pollen stored as described in (a) for 60 min. Each bar represents means and standard deviation of a minimum of five replicates. LSD5% values between storage conditions are provided above. (f) Relationship between pollen viability and pollen WC for the wheat lines 'Ferrum' (triangle) and TRI 9102 (square) stored as described in (a) for 60 min. Each bar represents means and standard deviation of a minimum of 5 biological replicates using a minimum of 1000 pollen. r is provided in the table above the plot.

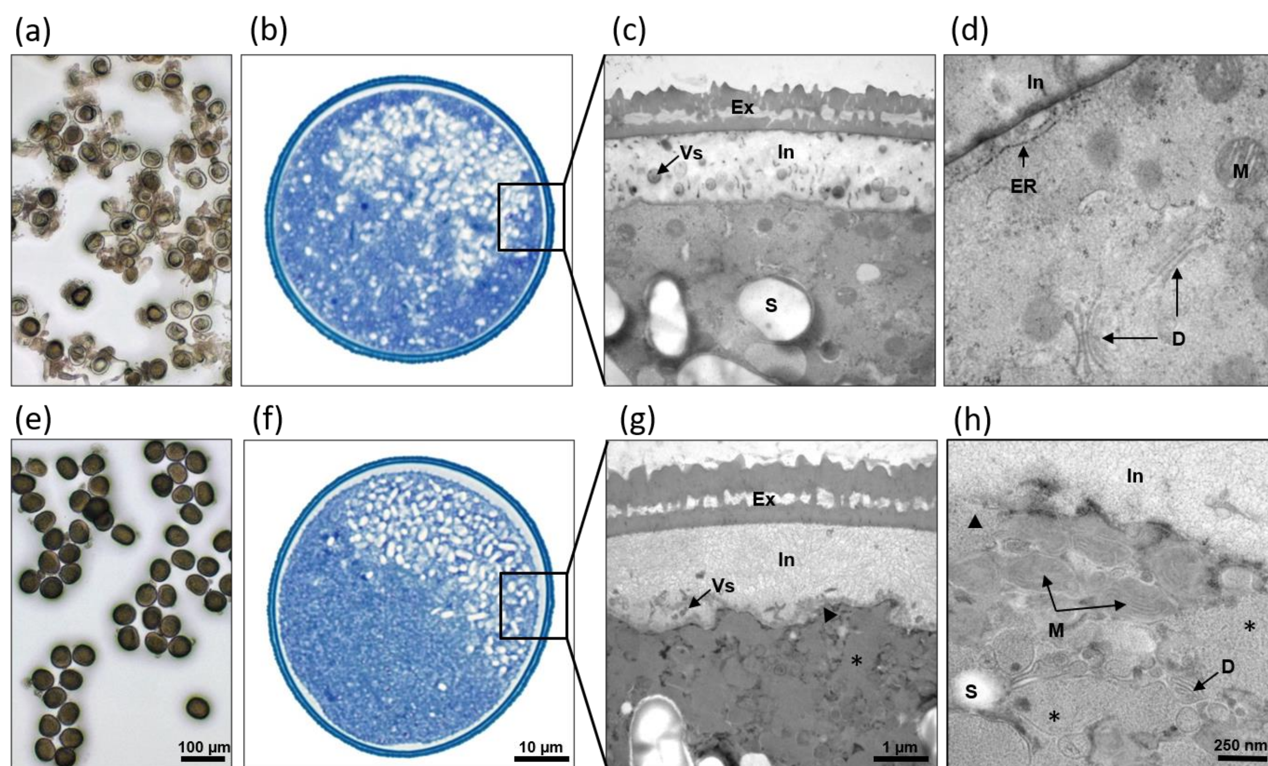


Figure 2 Storage of pollen at room temperature leads to ultrastructural changes. (a,e) Pollen germination on medium (b,f), complete pollen grains, (c,g) enlarged section of pollen wall with intine (In) including vesicels (Vs) and exine (Ex), (d,h) cytoplasmic organelles including starch granules (S), dictyosomes (D), endoplasmic reticulum (ER), and mitochondria (M) of (a-d) fresh and (e-h) stored pollen at room temperature and low RH (R-L) for 60 minutes are shown. Scale bars as in figure.

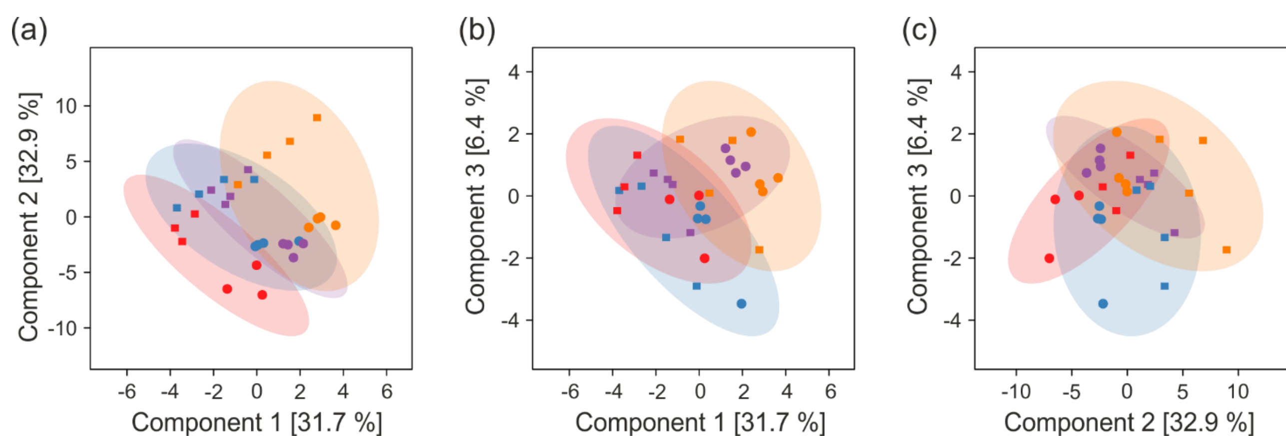
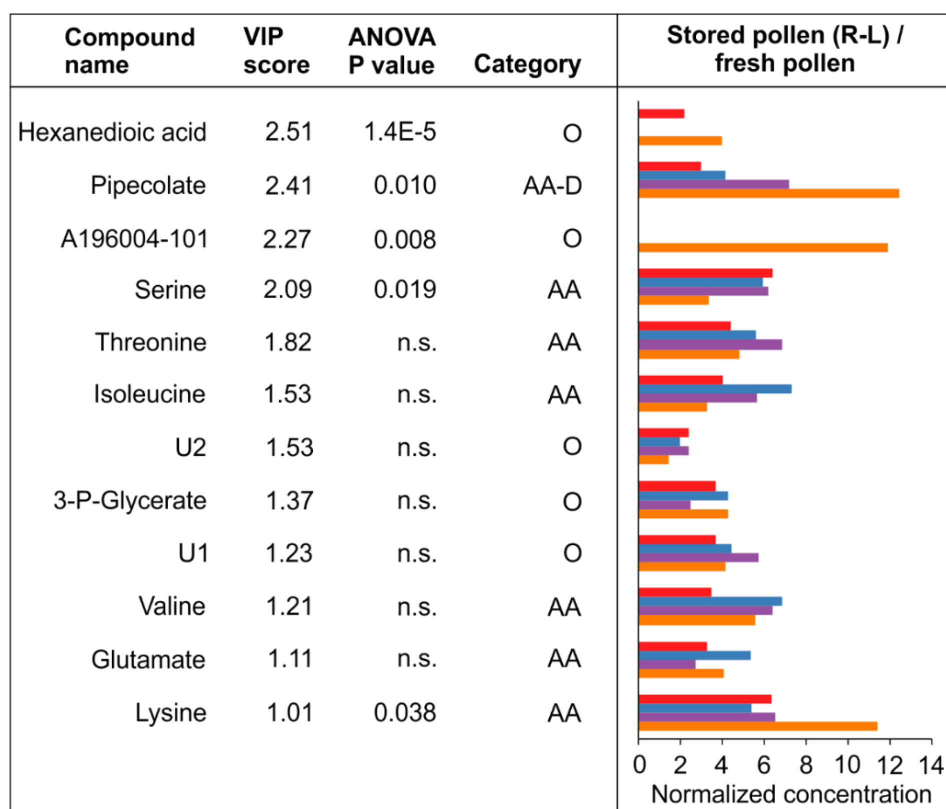


Figure 3 Pollen metabolite levels vary between wheat lines stored at room temperature as visualized by Partial Least Square – Discriminant Analysis (PLS-DA) (Genotype experiment). PLS-DA components for freshly collected pollen (dots) and pollen stored at room temperature at low RH (R-L, square symbols) for 60 minutes of the four wheat lines 'Ferrum' (red), 'Hermann', (blue), TRI 4399 (purple), TRI 9102 (orange) were compared. (a) Component 1 is plotted against component 2, (b) component 1 vs. component 3 and (c) component 2 vs. component 3. Four biological replicates were analysed for pollen from each wheat line and condition. Data were normalized, autoscaled and log transformed. The explained variances for each component is given in brackets. A validation of the PLS-DA model is given in **Supplemental Figure S6**.



Compound name	VIP score	ANOVA P value	Category
Hexanedioic acid	2.51	1.4E-5	O
Pipecolate	2.41	0.010	AA-D
A196004-101	2.27	0.008	O
Serine	2.09	0.019	AA
Threonine	1.82	n.s.	AA
Isoleucine	1.53	n.s.	AA
U2	1.53	n.s.	O
3-P-Glycerate	1.37	n.s.	O
U1	1.23	n.s.	O
Valine	1.21	n.s.	AA
Glutamate	1.11	n.s.	AA
Lysine	1.01	0.038	AA

Figure 4 Most metabolite levels are elevated in pollen stored at room temperature. On the **right panel**, bars show log-transformed, autoscaled ratios for metabolites differentially accumulated in pollen stored at room temperature at low RH (R-L) compared to freshly collected pollen of the four wheat lines 'Ferrum' (red), 'Hermann' (blue), TRI 4399 (purple) and TRI 9102 (orange). Four biological replicates were analysed for pollen from each wheat line and each condition. On the **left panel**, highly significant metabolites with a Variable Importance in Projection (VIP) score > 1.0 and their corresponding P-values analysed by Analysis of Variance (ANOVA) are provided. Metabolites are categorized in groups of amino acids (AA), amino acid derivatives (AA-D), and other compounds (O). The metabolite A196004-101 represent a repeatedly observed but not yet identified metabolite that is archived and made accessible through the search interface of the Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/search.aspx). Metabolites U1 and U2 are unknown.

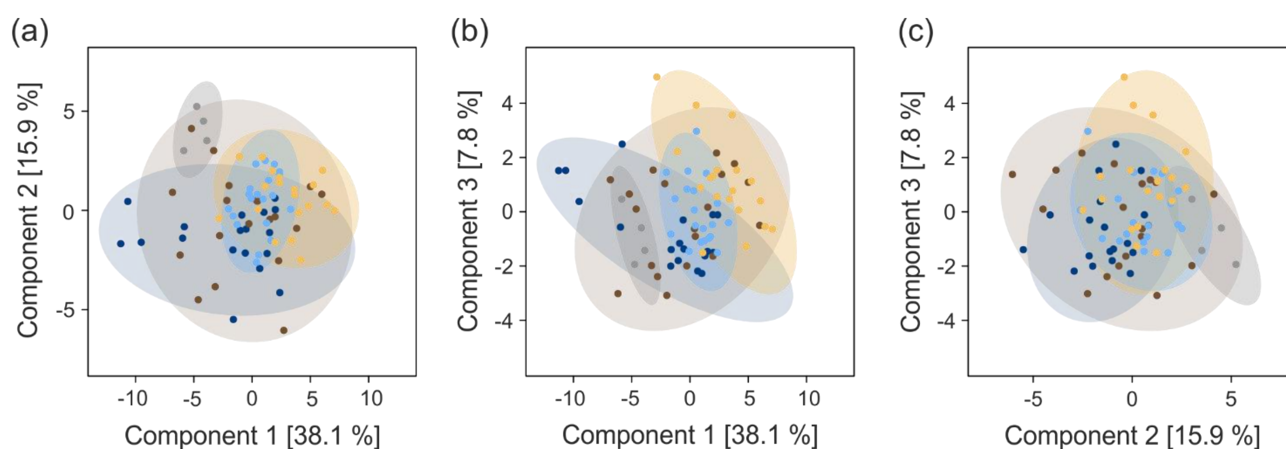


Figure 5 Pollen metabolite levels of the wheat line TRI 9102 vary across four storage conditions as visualized by Partial Least Square – Discriminant Analysis (PLS-DA) (Storage experiment). PLS-DA components for freshly collected pollen (grey), pollen stored at room temperatures at low RH (R-L, beige) and high RH (R-H, light blue) and pollen stored at cold temperatures at low RH (C-L, brown) and high RH (C-H, dark blue) for 10, 20, 30, 45 and 60 minutes (not specified in the plot) were compared. (a) Component 1 is plotted against component 2, (b) component 1 vs. component 3 and (c) component 2 vs. component 3. Four biological replicates were analysed for pollen from each condition. Data were normalized, autoscaled and log transformed. The explained variances for

each component is given in brackets. A validation of the PLS-DA model is given in **Supplemental Figure S8**. Separate PLS-DA for each storage condition are provided in **Supplemental Figure S11**.

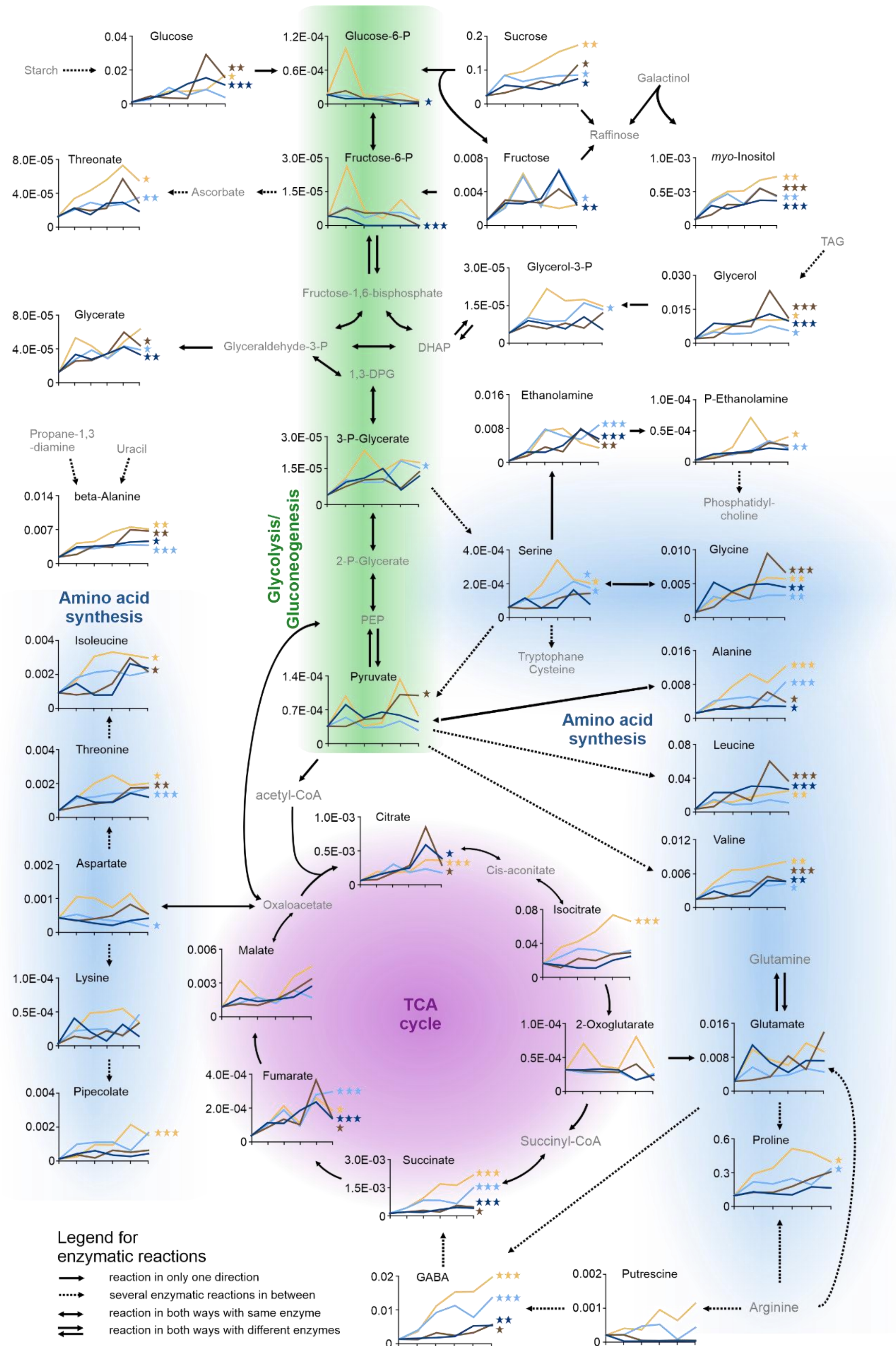


Figure 6 Changes of pollen metabolites traced across different metabolic pathways (Storage experiment). Metabolite levels of pollen of the wheat line TRI 9102 stored under room temperatures at low RH (R-L, beige line) and high RH (R-H, light blue line) and under cold temperatures at low RH (C-L, brown line) and high RH

(C-H, dark blue line) for 10, 20, 30, 45 and 60 minutes (symbolized by the scale line on the x-axis) are shown. Stars indicate significant differences over time for the respective storage conditions provided as Variable Importance in Projection (VIP) score. (***, VIP scores >1.3; **, VIP 1.2 to 1.3; * VIP 1.0 to 1.2). Normalized metabolite levels are provided in Supplemental Table S5. Arrow types as explained in the figure legend.

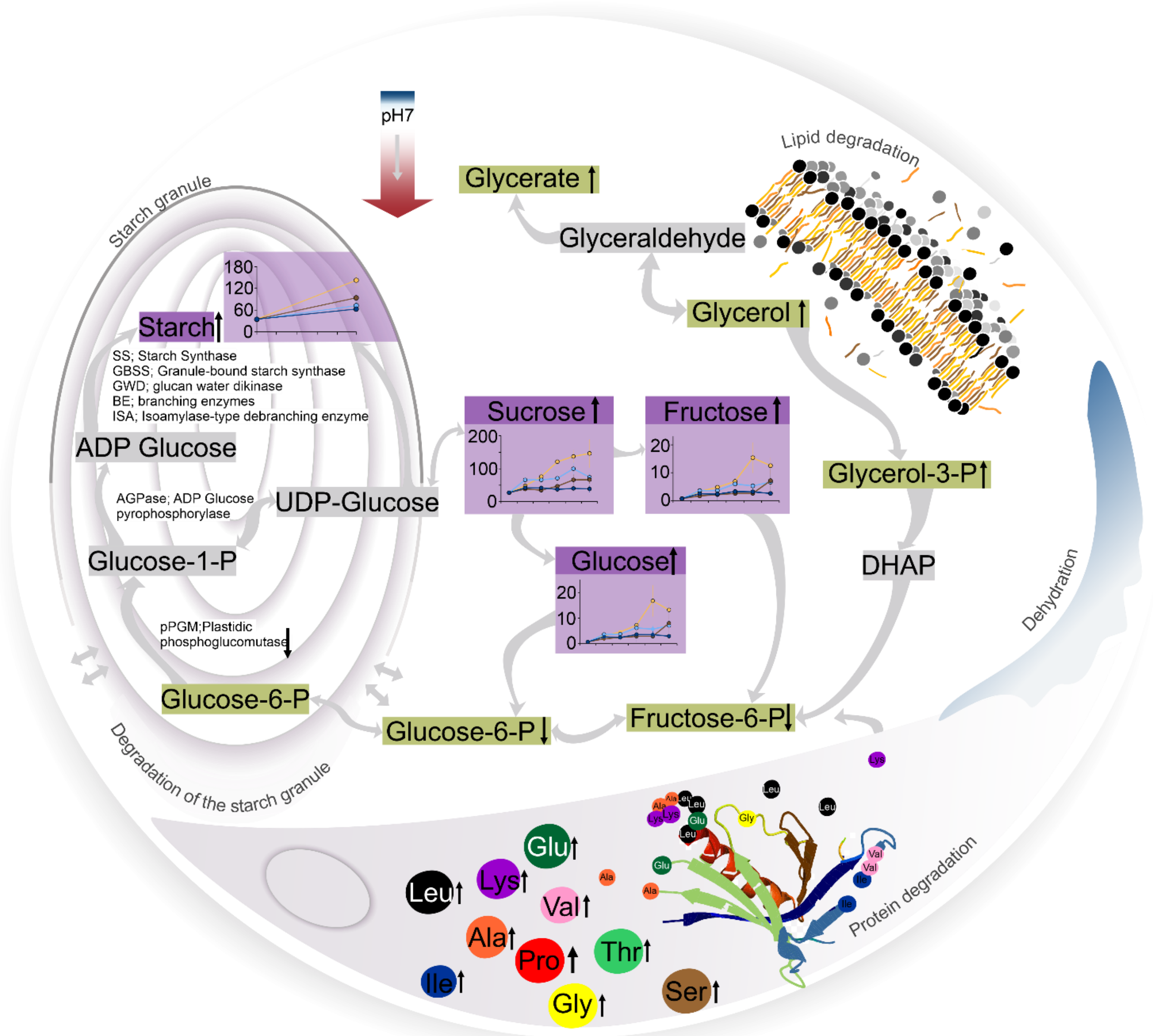


Figure 7 Model explaining increase in soluble carbohydrate and starch content during storage of wheat pollen. Content of glucose, fructose, sucrose and starch of wheat line TRI 9102 was analysed after storage under room temperatures at low RH (R-L, beige line) and high RH (R-H, light blue line) and under cold temperatures at low RH (C-L, brown line) and high RH (C-H, dark blue line) after 10, 20, 30, 45 and 60 min (symbolized by the scale line on the x-axis) and are shown in mg g^{-1} DW. Starch content was measured after 60 min. We hypothesize that high metabolic activity and continuous water loss may lead to lipid and protein degradation indicated by the rise of most metabolites in **Figure 6** and a potential shift in the pH. Degradation products may provide the source for the synthesis of starch according to Lee et al. (2016).

3.3.Supplementary Material

Supplemental Tables

Supplemental Table S1: Freeze substitution and resin infiltration of pollen for the ultrastructure analysis.

Freeze substitution in a Leica automated freeze substitution unit		
Reagent	**Temperature [°C]**	**Time [h]**
2% gluthar aldehyde in 100% acetone	-80	72
2% gluthar aldehyde in 100% acetone	-80 to -70	6
2% gluthar aldehyde in 100% acetone	-70	12
2% gluthar aldehyde in 100% acetone	-70 to -50	6
2% gluthar aldehyde in 100% acetone	-50	24
1% osmium tetroxide in 98% acetone	-50 to -35	6
1% osmium tetroxide in 98% acetone	-35	24
1% osmium tetroxide in 98% acetone	-35 to 10	6
98% Acetone	-10 to 20	3
Infiltration of Spurr resin with 100% acetone		
25% Spurr	21	2
50% Spurr	21	2
75% Spurr	21	2
100% Spurr	21	
Sample transfer into embedding moulds with fresh Spurr		
Polymerisation in heating cabinet	**70**	**48**

Supplemental Table S2 Multiplication factors to adjust amino acid concentrations

Compound peak	**Chosen Target m/z**	**Multiplication factor**
Valine 1TMS	72	1.86
Valine 2TMS	144	2.41
Isoleucine 1TMS	86	2.11
Isoleucine	232	45.2
Leucine 1TMS	86	1.97
Leucine 2TMS	158	2.23
Proline 1TMS	70	1.4
Proline 2TMS	142	1.8
glutamate 3TMS	246	3.07
Oxoproline	258	20
Aspartate 3TMS	232	3.17
Aspartate 2TMS	160	4.68
Threonine 2TMS	219	12.16
Threonine 3TMS	291	14.15
Asparagine 3TMS	231	8.54
Asparagine 2TMS	159	7.22

Supplemental Table S3 Correction factors to estimate the dry mass based on the measured fresh weights. Pollen of TRI 9102 was collected and pollen mass of fresh pollen and pollen stored under room temperatures at low RH (R-L) and high RH (R-H) and under cold temperatures at low RH (C-L) and high RH (C-H) for 10, 20, 30, 45 and 60 minutes was measured. Afterwards, pollen was dried at 100 °C for 24 h, pollen dry weight was registered and correction factors were estimated.

Storage condition	**Storage time [min]**	**Correction factor**
Fresh pollen	0	2.6
R-L	10	1.7
R-L	20	1.4
R-L	30	1.2
R-L	45	1.2
R-L	60	1.1
R-H	10	1.9
R-H	20	2.0
R-H	30	1.7
R-H	45	1.5
R-H	60	1.7
C-L	10	2.4
C-L	20	2.3
C-L	30	2.0
C-L	45	1.9
C-L	60	1.6
C-H	10	2.3
C-H	20	2.3
C-H	30	2.2
C-H	45	2.0
C-H	60	2.1

Supplemental Table S4 RAW data Genotype expriment. Fresh pollen and pollen stored at R-L for 60 minutes of four wheat lines were used for metabolite analysis using GCMS. After pre-processing the metabolite data, metabolites were divided by the highest relative values to obtain data in a range 0 to 1, and normalized for the dry weight by dividig through the correction factor 2.6 and 1.1 for fresh and stored pollen, respectively (**Supplemental Table S3**). **This is a preview and the original table is attached as CSV-file.**

ID	Genotype	Condition	2-Oxoglutarat	3-P-Glycerate	A178003-101	A196004-101	Alanine	Aspartate	beta-Alanine	Citrate
1	TRI 9102	fresh	0,000020033	0,000005679	0,000291411	0,000004794	0,001209868	0,000444379	0,000840112	0,000043203
2	TRI 9102	fresh	0,000032629	0,000005875	0,000188149	0,000000000	0,001475029	0,000520787	0,001283861	0,000064341
3	TRI 9102	fresh	0,000027663	0,000000000	0,000125925	0,000000000	0,001195218	0,000396045	0,001257200	0,000076686
4	TRI 9102	fresh	0,000044908	0,000005569	0,000389795	0,000005618	0,000877345	0,000304136	0,001810274	0,000055769
5	Hermann	fresh	0,000027399	0,000004284	0,000096695	0,000000000	0,001508987	0,000400111	0,002601228	0,000079063
6	Hermann	fresh	0,000037804	0,000014622	0,000117565	0,000000000	0,001284212	0,000467799	0,001970949	0,000091078
7	Hermann	fresh	0,000028082	0,000011444	0,000098630	0,000000000	0,001717499	0,000470785	0,001661799	0,000126757
8	Hermann	fresh	0,000012929	0,000000000	0,000044474	0,000000000	0,001481798	0,000267486	0,000907734	0,000316129
9	Ferrum	fresh	0,000041425	0,000011165	0,000168587	0,000000000	0,001693112	0,000365029	0,001567971	0,000057022
10	Ferrum	fresh	0,000068792	0,000006093	0,000162728	0,000000000	0,000624145	0,000243374	0,000048073	0,000027632
11	Ferrum	fresh	0,000003680	0,000004133	0,000012095	0,000000000	0,004219049	0,000121780	0,001472588	0,000036374
12	Ferrum	fresh	0,000054858	0,000006151	0,000286856	0,000000000	0,002413784	0,000254782	0,001704439	0,000057952
13	TRI 4399	fresh	0,000019992	0,000007269	0,000199113	0,000000000	0,001019674	0,000453258	0,001298311	0,000082025
14	TRI 4399	fresh	0,000012012	0,000004032	0,000109531	0,000000000	0,000587534	0,000266543	0,000816034	0,000048990
15	TRI 4399	fresh	0,000036879	0,000005641	0,000126096	0,000000000	0,000970439	0,000321334	0,001133897	0,000068178
16	TRI 4399	fresh	0,000010283	0,000000000	0,000166726	0,000000000	0,000808344	0,000365445	0,000904433	0,000080325
17	TRI 9102	R-L	0,000061132	0,000015108	0,001151369	0,000021220	0,013214187	0,000702418	0,008563047	0,000454660
18	TRI 9102	R-L	0,000017690	0,000048610	0,001892384	0,000000000	0,016028556	0,000294574	0,002969768	0,000339502
19	TRI 9102	R-L	0,000031184	0,000000000	0,000754543	0,000102662	0,007719190	0,000549176	0,007467869	0,000354209
20	TRI 9102	R-L	0,000029596	0,000009194	0,000692582	0,000000000	0,010841697	0,000515671	0,008935999	0,000257012
21	Ferrum	R-L	0,000000000	0,000000000	0,001458584	0,000000000	0,016895393	0,000000000	0,002165759	0,000869146
22	Ferrum	R-L	0,000054311	0,000026584	0,001355806	0,000000000	0,013876490	0,001726132	0,009890387	0,000372233
23	Ferrum	R-L	0,000000000	0,000032178	0,001020262	0,000000000	0,007753917	0,000365675	0,004429912	0,001718814
24	Ferrum	R-L	0,000025486	0,000027490	0,001277838	0,000000000	0,020923155	0,000445782	0,004749822	0,000284364
25	Hermann	R-L	0,000038236	0,000031469	0,000450024	0,000000000	0,008262431	0,001874348	0,009435354	0,000436667
26	Hermann	R-L	0,000000000	0,000000000	0,000510587	0,000000000	0,004990498	0,000532440	0,006439395	0,000705921
27	Hermann	R-L	0,000045577	0,000034685	0,000377199	0,000000000	0,010696216	0,000962358	0,005816908	0,000389321
28	Hermann	R-L	0,000050721	0,000062618	0,001252091	0,000000000	0,012498867	0,001229222	0,006419452	0,000390095
29	TRI 4399	R-L	0,000000000	0,000000000	0,000784001	0,000000000	0,006850267	0,001009645	0,005077457	0,001264816
30	TRI 4399	R-L	0,000032037	0,000019434	0,001056704	0,000000000	0,007276571	0,000674677	0,004865915	0,000226947
31	TRI 4399	R-L	0,000026979	0,000022479	0,001223372	0,000000000	0,012666923	0,001068320	0,005134152	0,000451373
32	TRI 4399	R-L	0,000030889	0,000000000	0,000661645	0,000000000	0,005863359	0,001774714	0,002959010	0,000441562

ID	Dimethylglycin	Ethanolamine	Fructose	Fructose-6-P	Fumarate	GABA	Glucose	Glucose-6-P	Glutamate	Glycerate	Glycerol
1	0,000137832	0,000513428	0,000511630	0,000006148	0,000030309	0,001023205	0,000933073	0,000018942	0,001503012	0,000008785	0,001011742
2	0,004006893	0,000234185	0,000442504	0,000003741	0,000026482	0,001538441	0,000715127	0,000021433	0,001073318	0,000013806	0,002423968
3	0,002767476	0,000573797	0,000891875	0,000000000	0,000047160	0,001192322	0,001289256	0,000000000	0,002903003	0,000015515	0,002524121
4	0,002238395	0,000158849	0,000836358	0,000006466	0,000040656	0,001715692	0,001421405	0,000024114	0,003536295	0,000012754	0,002195071
5	0,000688186	0,002977331	0,002441696	0,000000000	0,000057451	0,001550693	0,004187908	0,000000000	0,002976506	0,000016205	0,006409676
6	0,001600135	0,001567055	0,000871600	0,000000000	0,000055804	0,001841137	0,002057012	0,000006996	0,002433100	0,000015363	0,006045693
7	0,001380444	0,001351676	0,001065077	0,000000000	0,000061018	0,001630235	0,002065642	0,000005654	0,005515250	0,000015213	0,006231883
8	0,001019443	0,001441466	0,002152015	0,000000000	0,000034816	0,002069307	0,002986789	0,000000000	0,001169859	0,000013490	0,005459081
9	0,000748225	0,000915602	0,000415810	0,000004478	0,000028407	0,002320170	0,000531854	0,000013526	0,003758498	0,000012450	0,003854170
10	0,000949009	0,000030165	0,000434144	0,000004994	0,000028285	0,000063603	0,000625648	0,000011030	0,009478898	0,000007724	0,003869896
11	0,001014305	0,017555702	0,044191208	0,000002783	0,000479488	0,013672234	0,089569772	0,000011983	0,002622288	0,000477289	0,011921190
12	0,000726136	0,001059029	0,000759124	0,000002288	0,000040506	0,001954688	0,001207292	0,000010930	0,003584552	0,000010203	0,001841398
13	0,000553365	0,000361431	0,000660041	0,000009287	0,000022814	0,000863891	0,001375407	0,000030675	0,006034240	0,000014451	0,002367671
14	0,000625793	0,001053171	0,001064413	0,000000000	0,000015513	0,000452418	0,002177771	0,000009375	0,003469934	0,000012494	0,001901319
15	0,000802252	0,001030725	0,001177970	0,000005070	0,000026762	0,001183279	0,002132656	0,000025523	0,005231837	0,000017914	0,001821755
16	0,000872282	0,000173952	0,000354583	0,000005881	0,000016410	0,000740476	0,000670756	0,000021338	0,004648653	0,000014654	0,001939474
17	0,005473129	0,002256819	0,001955438	0,000012417	0,000161645	0,022808917	0,003820234	0,000026660	0,012397245	0,000082530	0,006081990
18	0,014063496	0,002185326	0,003308132	0,000000000	0,000161965	0,025680443	0,005966417	0,000000000	0,005067447	0,000082211	0,006914502
19	0,022781881	0,006557191	0,002728030	0,000000000	0,000255623	0,009463859	0,033044818	0,000000000	0,009036006	0,000042635	0,015313273
20	0,017490615	0,002430546	0,001623259	0,000000000	0,000154252	0,019258049	0,022943147	0,000000000	0,010039868	0,000041163	0,012923938
21	0,023897362	0,006019868	0,002283140	0,000000000	0,000000000	0,008686999	0,005250295	0,000000000	0,009395596	0,000000000	0,008902996
22	0,004427967	0,006719793	0,001366684	0,000005796	0,000133682	0,016355429	0,002410451	0,000012085	0,022891447	0,000037708	0,007245503
23	0,010965224	0,007768832	0,010665619	0,000000000	0,000188765	0,007092166	0,011254458	0,000000000	0,022092644	0,000082886	0,008051290
24	0,002872874	0,004406314	0,003402452	0,000000000	0,000148332	0,011356146	0,005740420	0,000007035	0,010159095	0,000061467	0,006356802
25	0,001965334	0,015379269	0,003874482	0,000000000	0,000196118	0,010180573	0,006371942	0,000000000	0,031696722	0,000046302	0,019148599
26	0,005610189	0,006717285	0,012495868	0,000000000	0,000259586	0,004933464	0,017594635	0,000000000	0,001752138	0,000079298	0,019878419
27	0,007835163	0,011675901	0,012401701	0,000000000	0,000419761	0,008870697	0,017765960	0,000000000	0,009057752	0,000105258	0,018815734
28	0,008231804	0,013252644	0,002719639	0,000000000	0,000203651	0,010936080	0,004020005	0,000000000	0,022230369	0,000048633	0,022662035
29	0,002394582	0,005375849	0,008782872	0,000000000	0,000170226	0,010230913	0,031553380	0,000000000	0,013844958	0,000037320	0,021011321
30	0,003161837	0,008885228	0,003190417	0,000000000	0,000108254	0,012189885	0,005135924	0,000000000	0,007099043	0,000029610	0,016968756
31	0,004089596	0,008209164	0,009696956	0,000000000	0,000218612	0,012686940	0,013032057	0,000000000	0,016790854	0,000086548	0,013233907
32	0,001257875	0,004285022	0,003272720	0,000000000	0,000119469	0,009383754	0,002380197	0,000000000	0,015400839	0,000041586	0,012055393

ID	Glycerol-3-P	Glycine	Hexanedioic a	Hexose-Phosp	Inositol-P	Isocitrate	Isoleucine	Leucine	Lysine	Malate
1	0,000005232	0,000439320	0,000017539	0,000003200	0,000006691	0,012422346	0,001920130	0,001910478	0,000007687	0,000331988
2	0,000005397	0,000989972	0,000001566	0,000000000	0,000010479	0,017256869	0,000560475	0,004566794	0,000004374	0,001025951
3	0,000001199	0,000983779	0,000001740	0,000000000	0,000006307	0,015294580	0,000486456	0,004970001	0,000000000	0,001070882
4	0,000004389	0,000714398	0,000005527	0,000000000	0,000006767	0,019064380	0,000612745	0,002226902	0,000000000	0,001050112
5	0,000003371	0,001777063	0,000000000	0,000000000	0,000011227	0,011890203	0,000743502	0,007709738	0,000017469	0,001001253
6	0,000011517	0,002010153	0,000000000	0,000000000	0,000019965	0,014360438	0,000937570	0,006347731	0,000023098	0,001054135
7	0,000010425	0,002002539	0,000000000	0,000000000	0,000015605	0,017782741	0,000653096	0,008231740	0,000020447	0,000810006
8	0,000000000	0,001873492	0,000000000	0,000000000	0,000010435	0,009461896	0,000537614	0,009929300	0,000000000	0,000759088
9	0,000009588	0,001221360	0,000000000	0,000004034	0,000006024	0,022411239	0,001135738	0,003104968	0,000012021	0,001131522
10	0,000005390	0,000202988	0,000001961	0,000000000	0,000006231	0,019509398	0,001593176	0,001207486	0,000000000	0,000983831
11	0,000003094	0,020630255	0,000003540	0,000000000	0,000004445	0,000145109	0,013393217	0,015863584	0,000266581	0,001384693
12	0,000004973	0,000989142	0,000000000	0,000000000	0,000003291	0,023552307	0,002557725	0,002167026	0,000020637	0,001012362
13	0,000005057	0,001503359	0,000000000	0,000000000	0,000009808	0,009817937	0,000590584	0,005941738	0,000034622	0,001116482
14	0,000003075	0,000987995	0,000000000	0,000000000	0,000005161	0,006705032	0,000336226	0,003807022	0,000017269	0,000531189
15	0,000005356	0,001321693	0,000000000	0,000000000	0,000007034	0,007992735	0,000490045	0,005111488	0,000034866	0,001120690
16	0,000000000	0,001195148	0,000000000	0,000000000	0,000007195	0,009113355	0,000602260	0,005128341	0,000017693	0,001211811
17	0,000013365	0,003837704	0,000035206	0,000004936	0,000018540	0,071919231	0,003129837	0,012030817	0,000071687	0,007142602
18	0,000044645	0,002918300	0,000005834	0,000000000	0,000032901	0,082619764	0,003203191	0,012716283	0,000011320	0,005720465
19	0,000000000	0,009299053	0,000029017	0,000000000	0,000051216	0,054148375	0,002306277	0,044485655	0,000000000	0,001949524
20	0,000000000	0,006440360	0,000034466	0,000000000	0,000035647	0,051125728	0,003015812	0,028436600	0,000054489	0,002690690
21	0,000000000	0,003465444	0,000000000	0,000000000	0,000000000	0,073191141	0,008332250	0,051499023	0,000000000	0,001389373
22	0,000022625	0,005828816	0,000004287	0,000000000	0,000017640	0,123738890	0,007334367	0,009277378	0,000040258	0,004066688
23	0,000000000	0,007472551	0,000000000	0,000000000	0,000102886	0,057644894	0,006905168	0,030777134	0,000068157	0,002285482
24	0,000021492	0,004338402	0,000000000	0,000000000	0,000007733	0,068501722	0,006945800	0,007599429	0,000098307	0,002736285
25	0,000025930	0,014352219	0,000011635	0,000000000	0,000058286	0,080858097	0,005577113	0,036893671	0,000142225	0,003317532
26	0,000000000	0,009128374	0,000000000	0,000000000	0,000051689	0,024401737	0,002695977	0,053800403	0,000032420	0,002254171
27	0,000000000	0,007720399	0,000000000	0,000000000	0,000063735	0,053761131	0,003564015	0,035537488	0,000074457	0,005719831
28	0,000054349	0,008196757	0,000000000	0,000000000	0,000090454	0,064432381	0,009132801	0,038076221	0,000079841	0,004353436
29	0,000000000	0,009993509	0,000000000	0,000000000	0,000029169	0,042168872	0,003004359	0,046000077	0,000207516	0,004021256
30	0,000000000	0,007851525	0,000000000	0,000000000	0,000026596	0,053416616	0,003038820	0,029967829	0,000174405	0,001132807
31	0,000000000	0,007245138	0,000000000	0,000000000	0,000029635	0,048053687	0,002530129	0,026544358	0,000195772	0,003867692
32	0,000000000	0,004062796	0,000000000	0,000000000	0,000028329	0,061893070	0,002825966	0,016844384	0,000102049	0,002897425

ID	myo-Inositol	P-Ethanolami	Phosphate	Pipecolate	Proline	Putrescine	Pyruvate	Serine	Succinate	Sucrose	Threonate	Threonine	U1	U2	Valine
1	0,000109762	0,000003595	0,014834869	0,000114571	0,030420123	0,000177622	0,000018076	0,000021293	0,000124891	0,009977478	0,000012053	0,000388512	0,000690382	0,000003851	0,001422586
2	0,000082167	0,000000000	0,016259567	0,000071062	0,126955930	0,000584164	0,000041356	0,000050964	0,000110600	0,029698435	0,000011538	0,000413201	0,000452603	0,000012346	0,001575430
3	0,000084342	0,000000000	0,019163867	0,000052047	0,104797845	0,000021595	0,000038606	0,000059303	0,000130499	0,023882075	0,000013706	0,000345738	0,000842302	0,000003199	0,001162748
4	0,000085339	0,000008309	0,021488613	0,000239936	0,113731540	0,000029740	0,000043664	0,000108562	0,000162038	0,034710539	0,000012184	0,000485010	0,000381667	0,000001691	0,001553287
5	0,000146286	0,000006910	0,017197800	0,000533528	0,138449843	0,000053864	0,000037043	0,000042068	0,000230329	0,039402059	0,000024131	0,000647825	0,000486909	0,000015515	0,001890160
6	0,000147237	0,000011423	0,021023515	0,000788814	0,159937591	0,000029451	0,000050098	0,000057787	0,000271174	0,031107143	0,000031346	0,000667413	0,000967509	0,000013417	0,001800799
7	0,000124984	0,000016127	0,023923999	0,001075478	0,128800744	0,000018323	0,000059276	0,000046311	0,000242330	0,033055046	0,000037557	0,000745465	0,000957382	0,000010144	0,001610947
8	0,000111776	0,000000000	0,017346066	0,000365388	0,084915195	0,000000000	0,000016091	0,000042257	0,000166864	0,016452550	0,000022259	0,000410641	0,000391937	0,000000000	0,001361372
9	0,000108730	0,000003341	0,017382149	0,000726888	0,138976264	0,000387679	0,000023464	0,000084096	0,000131507	0,032170503	0,000013656	0,000875960	0,000320257	0,000026488	0,004532070
10	0,000085624	0,000002567	0,019666649	0,000732425	0,120956914	0,000000000	0,000031333	0,000216334	0,000129029	0,017174431	0,000011426	0,001173910	0,000215778	0,000000000	0,001623938
11	0,005240134	0,000000000	0,021742856	0,001206138	0,085782051	0,000149946	0,000034703	0,000905965	0,000018433	0,000000000	0,000020732	0,012877350	0,000533696	0,000002134	0,019465794
12	0,000105307	0,000002793	0,013594617	0,001498850	0,084213322	0,000251311	0,000027699	0,000332093	0,000101132	0,020749927	0,000007286	0,001560974	0,000178594	0,000054380	0,005436660
13	0,000107890	0,000005235	0,017992609	0,000128552	0,102262920	0,000399499	0,000061959	0,000036289	0,000165483	0,041719071	0,000012143	0,000518666	0,000455174	0,000013486	0,001522923
14	0,000088791	0,000003720	0,012767982	0,000053901	0,078385733	0,000415984	0,000026037	0,000033328	0,000064652	0,021084944	0,000005129	0,000391775	0,000282539	0,000007556	0,001403369
15	0,000102468	0,000005493	0,015047219	0,000120790	0,077698263	0,000189210	0,000049024	0,000030558	0,000104525	0,036738929	0,000009993	0,000430115	0,000492946	0,000010863	0,001262736
16	0,000099131	0,000000000	0,014257552	0,000082841	0,089486989	0,000199929	0,000034899	0,000055822	0,000085981	0,046740046	0,000007149	0,000307720	0,000313853	0,000008931	0,001668527
17	0,000880119	0,000035538	0,071553607	0,000690074	0,374106527	0,001689510	0,000059124	0,000140854	0,002520176	0,143164156	0,000040766	0,002039129	0,002825456	0,000011421	0,008860126
18	0,000810633	0,000023287	0,050712752	0,000972785	0,284304465	0,002493102	0,000037439	0,000272863	0,002374725	0,127476882	0,000048308	0,002646490	0,001627843	0,000018394	0,009557989
19	0,000522229	0,000031513	0,086970787	0,002228663	0,455012913	0,000115261	0,000070226	0,000183821	0,001228804	0,114615561	0,000086330	0,001511054	0,003469298	0,000000000	0,005372138
20	0,000601841	0,000066292	0,107506516	0,002039487	0,442185128	0,000195478	0,000062098	0,000202253	0,002384343	0,294254985	0,000040962	0,001666628	0,001874028	0,000000000	0,007957072
21	0,000703405	0,000000000	0,066550484	0,000654432	0,808091445	0,048008752	0,000000000	0,002506620	0,000899466	0,217029420	0,000000000	0,006915503	0,000000000	0,000000000	0,018520672
22	0,000515759	0,000053876	0,079639639	0,004110677	0,476257688	0,000955819	0,000082143	0,000814759	0,001716898	0,129113867	0,000052025	0,005342196	0,001485903	0,000099865	0,014686590
23	0,000528704	0,000060970	0,064160617	0,001553041	0,659947157	0,012674178	0,000067406	0,002126135	0,000912437	0,155786344	0,000000000	0,005897807	0,000451990	0,000031276	0,011197979
24	0,000505179	0,000024716	0,044313554	0,003095796	0,330111603	0,000931290	0,000039629	0,001087623	0,001013832	0,097314256	0,000045026	0,004595022	0,000682820	0,000062728	0,014408033
25	0,000598613	0,000063292	0,129856625	0,003323812	0,648427228	0,000180822	0,000143278	0,000311915	0,001103960	0,188964243	0,000121904	0,005216674	0,002838310	0,000042479	0,009934266
26	0,000524242	0,000046366	0,064343129	0,000793787	0,292140887	0,000031582	0,000065248	0,000133070	0,000643962	0,109734101	0,000081128	0,002274057	0,003040850	0,000000000	0,006890927
27	0,000812538	0,000090759	0,118244282	0,001598406	0,482641819	0,000073314	0,000084880	0,000294396	0,001197655	0,163043322	0,000104953	0,002473549	0,003629545	0,000000000	0,008203442
28	0,000793824	0,000077001	0,134681683	0,005671002	0,909090909	0,000071942	0,000154207	0,000375413	0,001714764	0,302319798	0,000085252	0,003815291	0,002926866	0,000034369	0,020558400
29	0,000695694	0,000000000	0,087872888	0,000891849	0,518969921	0,000700649	0,000077772	0,000186367	0,001107129	0,352452936	0,000086605	0,001779395	0,003280683	0,000000000	0,008160302
30	0,000577517	0,000067204	0,114802489	0,000509028	0,635805387	0,000107079	0,000053445	0,000127577	0,001445225	0,138738802	0,000055440	0,002505342	0,002679024	0,000020696	0,006183159
31	0,000887745	0,000060915	0,102294928	0,000691713	0,536617257	0,000673091	0,000076161	0,000425483	0,001434239	0,235033664	0,000056968	0,003646222	0,001955473	0,000032023	0,011461623
32	0,000531938	0,000000000	0,091677526	0,000675199	0,530772206	0,000177492	0,000128090	0,000222710	0,000958618	0,161957154	0,000043036	0,003347356	0,000932723	0,000043970	0,011591060

Supplemental Table S5 RAW data Storage experiment. Pollen of wheat line TRI 9102 were stored under four condtions (R-L, R-H, C-L and C-H) for up to 60 minutes and used for metabolite analysis using GCMS. After pre-processing the metabolite data, metabolites were divided by the highest relative values to obtain data in a range 0 to 1, and normalized for the dry weight by dividing through the correction factor provided in **Supplemental Table S3. This is a preview and the original table is attached as CSV-file.**

ID	condition	Time	2-Oxogluta	3-P-Glycer	A178003-1	A196004-1	Alanine	Aspartate	beta-Alanir	Citrate	Dimethylgl	Ethanolam	Fructose	Fructose-6	Fumarate	GABA	Glucose	Glucose-6-	Glutamate	Glycerate	Glycerol	Glycerol-3-	Glycine	Hexanedio	Hexose-Ph	Inositol-P	Isocitrate	Isoleucine	Leucine	Lysine	Malate	myo-Inosit	P-Ethanola	Phosphate	Pipecolate	Proline	Putrescine	Pyruvate	Serine	Succinate	Sucrose	Threonate	Threonine	U1	U2	Valine
1	R-L	10 min	0,00016279	0,00002499	0,00124629	0,00006849	0,00491751	0,00171450	0,00639193	0,00019237	0,00539770	0,00068504	0,00301342	0,00009556	0,00018227	0,00514593	0,00401702	0,00035819	0,01007921	0,00008474	0,00633139	0,00002140	0,00344895	0,00016517	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	R-L	10 min	0,00005162	0,00000678	0,00045385	0,00000000	0,00287170	0,00114689	0,00336974	0,00020453	0,00278803	0,00173362	0,00249173	0,00000000	0,00008613	0,00321557	0,00288498	0,00001055	0,01253186	0,00003787	0,00420018	0,00000553	0,00240546	0,00000785	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
3	R-L	10 min	0,00002458	0,00000376	0,00038406	0,00000000	0,00236274	0,00088429	0,00330176	0,00012969	0,00399968	0,00182392	0,00148046	0,00000000	0,00007155	0,00236597	0,00207112	0,00000000	0,00792759	0,00003817	0,00624916	0,00000302	0,00214140	0,00000293	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	R-L	10 min	0,00004252	0,00001250	0,00041160	0,00002000	0,00353759	0,00044464	0,00358648	0,00023936	0,00281445	0,00236123	0,00247571	0,00000890	0,00007215	0,00385822	0,00318364	0,00002399	0,00841383	0,00005134	0,00536596	0,00001114	0,00246949	0,00001758	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
5	R-L	20 min	0,00004698	0,00006249	0,00207810	0,00003228	0,01044415	0,00189934	0,00654094	0,00028848	0,00478506	0,00712136	0,00744025	0,00002004	0,00050375	0,01401586	0,00688530	0,00004016	0,01303029	0,00007626	0,00810497	0,00005665	0,00350563	0,00006815	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
6	R-L	20 min	0,00004728	0,00001742	0,00104065	0,00003356	0,00777419	0,00076143	0,00411348	0,00015693	0,00496079	0,00847264	0,00717431	0,00000000	0,00017800	0,00983825	0,00795146	0,00000731	0,00354595	0,00004930	0,01062391	0,00001561	0,00338332	0,00000825	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
7	R-L	20 min	0,00003224	0,00000626	0,00045609	0,00001039	0,00583905	0,00074107	0,00332848	0,00012788	0,00525036	0,00534533	0,00271419	0,00000000	0,00007871	0,00913374	0,00363032	0,00000000	0,00889256	0,00002619	0,00668419	0,00000587	0,00347682	0,00000342	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
8	R-L	20 min	0,00002290	0,00000930	0,00065234	0,00000000	0,00570260	0,00062264	0,00404062	0,00013062	0,00529301	0,00857367	0,00719698	0,00000632	0,00009026	0,01176109	0,01101938	0,00001304	0,00407034	0,00002200	0,00685912	0,00000858	0,00351205	0,00002014	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
9	R-L	30 min	0,00002836	0,00002581	0,00106641	0,00001047	0,01180540	0,00132949	0,00885337	0,00030249	0,00430895	0,00423009	0,00182741	0,00001246	0,00010670	0,01768063	0,00339138	0,00003179	0,00841438	0,00002384	0,00514344	0,00002286	0,00319247	0,00003717	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
10	R-L	30 min	0,00001696	0,00001909	0,00064825	0,00000922	0,00662179	0,00059086	0,00356373	0,00011067	0,00658943	0,00501276	0,00303931	0,00000000	0,00010308	0,00992677	0,00342118	0,00000556	0,00151273	0,00002644	0,00824665	0,00001470	0,00339597	0,00000693	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
11	R-L	30 min	0,00003291	0,00000000	0,00082508	0,00000000	0,01198702	0,00067334	0,00746232	0,00018616	0,01522845	0,00734606	0,00343205	0,00000000	0,00013930	0,01710728	0,00441562	0,00000000	0,01068261	0,00002998	0,01325216	0,00001360	0,00526934	0,00000464	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
12	R-L	30 min	0,00005687	0,00001389	0,00112861	0,00000000	0,01129407	0,00032239	0,00609811	0,00016782	0,01179087	0,01528776	0,00142493	0,00000000	0,00009044	0,01640145	0,01840903	0,00001632	0,00376708	0,00003533	0,01560546	0,00001624	0,00685407	0,00003225	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
13	R-L	45 min	0,00014377	0,00003535	0,00120980	0,00004009	0,00823881	0,00181662	0,00866825	0,00040262	0,00934042	0,00205100	0,00309185	0,00003419	0,00042550	0,01832641	0,00325118	0,00004523	0,01797999	0,00006223	0,00813154	0,00003063	0,00489424	0,00006654	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
14	R-L	45 min																																												
15	R-L	45 min	0,00005800	0,00001265	0,00058250	0,00006011	0,00829750	0,00054292	0,00618140	0,00028689	0,02007159	0,00718306	0,00133299	0,00000000	0,00021115	0,01019587	0,01959703	0,00000000	0,00423885	0,00004064	0,01512426	0,00001233	0,00753681	0,00001631	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
16	R-L	45 min	0,00003997	0,00001149	0,00107997	0,00000000	0,00866927	0,00104132	0,00766433	0,00039928	0,00876191	0,00449800	0,00166181	0,00000000	0,00014396	0,01763097	0,00228839	0,00001153	0,01175095	0,00004290	0,00712427	0,00000925	0,00519176	0,00002299	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
17	C-L	60 min	0,00006226	0,00001539	0,00117269	0,00002161	0,01345889	0,00071543	0,00872162	0,00046308	0,00557448	0,00229861	0,00199165	0,00001265	0,00016464	0,02323130	0,00389098	0,00002715	0,01262682	0,00008406	0,00619462	0,00001361	0,00390877	0,00003586	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
18	C-L	60 min	0,00001802	0,00004951	0,00192743	0,00000000	0,01632538	0,00030003	0,00302476	0,00034579	0,01432393	0,00222579	0,00336939	0,00000000	0,00016496	0,02615601	0,00607691	0,00000000	0,00516129	0,00008373	0,00704255	0,00004547	0,00297234	0,00000594	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
19	C-L	60 min	0,00003176	0,00000000	0,00076852	0,00010456	0,00786214	0,00055935	0,00760616	0,00036077	0,02320377	0,00667862	0,00277855	0,00000000	0,00026036	0,00963912	0,03365676	0,00000000	0,00920334	0,00004343	0,01559685	0,00000000	0,00947126	0,00002955	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
20	C-L	60 min	0,00003014	0,00000936	0,00070541	0,00000000	0,01104247	0,00052522	0,00910148	0,00026177	0,01781452	0,00247556	0,00165332	0,00000000	0,00015711	0,01961468	0,02336802	0,00000000	0,01022579	0,00004193	0,01316327	0,00000000	0,00655963	0,00003510	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
21	R-H	10 min	0,00003773	0,00001030	0,00058631	0,00001331	0,00353550	0,00073855	0,00341320	0,00015910	0,00346966	0,00394273	0,00260913	0,00000925	0,00008530	0,00480162	0,00394279	0,00002490	0,00639992	0,00002797	0,00217655	0,00001016	0,00246639	0,00001726	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
22	R-H	10 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
23	R-H	10 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
24	R-H	10 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
25	R-H	20 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
26	R-H	20 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
27	R-H	20 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
28	R-H	20 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
29	R-H	30 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
30	R-H	30 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
31	R-H	30 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
32	R-H	30 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
33	R-H	45 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
34	R-H	45 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
35	R-H	45 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
36	R-H	45 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
37	C-H	60 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
38	C-H	60 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
39	C-H	60 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
40	C-H	60 min	0,00001976	0,00002256	0,00024869	0,00003346	0,00688831	0,00022852	0,00399936	0,00022834	0,01035599	0,00826249	0,00512487	0,00000000	0,00033471	0,01118862	0,00815794	0,00000000	0,00425948	0,00005493	0,00716105	0,00001914	0,00438864	0,00002244	0,00000000	0,00001889	0,02492146	0,00219537	0,01477269	0,00007501	0,00210435	0,00043457	0,00003697	0,06208127	0,00145055	0,37262355	0,00030577	0,00002598	0,00013099	0,00110026	0,09102760	0,00003821	0,00178422	0,00128535	0,00000000	0,00417582
41	C-L	10 min	0,00005509	0,00001461	0,00027801	0,00000468	0,00290269	0,00024048	0,00157909	0,00010101	0,00300673	0,00144416	0,00162629	0,00001186	0,00012173	0,00145021	0,00173327	0,00002669	0,00151525	0,00002204	0,00274830	0,00001363	0,00158675	0,00000751	0,00000423	0,00001667	0,01750764	0,00073661	0,00573260	0,00002578	0,00067241	0,00017396	0,00000589	0,02751251	0,00082350	0,14110657	0,00014233	0,00004424	0,00009115	0,00035979	0,02381569	0,00005137	0,00072836	0,00085068	0,00001296	0,00150277
42	C-L	10 min	0,00002822	0,00000312	0,00026719	0,00000145	0,00115035	0,00040665	0,00142566	0,00004692	0,00134344	0,00080007	0,00097061	0,00000000	0,00002886	0,00096111	0,00151997	0,00000415	0,00043615	0,00001190	0,00153456	0,00000233	0,00119543	0,00000264	0,00000000	0,00000525	0,00976574	0,00065758	0,00542206	0,00000000	0,00084480	0,00010017	0,00000000	0,01420834	0,00013969	0,12166291	0,00006452	0,00004201	0,00003774	0,00010136	0,03287577	0,00000808	0,00040609	0,00091976	0,00000661	0,00126667
43	C-L	10 min	0,00001496	0,00000613	0,00041812	0,00001278	0,00135598	0,00034839	0,00196335	0,00004730	0,00394380	0,00050298	0,00076254	0,00001415	0,00003959	0,00104313	0,00122104	0,00004847	0,00292423	0,00002104	0,00150216	0,00000545	0,00136792	0,00002051	0,00000672	0,00001364	0,00876915	0,00069627	0,00553393	0,00000844	0,00132599	0,00013325	0,00000672	0,01843027	0,00015712	0,12942058	0,00056870	0,00002947	0,00003140	0,00010707	0,04341810	0,00000812	0,00044613	0,00049408	0,00001154	0,00151687
44	C-L	10 min	0,00002202	0,00000816	0,00025271	0,00002421	0,00253597	0,00036383	0,00248018	0,00010003	0,00578505	0,00325731	0,00863589	0,00000475	0,00014913	0,00165803	0,01390946	0,00001387	0,00542044	0,00004762	0,00447878	0,00000695	0,00210510	0,00001375	0,00000000	0,00001375	0,00949141	0,00109583	0,00900441	0,00002099	0,00172809	0,00022916	0,00001160	0,02092420	0,00031637	0,11125518	0,00005925	0,00002591	0,00005582	0,00025160	0,03194942	0,00002284	0,00087446	0,00074728	0,00000000	0,00209425
45	C-L	20 min	0,00004371	0,00001699	0,00032047	0,00000473	0,00394069	0,00071304	0,00427361	0,00017006	0,00610037	0,00263545	0,00213138	0,00001288	0,00010252	0,00369992	0,00224458	0,00002708	0,00307985	0,00003131	0,00748836	0,00001335	0,00300918	0,00001516	0,00000000	0,00003692	0,02992465	0,00138680	0,01277683	0,00002501	0,00126469	0,00035796	0,00001336	0,04326722	0,00024269	0,20702467	0,00008324	0,00005823	0,00009616	0,00028995	0,07468039	0,00001965	0,00104828	0,00143092	0,00002339	0,00325368
46	C-L	20 min	0,00001098	0,00000000	0,00016290	0,00000000	0,00159828	0,00022775	0,00177519	0,00007240	0,00512935	0,00280862	0,00170634	0,00000000	0,00006774	0,00123074	0,00211792	0,00000000	0,00060861	0,00000817	0,00476351	0,00000000	0,00211241	0,00000469	0,00000000	0,00001212	0,01321241	0,00046896	0,01376849	0,00000000	0,00051628	0,00013683	0,00000000	0,02959048	0,00004762	0,07178121	0,00000641	0,00002630	0,00002646	0,00012517	0,01751385	0,00001878	0,00042860	0,00109788	0,00000000	0,00127663
47	C-L	20 min	0,00003026	0,00001209	0,00031872	0,00003526	0,00314276	0,00032521	0,00386983	0,00019708	0,00909872	0,00323605	0,00159396	0,00000919	0,00011609	0,00402259	0,00176760	0,00001353	0,00572072	0,00002125	0,00641469	0,00000959	0,00325504	0,00005116	0,00000000	0,00003453	0,03701349	0,00106194	0,01565008	0,00001678	0,00173674	0,00043316	0,00001515	0,06678591	0,00026574	0,14164808	0,00010048	0,00004361	0,00006686	0,00029790	0,07929943	0,00003971	0,00097516	0,00103636	0,00001295	0,00241475
48	C-L	20 min	0,00003142	0,00001443	0,00017207	0,00011386	0,00319233	0,00033485	0,00452703	0,00025709	0,02999530	0,00567212	0,00597093	0,00000000	0,00024967	0,00397468	0,00769934	0,00000000	0,00409912	0,00004437	0,01187689	0,00000000	0,00672258	0,00005639	0,00000000	0,00004226	0,00850801	0,00068217	0,04738073	0,00000000	0,00051096	0,00031043	0,00002574	0,03820945	0,00014517	0,07499466	0,00000000	0,00007364	0,00003404	0,00043054	0,02150138	0,00000000	0,00071737	0,00111897	0,00000000	0,00195736
49	C-L	30 min	0,00004280	0,00001871	0,00030451	0,00000086	0,00324107	0,00108284	0,00470318	0,00014007	0,00604429	0,00214986	0,00289803	0,00000845	0,00009867	0,00230363	0,00343113	0,00001693	0,00433555	0,00002816	0,00836504	0,00001362	0,00294521	0,00001338	0,00000000	0,00004703	0,02440724	0,00161874	0,01303308	0,00003680	0,00084441	0,00027742	0,00002056	0,04127782	0,00063968	0,22333595	0,00007273	0,00006123	0,00011267	0,00022976	0,05303078	0,00002517	0,00126387	0,00165823	0,00001858	0,00356800
50	C-L	30 min	0,00001670	0,00000518	0,00037950	0,00000000	0,00254029	0,00037150	0,00439734	0,00068502	0,00531072	0,00457829	0,00174839	0,00000000	0,00008122	0,00329583	0,00170654	0,00000000	0,00479236	0,00001874	0,00584455	0,00000540	0,00325166	0,00000421	0,00000000	0,00003326	0,01575359	0,00115379	0,01689059	0,00000303	0,00088970	0,00023237	0,00000537	0,03066157	0,00009313	0,20908124	0,00003552	0,00002993	0,00004339	0,00020986	0,06434246	0,00001883	0,00074508	0,00121831	0,00000000	0,00267870
51	C-L	30 min	0,00003692	0,00001373	0,00055730	0,00003133	0,00267946	0,00044642	0,00365964	0,00023986	0,00818920	0,00277584	0,00139319	0,00000768	0,00010918	0,00289360	0,00179086	0,00000943	0,01928158	0,00005280	0,00606106	0,00001279	0,00375355	0,00004557	0,00000594	0,00004269	0,03288689	0,00250646	0,01622577	0,00004844	0,00316070	0,00044389	0,00002135	0,09922125	0,00151944	0,24084714	0,00005711	0,00007192	0,00015285	0,00025331	0,11014513	0,00002898	0,00123382	0,00115345	0,00001257	0,00458956
52	C-L	30 min	0,00001519	0,00000758	0,00022629	0,00001632	0,00083662	0,00004426	0,00052210	0,00005544	0,00837332	0,00047114	0,00459173	0,00000614	0,00012026	0,00137581	0,00594738	0,00000674	0,00472601	0,00003891	0,00906428	0,00000000	0,00113879	0,00001839	0,00000000	0,00004059	0,00594504	0,00049644	0,00722083	0,00000000	0,00117936	0,00026004	0,00001316	0,02520297	0,00027249	0,03794995	0,00000000	0,00004708	0,00013664	0,00015852	0,04125009	0,00001658	0,00040371	0,00085719	0,00001204	0,00112553
53	C-L	45 min	0,00002699	0,00002173	0,00038289	0,00000000	0,00389269	0,00075790	0,00476781	0,00015996	0,00888676	0,00668903	0,00594990	0,00001163	0,00012830	0,00468461	0,00749868	0,00002101	0,00394919	0,00004034	0,01046644	0,00001803	0,00414493	0,00002059	0,00000000	0,00006845	0,02246546	0,00242456	0,01982656	0,00004070	0,00142626	0,00042773	0,00002228	0,05834594	0,00050206	0,21080974	0,00007716	0,00005410	0,00010879	0,00036755	0,08273962	0,00002255	0,00106403	0,00284780	0,00001194	0,00523336
54	C-L	45 min	0,00002520	0,00000000	0,00026386	0,00002521	0,00565740	0,00066002	0,00335326	0,00016369	0,03459478	0,00421122	0,00236564	0,00000000	0,00027611	0,00073032	0,03019621	0,00000000	0,00000000	0,00005341	0,01834785	0,00000000	0,00837990	0,00002126	0,00000000	0,00001948	0,00744688	0,00183659	0,05381580	0,00000508	0,00393990	0,00040908	0,00000000	0,07975880	0,00034601	0,12604308	0,00000000	0,00006779	0,00011087	0,00024715	0,03306301	0,00004774	0,00101472	0,00634731	0,00000000	0,00359201
55	C-L	45 min	0,00006857	0,00000000	0,00042184	0,00018419	0,00899136	0,00105117	0,01271559	0,00222457	0,05219612	0,01295424	0,00459722	0,00000000	0,00068965	0,00451696	0,04975913	0,00000000	0,01145839	0,00008506	0,04075452	0,00000000	0,01586855	0,00023409	0,00000000	0,00009943	0,05264901	0,00462927	0,10610513	0,00000000	0,00161072	0,00080298	0,00007031	0,16642738	0,00069922	0,41052338	0,00007960	0,00018204	0,00019269	0,00104314	0,04933867	0,00010065	0,00308937	0,00428073	0,00000000	0,00762177
56	C-L	45 min																																												
57	R-L	60 min	0,00000000	0,00001571	0,00028853	0,00000000	0,00319557	0,00033349	0,00620077	0,00047554	0,01809711	0,00357604	0,00243356	0,00000000	0,00011151	0,00375438	0,02030380	0,00000000	0,00040025	0,00002484	0,01179896	0,00001195	0,00616551	0,00002828	0,00000000	0,00006159	0,00898607	0,00096799	0,04259559	0,00000000	0,00054023	0,00024358	0,00000000	0,04884712	0,00013455	0,10549509	0,00003813	0,00007807	0,00005418	0,00035861	0,04963801	0,00000000	0,00091198	0,00223056	0,00000000	0,00293419
58	R-L	60 min	0,00002130	0,00000000	0,00060802	0,00000000	0,00518796	0,00072157	0,00575432	0,00016413	0,01408842	0,00374465	0,00132733	0,00000000	0,00013379	0,00653508	0,01146298	0,00000000	0,00659625	0,00003676	0,00879579	0,00000000	0,00660587	0,00001231	0,00000000	0,00003517	0,01984515	0,00259152	0,04085859	0,00000000	0,00217114	0,00053294	0,00000000	0,07391376	0,00054237	0,22964956	0,00002294	0,00006161	0,00011900	0,00053948	0,11283002	0,00003440	0,00166704	0,00401024	0,00000000	0,00634118
59	R-L	60 min	0,00000000	0,00001777	0,00089377	0,00006598	0,00391146	0,00048344	0,00749023	0,00018456	0,01422404	0,00780178	0,00429351	0,00000000	0,00015342	0,00618798	0,00516677	0,00001605	0,01403614	0,00004926	0,00931381	0,00001555	0,00622323	0,00008420	0,00000000	0,00007044	0,03678006	0,00161696	0,03267784	0,00003897	0,00189171	0,00048204	0,00003890	0,13628118	0,00051756	0,39772163	0,00007033	0,00007237	0,00012701	0,00047941	0,14245823	0,00002573	0,00174124	0,00246157	0,00000000	0,00356174
60	R-L	60 min	0,00004568	0,00002450	0,00088535	0,00006314	0,00316498	0,00063989	0,00733849	0,00032131	0,01650829	0,00409435	0,00266733	0,00000000	0,00017957	0,00634065	0,02614768	0,00000000	0,03427847	0,00006469	0,01553010	0,00002039	0,00775276	0,00005624	0,00000000	0,00009528	0,05003491	0,00353651	0,03103420	0,00009356	0,00880861	0,00050838	0,00006729	0,15508783	0,00131518	0,48989461	0,00005067	0,00018595	0,00026851	0,00052897	0,15050800	0,00005510	0,00271318	0,00327938	0,00003261	0,00582303
61	C-H	10 min	0,00003915	0,00001388	0,00020536	0,00001168	0,00317024	0,00032252	0,00393251	0,00012812	0,00778273	0,00264473	0,00401955	0,00000000	0,00016262	0,00230274	0,00436300	0,00000520	0,00409824	0,00004557	0,00921093	0,00001166	0,00358837	0,00001976	0,00000000	0,00002953	0,01670122	0,00100566	0,01982380	0,00002123	0,00194984	0,00039355	0,00001241	0,02948006	0,00015059	0,11419458	0,00003738	0,00005092	0,00006546	0,00031191	0,04511675	0,00002972	0,00093183	0,00188947	0,00000727	0,00262837
62	C-H	10 min	0,00000000	0,00000000	0,00007732	0,00000000	0,00149945	0,00008456	0,00166305	0,00016793	0,01238017	0,00309409	0,00260457	0,00000000	0,00010199	0,00129351	0,00422206	0,00000000	0,00065138	0,00002361	0,00965577	0,00000000	0,00450824	0,00000000	0,00000000	0,00001398	0,00216420	0,00030750	0,03484918	0,00000000	0,00055748	0,00014789	0,00000000	0,01245230	0,00000000	0,02381271	0,00000000	0,00003440	0,00005012	0,00016125	0,01212780	0,00001842	0,00000000	0,00015975	0,00000000	0,00127980
63	C-H	10 min	0,00002956	0,00001083	0,00096250	0,00003580	0,00202491	0,00047106	0,00421463	0,00015780	0,00808805	0,00169175	0,00165127	0,00001298	0,00010869	0,00144401	0,00205936	0,00001566	0,02888387	0,00003803	0,00890969	0,00001024	0,00953644	0,00005326	0,00000000	0,00005072	0,01847453	0,00342911	0,02069016	0,00011102	0,00205890	0,00029203	0,00002745	0,08033944	0,00095360	0,21347257	0,00003500	0,00018329	0,00028460	0,00020708	0,09062580	0,00001990	0,00317366	0,00128985	0,00001050	0,00549780
64	C-H	10 min	0,00005788	0,00001512	0,00030102	0,00000000	0,00188190	0,00052900	0,00379072	0,00010739	0,00749767	0,00229129	0,00224085	0,00000000	0,00007804	0,00157588	0,00284073	0,00001637	0,00982055	0,00002640	0,00743263	0,00001393	0,00315056	0,00001769	0,00000000	0,00004115	0,01969404	0,00109838	0,01631243	0,00002985	0,00209961	0,00033855	0,00001205	0,07423131	0,00060533	0,16865154	0,00003006	0,00005364	0,00006321	0,00017126	0,07400476	0,00002037	0,00092807	0,00133291	0,00001432	0,00211024
65	C-H	20 min	0,00002680	0,00000849	0,00022416	0,00001091	0,00193948	0,00020300	0,00292593	0,00038413	0,00655803	0,00167131	0,00232113	0,00000000	0,00006740	0,00136296	0,00231250	0,00000000	0,00170648	0,00001289	0,00662702	0,00000000	0,00283819	0,00001526	0,00000000	0,00002347	0,00665312	0,00038904	0,01936341	0,00000000	0,00030155	0,00012087	0,00001215	0,02614072	0,00008151	0,07707801	0,00002096	0,00005536	0,00003143	0,00014131	0,01854601	0,00000968	0,00053866	0,00138154	0,00000000	0,00144830
66	C-H	20 min	0,00001243	0,00000000	0,00015317	0,00000000	0,00095147	0,00006742	0,00109341	0,00015765	0,00584325	0,00087254	0,00104469	0,00000000	0,00005059	0,00021192	0,01403869	0,00000000	0,00000000	0,00001293	0,00679314	0,00000000	0,00339563	0,00000000	0,00000000	0,00000000	0,00239125	0,00034037	0,02102971	0,00000000	0,00046072	0,00009791	0,00000000	0,02157141	0,00010414	0,04337159	0,00000000	0,00001584	0,00003623	0,00007969	0,01581027	0,00000000	0,00035899	0,00062096	0,00000000	0,00091431
67	C-H	20 min	0,00003466	0,00001830	0,00043191	0,00007969	0,00343664	0,00031991	0,00602185	0,00011887	0,01339129	0,00395073	0,00311362	0,00000000	0,00021677	0,00279941	0,00330839	0,00002034	0,01722995	0,00004331	0,01195558	0,00001553	0,00566744	0,00007642	0,00000000	0,00006267	0,01896281	0,00149103	0,03363775	0,00005228	0,00233781	0,00041507	0,00003185	0,09992333	0,00101907	0,18570356	0,00004533	0,00008102	0,00007878	0,00032335	0,08465187	0,00002820	0,00171948	0,00227170	0,00001429	0,00341022
68	C-H	20 min	0,00005792	0,00002014	0,00035032	0,00000000	0,00238249	0,00046690	0,00401335	0,00013240	0,00938944	0,00296609	0,00383717	0,00000000	0,00010155	0,00286515	0,00506378	0,00001832	0,00782458	0,00004120	0,00760067	0,00001494	0,00348767	0,00002212	0,00000000	0,00005221	0,01591100	0,00088588	0,01759588	0,00002915	0,00231580	0,00034930	0,00001470	0,04312342	0,00120133	0,14386780	0,00003435	0,00005966	0,00008376	0,00022441	0,08246592	0,00002129	0,00091344	0,00140670	0,00001885	0,00210366
69	C-H	30 min	0,00004825	0,00002650	0,00036291	0,00001217	0,00238484	0,00041578	0,00352916	0,00011504	0,00470286	0,00268131	0,00265341	0,00000000	0,00016438	0,00310242	0,00232480	0,00001920	0,00356947	0,00003533	0,00816256	0,00001712	0,00335725	0,00001364	0,00000000	0,00003786	0,01537634	0,00095693	0,01836619	0,00002118	0,00161790	0,00031041	0,00001063	0,03693790	0,00027560	0,13846237	0,00004109	0,00007188	0,00008517	0,00029868	0,05866189	0,00003005	0,00094349	0,00135767	0,00000673	0,00259798
70	C-H	30 min	0,00000000	0,00000000	0,00015931	0,00000000	0,00188023	0,00002862	0,00133946	0,00004678	0,00576535	0,00335460	0,00340182	0,00000000	0,00010832	0,00020802	0,00502103	0,00000000	0,00099886	0,00002739	0,00697570	0,00000000	0,00327068	0,00000000	0,00000000	0,00000918	0,00282004	0,00027615	0,02239304	0,00000000	0,00077874	0,00014654	0,00000000	0,01913872	0,00005787	0,03791151	0,00000000	0,00002826	0,00002645	0,00018665	0,01976592	0,00001296	0,00030517	0,00087294	0,00000000	0,00112200
71	C-H	30 min	0,00004652	0,00002117	0,00019659	0,00010871	0,00354090	0,00016405	0,00657787	0,00057473	0,01913261	0,00582665	0,00344663	0,00000000	0,00028224	0,00331932	0,02815008	0,00000000	0,00865856	0,00004054	0,01476032	0,00000000	0,00787519	0,00010760	0,00000000	0,00006942	0,01405361	0,00111686	0,05040619	0,00000000	0,00217181	0,00047206	0,00004274	0,08787015	0,00069995	0,13319211	0,00004580	0,00009532	0,00006016	0,00051180	0,05161774	0,00004132	0,00139870	0,00214627	0,00000000	0,00222852
72	C-H	30 min																																												
73	C-H	45 min	0,00002718	0,00000000	0,00038936	0,00001974	0,00320349	0,00052358	0,00465191	0,00013138	0,00752752	0,00676852	0,00515607	0,00000000	0,00019073	0,00308117	0,00584195	0,00000000	0,01289146	0,00003921	0,00969799	0,00001502	0,00463843	0,00002192	0,00000000	0,00004447	0,01610000	0,00226664	0,02872289	0,00004769	0,00232928	0,00030484	0,00002697	0,06967328	0,00013587	0,11658925	0,00003329	0,00004909	0,00009966	0,00032252	0,05776005	0,00002211	0,00150342	0,00196783	0,00000000	0,00534696
74	C-H	45 min	0,00000000	0,00000000	0,00022048	0,00000000	0,00063425	0,00004124	0,00139457	0,00007300	0,00666834	0,00135228	0,00136959	0,00000000	0,00006479	0,00172242	0,02265494	0,00000000	0,00261434	0,00003209	0,01530941	0,00000000	0,00265554	0,00000732	0,00000000	0,00002472	0,00661557	0,00080814	0,01614343	0,00000000	0,00158124	0,00026291	0,00000000	0,04072570	0,00019869	0,09436106	0,00000000	0,00006776	0,00021314	0,00015632	0,06101696	0,00001249	0,00051733	0,00159657	0,00000000	0,00160997
75	C-H	45 min	0,00002325	0,00001924	0,00034906	0,00006977	0,00483498	0,00047004	0,00718180	0,00155751	0,01452714	0,01500612	0,01281562	0,00000000	0,00045033	0,01120209	0,01773552	0,00000000	0,00606972	0,00005549	0,01373321	0,00001626	0,00764983	0,00009413	0,00000000	0,00007607	0,03763489	0,00480056	0,04508045	0,00004669	0,00132499	0,00054687	0,00003961	0,10390829	0,00046980	0,31064152	0,00005003	0,00005896	0,00017733	0,00087213	0,05655424	0,00005355	0,00223101	0,00255027	0,00000000	0,00733647
76	C-H	45 min																																												
77	R-H	60 min	0,00001532	0,00001234	0,00032771	0,00000000	0,00302437	0,00036658	0,00442432	0,00014307	0,00679049	0,00423976	0,00130731	0,00000000	0,00010578	0,00454873	0,01106600	0,00000000	0,00543310	0,00002011	0,00964389	0,00001223	0,00424394	0,00001555	0,00000000	0,00003906	0,01859069	0,00118259	0,02603141	0,00002587	0,00110574	0,00033616	0,00002342	0,04436111	0,00018775	0,14091463	0,00003578	0,00005509	0,00007266	0,00042887	0,05290753	0,00002005	0,00106689	0,00119116	0,00000000	0,00305863
78	R-H	60 min	0,00004573	0,00000000	0,00021290	0,00000000	0,00211793	0,00064925	0,00223941	0,00023629	0,00289747	0,00339849	0,00196330	0,00000000	0,00014739	0,00487868	0,00221075	0,00000000	0,00538292	0,00005318	0,00535761	0,00000000	0,00243338	0,00000449	0,00000000	0,00002056	0,03815748	0,00552058	0,01350136	0,00001236	0,00657727	0,00037533	0,00000000	0,03492103	0,00036170	0,15594302	0,00002212	0,00003316	0,00013184	0,00038411	0,07375980	0,00003937	0,00147824	0,00118403	0,00000000	0,00999392
79	R-H	60 min	0,00000770	0,00001162	0,00036673	0,00002663	0,00187074	0,00025755	0,00337905	0,00008423	0,00431571	0,00638973	0,00289301	0,00000000	0,00011838	0,00481150	0,00415703	0,00000917	0,00487668	0,00002378	0,00502251	0,00000978	0,00265909	0,00002940	0,00000000	0,00004138	0,01318306	0,00115831	0,01328591	0,00001833	0,00108745	0,00029230	0,00001214	0,04634079	0,00055404	0,14828463	0,00003394	0,00002305	0,00004199	0,00029243	0,06743668	0,00001590	0,00069140	0,00105013	0,00000802	0,00218046
80	R-H	60 min	0,00002695	0,00002623	0,00037866	0,00000000	0,00411167	0,00037796	0,00822489	0,00107941	0,02503645	0,00812628	0,00355934	0,00000000	0,00018257	0,00742938	0,02760610	0,00000000	0,01284829	0,00003521	0,01949288	0,00000000	0,00859642	0,00004411	0,00000000	0,00008552	0,02824427	0,00159617	0,05471264	0,00000000	0,00199359	0,00046531	0,00004424	0,10428664	0,00061098	0,21512446	0,00003597	0,00007060	0,00007544	0,00053028	0,10149049	0,00000000	0,00155300	0,00266787	0,00004942	0,00329658
81	fresh	0 min	0,00002043	0,00000579	0,00029712	0,00000489	0,00123359	0,00045309	0,00085658	0,00004405	0,00014053	0,00052350	0,00052166	0,00000627	0,00003090	0,00104327	0,00095137	0,00001931	0,00153248	0,00000896	0,00103158	0,00000533	0,00044793	0,00001788	0,00000326	0,00000682	0,01266592	0,00195778	0,00194794	0,00000784	0,00033850	0,00011191	0,00000367	0,01512575	0,00011682	0,03101660	0,00018110	0,00001843	0,00002171	0,00012734	0,01017311	0,00001229	0,00039613	0,00070392	0,00000393	0,00145048
82	fresh	0 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
83	fresh	0 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
84	fresh	0 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Supplemental Table S6 Classification of the compounds identified in the metabolite analysis according to the scheme of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (KEGG, http://www.genome.ad.jp/kegg/pathway.html) and the human metabolome database (HMDB, https://hmdb.ca/).

Compound Name	KEGG ID	HMDB ID	Super Class	Class	Subclass	Pathways involved (abbreviated)
2-Oxoglutarate		HMDB0 0208	Organic acids and derivatives	Keto acids and derivatives	Gamma-keto acids and derivatives	Krebs cycle, Urea cylce, Gluconeogenesis, Glutamate Metabolism, Glucose-Alanine Cycle
3-Phospho-Glycerate	C0019 7	HMDB0 0807	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Glycolysis, gluconeogenesis, pyruvate metabolism, Glycine and Serine Metabolism, Glycerolipid Metabolism
A178003-101			unknown			
A196004-101			unknown			
Alanine	C0004 1	HMDB0 0161	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Alanine and Aspartate metabolism
Aspartate	C0004 9	HMDB0 0191	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Alanine and Aspartate metabolism
beta-Alanine	C0009 9	HMDB0 0056	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Alanine and Aspartate metabolism
Citrate		HMDB0 0094	Organic acids and derivatives	Carboxylic acids and derivatives	Tricarboxylic acids and derivatives	Krebs cycle, Transfer of Acetyl Groups into Mitochondria
Dimethylglycine	C0102 6	HMDB0 0092	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Glycine and Serine Metabolism, Betaine Metabolism, Methionine Metabolism
Ethanolamine		HMDB0 0149	Organic nitrogen compounds	Organonitrogen compounds	Amines	Phospholipid Biosynthesis, Phosphatidylcholine Biosynthesis
Fructose	C0009 5	HMDB0 0660	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Galactose, starch and sucrose metabolism, Fructose and Mannose degradation, Amino Sugar Metabolism
Fructose-6-Phosphate		HMDB0 0124	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Glycolysis, gluconeogenesis, Fructose and Mannose Degradation, Pentose Phosphate Pathway, Amino Sugar Metabolism
Fumarate	C0012 2	HMDB0 0134	Organic acids and derivatives	Carboxylic acids and derivatives	Dicarboxylic acids and derivatives	Krebs cycle, Phenylalanine and Tyrosine Metabolism, Arginine and Proline Metabolism, Aspartate Metabolism, Urea

						cycle, Mitochondrial Electron Transport Chain
GABA	C0033 4	HMDB0 0112	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Glutamate metabolism
Glucose	C0026 7	HMDB0 0122	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Glycolysis, gluconeogenesis, Galactose metabolism, pyruvate metabolism, Glucose-Alanine Cycle, Lactose Synthesis
Glucose-6-Phosphate		HMDB0 1401	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Glycolysis, Gluconeogenesis, Pentose Phosphate Pathway, Inositol Metabolism, Starch and Sucrose Metabolism
Glutamate	C0002 5	HMDB0 0148	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Glutamate metabolism
Glycerate	C0025 8	HMDB0 0139	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Glycolysis, gluconeogenesis, pyruvate metabolism, Glycine and Serine Metabolism, Glycerolipid Metabolism
Glycerol	C0011 6	HMDB0 0131	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Glycerolipid metabolism, Galactose Metabolism
3-Phospho-Glycerate		HMDB0 0807	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Glycolysis, Gluconeogenesis, Glycine and Serine Metabolism, Glycerolipid Metabolism
Glycine	C0003 7	HMDB0 0123	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Glycine, serine and threonine metabolism
Hexanedioic acid		HMDB0 0448	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates	
Hexose-Phosphate			Organic acids and derivatives	Organooxygen compounds		
Inositol-Phosphate	C0117 7	HMDB0 2985	Organic oxygen compounds	Organooxygen compounds	Alcohols and polyols	Inositol metabolism
Isocitrate		HMDB0 0193	Organic acids and derivatives	Carboxylic acids and derivatives	Tricarboxylic acids and derivatives	Krebs cycle
Isoleucine	C0040 7	HMDB0 0172	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Valine, leucine and isoleucine metabolism

Leucine	C00123	HMDB00687	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Valine, leucine and isoleucine metabolism
Lysine	C00047	HMDB00182	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Lysine metabolism
Malate	C00149	HMDB00156	Organic acids and derivatives	Hydroxy acids and derivatives	Beta hydroxy acids and derivatives	Krebs cycle, Pyruvate Metabolism, Transfer of Acetyl Groups into Mitochondria
myo-Inositol	C00137	HMDB00211	Organic oxygen compounds	Organooxygen compounds	Alcohols and polyols	Inositol metabolism, Galactose Metabolism, Phosphatidylinositol Phosphate Metabolism
Phospho-Ethanolamine	C00346	HMDB00224	Organic acids and derivatives	Organic phosphoric acids and derivatives	Phosphate esters	Glycerolipid metabolism, Sphingolipid Metabolism
Phosphate	C00009	HMDB01429	Homogeneous non-metal compounds	Non-metal oxoanionic compounds	Non-metal phosphates	Oxidative phosphorylation
Pipecolate	C00408	HMDB00070	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Lysine metabolism, Lysine Degradation
Proline	C00148	HMDB00162	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Urea cycle; arginine-, proline-, metabolism
Putrescine		HMDB01414	Organic nitrogen compounds	Organonitrogen compounds	Amines	Methionine Metabolism, Spermidine and Spermine Biosynthesis
Pyruvate		HMDB00243	Organic acids and derivatives	Keto acids and derivatives	Alpha-keto acids and derivatives	Krebs cycle, Urea Cycle, Glucose-Alanine Cycle, Glycine and Serine Metabolism, Pyruvate Metabolism, Alanine Metabolism, Amino Sugar Metabolism, Ammonia Recycling
Serine	C00065	HMDB00187	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Glycine, serine and threonine metabolism
Succinate		HMDB00254	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Krebs cycle, Glutamate Metabolism, Mitochondrial Electron Transport Chain, Phytanic Acid Peroxisomal Oxidation, Carnitine Synthesis

Sucrose		HMDB0 0258	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	Fructose, mannose, galactose, starch, and sucrose metabolism
Threonate		HMDB0 0943	Organic acids and derivatives	Organooxygen compounds	Carbohydrates and carbohydrate conjugates	
Threonine	C0018 8	HMDB0 0167	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Glycine, serine and threonine metabolism
U1			unknown			
U2			unknown			
Valine	C0018 3	HMDB0 0883	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides and analogues	Valine, leucine and isoleucine degradation, Propanoate Metabolism

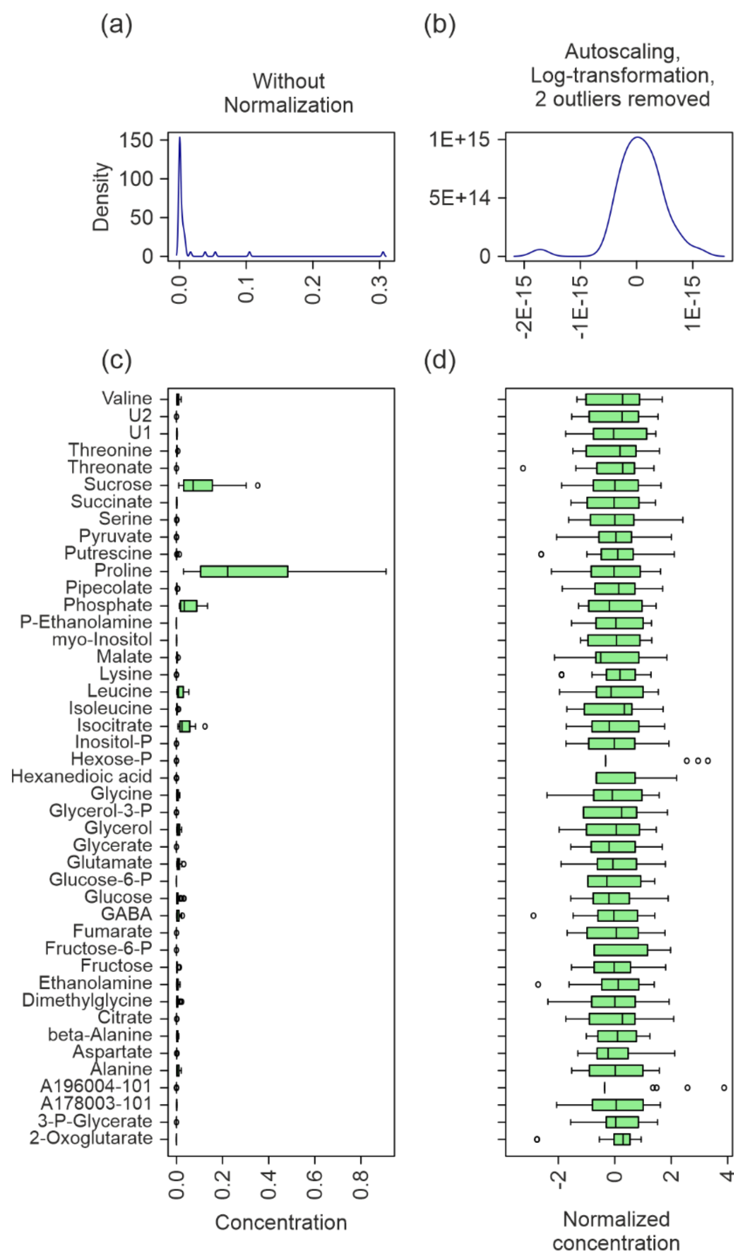
Supplemental Table S7 PLSDA cross validation result of the Genotype and Storage experiment. Q^2 values indicate how many componentes are required to explain the variance. The number of components showing highest Q^2 value (red) is chosen and respective R^2 and accuracies provided in addition.

Experiment name	**Performance measure**	**Components**				
		1	2	3	4	5
Genotype Experiment	Accuracy	0.18	0.37	0.54	0.69	0.75
	R^2	0.42	0.72	0.86	0.93	0.96
	Q^2	-0.19	0.35	0.56	0.57	0.64
Storage Experiment	Accuracy	0.36	0.41	0.38	0.38	0.34
	R^2	0.22	0.36	0.49	0.57	0.62
	Q^2	0.15	0.20	0.18	0.17	0.07

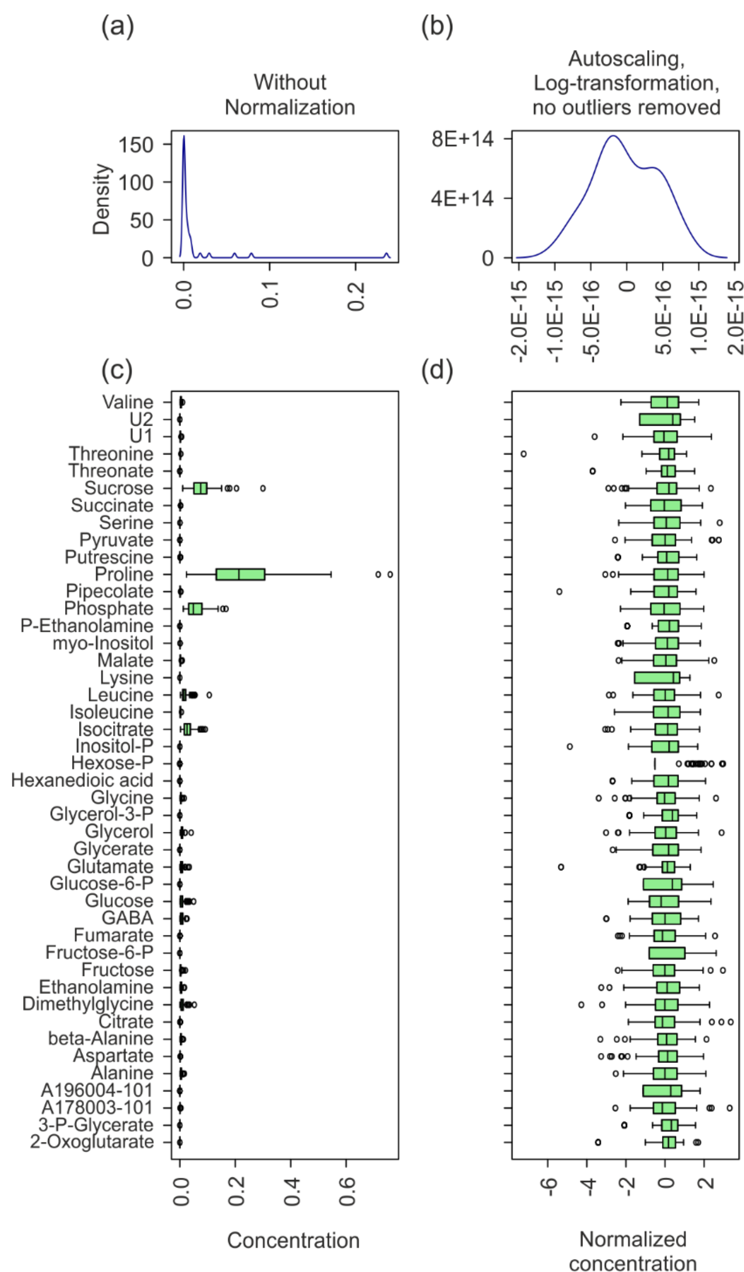
Supplemental Table S8 PLS-DA cross validation results of the Storage experiment with separate analysis for each storage group. Q^2 values indicate how many componentes are required to explain the variance. The number of components showing highest Q^2 value (red) is chosen and respective R^2 and accuracies provided in addition.

Storage condition	**Performance measure**	**Components**				
		1	2	3	4	5
R-L	Accuracy	0.17	0.17	0.30	0.39	0.48
	R^2	0.71	0.83	0.96	0.97	0.98
	Q^2	0.65	0.69	0.70	0.78	0.79
R-H	Accuracy	0.17	0.17	0.54	0.5	0.5
	R^2	0.67	0.81	0.87	0.92	0.97
	Q^2	0.60	0.57	0.46	0.28	0.25
C-L	Accuracy	0.17	0.04	0.17	0.13	0.13
	R^2	0.76	0.88	0.97	0.98	0.99
	Q^2	0.68	0.69	0.73	0.80	0.82
C-H	Accuracy	0.18	0.18	0.23	0.23	0.18
	R^2	0.48	0.64	0.83	0.92	0.95
	Q^2	0.33	0.44	0.47	0.51	0.43

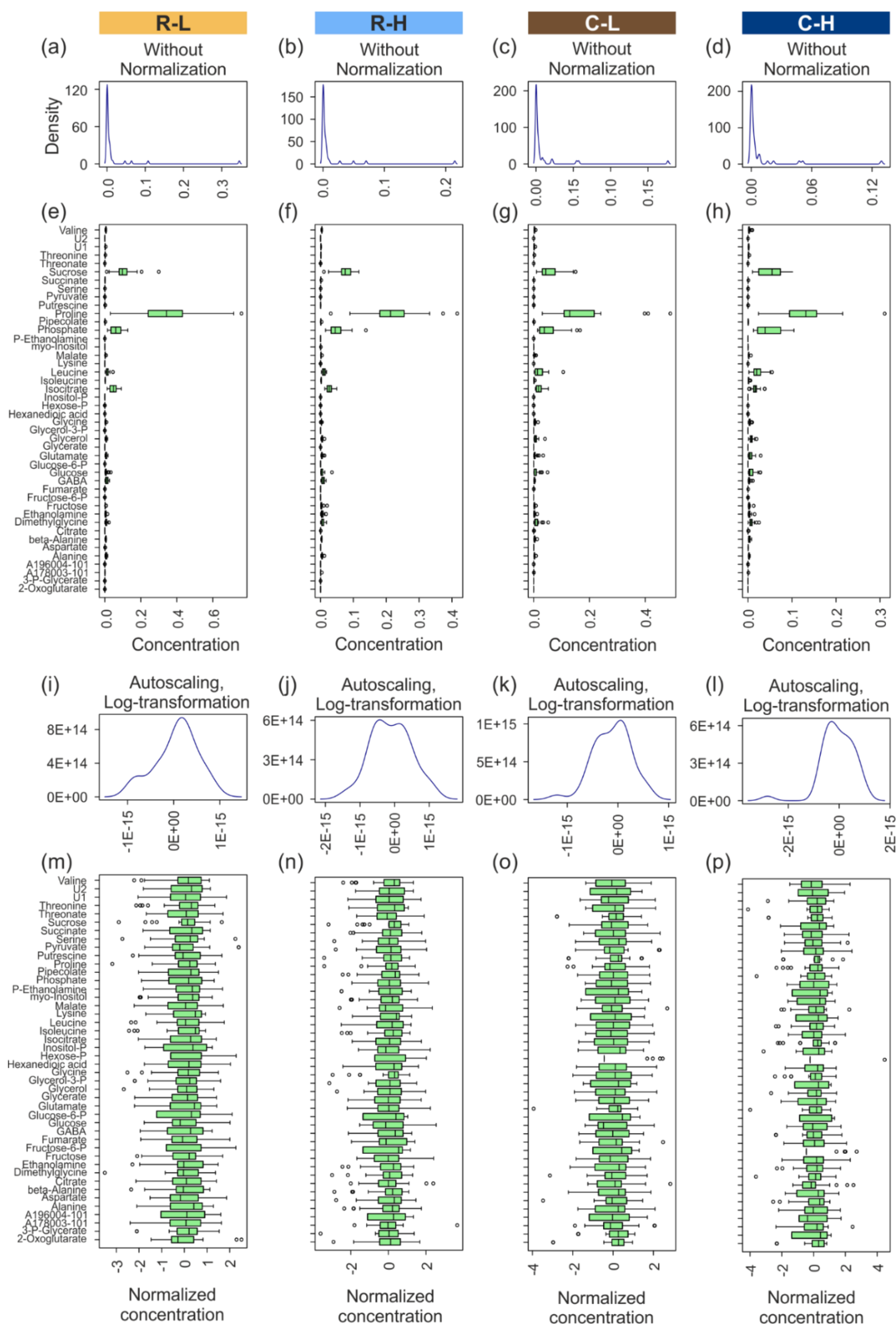
Supplemental Figures



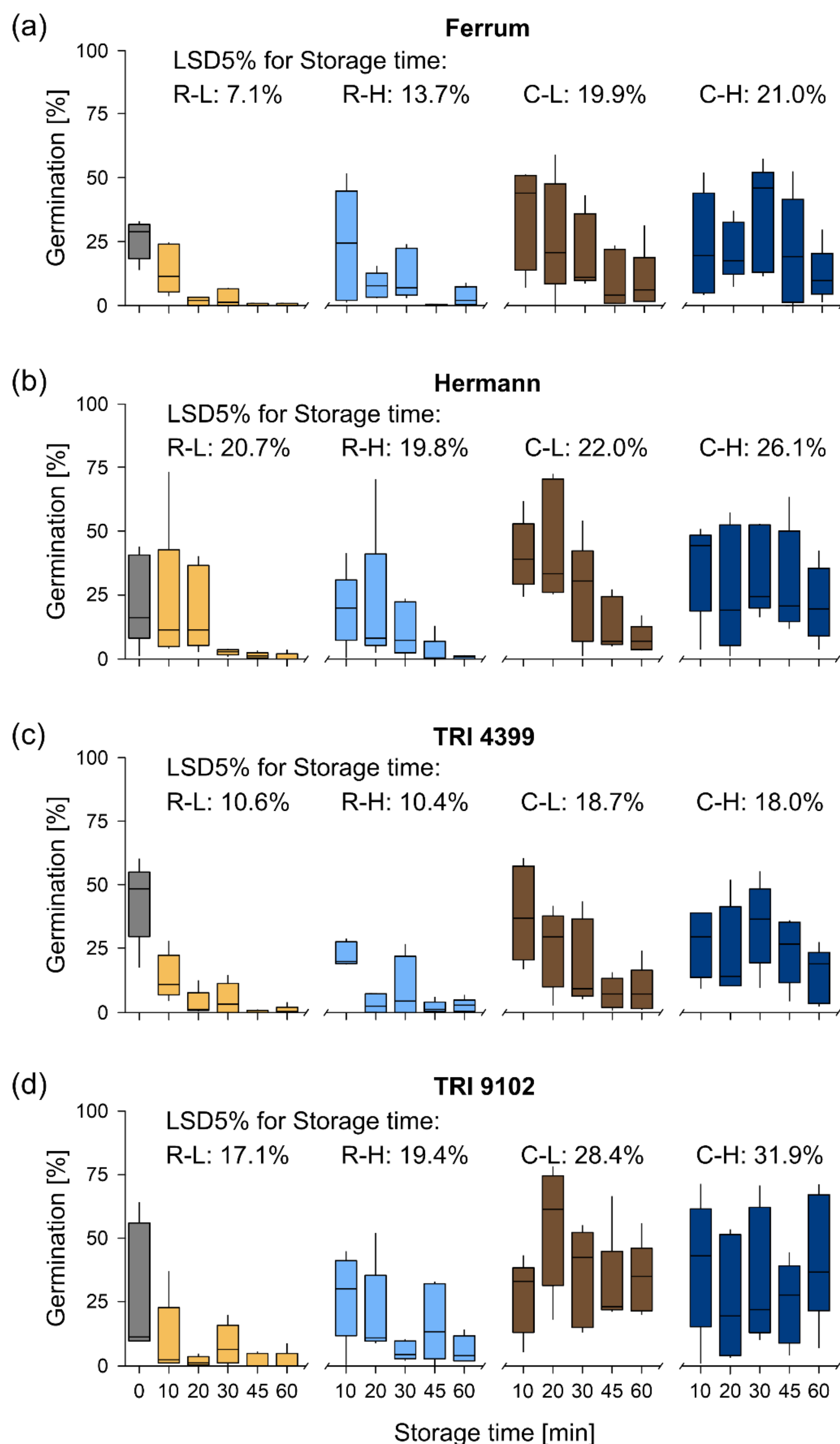
Supplemental Figure S1 Log-transformation to normalize metabolite levels (Genotype experiment). Distribution of (a) non-normalized and (b) normalized metabolite data using autoscaling and Log-transformation provided by the Metaboanalyst software package (www.metaboanalyst.ca). Whisker box plots show (c) non-normalized and (d) normalized concentrations of detected metabolites for freshly collected pollen and pollen stored at room temperature at low and high RH for 60 minutes of the four wheat lines ‘Ferrum’, ‘Hermann’, TRI 4399, TRI 9102. In (b) and (d) two outliers were removed.



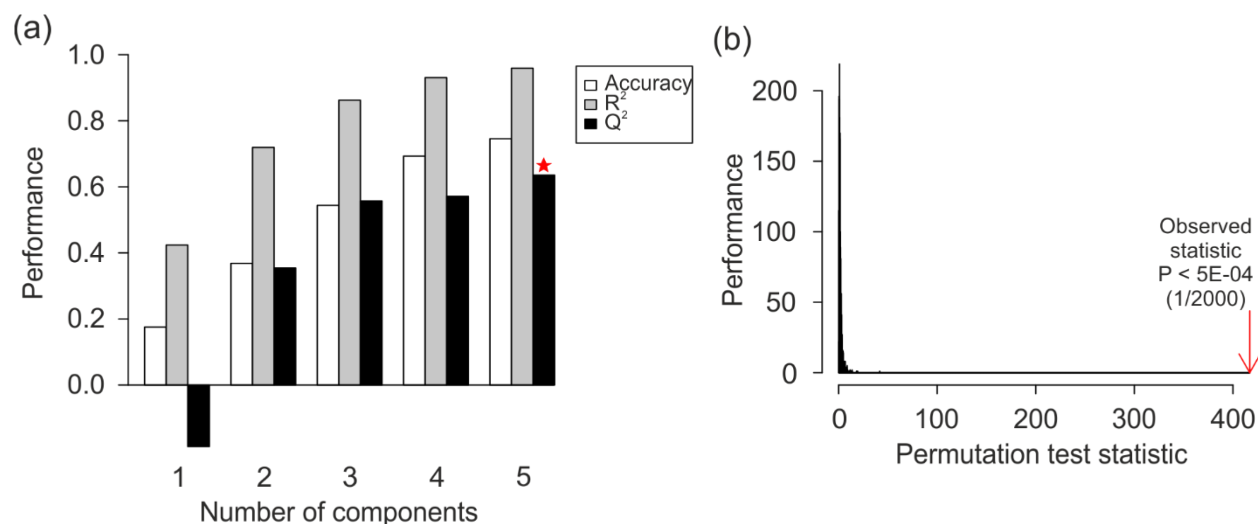
Supplemental Figure S2 Log-transformation to normalize metabolite levels (Storage experiment). Distribution of (a) non-normalized and (b) normalized metabolite data using autoscaling and Log-transformation provided by the Metaboanalyst software package (www.metaboanalyst.ca). Whisker box plots show (c) non-normalized and (d) normalized concentrations of detected metabolites for freshly collected pollen and pollen stored at room temperatures at low and high RH and at cold temperatures at low and high RH for 10, 20, 30, 45 and 60 minutes of the wheat line TRI 9102. In (b) and (d) no outliers were removed.



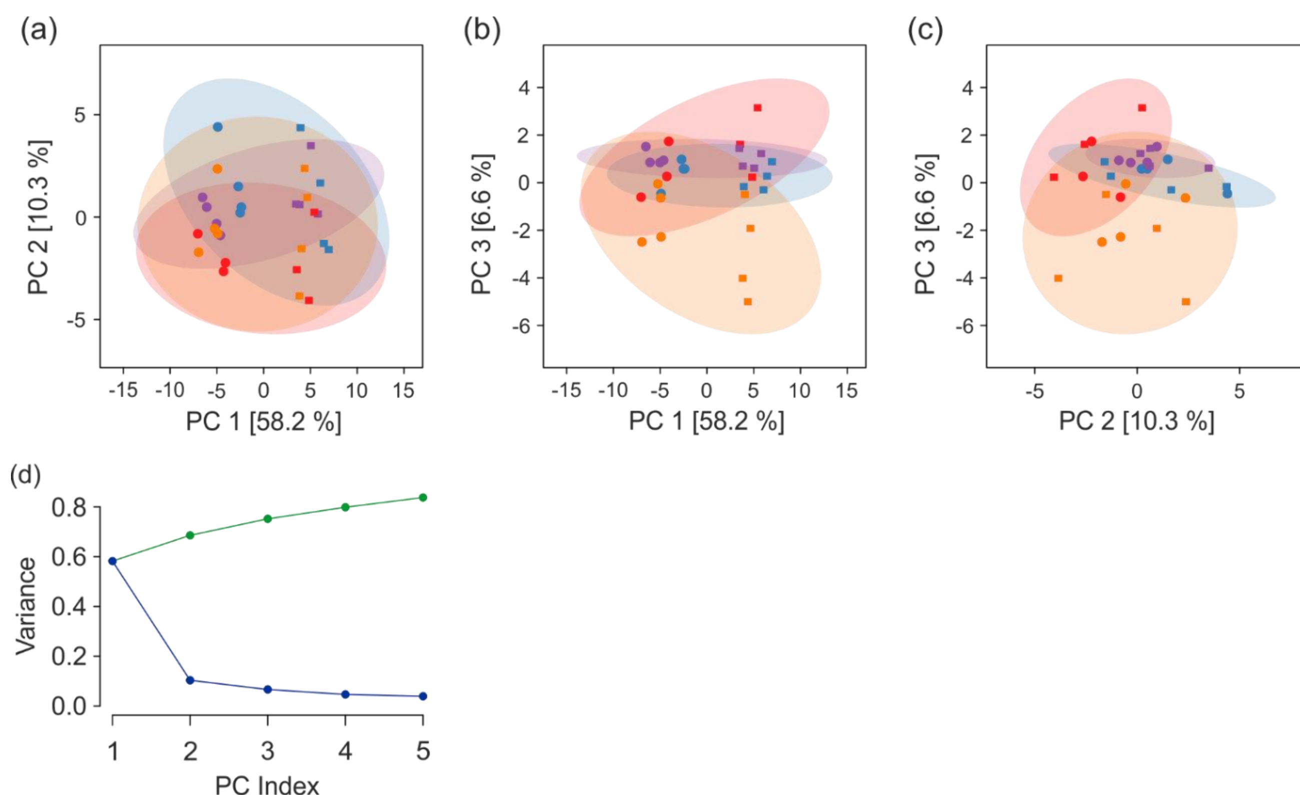
Supplemental Figure S3 Log-transformation to normalize metabolite levels (Storage experiment). Distribution of (a-d) non-normalized metabolite data and (i-l) normalized data using autoscaling and Log-transformation provided by the Metaboanalyst software package (www.metaboanalyst.ca). Whisker box plots show (e-h) non-normalized and (m-p) normalized concentrations of detected metabolites for a combination of freshly collected pollen of the wheat line TRI 9102 and pollen stored at room temperatures at (a,e,I,m) low and (b,f,j,n) high RH and at cold temperatures at (c,g,k,o) low and (d,h,l,p) high RH for 10, 20, 30, 45 and 60 minutes. In (i) to (p) no outliers were removed.



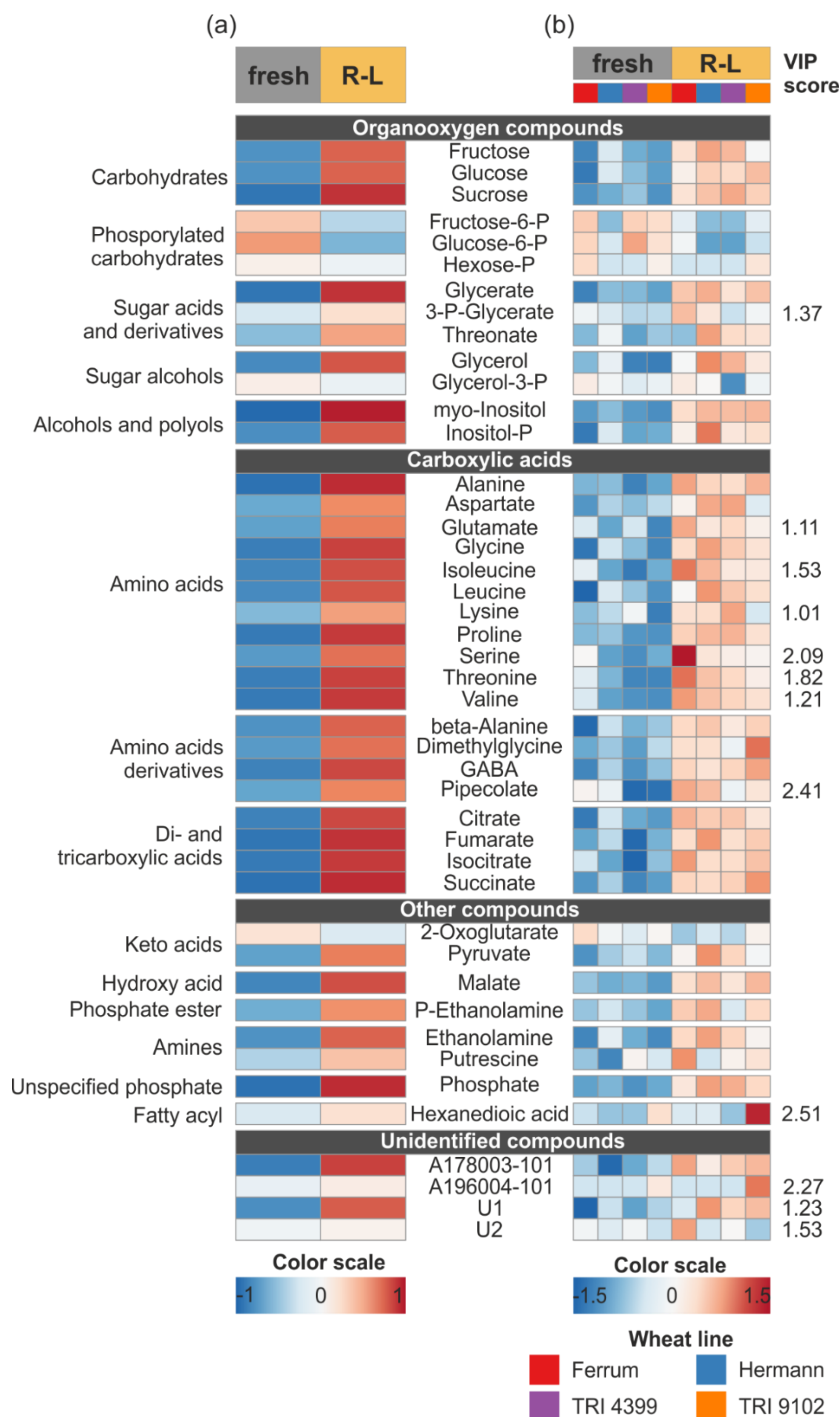
Supplemental Figure S4 Pollen germination of the individual wheat lines (a) ‘Ferrum’, (b) ‘Hermann’, (c) TRI 4399 and (d) TRI 9102. Whisker-box plots show pollen germination of five biological replicates using between 2700 and 7700 pollen each for fresh pollen (grey) and after storage at room temperatures at low RH (R-L, beige) and high RH (R-H, light blue) and at cold temperatures at low RH (C-L, brown) and high RH (C-H, dark blue) for 10, 20, 30, 45 and 60 minutes. Least significant differences at $P < 0.05$ (LSD5%) between storage times is provided for each condition above.



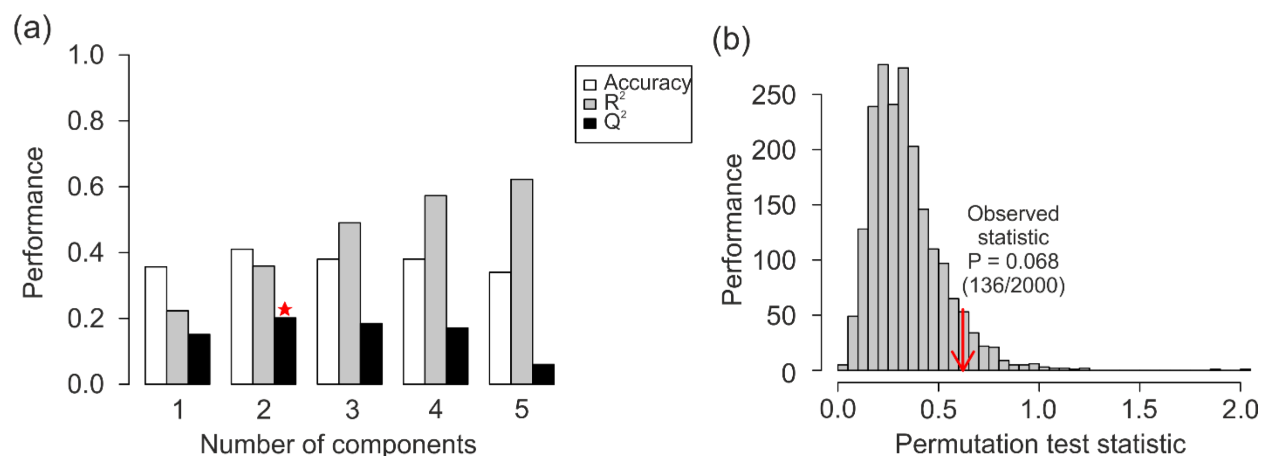
Supplemental Figure S5 Validation of the Partial Least Square Discriminant Analysis (PLS-DA) model in the Genotype experiment. (a) Cross validation was performed for five components analysed for freshly collected pollen and pollen stored at room temperature at low and high RH for 60 minutes of the four wheat lines 'Ferrum', 'Hermann', TRI 4399, TRI 9102. The red star marks the highest value for the cross-validated sum of squares (Q^2) and indicate the best fit of the model when five components are used. (b) The permutation test shows highly significant assignment of group membership. The red arrow indicates that pollen samples are located outside the distribution of randomly permuted samples.



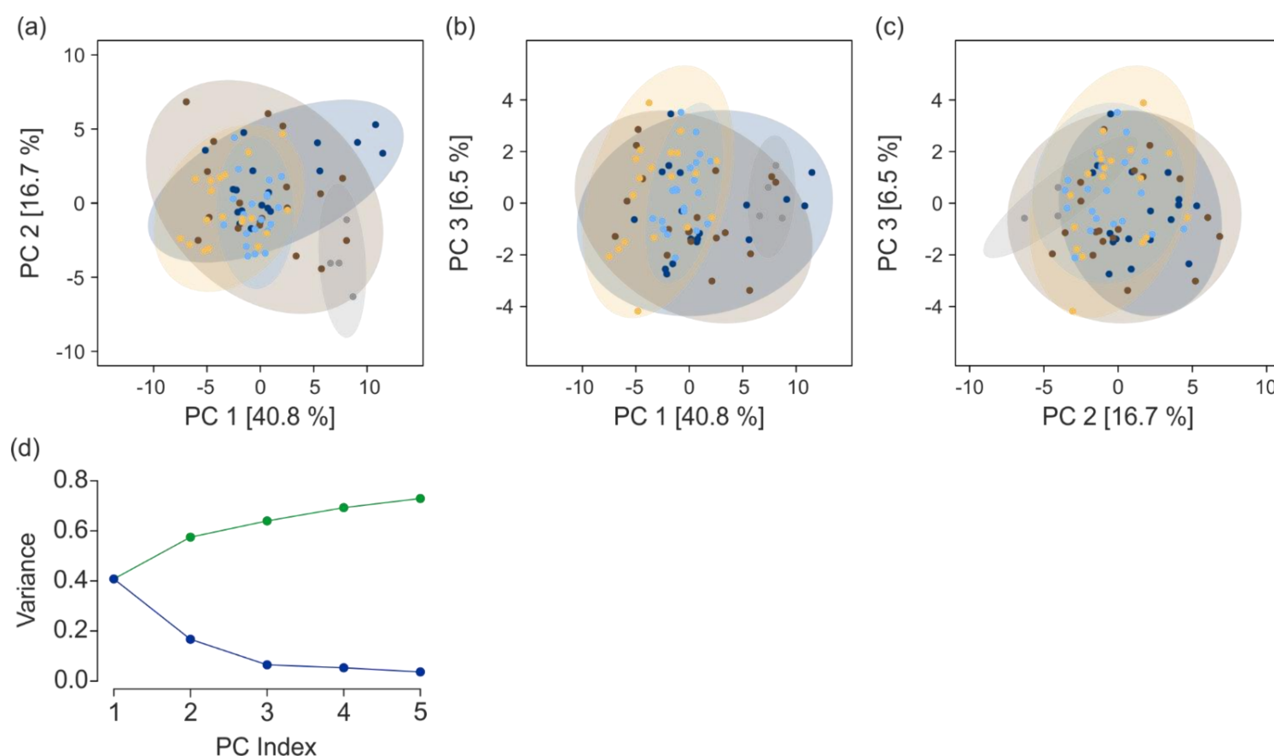
Supplemental Figure S6 Principal components pollen metabolites separate pollen samples of four wheat lines according to the room temperature storage conditions (Genotype experiment). Principal components (PC) for freshly collected pollen (dots) and pollen stored at room temperature at low RH (R-L, square symbols) for 60 minutes of the four wheat lines 'Ferrum' (red), 'Hermann', (blue), TRI 4399 (purple), TRI 9102 (orange) were compared. (a) PC1 is plotted against PC2, (b) PC1 vs. PC3 and (c) PC2 vs. PC3. The explained variances for each component is given in brackets. (d) The scree plot shows explained (blue) and cumulative variances (green) of each of the first PCs. Four biological replicates were analysed for pollen from each wheat line and condition. Normalized, autoscaled and log transformed data were used.



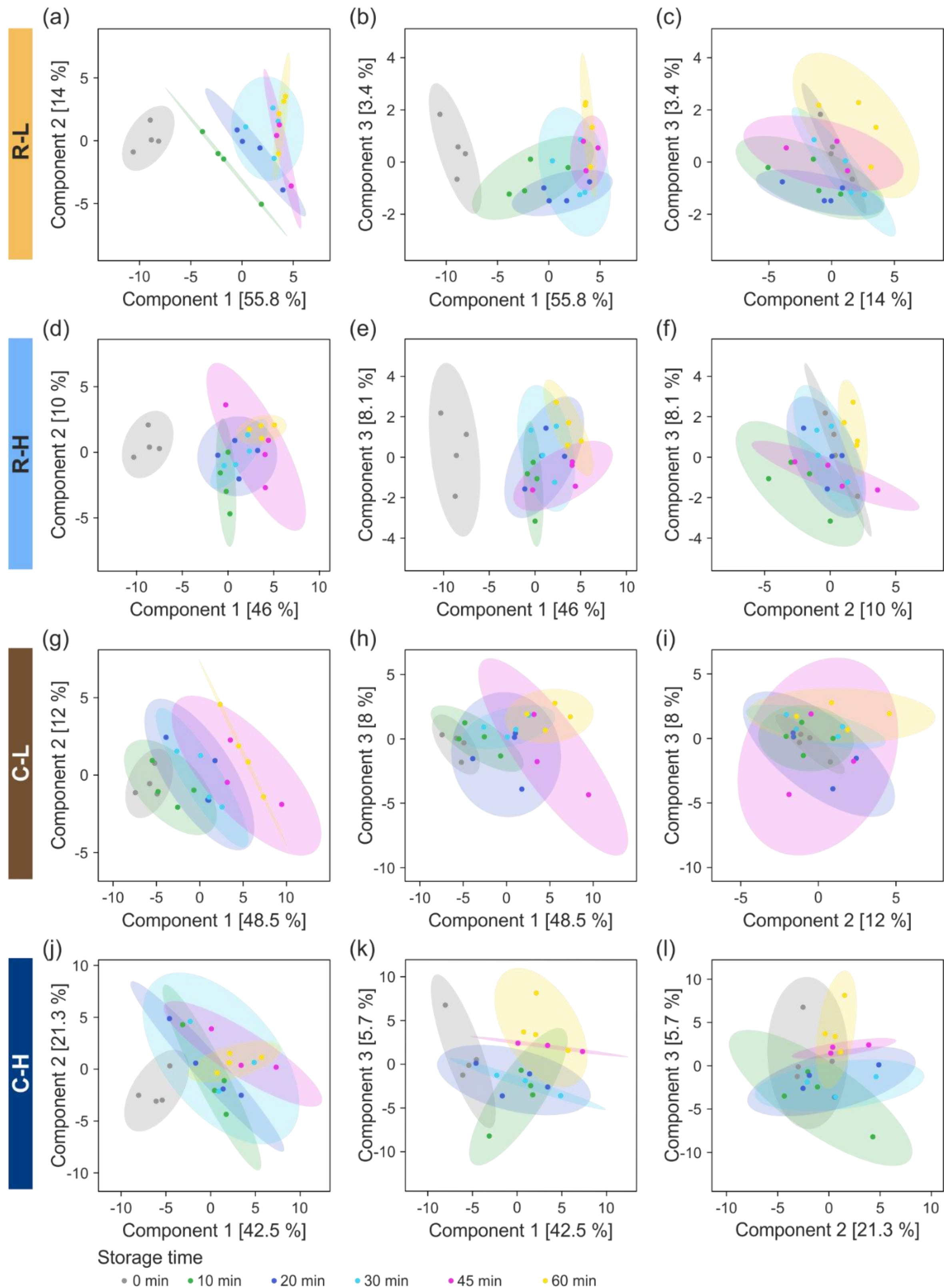
Supplemental Figure S7 Heat maps of metabolites of pollen collected from different wheat lines and stored under room temperature (Genotype experiment). Log-transformed, autoscaled values for metabolites differentially accumulated are shown in fresh pollen and in pollen stored at room temperature at low RH (R-L). (a) The data show means of four wheat lines (‘Hermann’, ‘Ferrum’, TRI 9102 and TRI 4399, each with 4 biological replicates). (b) Mean values of four replicates are shown for the wheat lines ‘Ferrum’ (red), ‘Hermann’ (blue), TRI 4399 (purple) and TRI 9102 (orange). The heat maps were constructed with the online-tool ClustVis (https://biit.cs.ut.ee/clustvis/). Metabolites are sorted according to their chemical class. Variable Importance in Projection (VIP) score > 1.0 for metabolites differentially abundant in wheat lines are provided. Sub-classifications are provided on the left side. Colour scales are given below the panels.



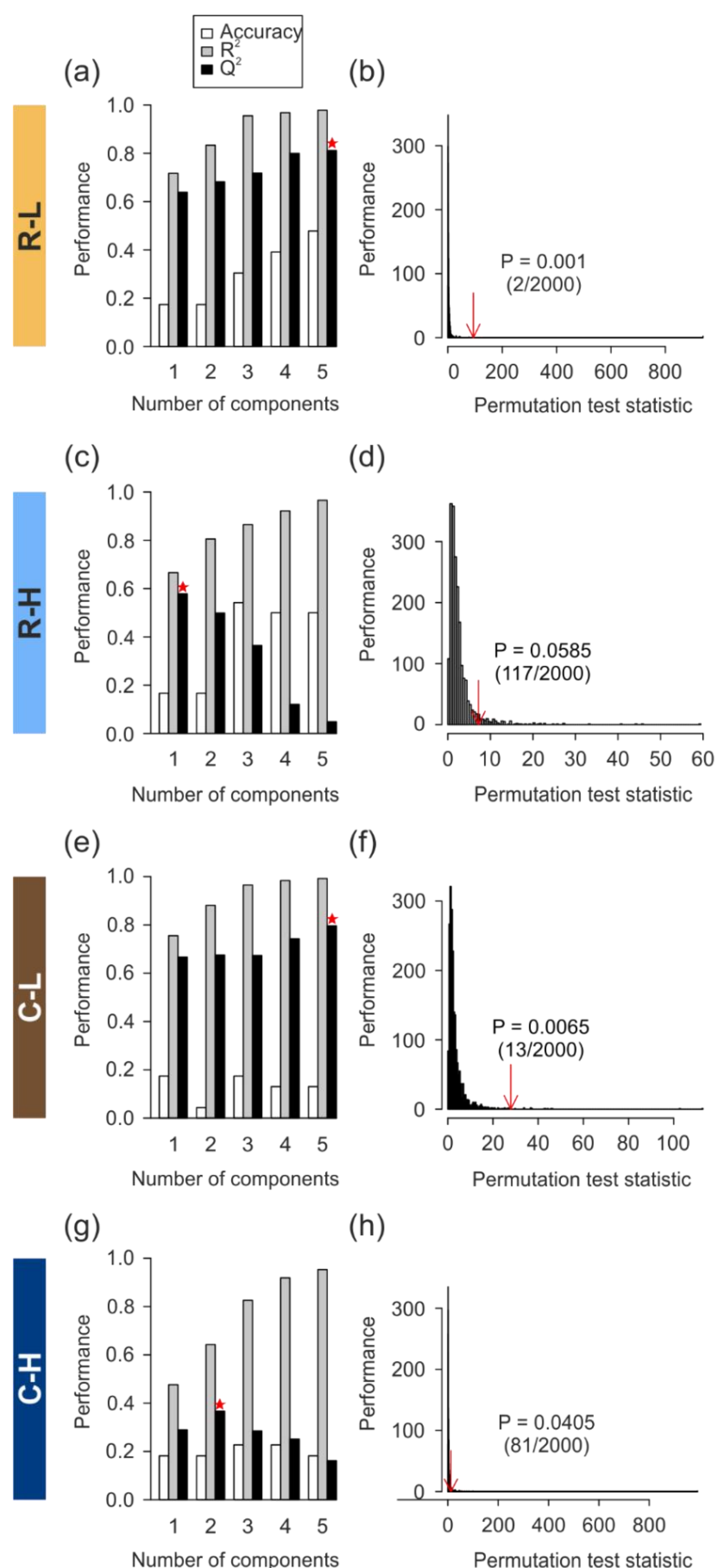
Supplemental Figure S8 Validation of the Partial Least Square Discriminant Analysis (PLS-DA) model in the Storage experiment. (a) Cross validation was performed for five components analysed for freshly collected pollen and pollen stored at room temperatures at low and high RH and pollen stored at cold temperatures at low RH and high RH for 10, 20, 30, 45 and 60 minutes. The red star marks the highest value for the cross-validated sum of squares (Q^2). The value of Q^2 (0.2) shown at component 2 indicates that the model does not fit (Lundstedt et al., 1998). (b) The permutation test shows assignments of the group membership. The red arrow indicates that pollen samples are located within the distribution of randomly permuted samples, hence, are not assigned significantly to any group. Separate validation of PLS-DA for each storage condition are provided in **Supplemental Figure S11**.



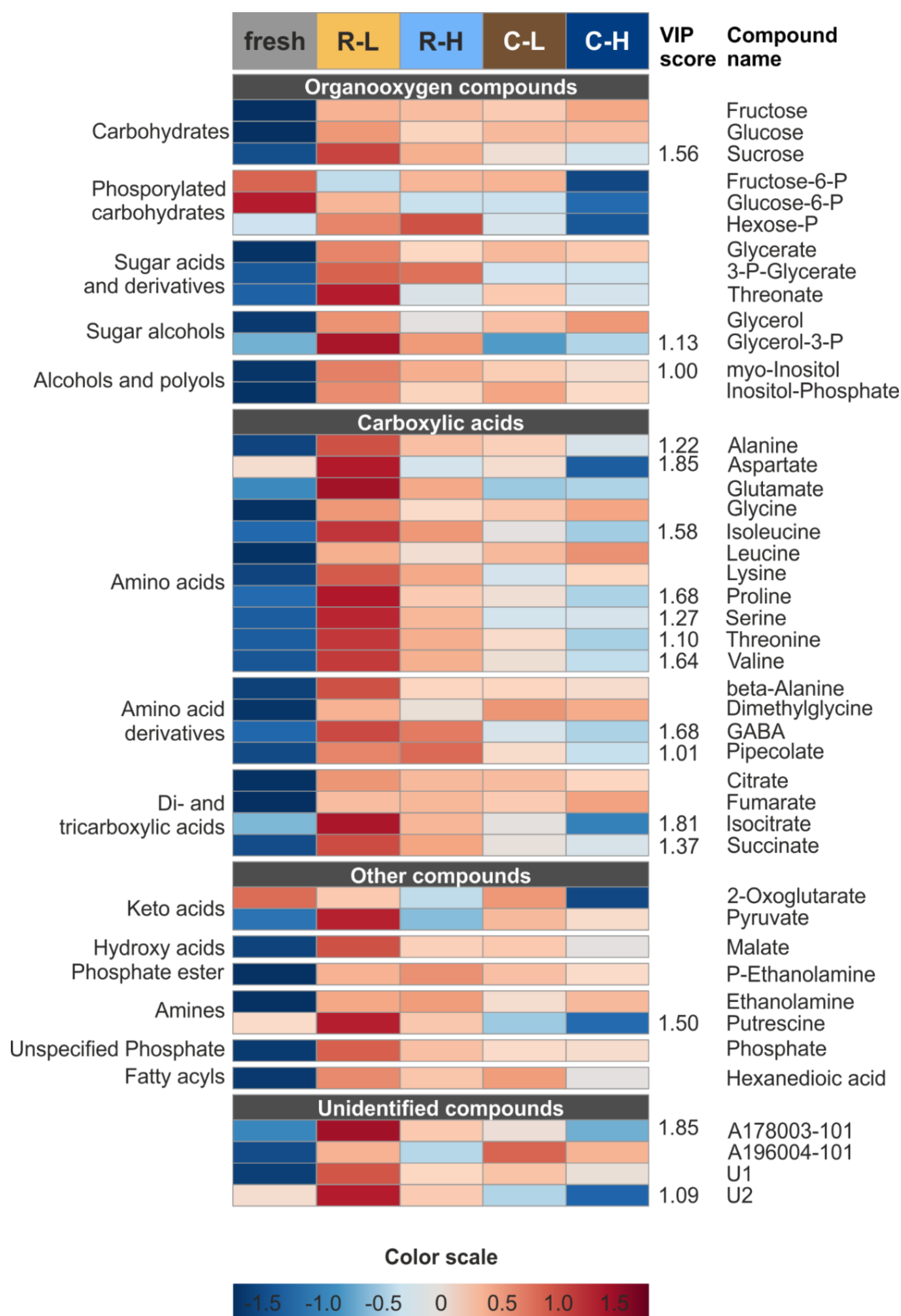
Supplemental Figure S9 Principal component analysis of pollen metabolites shows separation of pollen samples based on the conditions in the Storage experiment. Principal components (PC) of the wheat line TRI 9102 for freshly collected pollen (grey) and pollen stored at room temperature at low RH (R-L, beige) and high RH (R-H, blue), and at cold temperatures at low RH (C-L, dark brown) and high RH (C-H, dark blue) for 10, 20, 30, 45 and 60 minutes (not specified) were compared. (a) PC1 is plotted against PC2, (b) PC1 vs. PC3 and (c) PC2 vs. PC3. The explained variances for each component is given in brackets. (d) The scree plot shows explained (blue) and cumulative variances (green) of each of the first PCs. Four biological replicates were analysed for pollen from each wheat line and condition. Normalized, autoscaled and log transformed data were used. Separate score plots for each storage condition and storage time are provided in **Supplemental Figure S13**.



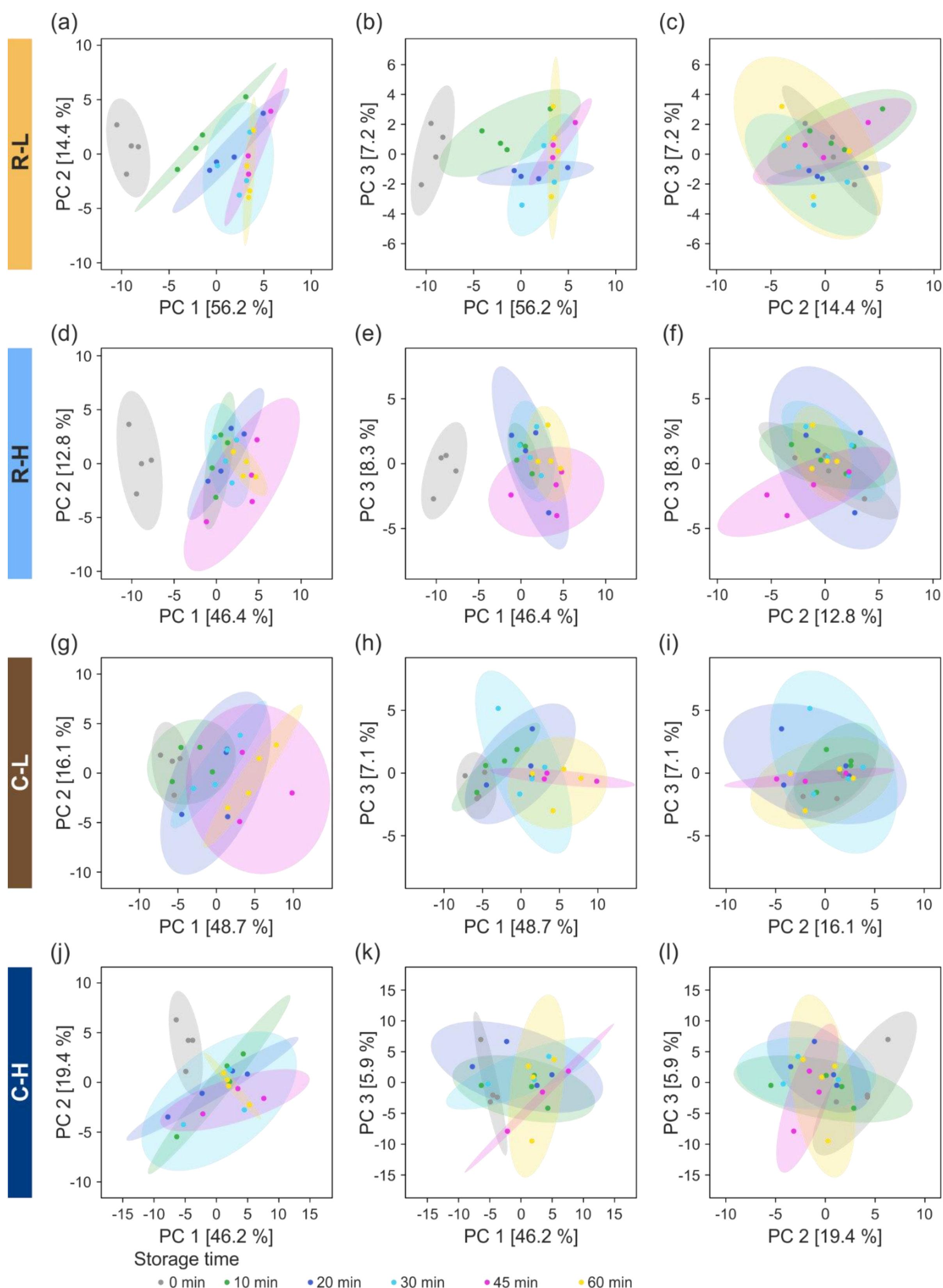
Supplemental Figure S10 Partial Least Square – Discriminant Analysis (PLS-DA) of pollen metabolite show components seperating storage period for each condition (Storage experiment). Components of the wheat line TRI 9102 were compared for freshly collected pollen (grey) and pollen stored at room temperature at (a-c) low RH (R-L) and (d-f) high RH (R-H), and at cold temperatures at (g-i) low RH (C-L) and at (j-l) high RH (C-H) for 10 (green), 20 (dark blue), 30 (light blue), 45 (pink) and 60 minutes (yellow). (a,d,g,j) Component 1 is plotted against Component 2, (b,e,h,k) Component 1 vs. Component 3 and (c,f,i,l) Component 2 vs. Component 3. The explained variances for each component is given in brackets. Four biological replicates were analysed for pollen from each condition and storage period. Normalized, autoscaled and log transformed data were used.



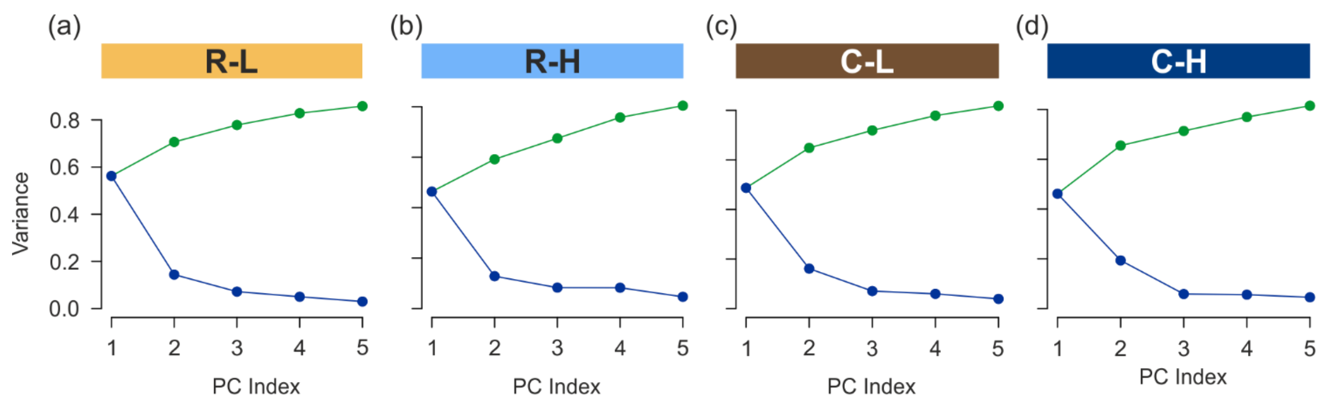
Supplemental Figure S11 Validation of the Partial Least Square Discriminant Analysis (PLS-DA) model of pollen metabolites studied in the Storage experiment and are shown for each storage condition separately. (a,c,e,g) Cross validation and (b,d,f,h) permutation test was performed for five components and 2000 permuations, respectively. Analysis was conducted for a combination of freshly collected pollen and pollen stored at room temperatures at (a,b) low RH and (c,d) high RH and pollen stored at cold temperatures at (e,f) low RH and (g,h) high RH for 10, 20, 30, 45 and 60 minutes. The red star marks the highest value for cross-validated sum of squares (Q^2). Q^2 values higher than 0.4 indicate the best fit of the model (Lundstedt et al., 1998). The permutation test shows assignments of the group membership. Pollen samples located outside the distribution of randomly permuted samples are significantly assigned to a group.



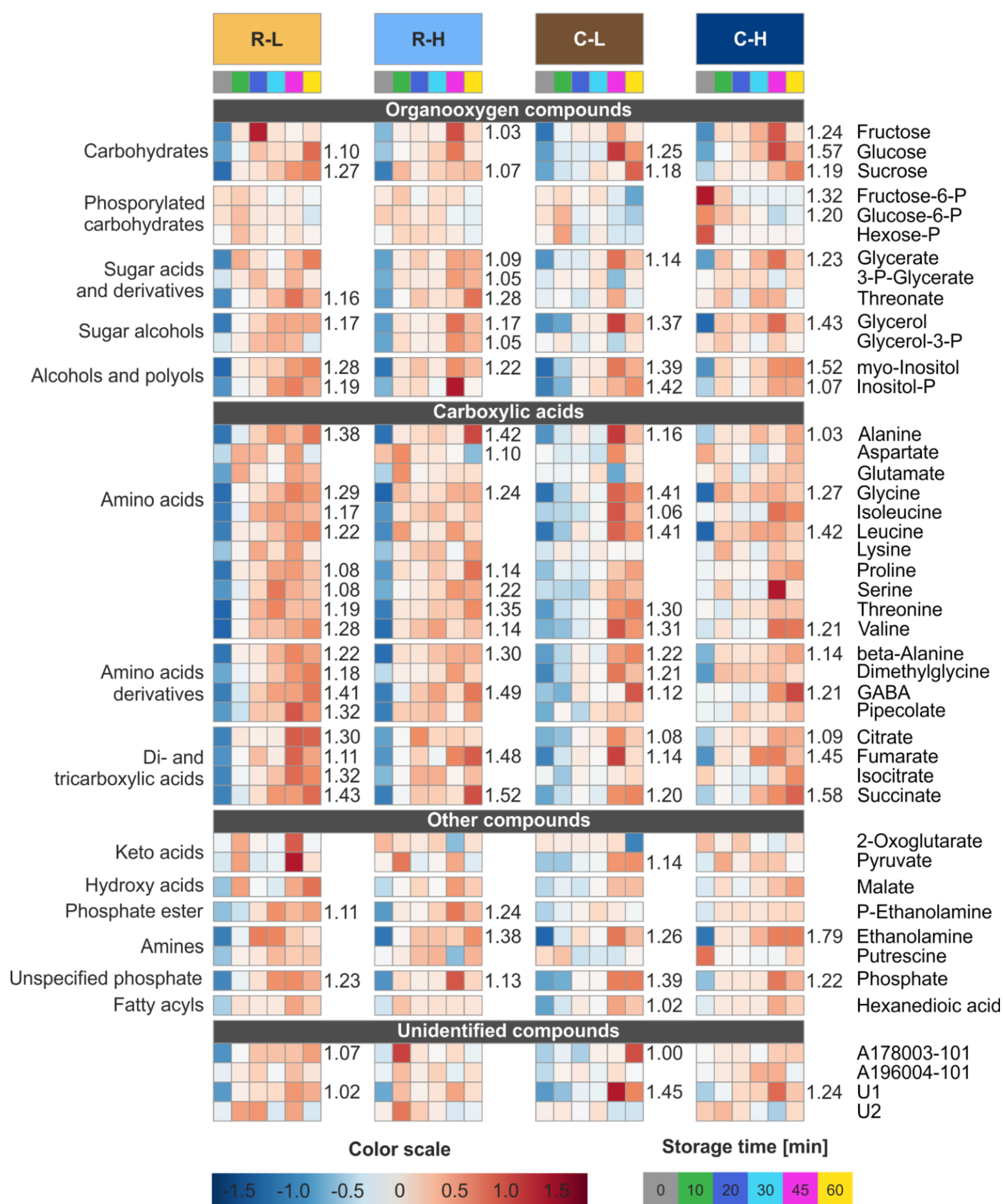
Supplemental Figure S12 Heat map of metabolites of wheat pollen stored under four conditions (Storage experiment). Log-transformed, autoscaled values for metabolites differentially accumulated in pollen of the wheat line TRI 9102 are shown. Data represent mean values of four biological replicates and storage periods (10, 20, 30, 45 and 60 minutes) of pollen stored under room temperatures at low RH (R-L, beige) and high RH (R-H, light blue) and under cold temperatures at low RH (C-L, brown) and high RH (C-H, dark blue) and were compared with fresh pollen (grey). The heat map was constructed with the online-tool ClustVis (https://biit.cs.ut.ee/clustvis/). Metabolites are sorted according to their chemical class. Sub-classification are provided on the left side. Variable Importance in Projection (VIP) scores for metabolites differing between storage groups are provided next to metabolite names. A colour scale is given below the panel. Separate heat maps for each storage condition and storage period are provided in **Supplemental Figure S15**.



Supplemental Figure S13 Principal Components (PC) of pollen metabolites seperate pollen samples according to storage period and are shown for each condition seperately (Storage experiment). PCs of the wheat line TRI 9102 were compared for freshly collected pollen (grey) and pollen stored at room temperature at (a-c) low RH (R-L) and (d-f) high RH (R-H), and at cold temperatures at (g-i) low RH (C-L) and (j-l) high RH (C-H) for 10 (green), 20 (dark blue), 30 (light blue), 45 (pink) and 60 minutes (yellow). (a,d,g,j) PC1 is plotted against PC2, (b,e,h,k) PC1 vs. PC3 and (c,f,i,l) PC2 vs. PC3. The explained variances for each component is given in brackets. Four biological replicates were analysed for pollen from each condition and storage period. Normalized, autoscaled and log transformed data were used.



Supplemental Figure S14 Explained (blue) and cumulative variances (green) are shown for each storage group separately (Storage experiment). The scree plots show variances of each of the first Principles Components (PC) of detected metabolites for a combination of freshly collected pollen and pollen stored at room temperatures at (a) low and (b) high RH and at cold temperatures at (c) low and (d) high RH for 10, 20, 30, 45 and 60 minutes of the wheat line TRI 9102.



Supplemental Figure S15 Heat map of metabolites of wheat pollen stored under four conditions for different intervals (Storage experiment). Log-transformed, autoscaled values for metabolites differentially accumulated in pollen of the wheat line TRI 9102 are shown. Data represent mean values of four biological replicates of pollen stored under room temperatures at low RH (R-L, beige) and high RH (R-H, light blue) and under cold temperatures at low RH (C-L, brown) and high RH (C-H, dark blue) for periods of 0 (grey), 10 (green), 20 (dark blue), 30 (light blue), 45 (pink) and 60 minutes (yellow). The heat map was constructed with the online-tool ClustVis (https://biit.cs.ut.ee/clustvis/). Metabolites are sorted according to their chemical class. Sub-classification are provided on the left side. Variable Importance in Projection (VIP) scores > 1.0 of metabolites with different concentration across storage period in each storage condition are provided. A colour scale is given below the panel.

4. Impact of drying and cooling rate on the survival of the desiccation-sensitive wheat pollen

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Type of authorship:	First author
Type of article:	Research article
Contribution to article:	design and conduction of experiments, data analysis, manuscript preparation
Co-authors contribution:	All authors conceived and designed the research. DI conducted experiments and wrote the first draft of the manuscript. All authors commented on previous versions and approved the final manuscript.
Journal:	Plant Cell Reports
Impact Factor:	4.57
Date of Publication:	2022-01-31
DOI:	10.1007/s00299-021-02819-w

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KEY MESSAGE

Fast-drying and cooling induce fast intracellular water loss and reduced ice-crystal formation, which may promote the formation of intracellular glasses that might improve the likelihood of wheat pollen survival.

ABSTRACT

Long-term storage of pollen is important for the fertilization of spatially or temporally isolated female parents, especially in hybrid breeding. Wheat pollen is dehydration-sensitive and rapidly loses viability after shedding. To preserve wheat pollen, we hypothesized that fast-drying and cooling rates would increase the rate of intracellular water content (WC) removal, decrease intracellular ice-crystal formation, and increase viability after exposure to ultra-low temperatures. Therefore, we compared slow air-drying with fast-drying (dry air flow) and found significant correlations between pollen WC and viability ($r = 0.92$, $P < 0.001$); significant differences in WCs after specific drying times; and comparable viabilities after drying to specific WCs. Fast-drying to WCs at which ice melting events were not detected ($\Delta H = 0$ J mg^{-1} DW, < 0.28 mg H_2O mg^{-1} DW) reduced pollen viability to 1.2 ± 1.0%, but when drying to 0.39 mg H_2O mg^{-1} DW, some viable pollen was detected (39.4 ± 17.9%). Fast cooling (150 °C min^{-1}) of fast-dried pollen to 0.91 ± 0.11 mg H_2O mg^{-1} DW induced less and a delay of ice-crystal formation during cryomicroscopic-video-recordings compared to slow cooling (1 °C min^{-1}), but viability was low (4.5–6.1%) and comparable between cooling rates. Our data support that the combination of fast-drying and cooling rates may enable the survival of wheat pollen likely due to (1) a reduction of the time pollen would be exposed to drying-related deleterious biochemical changes and (2) an inhibition of intracellular ice-crystal formation, but additional research is needed to obtain higher pollen survival after cooling.

Keywords (4 to 6): Cryomicroscopy, Differential scanning calorimetry, Hybrid breeding, Impedance flow cytometry, Pollen viability

INTRODUCTION

Preservation of pollen from flowering plants is an important way to complement plant biodiversity conservation efforts and to widen genetic diversity in breeding programs. The storage of haploid male gametophytes allows fertilization of spatially or temporally isolated parents and supports reproduction in the absence of efficient and effective pollinators (Dinato et al. 2020). Wheat (*Triticum aestivum*), the second most produced crop (http://www.fao.org/faostat/) develops desiccation-sensitive pollen which has completed second pollen mitosis and is tricellular before shedding. This pollen type is ready to germinate upon landing on the stigma (Franchi et al. 2002b; Brewbaker 1967) and loses its viability when exposed to ambient laboratory conditions for 60 minutes or to field conditions for 30 min (D´Souza 1970). So far, the time to pollinate the female parents during crossing and hybrid breeding trials is limited to a short time window and only little information has been reported on its desiccation and low-temperature stress tolerance. Therefore, studies on long-term storage of viable wheat pollen would be of high interest to support preservation, accelerate breeding programs, and to promote new breeding options.

Most pollen grains have water contents (WCs) below 30% (fresh weight (FW) basis) at dispersal and are termed desiccation-tolerant, partially dehydrated or orthodox. This pollen type is often small, between 30 µm and 100 µm (Pacini and Franchi 2020), and is able to be stored at low WCs (< 15% FW basis) and low temperatures for longer periods (Dinato et al. 2020). The low WCs do not favour ice-crystal formation and, thus, freeze-injuries during storage at sub-zero temperatures or ultra-low temperatures (< – 150 °C) (Dinato et al. 2020). To achieve low WCs, pollen is often dried until the cytoplasm enters to a metastable solid state known as the glass (Buitink et al. 1996). Drying may be performed naturally at anthesis, or forced at ambient conditions (if relative humidity (RH) < 75%), above salt solutions (Connor and Towill 1993) or silica gel (Walt and Littlejohn 1996). Protocols for dry storage at low or ultra-low temperatures have been employed for pollen of various crops (Souza et al. 2018; Hecker et al. 1986; Sacks and Clair 1996), medicinal plants (Gaudet et al. 2020a), woody trees (Alba et al. 2011; Zhang et al. 2017; Maryam et al. 2017), ornamental plants (Xu et al. 2014; Geng et al. 2013), and endangered species (Rajasekharan et al. 2013).

Pollen grains that have WCs above 30% (FW basis) at dispersal, termed desiccation-sensitive, partially hydrated or recalcitrant, are often bigger (15 to 150 µm) (Pacini and Franchi 2020) and hardly tolerate water loss below 30% (FW basis). To store dehydration-sensitive pollen, the levels of WC reached must be carefully balanced, and stay higher to those that generate

desiccation damage and lower or close to the limit to those in which water can freeze (Nebot et al. 2021). For desiccation-sensitive pollen of some members of the *Poaceae* family, e.g. maize (*Zea mays*) (Nath and Anderson 1975; Barnabás and Rajki 1976a), pearl millet (*Pennisetum glaucum*) (Hanna 1990), protocols for cryogenic storage were successfully developed. The additional application of rapid dehydration by a stream of dry air showed to increase the survival to low WCs and to improve the cryostorage success of embryonic axes of various desiccation-sensitive seeds (Pammenter et al. 1991; 1998; Berjak et al. 1993; Wesley-Smith et al. 2001a) and has been applied to pollen of maize (Nebot et al. 2021; Buitink et al. 1996).

Besides drying, pollen viability can be affected by cooling speed, i.e., slow vs. fast cooling, during the cryogenic procedure (Dinato et al. 2020). In general, for cells, tissues, or small organs, slow cooling is often conducted in two steps. After samples are cooled at 0.1–5 °C min-1 down to – 35 to – 40 °C which is above the ice nucleation temperature, samples are exposed to liquid nitrogen (LN) for long-term storage. Most intracellular water is removed by freeze dehydration. If applied, cryoprotective substances such as dimethyl sulfoxide (DMSO) interact and modulate the distribution of water in- and outside the cell. Fast cooling applies cooling rates of > 100 °C min-1 to enable vitrification processes (Wolkers and Oldenhof 2021). Biological systems vitrify when cell viscosity increases, glassy structures are formed, and water molecules are prevented from aggregating into larger ice-crystals (Ganeshan et al. 2008). To increase intracellular viscosity, often cells or small organs are exposed to dehydrating agents such as Plant Vitrification Solutions (PVS) which consist of sucrose, glycerol, and DMSO (Fahy and Wowk 2015). Nevertheless, re-crystallization events can occur during warming and affect the viability (Mazur 1984; Bajaj 1985; Meryman and Williams 1985). Therefore, rapid warming by immersing the samples in a warm water bath at 37 to 40 °C for 1 to 5 min is often applied (Ganeshan et al. 2008). The kinetics of intracellular ice formation accompanying cooling and warming can be recorded by advanced video-cryomicroscopy (Karlsson 2015) introduced in 1971 (Diller and Cravalho 1971). This technology has been frequently used in cryopreservation studies of mammalian cells (Stott and Karlsson 2009; Scheiwe and Korber 1984), but its application in plant cryopreservation is still novel. As it provides additional data to assess the risk of cryoinjury (Karlsson 2015), it may guide further advancements in the cryopreservation procedure of, i.e., dehydration-sensitive pollen.

The aim of our study is to investigate systematically the relationship between drying rate, pollen WC, and cooling rates on wheat pollen viability before and after exposure to ultra-low temperatures. Video-cryomicroscopy and differential scanning calorimetry (DSC) were

employed to monitor events of ice crystallization in wheat pollen dried at two drying rates, to test the hypotheses that (1) pollen desiccation tolerance increases in fast-dried compared to slow air-dried pollen, (2) ice crystallization can be reduced in fast-dried and fast-cooled desiccation-sensitive pollen, and (3) the higher desiccation tolerance of fast-dried pollen allows a higher survival after rapid cooling. Results obtained are discussed in relation to main reasons for wheat pollen damage during dehydration and cooling, and are used to recommend further protocol improvements for the cryopreservation of wheat pollen.

MATERIALS AND METHODS

Plant material and pollen sampling

Seeds of the spring wheat lines TRI 9102 (https://doi.org/10.25642/IPK/GBIS/9074) and TRI 3633 (https://doi.org/10.25642/IPK/GBIS/3633) were provided by the Federal *Ex situ* Gene Bank of agricultural and horticultural plants at IPK Gatersleben. Seeds of the winter wheat line 'Ferrum' (KWS, licensed since 2012) were commercially available. Seeds were germinated in a standard culture medium (Substrate1, Klasmann-Deilmann GmbH, Geeste, Germany) at 20 ± 2 °C. One-week old seedlings were subjected to 4 ± 1 °C for 4 weeks. Vernalized plants were transferred into pots containing a sand/soil mixture (70% compost soil, 20% white peat, 10% sand) and grown under optimum conditions (regular watering and fertilization, 16 hours light) at 20 ± 2 °C in the greenhouse.

At the beginning of anthesis, spikes were cut between 8:00 and 10:00 a.m., kept in water and used within 6 h. Only mature pollen was used for all experiments. To stimulate pollen maturation, awns, glumes, and lemmas were carefully removed and pollen was sampled when lodicules swelled, the stigma fanned out, filaments elongated, and anthers enlarged and turned greenish to bright yellow (Impe et al. 2020). Before the tip of the anther opened, at minimum three anthers were taken and pollen shedding was supported by opening gently with a needle. Mature pollen was used immediately after anthesis as a control and termed 'fresh'. Due to different flowering times, pollen of different wheat lines had to be used for different experiments.

Pollen treatment and water content

Fresh mature pollen was collected from 5 to 8 anthers of one spike, transferred to a mesh of pore size 30 µm, and fixed with a second layer of mesh in a so-called flash-dryer according to Buitink et al. (1996) and Nebot et al. (2021). The pollen was exposed to a stream of dry air (10.7 ± 0.1% RH) equilibrated above 250 g silica gel at room temperature which reached

equilibrated RH 4 min after opening. By this method, pollen was fast-dried for 1, 2, 3, 5, 7, 10, 12, 15, 20, and 60 min and corresponding RHs are provided in **Supplemental Figure 1**. Pollen air-dried at 60.0 ± 0.1% RH and room temperature (23.0 ± 0.4 °C) for 10, 20, 30, 40, and 60 min was used as a reference of slow-dried pollen, and fresh pollen served as a reference of non-dried pollen.

Pollen WC was measured on a set of fresh, fast-dried, and air-dried pollen. Pollen was transferred in an aluminium pan, hermetically sealed, and weighted. To determine pollen WC, pans were perforated and exposed to 100 °C for 24 h. Afterwards, pans were re-weighted and the difference between FW and dry weight (DW) was calculated and expressed as mg H_2O mg^{-1} DW (1) or percentage of FW (2).

$$\text{WC [mg H}_2\text{O mg}^{-1}\text{ DW}^{-1}] = \frac{(Weight\ of\ fresh\ pollen\ [mg] - weight\ of\ dry\ pollen\ [mg])}{weight\ of\ dry\ pollen\ [mg]} \quad (1)$$

$$\text{WC [\%]} = \frac{(Weight\ of\ fresh\ pollen\ [mg] - weight\ of\ dry\ pollen\ [mg])}{weight\ of\ fresh\ pollen\ [mg]} \times 100 \quad (2)$$

Then WC values were plotted against drying time and drying curves were built for each wheat line and drying method. Drying rates were calculated for the first part of the drying curve (0 to 10 minutes) in terms of mg H_2O mg^{-1} DW lost per minute (Ballesteros et al. 2014).

Pollen viability

Pollen viability was assessed by pollen germination and impedance flow cytometry (termed IFC viability) using at minimum four biological replicates each if not otherwise stated. Pollen was germinated on a solid medium containing 594 mM raffinose, 0.81 mM H_3BO_3, 2.04 mM $CaCl_2$ at pH 5.8. Pollen tubes exceeding the lengths of the pollen radius were counted manually and expressed as percentage of germination. To determine IFC viability, pollen was transferred into 1 mL IFC buffer (AF6, Amphasys, Lucerne, Switzerland), filtered using 100 µm pore size, and loaded onto a chip of 120 µm channel size. The chip was inserted in the IFC (Ampha Z32, Amphasys, Lucerne, Switzerland) and measurements were carried out at 1 MHz at the default settings for wheat pollen. IFC viability of at minimum 1000 pollen was analysed using AmphaSoft 2.0 version (Amphasys, Lucerne, Switzerland) and given as a percentage.

Differential scanning calorimetry

Fresh, air-dried and fast-dried pollen of TRI 9102 and 'Ferrum' were hermetically sealed in aluminium pans, weighted, and scanned using a DSC-Q2000 (Thermal Analysis Instruments, New Castle, USA) equipped with an LN cooling device. Measurements were carried out between 23 °C and − 150 °C using cooling/warming rates of 10 °C min^{-1}. The onset of crystallization and melting events were determined from the intersection between the baseline and a line drawn from the steepest segment of the transition peak using the software Thermal Analysis v. 4.4. (Thermal Analysis Instruments, New Castle, USA). Baselines were determined using an empty aluminium pan as a reference. The enthalpy (ΔH) of the transition was determined from the area encompassed by the peak and the baseline (Ballesteros and Walters 2007). Exothermic and endothermic changes are expressed on a DW basis and were plotted against the pollen WC. Results were obtained from at least four replicates each for each drying treatment.

Cryomicroscopy-video-recordings

Fresh, air- and partially fast-dried pollen were used. Partially fast-dried pollen was dried for 5 minutes and kept still a high IFC viability (~ 80%) after drying, while the WC decreased to 0.91 ± 0.11 mg H_2O mg^{-1} DW. Although WC was likely above the levels of unfrozen WC (measured at about 0.20–0.40 mg H_2O mg^{-1} DW for seeds, fern spores, *Typha latifolia* or maize pollen (Buitink et al. 1996; Ballesteros and Walters 2007)), we presumed that this would reduce the risk of over-drying of pollen (< 40% IFC viability) which may occur when pollen is dried for 10 minutes, down to the unfrozen WC. For comparison, we also used fresh pollen and pollen dried for 60 min (WC < 0.06 mg H_2O mg^{-1} DW), which was at WC below the unfrozen WC (< 0.34 mg H_2O mg^{-1} DW), but was completely dead. Pollen from different treatments were dusted onto glass plates and placed in the cryomicroscopic stage system (BCS196, Linkam Scientific Instruments, Tadworth, United Kingdom) which was mounted on a light microscope (Eclipse LV100, Nikon, Tokyo, Japan). The stage was connected to a T95-PE temperature control unit (Linkam Scientific Instruments, Tadworth, UK) and a Dewar vessel containing LN. The temperature program and flow rates were controlled using LinkSys32 software (Linkam Scientific Instruments, Tadworth, UK). Pollen were exposed to slow (1 °C min^{-1}) and fast cooling/warming (150 °C min^{-1}) in a range between 0 to − 40 °C. Details are provided in **Table 1**. Due to the high rates during fast cooling/warming, the means and standard deviations of temperatures for structural changes of pollen (1. Darkening, pollen turn black and thawing) were interpolated based on temperature at the start, within the holding phase and at the end of

the cooling program and a few temperature points in between taken at the time points when structural changes were visible.

Time-lapse and real-time videos recorded with a camera attached to the optical output of the microscope were used to monitor structural changes during cooling/warming. To evaluate significant events, NIS software (v. 4.11, Nikon Metrology, Brighton, USA) allowed the extraction of specific images for an exact time point. Three-to-six replicates for each of the six treatments including two cooling/warming programs and three drying treatments were prepared.

Statistical analysis

Means and standard deviations (SD) were calculated in Excel 365 (Microsoft, Richmond, CA, USA) and data are shown as mean ± SD. Statistical analyses were performed based on unpaired Student's *t* test. *P* values < 0.05 were considered significant; in the figures, *, **, and *** indicate significant differences at the 0.05, 0.01, and 0.001 levels of confidence, respectively, while no label indicates no significance. Using GenStat 19.1 (VSN International Ltd., 2016), Pearson correlation analysis was performed to find significant relationships at $P < 0.05$ between drying method, WC and viability. To compare the drying rates, slopes of WCs were estimated by linear regression within the first 10 min and 20 min of drying. WC limits for pollen viability and germination were calculated using the effective dose function (probit analysis) in Genstat 19.1 (VSN International Ltd., 2016). Effective dose of WC was estimated at 60% IFC viability and 20% pollen germination.

RESULTS

The viability of wheat pollen is dependent on the drying rate and water content

Immediately after shedding, pollen of the wheat lines 'Ferrum' and TRI 9102 had a steady high WC of 1.72 ± 0.01 mg H_2O mg^{-1} DW (63.3% of FW) and 1.57 ± 0.10 mg H_2O mg^{-1} DW (61.1% of FW), respectively. After 10 min of drying, air-dried pollen of 'Ferrum' and TRI 9102 lost on average 62.9% (0.64 ± 0.72 mg H_2O mg^{-1} DW) and 54.2% (0.72 ± 0.20 mg H_2O mg^{-1} DW) of the FW, respectively. This slow-drying method resulted in a wide range of pollen WCs across replicates as shown by the standard deviation, indicating an inhomogeneous drying of the pollen. The pollen grains dried on a plate, so there was probably passive drying on the surface of the pollen grains exposed to drying air at 60% RH. In comparison, fast-drying resulted in an average loss of 79.7% (0.32 ± 0.15 mg H_2O mg^{-1} DW) and 67.7% of FW (0.51 ± 0.13 mg H_2O mg^{-1} DW), respectively (**Figure 1**), with more homogenous drying including smaller standard deviation observed. Fast-drying was carried out on pollen "floating" on a stream of 10% RH dry air, which likely dried the whole grain and not just the exposed area. The loss of water during the first 10 min followed a near-linear trend **(Figure 1**) and the drying rates (slopes of linear regression curves) were significantly different between the drying approaches ($P \leq 0.001$) but not between the wheat lines ($P = 0.133$). Fast-dried pollen lost − 0.116 ± 0.080 mg H_2O min^{-1}, while air dried pollen lost − 0.097 ± 0.086 mg H_2O min^{-1}. Overall, fast-drying led to a homogenous, significant ($P \leq 0.01$) and 1.8- and 1.4-times larger loss of water of 'Ferrum' and TRI 9102, respectively, compared to slow air-drying after 10 min. Comparable significant differences ($P \leq 0.001$) were also found after 20 min of drying (**Figure 1**). However, after 60 min, no significant differences were found in the WC of pollen from both lines exposed to air- and fast-drying, which reached steady WC values of 0.08 ± 0.04 mg H_2O mg^{-1} DW and 0.06 ± 0.02 mg H_2O mg^{-1} DW (95.2% WC loss from FW), respectively. In summary, the flow of dry air during fast-drying caused a larger, faster and more homogenous water loss on average compared to slow air-drying, particularly in the first 20 min.

Viability of fresh pollen determined by IFC (IFC viability) was high, 93.2 ± 5.5% and 87.3 ± 6.7% for 'Ferrum' and TRI 9102, respectively (**Figure 2a**). Pollen germination evaluated on raffinose-based media was lower, ranged between 28.8 ± 15.3% and 38.5 ± 19.3%, respectively (**Figure 2c**). After 5 min of fast-drying ($WC_{'Ferrum'}$ = 0.87 ± 0.13 and $WC_{TRI9102}$ = 0.96 ± 0.09 mg H_2O mg^{-1} DW), IFC viability was 83.9 ± 6.4% and 78.8 ± 14.2% and pollen germination was 4.5 ± 0.9% and 10.9 ± 1.0% for 'Ferrum' and TRI 9102, respectively. After 10 min of fast-drying ($WC_{'Ferrum'}$ = 0.35 ± 0.11 and $WC_{TRI9102}$ = 0.51 ± 0.13 mg H_2O mg^{-1} DW), IFC viability

decreased further to 29.6 ± 16.3% and 49.1 ± 15.3% for 'Ferrum' and TRI 9102, respectively, and pollen germination to 1.9 ± 3.9% for both wheat lines. For comparison, after 10 min of air-drying, pollen germination of both wheat lines dropped to 12.2 ± 12.3% (**Figure 2c**), but in this case, WC was significantly higher (0.68 ± 0.22 mg H_2O mg^{-1} DW). After 60 min of air- and fast-drying, when WC was below 0.08 ± 0.04 and 0.06 ± 0.02 mg H_2O mg^{-1} DW, respectively, both pollen germination and IFC viability dropped to 0% (**Figure 2c, d**), although after 20 min of drying, viability was near zero in both drying regimes. The decrease of viability during drying period (or the reduction of pollen WC) followed a sigmoidal trend (**Figure 2a, b**). To estimate the "damaging" WC during drying, we assumed that at this point the IFC viability dropped by one-third to 66% and calculated the WCs using probit transformation. Finally, the damaging WCs were estimated at 0.83 ± 0.11 and 0.94 ± 0.11 mg H_2O mg^{-1} DW for 'Ferrum' and TRI 9102, respectively, during fast-drying. When pollen germination dropped by one-third, at about 20%, the damaging WCs was at 1.29 ± 0.16 and 1.29 ± 0.13 mg H_2O mg^{-1} DW for air- and for fast-drying, respectively, indicating that damaging WCs were similar between both drying methods and wheat lines. Coefficients of correlation between WC and IFC viability or pollen germination were 0.92 ($P < 0.001$) and 0.94 ($P < 0.001$), respectively, suggesting that pollen viability is tightly linked to WC.

Thermo-physical properties of wheat pollen are altered by drying procedures before ultra-low freezing

Differential scanning calorimetry detected diverse first- and second-order phase transitions in both the cooling and warming scans of samples with varying WCs. During cooling scans, broad exothermic events (crystallization) were detected between −12 to − 56 °C for wheat pollen having WC between 0.27 and 1.77 mg H_2O mg^{-1} DW. In some cooling thermograms, multiple broad crystallization peaks were observed in pollen samples dried to a maximum of 5 min (WC between 0.91 and 1.77 mg H_2O mg^{-1} DW). Additionally, a second small peak, presumably eutectic formations (Sun 2021), was found at ~ − 100 °C and 'crystallization loops' appeared in most pollen samples with high WC (> 1 mg H_2O mg^{-1} DW corresponding to < 5 min fast-drying) (**Supplemental Figure S2**). Broad endothermic events (melting transitions) during the warming program were observed between − 3 to − 32 °C. When the WC of fresh pollen of both wheat lines (1.64 ± 0.11 mg H_2O mg^{-1} DW) was reduced by fast-drying for up to 15 min (≥ 0.22 ± 0.05 mg H_2O mg^{-1} DW) or by air-drying for up to 60 min (0.08 ± 0.02 mg H_2O mg^{-1} DW), the size of the broad crystallization and melting peaks (measured by the enthalpy [ΔH] of transition) declined, indicating that these phase transitions are ice-crystal formation and melting events (**Figure 3a**).

By plotting WC against onset and peak temperature (**Figure 3b**), a moderate decrease of the regression line was shown until WC dropped to 0.74 mg H_2O mg^{-1} DW. This would be expected for a dilute solution of increasing concentration and was observed in embryos of recalcitrant seeds (Pammenter et al. 1991). Below, a rapid decrease in the onset and peak temperatures of the water melting transitions with WC was observed, indicating a change in the water properties of the cells as the solute concentration continues to increase which strongly affects the melting activity of the ice crystals. Furthermore, the ΔH of melting transitions dropped linearly (**Figure 3c**) and reached 0 J mg^{-1} DW at < 0.34 mg H_2O mg^{-1} DW after more than 12 min fast-drying. The value for ΔH of melting (slope of linear regression) was 106.6 and 119.1 J mg^{-1} H_2O for 'Ferrum' and TRI 9102, respectively (**Figure 3c**). The amount of water that did not freeze was calculated from the intersection of the X-axis and the sloped line drawn for water transitions. Unfrozen WCs were achieved at 0.24 and 0.28 mg H_2O mg^{-1} DW for 'Ferrum' and TRI 9102, respectively. Coefficients of correlation of the regression lines constructed to calculate the unfrozen WC showed high-quality fit for 'Ferrum' (r^2 = 0.97) and TRI 9102 (r^2 = 0.95). At WCs below the unfrozen WC pollen showed very low or no viability (**Figure 2b**), however, at WCs near the unfrozen WC, some pollen was still viable (39.8 ± 24.8% IFC viability at < 0.39 mg H_2O mg^{-1} DW corresponding to 12 min drying time), indicating that under these conditions some viable pollen could be exposed to sub-zero temperatures without a lower risk of ice-crystal formation. However, to avoid over-drying of pollen when drying to WCs below the unfrozen WC, in further freezing experiments, we applied partially fast-drying for 5 min which provided > 50% IFC viability.

Partial fast-drying and fast cooling/warming may contribute to pollen survival by reducing intracellular ice-crystal formation

Changes in structure and colour were observed in fresh and partially fast-dried pollen (with a WC above the unfrozen WC) but not in fully dried pollen (to WCs below the unfrozen WC), during both slow and fast cooling/warming. During slow cooling, both fresh and partially fast-dried pollen suddenly 'flashed' and turned dark at − 31.6 ± 1.0 °C and − 38.0 ± 0.8 °C, respectively. The so-called 'darkening' was followed by the formation of ice crystals on the surface of the pollen grains (Figs. 4a, b, 5a, Supplemental video 1−2). During slow warming, pollen turned completely black at − 32.4 ± 6.1 °C and − 31.5 ± 1.7 °C, respectively, and thawing of ice crystals began at − 12.6 ± 4.0 °C for fresh pollen and at − 17.6 ± 1.2 °C for partially fast-dried pollen. At the end of the program, fresh pollen shrunk massively and had an IFC viability of 0.9 ± 0.7% while the partially fast-dried pollen also shrunk but had an IFC viability of 4.5 ± 7.0%. During fast cooling, less and/or smaller ice crystals were observed

around the pollen. Darkening occurred in both fresh and partially fast-dried pollen, when temperature was hold at − 40 °C for 3 min and turned completely black at − 19.6 ± 11.6 °C and − 18.6 ± 5.0 °C during fast warming, respectively (**Figures 4a, b, 5b, Supplemental video 3−4**). At the end of the fast-warming step, fresh pollen and partially fast-dried pollen appeared rounder, were less dehydrated and showed some viability (but not germination) (fresh pollen: 1.5 ± 1.7%, partially fast-dried pollen: 6.1 ± 8.8%) compared to slow cooling/warming (**Figure 6**). In addition, the change in colour and thawing of ice crystals (fresh pollen: − 6.0 ± 8.8 °C, partially fast-dried pollen: − 4.9 ± 4.6 °C) occurred at about 10 °C higher temperatures compared with slow cooling/warming (**Figure 5b**). Pollen dried below the unfrozen WC had an IFC viability of 0% (**Figure 2**) and showed no darkening or ice-crystal formation at either slow or fast cooling (**Figure 4c, Supplemental video 5−6**). Overall, pollen treated with a combination of partially fast- drying and fast cooling/warming tend to have visually less and/or smaller ice crystals and higher IFC viability than fresh pollen.

DISCUSSION

The preservation of wheat pollen is important for conventional breeding, hybrid breeding and production and the conservation of wheat genetic diversity. Currently, any prolonged storage, more than several hours, is not possible due to the high desiccation sensitivity of mature pollen after shedding. In this paper, we investigated how drying rate and cooling/warming rate affect pollen viability and the physicochemical properties of the water in their cells. We found that viability in pollen was lost soon during the drying process, even before any changes in the structural properties of water are detected by DSC. However, about 40% of wheat pollen retained viability at WCs > 0.28 mg H_2O mg^{-1} DW, a WC slightly above the limit at which ice freezing/melting events occur. At this WC, pollen grains appear to have highly concentrated cytoplasm based on the thermal responses of the freezing water, which open the option for the development of successful ultra-low-temperature storage. We also measured that the combination of fast-drying, fast cooling, and warming rates during video-cryomicroscopy resulted in a reduction and delay of ice-crystal formation, a reduction of the temperature for intracellular water crystallization, and an increase in the temperature of intracellular ice melting. All three properties may favour the formation of intracellular glasses and could contribute to the success of wheat pollen cryopreservation, however, a fine-tuning of the combination of fast partial-drying and cooling rates is needed to achieve higher survival/germination rates.

Desiccation damages prior cooling can be reduced by rapid drying

Dependent on the drying rate and the amount of water removed, different types of damages compromise survival in desiccation-sensitive pollen and tissues (Pammenter and Berjak 2014). At high WC, dehydration causes physical damages by reducing cell volume (Pammenter and Berjak 2014). In wheat, we observed some shrinkage of pollen when dried to 0.91 mg H_2O mg^{-1} DW (**Figure 4b**). Further intense drying causes "*desiccation damage sensu stricto*" (Walters et al. 2001). The WC at which 33% of the initial wheat pollen viability is lost was estimated at between 0.83 ± 0.11 and 1.29 ± 0.16 mg H_2O mg^{-1} DW depending on the viability testing method used. We considered these WC ranges to be an indication of the WC limit at which the pollen was significantly damaged and refer to them as damaging WC. Compared to embryonic axes of recalcitrant seeds that retain high viability (over 90%) upon drying to near freezable WCs at ~ 0.25−0.50 mg H_2O mg^{-1} DW (Wesley-Smith et al. 2001b), the damaging WC limit determined for wheat pollen in the current study was very high. By contrast, maize pollen dried to 0.55 mg H_2O mg^{-1} DW can still retain over 75% germination (Buitink et al. 1996). In wheat pollen, the damaging WC determined fell above the break in the relation between WC and water

melting peak/onset temperature (**Figure 3b**) detected at 0.74 mg H_2O mg^{-1} DW. Although conjectural, this break might be due to a change in the water properties of the cytoplasm that could be related to a threshold in the concentration of solutes during drying that alter the physicochemical properties of ice-crystal formation (Berjak et al. 1993) and may lead to deleterious molecular interactions (Oliver et al. 2020). When WC is reduced below a critical value, the removal of water from macromolecular and membrane surfaces causes metabolism-linked damages (Pammenter and Berjak 2000). In wheat, pollen lost most of its initial viability around freezable WC at < 0.28 mg H_2O mg^{-1} DW, ~13% FW basis. Extensive membrane damages were found in desiccation-sensitive pollen of *Pennisetum typhoides* and maize dried to 3% and 7 to 8% WC, respectively (Hoekstra et al. 1989a; Kerhoas et al. 1987). In wheat pollen, at WC below 0.06 mg H_2O mg^{-1} DW (2.4%), viability was completely lost and accompanied with the strong shrinkage of wheat pollen (**Figure 4c**). At this stage, cells, organelles, and membranes may collapse (Buitink et al. 1998) resulting in cell/pollen death (Bajaj 1985; Pammenter et al. 2003; Pammenter and Berjak 2000).

However, drying damage can be partially avoided depending on the drying rate. When drying is conducted slowly, electron transfer in mitochondria and plastids is impaired that facilitates the formation of reactive oxygen species (ROS) (Halliwell and Gutteridge 2015; Smirnoff 1993; Hendry et al. 1992) reacting with proteins, lipids, and nucleic acids (Halliwell and Gutteridge 2015). Additionally, the reduced fluidity of the cytoplasm at intermediate WC limits the efficiency of corresponding detoxifying processes (Vertucci and Farrant 1995; Bailly 2004; Berjak 2006). When drying is conducted sufficiently rapid, i.e., by a stream of dry air (Buitink et al. 1996), and WC in dehydration-sensitive cells remain above the critical points, the time in which cells pass through intermediate WC ranges is shorter. Thus, fast-drying may reduce the overall occurrence of metabolism-derived damages (Farrant et al. 1993; Kioko et al. 1998; Pritchard and Manger 1998; Pammenter and Berjak 1999; Pammenter et al. 2002). However, despite the faster and more homogeneous drying induced by the fast-drying method applied in this work (**Figure 1**), we found that both the damaging WC calculated by the viability/WC relations (**Figure 2**) or the viability loss observed at given WCs was comparable for the air- and fast-drying methods used, suggesting that the different drying rates applied in this study were not sufficient to detect drying-related differences in viability prior to the cooling stage. Nonetheless, fast-drying reduces the time the pollen is exposed to stressful metabolic processes. Due to the additional stress on the pollen by cooling, we think that fast-drying prior cooling increases the chances that the desiccation-sensitive wheat pollen will survive the whole cryopreservation process.

Wheat pollen can partly survive the loss of freezable water

When cells lose the unbound, freezable water, intracellular viscosity increases (Hoekstra et al. 2001). Upon further drying and/or cooling the fluid cytoplasm develops properties of a solid structure (Buitink and Leprince 2004; Ballesteros and Walters 2007; Ballesteros and Walters 2011) which is non-crystalline (unlike the intracellular ice that forms in the presence of freezable water) and is referred to as a glass. A glass is an amorphous solid that combines properties of a solid and a liquid without any defined structures (Buitink and Leprince 2004). The increase in cytoplasmic viscosity correlates with decreases in molecular mobility (Sun 2000; Ballesteros and Walters 2011; Leprince and Hoekstra 1998) and metabolic activity where most chemical reactions are ceased (Benson 2008). The extremely high cytoplasmic viscosity of the glass and the binding of most water molecules to macromolecules prevent the formation of ice crystals during cooling (Benson 2004). For diverse pollen, ice crystallization events are not detected by DSC typically at WC < 20% (0.21−0.26 mg H_2O mg^{-1} DW). Below this WC, cryo-injuries can be prevented during freezing and thawing (Dinato et al. 2020). For wheat pollen, we demonstrated that depending on the drying rate, pollen reduced the fraction of freezable water in less than 30 min, but viability was severely impaired, when pollen dried to the freezable WC at 0.28 mg H_2O mg^{-1} DW, ~13% FW basis. However, near the freezable WC limit, wheat partially fast-dried pollen retained IFC viability at 39.8 ± 24.8%. Although both, desiccation-sensitive and -tolerant, plant tissues are capable of forming glasses upon drying and/or cooling (Buitink et al. 1996), most dehydration-sensitive plant material cannot survive the low WC at which glasses typically form before exposing the samples to sub-zero temperatures (Berjak and Pammenter 2004). Nevertheless, cryopreservation was successfully implemented for desiccation-sensitive maize pollen. Dependent on genotype, some varieties retained high viability and seed set after drying to between 12 and 20% WC corresponding to 0.14 and 0.25 mg H_2O mg^{-1} DW (Barnabás and Rajki 1976a, 1981). Inagaki and Mujeeb-Kazi (1994) achieved about 22.1% of maize pollen germination after storage at – 80 °C and 9.5% WC for 4 weeks which was likely due to the removal of the freezable water fraction and the formation of a glassy state below – 25°C at such WCs (Buitink et al. 1996). To cryopreserve desiccation-sensitive wheat pollen with high viability, existing procedures (Nebot et al. 2021) must be modified to favour the formation of a glassy state while overcoming the constraints of the low viability when WC is reduced below the freezable WC limit. This balance between vitrification without ice formation may be achieved by increasing cooling rates at the WCs at which wheat pollen still retains high viability.

Fast cooling limits and delays ice-crystal formation compared to slow cooling

The survival of plant cells after freezing is dependent on the lethal effects of intracellular ice. Ice crystals affect the organization of the cytoplasm and damage the membranes of cell organs by increasing their volume (Wolkers and Oldenhof 2021). The formation of intracellular ice can be effectively minimized by the cooling rate (Mazur 2004). At very slow cooling rates (~ 0.1 to 5 °C min^{-1}), ice is formed intercellularly. The chemical potential between cells inside and outside leads to water efflux, cell dehydration (Wolkers and Oldenhof 2021), and eventually vitrification of the cytoplasm. However, the dehydration during slow cooling may severely damage desiccation-sensitive cells (Wesley-Smith et al. 2001b). When the cooling rate increases (~ 100 °C min^{-1}), the time for the water to flow out the cell is not sufficient to prevent supercooling, spontaneous intracellular freezing, and the aggregation of larger ice crystals (Mazur 1984). At very high cooling rates (> 1000 °C min^{-1}), more, smaller and evenly distributed ice crystals appear (Dumont et al. 2003) and might be more beneficial for desiccation-sensitive cells compared to dehydration at slower cooling rates. Therefore, in desiccation-sensitive embryonic axes of *Aesculus hippocastanum* L., a combination of fast-drying followed by cooling at very high rates reduced deteriorative processes and enabled the survival of the tissue (Wesley-Smith et al. 2001b). When wheat pollen was fast-dried for 5 min and rapidly cooled and warmed in the Linkam chamber using 150 °C min^{-1} (**Figure 3, Supplemental videos 2, 3**), we observed a reduction of intracellular ice-crystal formation and the limitation of the ice-crystals formation to the isothermal step at − 40°C. Under these conditions, some wheat pollen still showed IFC viability of 6.1 ± 8.8%. To observe processes of ice formation in wheat pollen by cryomicroscopy, we were limited to the rapid cooling rates at max 150 °C min^{-1} and an experimental design conceived to study ice formation in the first stages of cooling. However, higher very rapid cooling rates to < − 130 °C temperatures should be applied in future experiments to combine the effects of fast-drying, evenly distributed ice crystals, and the supercooling of the system which may allow the formation of a glassy state and an even higher survival after cryopreservation.

During cryomicroscopy, a sudden change in opacity of the cytoplasm is often described as "blackening" or "flashing" (Smith and Smiles 1953; Scheiwe and Korber 1984; Day et al. 2000; Acharya and Devireddy 2010). The cause for blackening is not completely understood. It may occur as a result of light scattering from the surfaces of ice crystals (Smith 1961; Körber et al. 1991), from tiny gas bubbles formed in parallel (Steponkus and Dowgert 1981) or from rearrangement and aggregation of the intracellular organic matter (Dong et al. 2010; Stott and Karlsson 2009). Mazur et al. (2005) speculated that the darkening is the result of glass transition

of intracellular water. In wheat pollen studied, the '1. darkening' appeared before ice crystals were visible and indicate that ice crystals alone or combined with vitrification events may be the cause of darkening in fresh and fast-dried pollen (**Figure 4a, b, Supplemental videos 1, 2**). Interestingly, an abruptly second darkening termed 'blackening' occurred during warming. This phenomenon was also observed in mouse embryos and was suggested to be related with devitrification, but more likely with re-crystallization events of small intracellular crystals (Mazur et al. 2005). In general, cooling and warming rates are intertwined (Pegg 2007) and both processes affect the viability (Normah and Makeen 2008). During re-crystallization, ice crystals merge and damage the cells (Meryman 1966a; Mazur 1984). However, there was a clear difference in the temperature at which the '1. darkening' and 'pollen turn black' occurred between slow and fast cooling/warming and between fresh- and fast-dried pollen. Here, we speculate that at slower cooling/warming and at higher WCs, ice crystals had more time and volume to aggregate which facilitated an earlier '1. darkening and 'blackening' compared to fast cooling/warming and fast-drying. Nonetheless, these effects on the delay of ice-crystal formation did not favour a higher survival of the pollen grains. In further experiments, it has to be clarified if the 'darkening' of cells is related with pollen viability changes and could be used to optimize the cryogenic procedure for wheat pollen preservation.

CONCLUSION

Wheat is among the most important crops worldwide and the preservation of viable pollen would be beneficial for breeding programs. By analysing the change in pollen viability after exposure to different drying treatments, we observed that wheat pollen lost viability extremely fast (after > 12 minutes drying) and that the increased drying rate did not show the expected benefits. However, when fast-dried pollen was exposed to fast cooling/warming rates, we found some viable pollen, a reduction of the ice crystallization temperature, and a lower amount of ice crystals formed. Nevertheless, these results were not enough to achieve a high pollen survival. Long-term wheat pollen storage is still not efficient due to its extreme sensitivity to the removal of water molecules and osmotic concentration. Further investigations of the water properties of wheat pollen at high WCs, a better control of the ice crystallization and re-crystallization events during freezing and warming, and a better understanding of the vitrification of pollen at high water contents will help to develop more optimal cryopreservation protocols. Thereby, it should be considered that cooling/warming procedures should be extremely fast at WCs where viability is high and, eventually, cryoprotective chemicals, if entered, may stabilize the pollen without ice formation. These protocols and further testing in

the fertilization of plants with cryopreserved pollen will elucidate if wheat pollen cryogenic storage can be an efficient tool for wheat conservation and breeding.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1007/ s00299- 021- 02819-w.

ACKNOWLEDGEMENTS

We like to acknowledge Kerstin Wolf, Jürgen Marlow, and the gardeners of IPK and Kew garden/Wakehurst for their excellent technical support.

AUTHOR CONTRIBUTION STATEMENT

DI, DB, and MN conceived and designed research. DI conducted experiments and wrote the first draft of the manuscript. All authors commented on previous versions and approved the final manuscript.

FUNDING

The work is part of a PhD project and was supported by Leibniz Institute of Plant Genetics and Crop Plant Research (IPK). The Royal Botanic Gardens, Kew receives grant-in-aid from Defra.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

DECLARATION

- Funding: The work is part of a PhD project and was supported by Leibniz Institute of Plant Genetics and Crop Plant Research (IPK). The Royal Botanic Gardens, Kew receives grant-in-aid from Defra.
- Conflicts of interest/Competing interests: The authors have no conflicts of interest to declare that are relevant to the content of this article.
- Availability of data and material: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request
- Code availability: not applicable
- Ethics approval: not applicable

- Consent to participate: not applicable
- Consent for publication: not applicable

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4.2. Tables and Figures

Table 1: Cooling / warming programs used to monitor wheat pollen during cryogenic treatments.

Temperature program	Type of video recording	Ramp	Temperature [°C]	Flow rate [°C min^{-1}]	Holding time [min]
Slow cooling	Time lapse video 1 frame per 5 seconds	1	20	100	
		2	0	1	
		3	-40	1	2
		4	0	1	
		5	20	100	
Fast cooling	Real-time video 30 frames per sec	1	20	150	
		2	-40	150	3
		3	20	150	

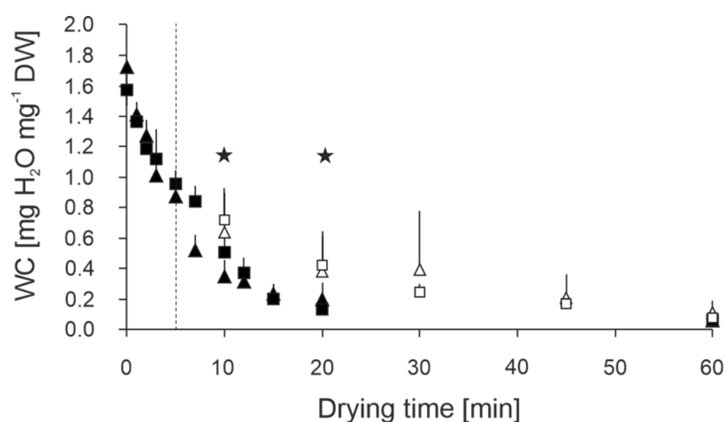


Figure 1 Fast-drying resulted in a faster reduction in pollen water content (WC). Pollen of the wheat lines 'Ferrum' (triangles) and TRI 9102 (squares) were slow air-dried (white symbols) or fast-dried (black symbols) at room temperature at low relative humidity for up to 60 min. Mean and standard deviation are shown for each drying time representing for 4 to 5 replicates each. Stars mark significant differences in WC between fast and air-dried pollen at $P < 0.05$ (comparison made only for available sampling points, here after 10, 20 and 60 minutes of drying). The dashed line indicates WC after 5 min drying time used for following cooling experiments. DW, dry weight

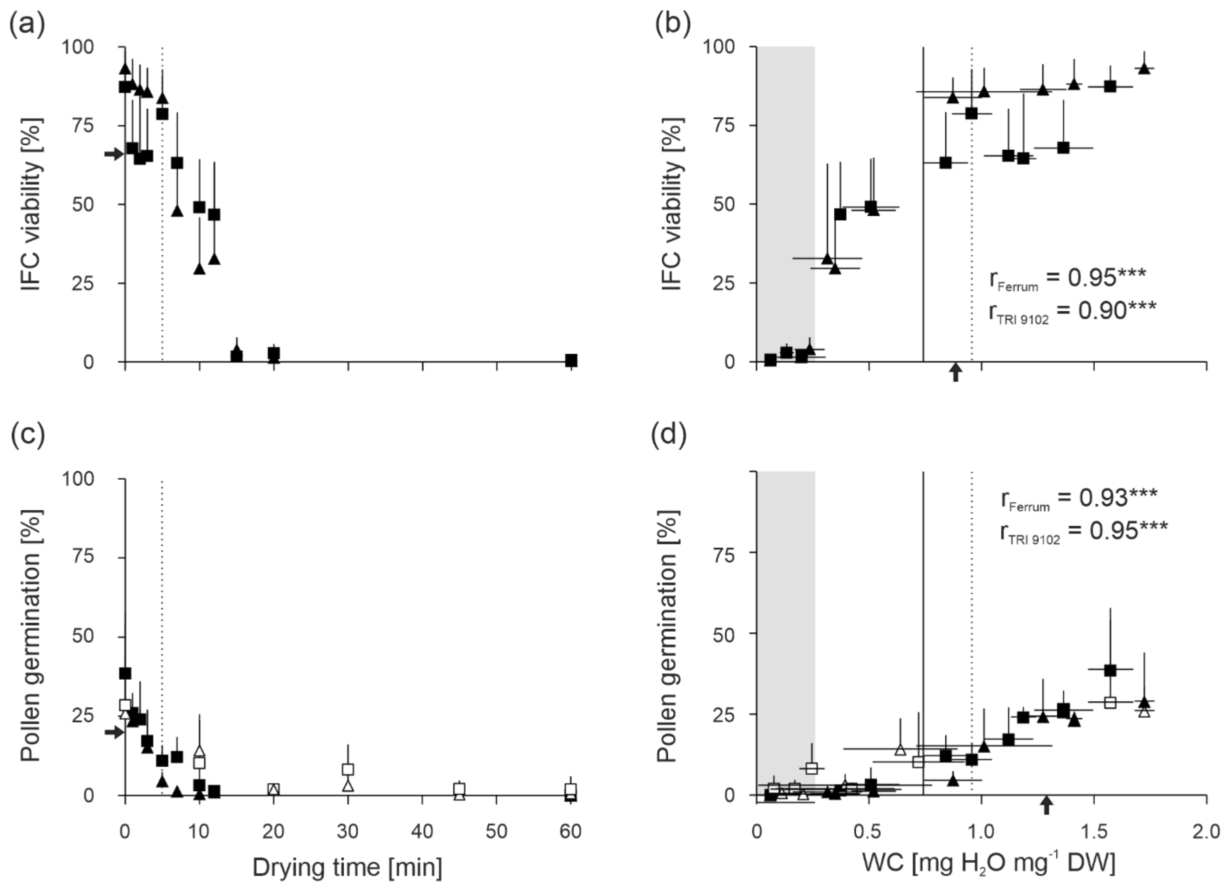


Figure 2 Reduction in pollen water content (WC) affects pollen viability. Pollen of wheat lines 'Ferrum' (triangles) and TRI 9102 (squares) were slow air-dried (white symbols) or fast-dried (black symbols) for 1 to 60 min. **a, c** Drying time and **b, d** pollen WC was plotted against (**a, b)** pollen viability determined by impedance flow cytometry (IFC viability) and **c, d** pollen germination determined on a raffinose-based medium. Mean and standard deviation are shown for each drying time and respective WCs representing 4 to 5 replicates each. The dashed lines indicate pollen viability/germination and WCs after 5 min drying time used for following cooling experiments. Shaded areas show WCs at which no frozen water was detected. Solid lines indicate a transition of onset/ peak temperatures in the melting curves (compare with **Figure 3b**). Black arrows indicate damaging WC (**b, d**), at which IFC viability and pollen germination dropped by one third (**a, c**). DW, dry weight

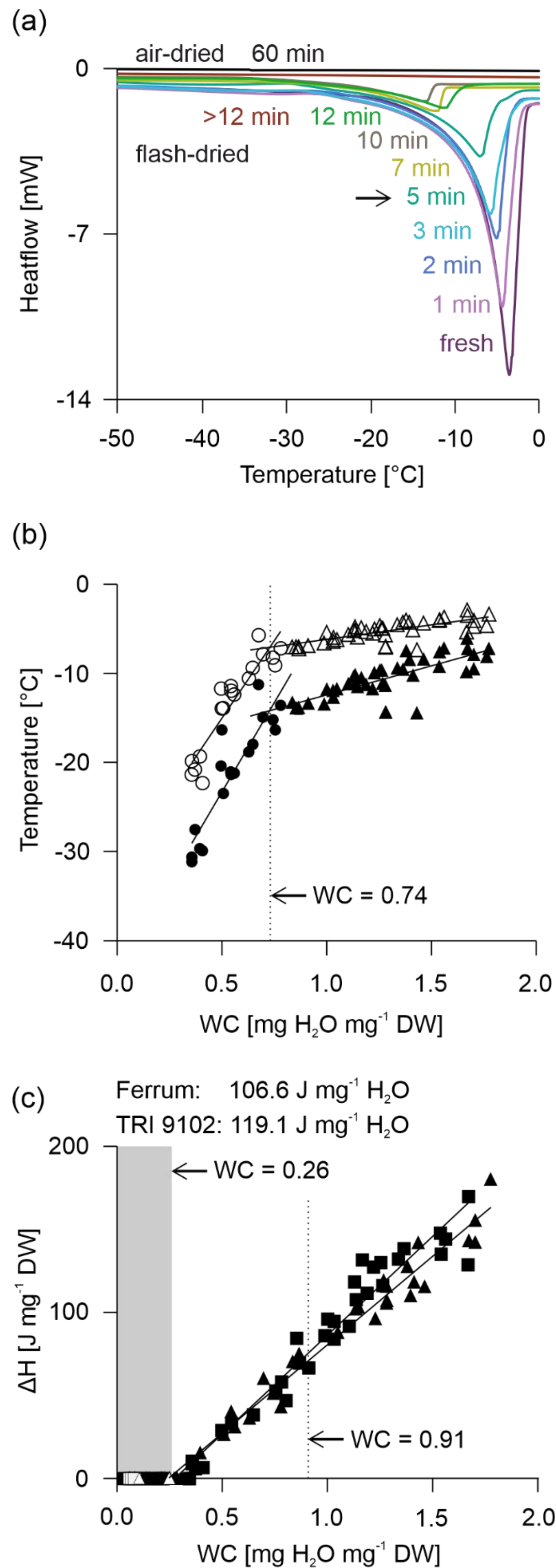


Figure 3 Heat flow decreases when pollen water content (WC) declines and presumably causes fewer crystallization events. Pollen of wheat lines 'Ferrum' (triangles) and TRI 9102 (squares) were slow air- (white symbols) and fast-dried (black symbols) for up to 60 min and heat flow measured using Differential Scanning Calorimetry (DSC). **a** Heating thermograms

represent warming curves of selected replicates of pollen from wheat line 'Ferrum'. **b** Relationship between onset temperature (closed symbols) and peak temperatures (open symbols) of the melting transitions with WC. The dashed line indicates the WC (corresponding to > 5 min partially fast-drying time) from which we observed a steep viability decline (compare **Figure 2a, c**). **c** Relationship between the pollen WC and the enthalpy (ΔH) calculated on basis of the area covered by the warming curves is shown for air- and fast-dried pollen. The dashed line indicates the WC and respective enthalpy after 5 min drying time used for following cooling experiments. Shaded areas show WCs at which no frozen water was detected for both wheat lines and drying methods.

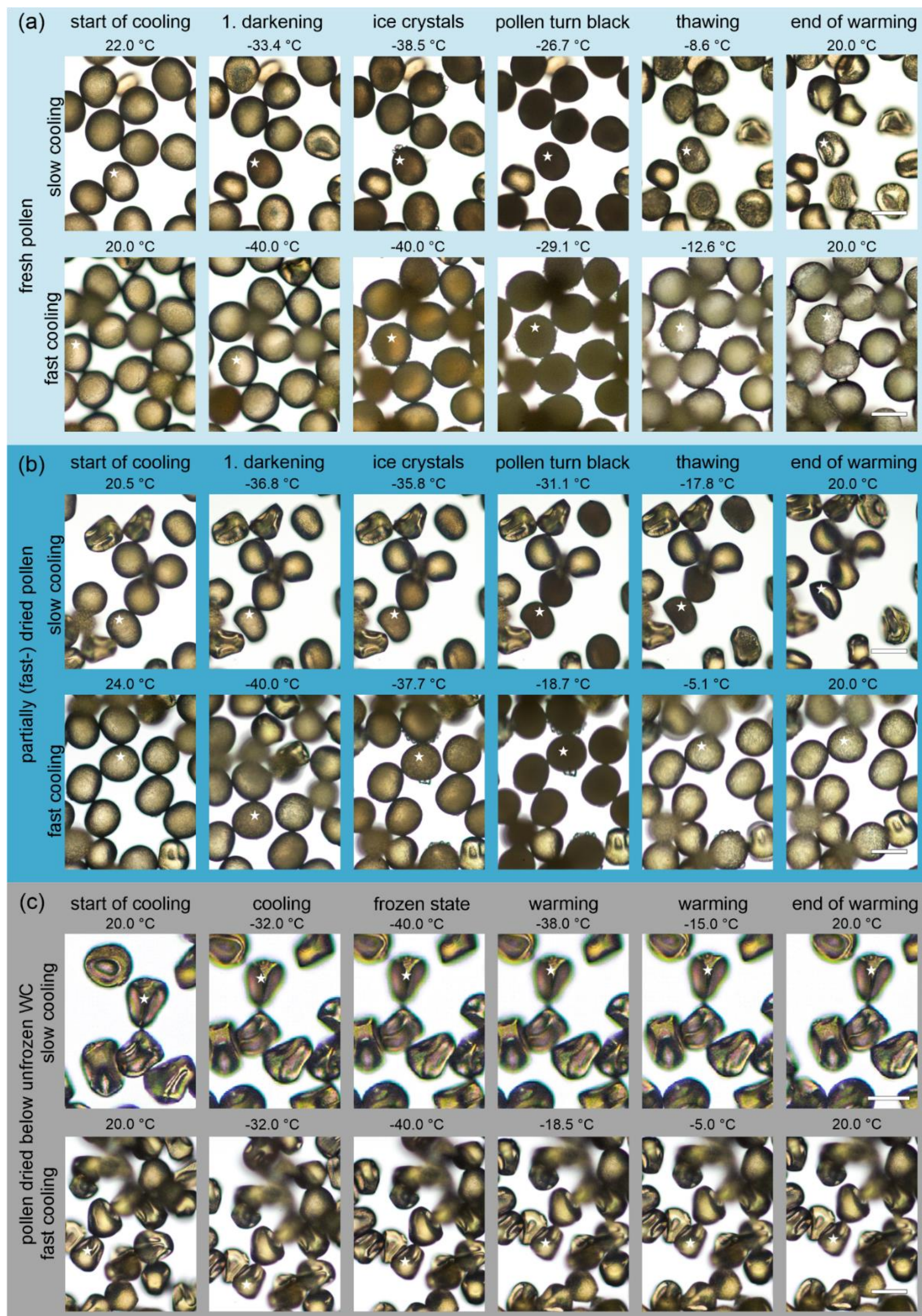


Figure 4 Slow and fast cooling/warming combined with various drying treatments infers a variation in the structural changes in wheat pollen that occur at different temperatures. **a** Fresh pollen (light blue panel), **b** pollen partially fast-dried for 5 min (dark blue panel), and **c** pollen air-dried to WCs below the unfrozen WC for 60 min (grey panel) of wheat line TRI 3633 were cooled and warmed at 1 °C min^{-1} (slow cooling) and 150 °C min^{-1} (fast cooling). Significant events (i.e.,. 1. darkening, ice crystallization) were visualized by extracting images from the videos in Supplementary video 1 to 6; the range of temperatures is shown in **Figure 5**. Due to movements in the Linkam chamber, for better orientation we marked the same pollen in each series (row) with a star. Scale bars indicate 50 µm, size and magnification were identical for all images.

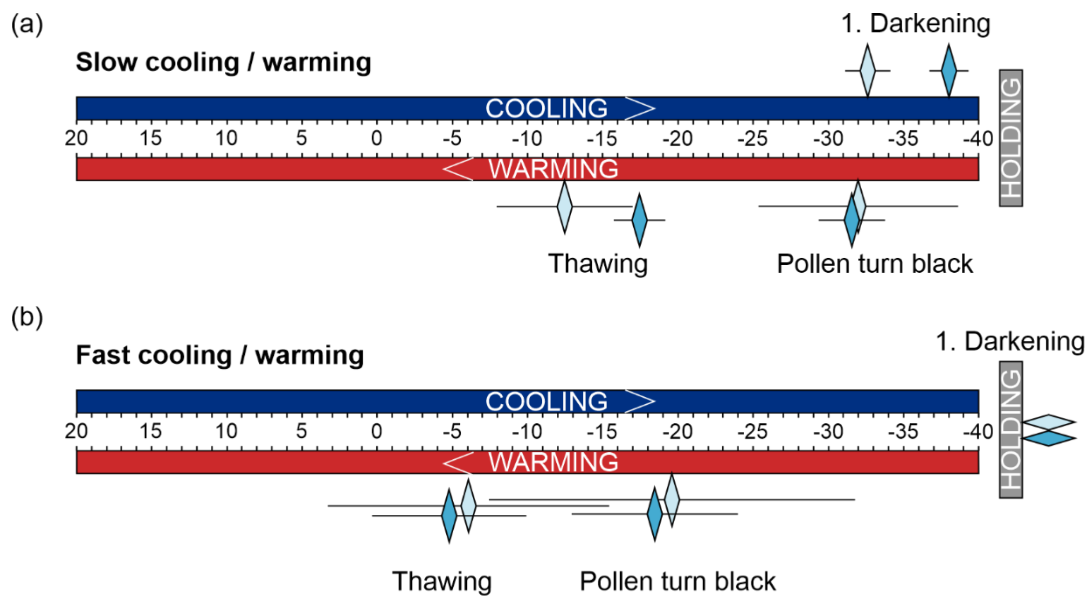


Figure 5 Cooling/ warming rate and pollen water content affect crystallization and melting events. Fresh pollen (light blue diamonds) and pollen partially fast-dried for 5 min (petrol blue diamonds) of the wheat line TRI 3633 was used to evaluate the significant events during **a** slow cooling/warming at 1 °C min^{-1} and **b** fast cooling/warming at 150 °C min^{-1}. Temperature ranges (diamonds and lines) are given for significant structural changes of the pollen (1. Darkening, Pollen turn black, Thawing) exposed to different cooling/warming approaches. Diamonds and horizontal lines represent means and standard deviations of 5 biological replicates for each condition. For fast cooling/warming and due to the high cooling rate, means and standard deviations were interpolated based on temperature at the start, within the holding phase, and at the end of the cooling/warming program and a few temperature points in between.

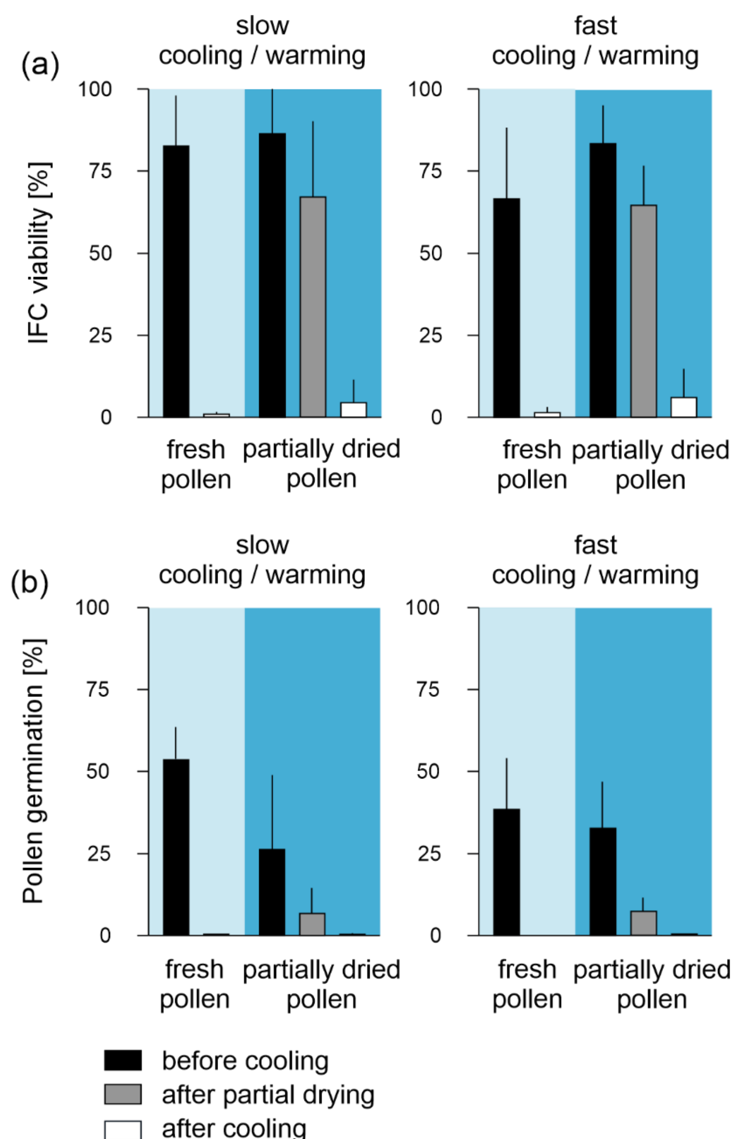
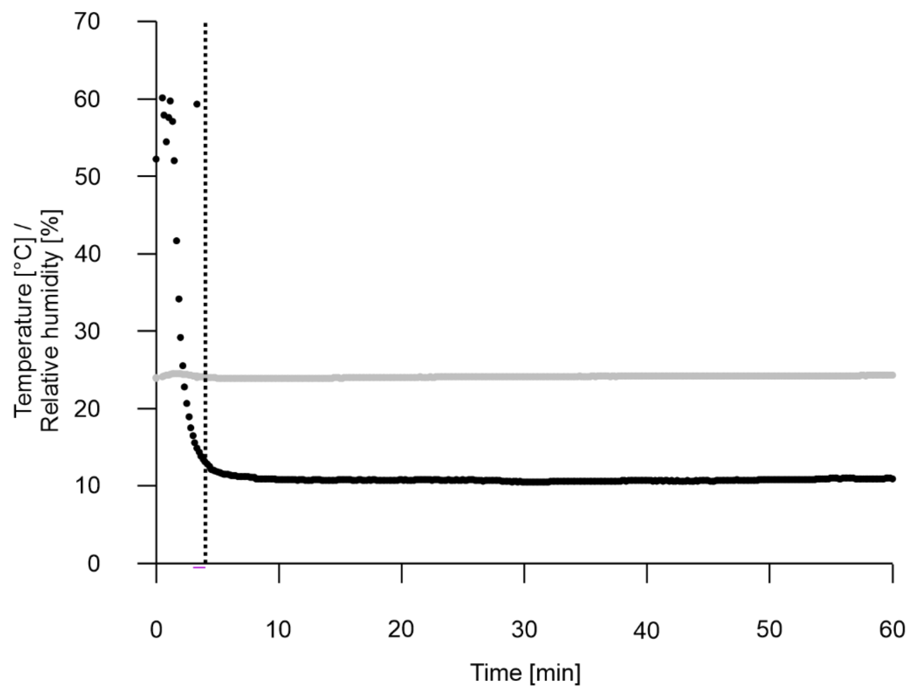
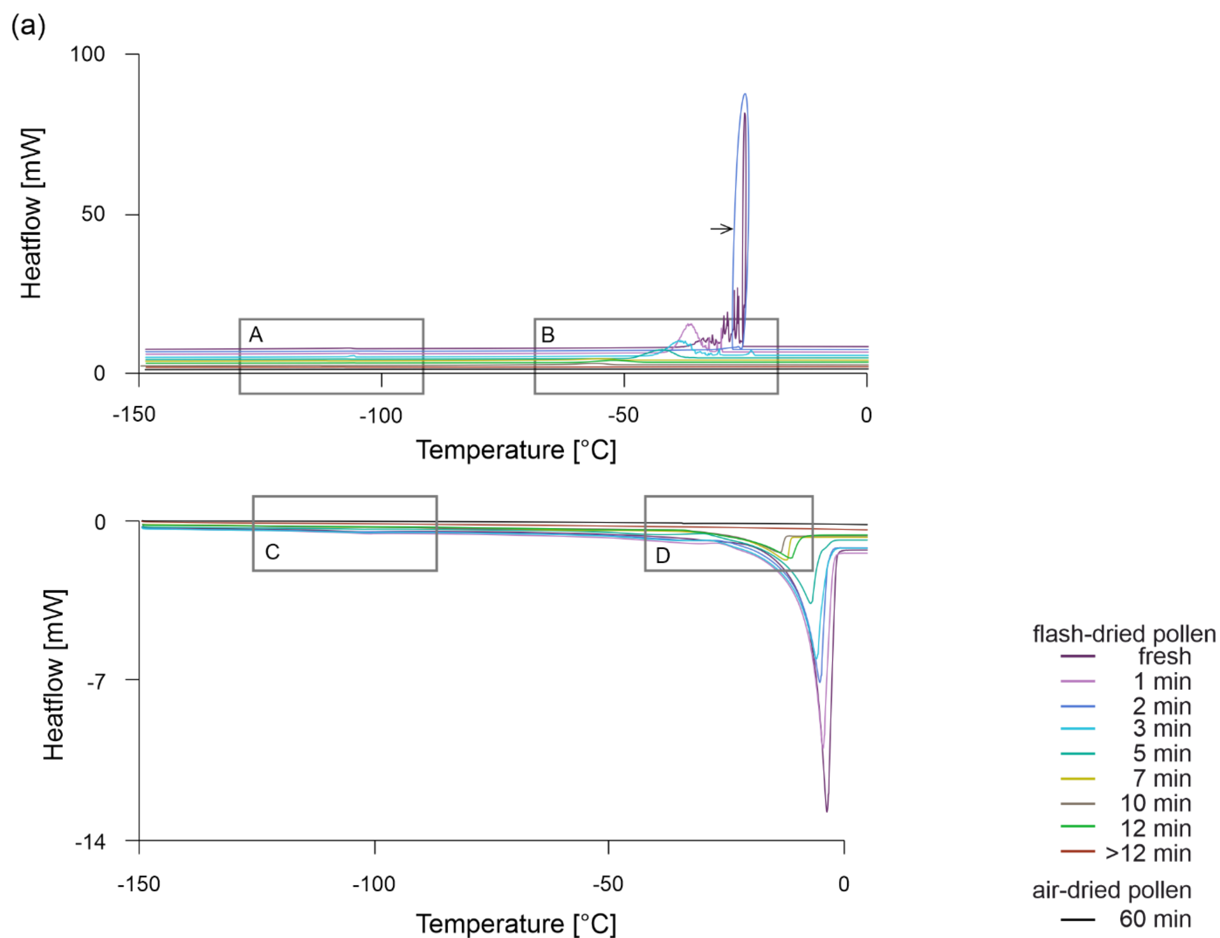


Figure 6 Partial fast-drying increases the chance of pollen survival after cooling/warming. Fresh pollen (black bars), and pollen flash-dried for 5 min (grey bars) of the wheat line TRI 3633 was exposed to slow cooling/warming at 1 °C min^{-1} and fast cooling/warming at 150 °C min^{-1}. Pollen viability was analysed by **a** Impedance Flow Cytometry (IFC viability) and **b** as pollen germination on a raffinose-based media before (black bars), after fast-drying (grey bars) and after cooling (white bars). Bars and lines show means and standard deviations of 5 biological replicates.

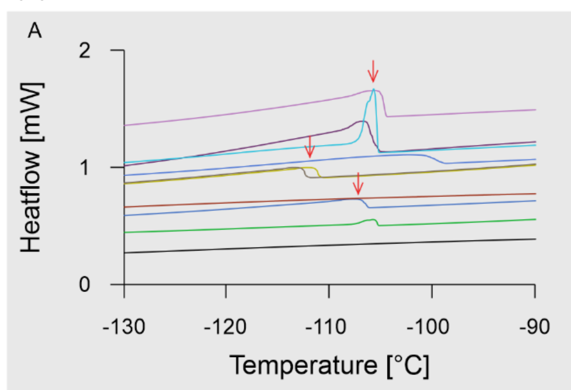
4.3.Supplementary Material

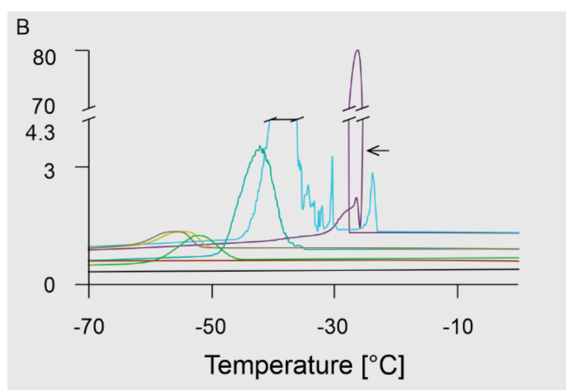


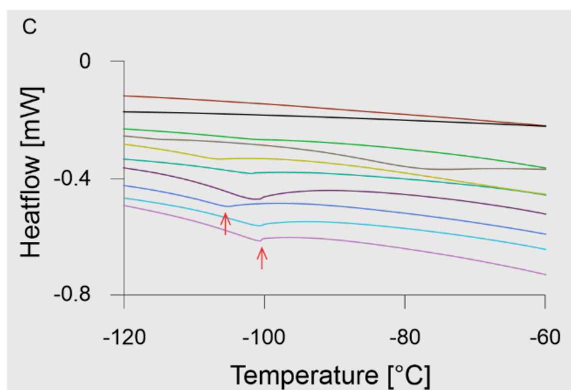
Supplemental Figure S1: Temperature (grey) and relative humidity (RH, black) were measured inside the pollen flash dryer using a data logger. Temperature was held constant at 24.0 ± 1.0 °C. Equilibration of RH was achieved 4 minutes (vertical dashed line) after opening the flash drier to place pollen.

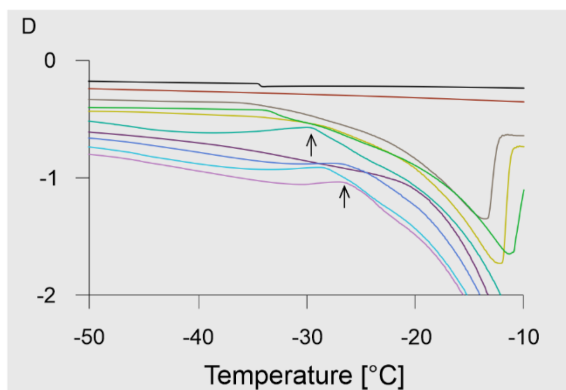


(b)









Supplemental Figure S2 Thermograms with enlarged sections show additional thermal transition events. Pollen of wheat lines 'Ferrum' and TRI 9102 were used as fresh pollen or were air- or flash-dried for up to 60 min and heat flow measured using DSC. **a** Cooling and **b** warming thermograms represent cooling and warming curves of selected replicates of pollen from wheat line 'Ferrum'. Enlarged sections in the cooling thermograms point to eutectic formations (red arrows) and crystallization loop (black arrow) and broad crystallization peaks. Enlarged sections in the warming thermograms show possible glass transitions (red arrows) and revitrifications (black arrows).

Supplemental videos

Supplemental Video 1: Time-lapse video of fresh pollen of wheat line TRI 3633 during slow cooling with 1 °C min^{-1} cooling/warming rate.

Supplemental Video 2: Time-lapse video pollen of wheat line TRI 3633 fast-dried for 5 min during slow cooling with 1 °C min^{-1} cooling/warming rate.

Supplemental Video 3: Time-lapse video of pollen of wheat line TRI 3633 air-dried for 60 min during slow cooling with 1 °C min^{-1} cooling/warming rate.

Supplemental Video 4: Real-time video of fresh pollen of wheat line TRI 3633 during fast cooling with 150 °C min^{-1} cooling/warming rate.

Supplemental Video 5: Time-lapse video of pollen of wheat line TRI 3633 fast-dried for 5 min during fast cooling with 150 °C min^{-1} cooling/warming rate.

Supplemental Video 6: Time-lapse video of fresh pollen of wheat line TRI 3633 air-dried for 60 min during fast cooling with 150 °C min^{-1} cooling/warming rate.

5. General discussion

5.1. Pollen storage as tool for wheat hybrid breeding

5.1.1. Expectations of hybrid wheat breeding

Bread wheat plays an important role in feeding the world's population. In the past seven decades wheat yields and the plants resistances against abiotic and biotic stresses had constantly improved through breeding successes. However, nowadays the increase in yields have reached a plateau and concerns grow about feeding the world's rising population. Furthermore, genetic variability decreased through breeding with elite wheat lines (Ray et al. 2013; Ray et al. 2012a). Thus, interest in researching heterosis in wheat and exploiting the potential benefits of hybrid wheat lines rise. The recent release of the wheat genome data created a boost in the amount of knowledge, and new technologies accumulated along with the rapid development of gene-editing/genomic modification tools such as CRISPR-Cas9 which have high potential use in the development of hybrid wheats (Milner et al. 2020; Zhao et al. 2015). In addition, the use of plant genetic resources can help to diversify wheat breeding (Feuillet et al. 2008; Mascher et al. 2019). The flower morphology and the nature of flowering in wheat suggest that adaptation to cross-pollination prevailed in its ancestors (De Vries 1971). Thus, as a sources of genetic variation, landraces, heirloom varieties, crop wild relatives and wheat-related species have a high potential to inherit advantageous alleles to generate new high-performing hybrid varieties (Schneider et al. 2021). There are great expectations that hybrid varieties can be grown on the same amount of arable land as inbred lines, a commodity that is shrinking. In this case, growers could expect better financial returns through increased yields even with higher expected seed costs over conventional varieties. Nonetheless, so far, global hybrid production is a niche sector and is below 1% (Longin et al. 2012; Kempe et al. 2014). A remaining challenge hybrid breeding programs are faced with may lay in the biology of the wheat's flowers and pollen and will be discussed in the next section.

5.1.2. The male floral architecture as barrier for wheat hybrid production

A challenge of the wheat breeding is the floral architecture and cleistogamous flowering of wheat. As hybrid breeding requires a high percentage of outcrossing, including the physical movement and distribution of pollen and beneficial pollen aerodynamics, these may be limiting factors for wheat (Hegde and Waines 2004).

De Vries and Ie (1970) showed a great variability for wheat pollen mass, which may affect aerodynamic properties of the pollen. The differences in weight may contribute to different fall rates and pollen travel distances from the source plant. Pollen of hexaploid wheat are relatively big with around 58 µm in diameter (Goss 1968; De Vries 1971), and heavy (De Vries 1971), and have a sink rate of 55-60 cm sec^{-1} (Lelley 1966). In contrast to Lelley (1966), D'Souza (1970) reported a lower sink rate with 19 cm sec^{-1} for wheat pollen which may be largely influenced by its WC that changes according to RH of the surrounding environment (Faegri and Van Der Pijl 1979). In agreement with the high weight and fast sink rate, it was stated that more than 90% of wheat pollen fall within three metres of the source (Hegde and Waines 2004). However, some researchers have reported that wheat pollen is able to travel long distances. For instance, seed set on male sterile wheat could be recorded up to 20 m (De Vries 1974) or even 50 m from the source floret (D´Souza 1970). In field experiments, pollen-mediated gene flow was reported even up to a 100 m distance (Loureiro et al. 2012), although usually below 1% (Waines and Hegde 2003; Matus-Cádiz et al. 2007; Matus-Cádiz et al. 2004; Hanson et al. 2005; Griffin 1987; Gatford et al. 2006). Depending on varieties and environmental conditions, rates above 1% can be hardly observed (Hucl and Matus-Cádiz 2001; Hucl 1996) and, thus, the gene flow rate decreases with greater distance from the pollen source (Hegde and Waines 2004).

The time required for floret opening, elongation of anthers and their filament, anther tip rupture, pollen shedding and maintenance of pollen viability affect the rate of outcrossing (Waines and Hegde 2003; Joppa et al. 1968; Beri and Anand 1971; De Vries 1974). In addition, anther length and length of the elongated filament affect how far the anther can be pushed outside the floret, and, thus, how much pollen can be shed outside the floret. Although some varieties can shed more than 80% of their pollen outside the floret (D´Souza 1970), there is a tendency for tall varieties to have more pollen grains per anther, and for anthers with longer filaments to shed greater amount of pollen outside the floret (Beri and Anand 1971). Furthermore, the time it takes for the anthers to burst open and for the filament to elongate influences how much pollen is discharged outside the flower. Anthers take about 2 to 5 minutes to fully emerge and filaments take 15 to 40 minutes to fully elongate (Briggle 1967). In a supplemental experiment we found that flowering of individual florets, i. e. opening of palea and lemma, anther extrusion and tip opening, and closing of palea and lemma, varies and can last between 30 and 195 min (**Appendix, Supplemental Experiment, Video X1, Table X3**). Depending on the variety, the time for anther extrusion may be longer than the actual opening of the anthers and/or the elongation of the filament may occur after the anther tip has opened. In addition, given the short viability of wheat pollen, the pollen may lose some of its viability before the anthers can leave

the flower. In our experiments, anther tips usually ruptured before the elongation of the filament took place (**Manuscript 1, Supplementary Video S1**). Also, the mean time that florets were open varied depending on floret position (**Appendix, Supplemental Experiment, Video X1, Table X3**).

Another limiting factor for wheat hybrid production may be the low pollen production per inflorescence compared to other grasses. In wheat, there are only about 1,000 to 3,800 pollen per anther (approximately 450,000 pollen grains per plant) produced depending on variety (De Vries 1971). In comparison, allogamous rye (*Secale cereale* L.) has nine times as much pollen per anther (four million pollen grains per spike), and maize even 40 times as much (18 million pollen grains per maize tassel) (De Vries 1971). Taken together, the success of outcrossing thus seems to depend largely on male floral characteristics and flowering kinetics.

5.1.3. Environmental, economic, and genetic barriers

Floret opening, anther extrusion and pollen release are strongly influenced by the prevailing environmental conditions such as RH and temperature (D´Souza 1970; Früwirth 1905; De Vries 1971). Floret opening and anther extrusion in wheat are promoted by warm temperatures and low humidity, whereas rainy conditions tend to reduce anther extrusion (Emecz 1962). However, as temperature increase and RH decrease towards mid-day, florets would not open. This is reflected in the anther extrusion that varies greatly over the day. Flowering in wheat happens mostly in early morning, particularly around 5.00 a.m. where around 30 to 40% of anther extrusion takes place. At late afternoon (5.00 to 8.00 p.m.) a second flowering period can be observed for most wheat lines, whereas at noon flowering takes a halt (Zajączkowska et al. 2021). Considering the overall short flowering period of wheat, synchronisation of the flowering times of different wheat lines is often difficult to achieve. Thus, an extended duration of flowering is favourable in order to increase the likelihood of cross pollination. Generally, it is favourable that the male lines should flower two days later on average than the female lines in order to extend the time for complete opening of the florets and full exposure of the stigma (Pickett 1993).

Hybrid wheats generally require male sterility which is introduced into wheat plants by three main methods: cytoplasmic genes, nuclear genes, or chemicals. Cytoplasmic sterility and chemical hybridization agents are widely used approaches as pollination control systems in wheat (Singh et al. 2010). The use of genetic or mechanical methods to produce male sterile plants, the isolation of female plants from unwanted pollen sources and overall field

management lead to a huge increase in production efforts and cost. This brings another problem with it: seed production from hybrid lines requires significantly more space than seed production with inbred lines, because male plants are grown in addition to the female plants, but these do not contribute to the seed yield (Longin et al. 2013). In addition, the huge genome size, complexity, and polyploid nature (16 Gbp, with > 85% of repetitive sequences) (Martín et al. 2018) have hampered screens for the identification of marker genes for desirable attributes. Thus, the identification of suitable germplasm for compatible parent plants constitutes a challenge. Due to genetic redundancy in wheat, it is still difficult to combine important traits into beneficial allele combinations (Longin et al. 2014), and phenotyping in field trials is very complex and expensive (Montes et al. 2007).

Establishing and improving pollen storage technologies can potentially reduce or eliminate some of the technologies above. Male plants can be grown in separate small fields and pollen collected at an optimum time point. Female plants can be grown with more space. The laborious field management of planting in alternate rows, and the need to synchronise the flowering time of male and female varieties becomes redundant. In sum, the application of stored pollen in the field could boost production of hybrid seeds.

5.1.4. Reliable pollen viability tests and pollen storage can support hybrid breeding

Thus far, most wheat hybrid breeding programs have not focussed on pollen biology and fertility. However, to achieve successful cross-pollination between two desired parental lines, it is important that viable pollen of one line lands on the stigma of the other line. However, wheat pollen survives only short periods after release from the anther. Therefore, rapid and reliable tests for pollen viability, and improvements in protocols enabling pollen storage, extending the pollen shelf-life and alleviating pollen application in the field would be of great value for the agricultural industry.

With regards to pollen viability assessment, we showed that pollen germination *in vitro* on a raffinose-based medium, FDA staining, or impedance flow cytometry can give reliable estimates of the wheat pollen's viability (**Manuscript 1, Figure 3 and 5**). Here, it is important to work quickly after the pollen is released from the anther, because the time pollen can survive at ambient conditions (RT, low RH) is one hour at maximum (**Manuscript 2, Figure 1b**). Recently, a new method was published for the assessment of pollen viability in wheat (Biswas et al. 2020). The principle of this test is based on the reducing activity of cytoplasmic compounds (carbohydrates, lipids, enzymes) within the pollen that turn silver ions (Ag^+) in the

presence of light into nanoparticles. Thus, pollen grains are stained with a brown colour which can be measured photometrically or estimated by counting stained pollen grains. This test might be a good alternative to the ones presented in this thesis. One advantage of this test is that it can give a quick qualitative estimation whether a pollen sample is viable. However, if quantitative measurements are to be performed, then the requirements of a microscope for pollen counting or photometric instruments still render this test less suitable for field application. With regards to pollen counting, a further improvement may be the implementation of automated counting techniques using intelligent imaging software. Such a counting protocol has already been developed with a free imaging software (ImageJ) (Costa and Yang 2009). This protocol could be adapted to above mentioned staining method (FDA staining) or to the newly developed Ag^{+}-test by Biswas et al. (2020). However, it would be again only applicable if pollen stays intact during the staining procedure. A further innovative improvement would involve an automatic recognition of pollen tubes. Perhaps this could be realized with the help of intelligent, machine-learning approaches.

Pollen cryopreservation is a promising method for long-term conservation of wheat pollen. The use of stored, viable pollen is valuable in many ways. A wheat pollen cryobank could provide a constant supply of viable and fertile pollen which allows pollinations of spatially or temporally isolated parent plants in wheat hybrid breeding programmes. Further, it allows pollination of different inbred lines to combine unique traits that improve plant characteristics and seed quality. In addition, pollination from undesirable sources could be reduced. Thus, conservation of wheat pollen could reduce wheat seed production costs and eliminate many of the production challenges in the wheat hybrid seed industry. Finally, a pollen cryobank can be an important component for the conservation of wheat genetic resources in gene banks.

5.1.5. Hybrid production using grass pollen

Production of hybrid varieties with the use of stored pollen has been reported for many crops for which pollen storage is easily facilitated such as sunflower (Fick and Miller 1997), sugar beet (Smith 1980), hot pepper (Mathad et al. 2013), cucumber (Palupi et al. 2017). However, only very few reports exist where hybrid productions in cereals is based on the use of stored pollen.

Some progress to produce maize hybrids from stored pollen has been made by the company PowerPollen® that received a patent for their 'On-Demand Pollination Technology' (https://powerpollen.com/news/company-news-powerpollen-recieves-patent-for-on-demand-

pollination-technology/, accessed on 5.8.2021). In 2017, pollen of *Imperata cylindrica*, a wild perennial grass of the *Poaceae* family, had been stored for one month at -20 °C and used to be crossed with wheat plants for the production of double haploids (Rather et al. 2017). These reports show that some grass pollen can be stored over longer periods. However, the scarce reports and the results of this work showed, that it is extremely difficult to adapt methods for pollen cryopreservation for this species, particularly in wheat.

In the following sections an attempt was made to construct a model about which factors may have an influence on pollen viability during the drying and storage. Processes resulting in viability decline in other pollen or recalcitrant seeds have been compared and used to help explain phenomena and processes found in this study.

5.2. Effects of dehydration and ultra-low temperature on pollen grains

5.2.1. Sensitivity of wheat pollen and types of damages introduced by dehydration

Water loss in pollen grains occurs immediately before and during anther extrusion. Grass pollen usually loses around 20% of its original weight within one hour after release at normal temperatures and humidity levels (Heslop-Harrison 1979c). In contrast to Heslop-Harrison (1979c), we reported a loss of 94% of the wheat pollen's original WC in RT (23.0 ± 0.4 °C) after one hour exposure and germination completely ceased when pollen were exposed to low RH (60.0 ± 1.0%) for one hour (**Manuscript 2, Results and Figure 1**). Water loss causes physical, molecular, and biochemical changes in the pollen cell. At the physical level, molecules move closer together due to the decreasing hydrate envelopes around proteins, membranes, RNA, and DNA, leading to new interactions between biomolecules. In wheat pollen, we observed different structural, physicochemical, and biochemical (**Manuscript 2, Figures 1 to 6**) changes when pollen was exposed to a dry environment (60 ± 1.0% RH) and room temperatures (23 ± 0.4 °C). In the following sections, potential vulnerabilities of wheat pollen in relation to dehydration and the damage it causes are discussed in detail.

5.2.1.1. Structural damage on membranes and cytoskeleton caused by water loss

Membranes

In fully hydrated cells, phospholipids in a membrane are in a liquid-crystalline state (**Figure 4A**) (Hoekstra et al. 2001). Cells lose turgor when water potential fall below about -2 MPa (98.5% RH) (Walters and Koster 2007; Wood and Jenks 2007). Water potential (Ψ, Psi) is the measure of the relative tendency of water to move from one area to another. When RH is at 100%, water potential is 0 MPa and water is in an equilibrium. With decreasing RH, water potential decreases and a gradient is generated which leads to evaporative loss of water from plant cells (Wood and Jenks 2007). The plasma membrane of the vegetative pollen cell and organellar membranes are highly stressed by water loss. In dehydration-tolerant species, stabilisation of the subcellular membranes and maintenance of membrane fluidity is important for drought stress tolerance and may contribute to prolonged viability in the dehydrated state (Oliver et al. 2020). Membranes and other macromolecules (DNA, proteins) in dehydration-tolerant species are stabilized by replacing structural water with sugars, sugar alcohols, or proteins such as late embryogenesis abundant (LEA) or heat shock proteins (HSP) to maintain the hydrogen bonds upon water loss (Hoekstra et al. 2001). The distance between the

phospholipid molecules in membranes are maintained and the bilayer remains in the liquid crystalline phase. In dehydration-sensitive cells, however, the lower concentration or absence of these sugars and proteins could lead to membrane fusion during dehydration. Thus, phospholipid molecules of membranes aggregate when water is withdrawn from the hydration shell, which disturbs the structure and function of membranes (Hoekstra et al. 2001).

Furthermore, in dehydration-sensitive cells, dehydration causes a separation of lipids into distinct domains. For example, the fatty acid domains in the bilayers of membranes become more rigid and the membrane fluidity is altered. This can also lead to phase changes within the membrane and a transition from a liquid to a gel phase (water potential at about -12 MPa or 91.5 % RH) (Walters and Koster 2007; Wood and Jenks 2007). Connections between the plasma membrane and the pollen cell wall can become loose and cause collapse of membranes (Walters and Koster 2007). Membrane material can get internalized through vesiculation and gets separated from the plasmalemma. Indeed, we observed that the plasma membrane appeared undulated and damaged in wheat pollen when they were heavily dehydrated as shown in **Manuscript 2** (**Figure 2**). Also, we saw the internalization of the plasma membrane which was progressing in dehydrated wheat pollen (**Manuscript 2, Figure 2**). When the shrunken cell is returned to the original water potential through re-hydration, e.g. after landing on the stigma, the pollen cell may bursts because there is insufficient membrane surface area to accommodate the swelling (Walters and Koster 2007). This may be a possible explanation for poor pollen tube growth rates of dehydrated pollen samples both, *in vitro* and on stigma as shown in **Manuscript 1 (Figures 2, 4)**. Additionally, we observed that different cell organelles like mitochondria and dictyosomes shrivelled and were less visible, which may be a hint for irreversible damages in the plasma membrane and membranes of those organelles (**Manuscript 2, Figure 2**).

Cytoskeleton

The leakage of water can rapidly cause cytoskeletal damages and progresses the more the pollen is dehydrated (**Figure 4 B, C, D and E**). In hydrated mature wheat pollen, the actin fibrils of the vegetative cell is highly organized and points towards the apertural pole (Heslop-Harrison and Heslop-Harrison 1992). Intracellular movement of organelles, P-particles, that likely transport precursor-wall elements of the pollen tube, and lipid bodies takes place already when pollen is still in the anther and continues after anthesis. The movement of the cell compartments is directed along actin fibrils and point toward the pollen pore evidently holding the pollen in a ready-to-germinate state (Heslop-Harrison and Heslop-Harrison 1992). However, only after 1h

exposure of wheat pollen to low RH (55%), organelle movement rapidly stopped, and germinability declined to 12.5% from an initial 85.5% (Heslop-Harrison and Heslop-Harrison 1992). In agreement, in **Manuscript 2** (**Figure 2**), we found that the wheat pollen cytoskeleton was highly disordered after 1h dehydration at ambient air and pollen germination completely ceased. Therefore, it is assumed that dehydration leads to some irreversible damages on the actin cytoskeleton which cannot be fully restored upon rehydration.

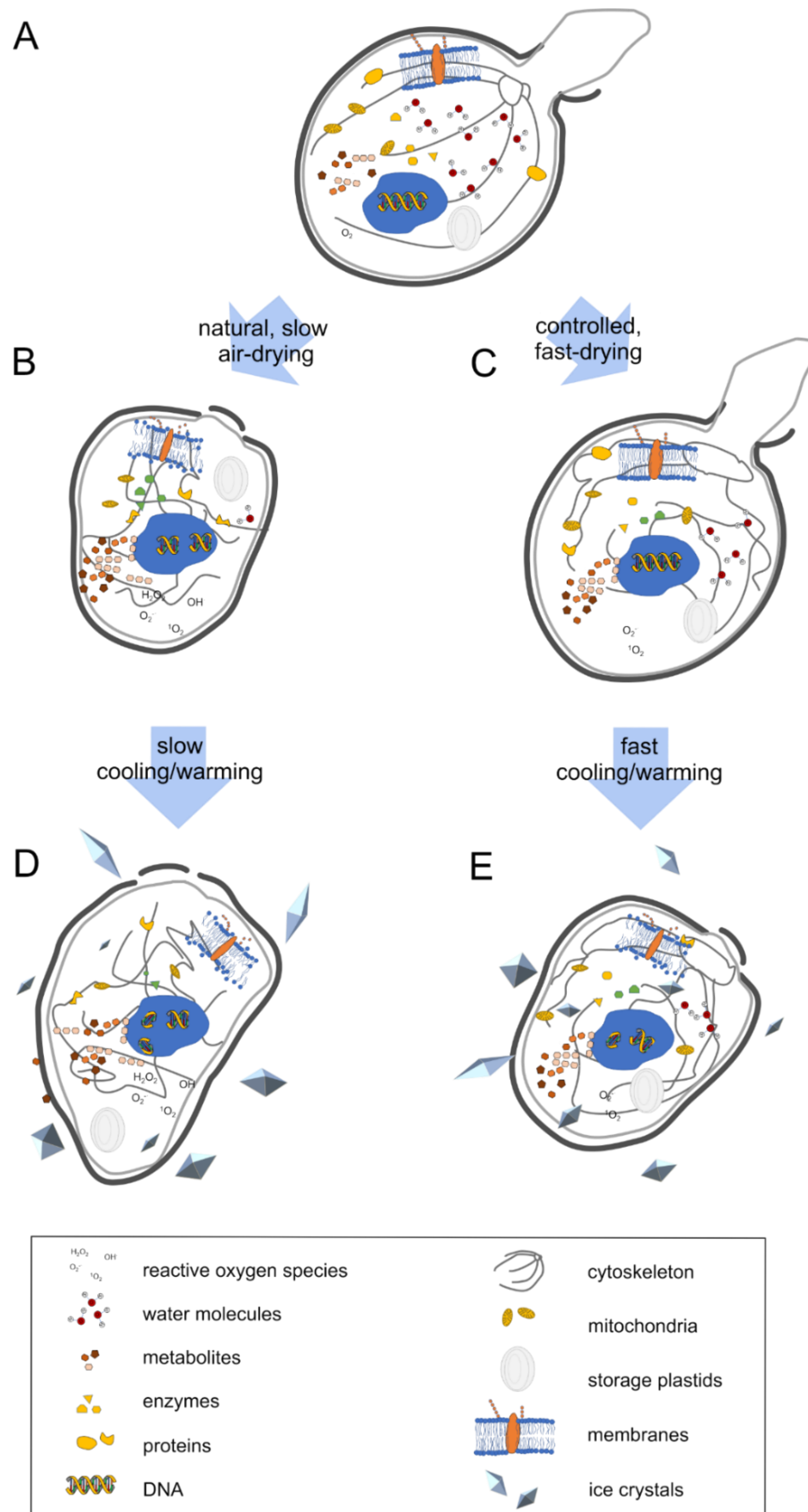


Figure 4: Possible cellular damages in wheat pollen induced by dehydration and exposure to ultra-low temperatures. A) Fresh wheat pollen is in a hydrated state during/after anthesis and has an organized cytoskeleton along. At this state, cell organelles are oriented towards the apertural pole (top) (Heslop-Harrison and Heslop-Harrison 1992). Macromolecular structures like DNA, proteins, and membranes as well as cell organelles function normally and show no damages. The oxidative status of the cell is balanced. The pollen tube grows when pollen lands on the stigma. B) Exposure of the pollen to natural air induces uncontrolled loss of water leading to damages of the cytoskeleton, fragmentation of DNA, structural changes in proteins and membranes, disorganization of cell organelles and eventually a complete loss of germinability. C) During fast dehydration, metabolic damages may be reduced in wheat pollen since reactive oxygen species do not have much time to damage macromolecules and organelles. Some germinability can be retained if the water content stays above a specific threshold. D) Exposure of dehydrated pollen to ultra-low temperatures causes further dehydration due to water outflow that is caused by the formation of extracellular ice crystals. The solutes in the cytoplasm become concentrated and only few ice crystals may form intracellularly and reduce ice-crystal-induced damages. Nonetheless, dehydration causes structural damages on cytoskeleton, membranes and macromolecules like DNA and proteins leading to cell death. E) Fast-freezing of fast-dried pollen does not dehydrate the cell as much as the slow cooling method. Theoretically pollen may have a better chance to survive the procedure, but the protocol needs to be further optimized to increase the number of wheat pollen surviving the procedure.

5.2.1.2. Porous pollen wall as weak point

Exine and Intine

The morphology of the pollen wall may play an important role in protecting the pollen grain from dehydration. Pollen walls consist mainly of three parts, the intine, the exine and a pollen coat. The intine is permeable to water (Katifori et al. 2010) and will allow water passing into or out of the vegetative cell (Heslop-Harrison 1979c). Exine, the outer part of the pollen wall, consists of sporopollenin, which is chemically resistant and water impermeable (Katifori et al. 2010; Heslop-Harrison 1979c). However, the presence and amount of microchannels within the exine in some *Poaceae* pollen seem to be involved in the dehydration after pollen release and, thus, in pollen longevity (Fu et al. 2001). In wheat pollen, using transmission electron microscopy, we observed that the exine is permeated with many microchannels (**Manuscript 2, Figure 2c**). The microchannels in wheat pollen are about 40 to 50 nm in diameter in the microspore stage (Rowley et al. 2001), and progressively decrease in thickness as the sporopollenin is built up in the exine until mature pollen stage (El-Ghazaly and Jensen 1986). Similarly, in rice pollen (Fu et al. 2001) and rye pollen (Rowley et al. 1959), the exine is perforated by numerous channels. The microchannels may be of great importance for the rapid germination as observed in many grass pollen. With numerous microchannels present, water and metabolites can be rapidly transported across the exine (Rowley et al. 1987; Shivanna and Johri 1985). Additionally, not only water is transported but also nutrients and likely enzymes. In rye pollen, outflow of a wide range of proteins via the microchannels was observed freely over the whole surface of the pollen grain right before germination (Heslop-Harrison 1979a). Using tracer experiments, the routes of nutrients into the pollen cytoplasm via microchannels

were demonstrated in different species (Rowley et al. 1987; Rowley et al. 2003; Fernandez and Garcia 1990). The release of proteins may play a role for the recognition of the presence of pollen grains by the stigmas (Lin et al. 1979 cited by Fu et al. 2001). However, due to the numerous microchannels in the exines of wheat, rye and rice pollen, the pollen walls are also porous towards the outside, which leads to faster dehydration and can contribute to the loss of viability (Edlund et al. 2004). In comparison, maize pollen has fewer microchannels than rice, and *Pennisetum alopecuroides* pollen completely lacks microchannels (Fu et al. 2001). This may explain the higher resistance of maize and *Pennisetum* pollen to dehydration and the better storage properties as these two pollen species can be easily stored at low temperatures (Fu et al. 2001) or in LN (Barnabás and Rajki 1976).

The thickness of the pollen wall may also be connected to the short livespan of wheat pollen grains. In general, tricellular pollen grains typically have thinner walls than bicellular pollen (Fu et al. 2001). A positive correlation between pollen longevity and pollen wall thickness has been reported in some grass species (Fu et al. 2001). The exine of mature wheat pollen is around 750 nm thick (El-Ghazaly and Jensen 1986). Likewise, the rice pollen wall has an exine that is similar in thickness (700 nm). Like wheat pollen, rice pollen usually loose viability within 5 minutes after shedding (Koga et al. 1971), and at maximum can live for 70 minutes (Brena et al. 2019). In comparison, pollen grains of maize have an exine that is up to 900 nm (Fu et al. 2001) and can live for up to 2 h under atmospheric exposure (Luna et al. 2001), up to 8 days when stored in a beaker (Jones and Newell 1948), or even made amenable for long-term storage when stored in liquid nitrogen (Barnabás and Rajki 1976). The pollen of the wild relative *P. alopecuroides* has an exine that is 850 to 950 nm in thickness and can live for several months and can easily be stored for a long time (Zhao 1983 cited by Fu et al. 2001; Li and Chen 1998). Thus, it appears, that thicker pollen walls provide a better protection from prevailing environmental conditions.

Aperture

Like the cell wall, the aperture may be a point of increased water loss. It is the primary site through which a pollen tube germinates (Wang and Dobritsa 2018a). Apertural sites are areas of the wall where the exine is much reduced. Thus, the underlying intine is much more exposed, presenting a surface that is therefore much less protected from water loss than elsewhere. During dehydration it may be the site through which most water is lost (Heslop-Harrison 1979c; Vieira and Feijó 2016), whereas during re-hydration it is the site, where pollen takes up water. In barley pollen, for example, rapid swelling within seconds upon contact with germination

medium was observed accompanied by an accumulation of potassium ions at the aperture side (Rehman et al. 2004). Wheat and other grass pollen have a single aperture which is covered by a lid-like structure called operculum (Heslop-Harrison 1979b). These structures act as lids that opens to allow pollen tube growth (Hesse et al. 2009). Opercula seal the grains during dehydration to prevent water loss, and can lift during the re-hydration phase to allow pollen hydration and pollen tube emergence (Wang and Dobritsa 2018b). Nevertheless, the pores are in general a weak spot of the grain and likely to rupture first (Božič and Šiber 2021). The aperture may be an order of magnitude weaker than the regions covered by regular exine (Edlund et al., 2016). During rehydration, for example, pollen undergo a rapid inflation and the pore bulges out and assumes a hemispherical shape. If the grain hydrates in a surrounding with an inadequate osmolarity and ionic content, as might happen in the atmosphere, the grain ruptures at the apertural side and cytoplasm is released (Božič and Šiber 2021). In agreement, in **Manuscript 1 (Supplementary Table S2)**, wheat pollen almost exclusively bursted on the apertural side when the medium did not have the correct osmotic adjustment. Thus, it can be assumed that wheat pollen may lose parts of their water through the apertural sides which contributes to viability decline.

Absent pollen wall structures

Desiccation-sensitive pollen usually lacks specific pollen wall structures, resulting in further reduced resistance and flexibility of pollen walls to volume changes, uncontrolled water loss, and dehydration (Katifori et al. 2010). In *Poaceae* the endexine, a distinct exine layer between ektexine and intine (Hesse et al. 2009), is absent (Pacini and Hesse 2012; Diethart et al. 2007). The endexine mainly consists of sporopollenin, lipids and proteins (Heslop-Harrison 1968a). A lacking endexine layer may reduce the pollens' flexibility to volume changes. In addition, most *Poaceae* pollen do have only little or no tapetum-derived coating on the surface of the pollen (Heslop-Harrison 1979c; Pacini and Hesse 2005). The pollen coat fills the cavities of the pollen exine and protects the male gametophyte from dehydration, pathogen attack and UV damage (Pacini and Hesse 2005). Mutant rice pollen with a defect in the pollen coat showed rapid dehydration, leading to humidity-sensitive male sterility (Xue et al. 2018). It is possible, that wheat pollen lacks this protective layer of a pollen coat. However, no data exist and thus this would need to be further investigated.

Pollen wall enzymes

The composition, number and activity of pollen wall enzymes may be another factor affecting pollen longevity. Pollen walls contain enzymes such as hydrolases, transferases, dehydrogenases, oxidases, liases and lyases (Zinger and Petrovskaya-Baranova 1961; Knox and Heslop-Harrison 1970, 1969). These enzymes play important roles in pollen dispersal, pollen and stigma recognition, initiation of pollen germination on the stigma surface, and pollen tube growth. The distribution of enzymes in pollen walls varies from species to species. Enzymes found in the exine, e.g. dehydrogenases (Knox et al. 1975), are closely related to the longevity of pollen grains. For example, in maize and *Pennisetum* pollen, lower activity of succinate dehydrogenase and glucose-6-phosphate dehydrogenase in the outer layers of pollen walls correlate with a longer pollen longevity compared to higher activity in rice pollen that has shorter longevity (Fu et al. 2001). To best knowledge there are also no data of pollen wall enzymes and their roles in pollen viability existing for wheat pollen and, thus, further research is required.

5.2.2. Damage through reactive oxygen species

The production and accumulation of ROS depend on the metabolic and physiological cell state (Jeevan Kumar et al. 2015; Foyer et al. 2017). ROS are constantly formed in a cell during normal metabolism, particularly during respiration and photosynthesis that include the formation of superoxide (O^{-2}), hydrogen peroxide (H_2O_2) and ultimately the hydroxyl radical (HO·), whereas singlet oxygen ($^1O_2$) is produced by direct energy-transfer to oxygen under light stress (Apel and Hirt 2004; Ray et al. 2012b). In vital cells of seeds, ROS production and scavenging are under strict control (Halliwell 2006). ROS scavenging is controlled by the action of ROS-processing enzymes such as superoxide dismutase, catalase, and peroxidases (Bailly 2004), and by molecular antioxidants (Kranner and Birtić 2005) balancing out an overproduction of ROS to maintain an equilibrium in the oxidative state (**Figure 4A**). However, water shortage can lead to accumulation of ROS (Kranner and Birtić 2005). In a dehydrated cell, oxidative phosphorylation in mitochondria and peroxisomes, and oxidations at the plasma membrane can lead to supraphysiological ROS concentrations in cells (**Figure 4B, C, D, E**) (Ratajczak et al. 2019; Bailly 2004). Additionally, ROS can be produced through nonenzymatic reaction in the dehydrated state (Bewley et al. 2012), and accumulate in the cell when activity of the antioxidant system and/or the content of LEA proteins are low (Berjak and Pammenter 2013). Through imbalance in the intracellular ROS status oxidative stress in cells gradually increases which drives processes leading to the structural and functional damages and

ultimately to the loss of viability (Bailly 2004). The role of ROS during dehydration has been studied extensively in desiccation-tolerant as well as desiccation-sensitive seeds. Briefly, desiccation-tolerant seeds developed adaptive mechanisms to cope with water shortage. This involves for example slowing down of metabolism to avoid unregulated ROS production; accumulation of insoluble proteins, starch and lipid reserves to provide volume buffering capacity during dehydration and rehydration; increased ROS scavenging through increased action of antioxidant-mechanisms; accumulation of LEA proteins, and accumulation of sucrose and/or raffinose-family oligosaccharides as major constituents contributing to the intracellular vitrified (glassy) state (Berjak and Pammenter 2013). In recalcitrant seeds, however, these mechanisms are often insufficient or fail which leads to viability loss when cells reach to a critical low water content (≤ 0.35 g H_2O g^{-1} DW) (Berjak and Pammenter 2013). Till now, there is not much known in recalcitrant pollen about ROS accumulation or scavenging during dehydration. But it is likely that recalcitrant pollen, like recalcitrant seeds, have insufficient or even lacking mechanisms to counteract oxidative stresses. Further research may reveal whether recalcitrant pollen such as wheat have insufficient antioxidant mechanism and if external application of antioxidants could enhance the pollens' ability to survive these stresses (see section 5.2.5.6 for further suggestions and discussion).

An increase in intracellular ROS and a decrease in intracellular antioxidant mechanisms is a common phenomenon also occurring during cryopreservation. Moreover, ROS-induced oxidative stress has been stressed out as a major contributing factor to viability loss after cryopreservation (Benson and Bremner 2004). The accumulation of ROS after rewarming has been demonstrated in pollen of different species (Jia et al. 2017; Ren et al. 2019a; Jia et al. 2018; Jiang et al. 2019). It was shown that the intracellular concentration of non-enzymatic antioxidants and the activity of antioxidative enzymes are related to differences in survival after rewarming (Ren et al. 2021). Depending on the species and variety, the levels of ROS can increase differently and correlate with different viability after cooling and rewarming (Ren et al. 2021). In *Paeonia lactiflora* pollen, it was further shown that the activity of intracellular antioxidant enzymes (e.g. SOD activity) decreased significantly after cryopreservation and was related to the decrease in viability after storage in LN (Ren et al. 2019b). A change in the levels of malondialdehyde (MDA) and protein carbonyl (PCO) may be another indicator of oxidative damage. In *Paeonia suffruticosa* pollen, intracellular levels of MDA and PCO were significantly lower in the variety with high viability than in other varieties with lower post-LN viability (Ren et al. 2021).

The large amount of ROS produced during dehydration and cooling/rewarming may cause irreversible damages to cells and ultimately lead to cell death. ROS remove electrons from other organic molecules and are thus reduced, while the molecule that has lost an electron is oxidised (Ballesteros et al. 2020). Oxidation of lipids (phospholipids in the cell membrane), proteins (structural proteins and enzymes) and genetic material (DNA, RNA) lead to changes in their structure that disrupt their function in the cell (compare cell compartments and cellular structures of **Figure 4A with 4B,C,D, E**) (Sharma et al. 2012). As discussed in **Manuscript 3**, particularly during slow dehydration electron transfer in mitochondria and plastid is impaired facilitating the formation of ROS. In addition, membrane fluidity decreases with water loss, so that any detoxification processes that may be present can only act inefficiently. Membranes are a major site of ROS attack where lipid peroxidation modifies membrane permeability (Ren et al. 2021; Kaczmarczyk et al. 2012b). The peroxidation of fatty acids (FA) in phospholipids can lead to large areas of the membranes showing altered semi-permeability. The cell membrane is thus significantly damaged and can no longer function normally (Benson et al. 1992; Halliwell and Gutteridge 2015). Specific ROS (hydroxyl radicals, peroxyl radicals and singlet oxygen) oxidise mainly polyunsaturated fatty acids (Møller et al. 2007). The attack of ROS on proteins cause protein modifications, structural and conformational changes and ultimately leads to protein dysfunction (Møller et al. 2007). Mitochondria are also significantly exposed to oxidative damage as they are a site of ROS production (Kurek et al. 2019). The accumulation of ROS in mitochondria leads to a dysfunction of their membranes as well as damage to mitochondrial proteins and DNA (Chen et al. 2013). As a result, phosphorylation is inhibited (Liberatore et al. 2016) and the activity of the antioxidant system is reduced (Xin et al. 2014). Overall, it is likely that the damage to the biomolecules inside the wheat pollen cell is caused by the production of ROS during dehydration and cryopreservation. Further studies are needed to clarify the possible accumulation of ROS and to detect damage to biomolecules.

5.2.3. DNA damages and insufficient DNA repair mechanisms as a cause of dehydration and storage sensitivity

DNA assumes different conformational states depending on water activity (Osborne and Boubriak 1994). Under conditions of high-water activity, the B conformation of DNA prevails. Upon dehydration conformational changes can appear; when water is withdrawn from the individual hydrated phosphate groups of the DNA, base pairs move closer together to form supercoiled DNA. Under these conditions, the usual B form (right-handed) changes into the A (right-handed and with shorter base pair spacing) or Z form (left-handed) (Harvey 1988; Wells

et al. 1988; Saenger et al. 1986). Such conformational changes of DNA are of particular importance for the recognition of specific base sequence regions by proteins acting as repressors, promoters or enzymes e.g. in different DNA repair mechanisms. Glycation of proteins, i.e. non-enzymatic addition of reducing sugars to DNA binding proteins (e.g. histones), and non-enzymatic methylation of cytosine can occur, which would also favour the Z form (Osborne and Boubriak 1994). Desiccation tolerance of bacterial spores, for example, depends upon the conversion of B-form to the more condensed A-form (Setlow 1992). To retain structural integrity of the DNA in the A-form in the dry spore of *Bacillus subtilis*, a specific DNA binding protein is synthesised during dehydration (Setlow 1992). Alternatively, specific DNA repair pathways are required for the acquisition of desiccation tolerance in *Streptococcus pneumoniae* (Matthews et al. 2021). In orthodox seeds, DNA damages like single and double strand breaks, base loss, and base modifications progress during the dehydrated state (Cheah and Osborne 1978; Dourado and Roberts 1984). But if the functionality of the DNA repair enzymes is retained, then re-ligation of breaks and major restoration of genomic integrity retain germination capacity in desiccation-tolerant seeds (Elder et al. 1987), whereas failure in DNA repair has been suggested as one explanation for disability of tomato seed germination (van Pijlen et al. 1996). These examples demonstrate the importance of maintaining genome integrity during dehydration. In recalcitrant seeds, mechanisms for DNA repair are significantly impaired or inefficient (Pammenter and Berjak 1999; Berjak and Pammenter 1997; Boubriak et al. 2000). In seeds of *Avicenna marina*, DNA was severely damaged after slight dehydration, followed by an inability for its repair after loss of 22% of the water present at shedding (Boubriak et al. 2000). To date, little is known about the effects of dehydration on DNA, chromatin, and nuclear architecture in desiccation-sensitive pollen like in wheat. Naturally, grass pollen is shed in highly hydrated state. It is thus likely, that the DNA takes up other conformations than the B form if pollen is exposed to dehydrating conditions over long periods which may impose DNA damages. Further, wheat pollen naturally reaches the stigma in a matter of minutes or few hours. In recalcitrant seeds of tropical environments, mechanisms for the maintenance of nuclear and DNA integrity could have been reduced over evolution (Marques et al. 2018). Likewise, these mechanisms could be imperfectly expressed or completely absent in wheat pollen. Further research is required to gain a deeper understanding to what extent DNA damages occur and whether DNA repair is insufficient and thus is one factor of the quick loss of viability after shedding in recalcitrant wheat pollen.

5.2.4. Sugar content and biochemical changes

The loss of viability during exposure to different dehydrating environments may be related to the type of carbohydrates that are stored in the cytoplasm (Pacini 1996). Whereas the content of glucose and fructose does not seem to have a great influence on pollen viability, sucrose seems to be an important regulator for the acquisition of tolerance against dehydration. For example, maize only contains 5% sucrose and loses viability rapidly through dehydration, whereas *Pennisetum typhoides* contain 14% sucrose and survives much longer (Hoekstra et al. 1989). By replacing water at hydrogen-bonding sites of the polar head groups of phospholipid with sugars and proteins, the spacing between phospholipids is maintained and the overall integrity of the membrane is retained (Hoekstra et al. 1992). Pacini and Viegi (1995) and Franchi et al. (1996) suggest that cytoplasmic polysaccharides are also involved in resistance to dehydration.

Further changes involve metabolites of important metabolic pathways. In wheat pollen, for example, the concentration changed for many different metabolites of the glycolytic pathway and the TCA cycle. The most significant changes were found in amino acid composition and sugar content. Here, the changes between wheat lines were less striking than between the different environmental conditions, indicating severe damage and a general loss of viability in all wheat lines tested.

5.2.5. Can improvements in short-term storage already help?

In cases where short-term storage is effective, the shelf-life of cereal pollen might be extended for a few hours or days to bridge asynchrony of flowering or for short-distance shipping. For example, for maize pollen, a liquid medium has been developed that keeps the pollen stable without stimulating it to germinate. This medium contains 1000 mM betaine, 160.0 g L^{-1} sucrose, 0.3 g L^{-1} calcium chloride dihydrate, 0.1 g L^{-1} boric acid, 0.25x phosphate-buffered saline (PBS) and at least one pollen encapsulating agent (mineral oil, honey, phenolic acids, flavonoids) adjusted at pH 9. Maize pollen thus could be stored in this medium at 4 °C for 15 days and showed a viability of 85 to 90% using the FDA assay. Germination of the stored maize pollen could be induced by removal of the storage medium and replacing it with a germination medium (Arling et al. 2020, WO 2020/055647 A1). This storage medium or adaptations of it might as well stabilize other cereal pollen such as wheat.

Another possibility that may extend the pollen shelf-life for short-term storage could be the addition of specific substances to prevent the interaction between viable and non-viable pollen.

However, the possible leakage of cytoplasmic compounds from dead pollen may interfere with surrounding viable pollen causing viability loss and pollen death (Etter et al. 2019, US2019/0008144 A1). Accordingly, various solid, liquid and gaseous substances can serve as space filling and absorb additives for pollen. Particularly hydrophilic substances have good absorbing properties. A vast variety of substances were suggested including inorganic and organic particles, isotonic buffers, osmotic regulators, dormancy regulators, cellular respiration inhibitors and disruptors, electron transport inhibitors, uncouplers of membrane electron transport, membrane stabilizers and conditioners, cell wall stabilizers and conditioners, plant growth regulators, cryoprotectants, carbohydrates of various classes (e.g. potato starch, corn starch, lactose, cellulose), anti-flocculants, dispersal agents, bentonite, sand, desiccants, flow agents, mineral oil, paraffin oil, activated charcoal, calcium silicate (e.g. Sipernat®), and combinations thereof. The authors of this patent further claim that spatial separation of viable from dead pollen grains is best achieved when these substances surround each individual pollen grain in a ratio of at least 3:1 and the particle size is in a range between ten times smaller or larger than the pollen grains. Continuous mixing using vibration, shaking, rotation, stirring, or other means should be applied to ensure homogenous distribution. Additionally, the solid substances may maintain pollen moisture content at 15 - 60%, depending on pollen species. The authors suggested further the use of empty pollen grains that have been washed free or otherwise emptied of their internal composition. Referring to maize as an example, the use of a mix of 96% lactose and 4% Aerosil® in a ratio of 3:1 showed an astonishing good retainment of maize pollen viability at 56% IFC viability after storage at 4 °C for 17 days. Alternatively, liquids like isotonic buffers, mineral oil and other liquids that do not readily exchange fluid across the pollen membrane, or gases such as nitrogen, carbon dioxide, nitrous oxide, oxygen, or a mixture may serve as an excellent medium for short-term storage.

5.2.6. Types of damages caused by cryopreservation and means for reducing them

5.2.6.1. Finding the balance between intracellular ice formation vs. dehydration

During cryopreservation, the occurrence of ice crystals depends on the available volume of water, the concentration of salts and other solutes, and the speed of cooling. Combinations of these factors affect the location and extent of ice crystal formation when sub-zero temperatures are reached. Ice-crystal formation is more likely to be initiated in a large volume than in a small volume. Because the volume of a single cell is much smaller than the volume of the extracellular aqueous environment, ice crystal formation is favoured in the extracellular space (Karow 1969). The formation of extracellular ice results in change in concentration of solutes and osmosis leading to the deprivation of available water molecules from the cell and a dehydration. In order to form a crystalline ice structure, water molecules must be very specifically aligned with each other. The inclusion of any salts or other dissolved substances in the cytoplasm disturbs the alignment of the water molecules to each other and thus the formation of a crystalline ice structure. Hence, an ice crystal consists of relatively pure water ice with very low concentrations of other molecules (Dai et al. 2021). For a cell this means, that the fewer water molecules are present the less likely intracellular ice formation will occur. Further, the cooling rate influences the time available for ice crystal formation. The effects of combining different pollen states (fresh pollen, partially and extensively dehydrated pollen) with different cooling and warming rates have already been shown in the **Manuscript 3**. The damage caused by dehydration and exposure to sub-zero temperatures is lethal for wheat pollen if the pollen is dried inhomogeneously (slow air drying) and cooled only slowly. The different types of damage that occur in the pollen during this process have been discussed in **Manuscript 3**. The following sections attempt to give further information and suggestions for optimisation of a cryopreservation protocol for wheat pollen. The modification of the cooling and heating rates and addition of cryoprotectants or antioxidants are discussed. This may potentially reduce cryopreservation damage and increase the chance of obtaining viable and germinable pollen after cryopreservation.

5.2.6.2. Water content prior to freezing

The formation of intracellular ice crystals and the associated damage during cooling can be reduced by drying before freezing. Drying increases the viscosity of the cytoplasm (Hoekstra et al. 2001) and reduces the intracellular mobility of water (Buitink et al. 2000). However, as

shown in the **Manuscript 3**, the extent of dehydration is limited in recalcitrant pollen such as wheat. Below a certain WC, wheat pollen suffers desiccation damage (results of **Manuscript 3**). If, on the other hand, freezing occurs at WC above this value, damage due to intracellular ice crystal formation is to be expected (Becwar et al. 1983). However, there is a window of optimal WC at which dehydration-intolerant pollen such as wheat could be cryopreserved. In various recalcitrant pollen and seeds, rapid drying can extend this window (Berjak et al. 1990). In **Manuscript 3 (Figure 4, 6)**, we showed that a rapid drying rate can help to positively influence the vitality of wheat pollen after freezing and thawing. We assume that the fast drying is necessary to reduce the dehydration-induced damages before cryopreservation. Nevertheless, we believe that a further optimisation in the cooling/warming rates are necessary.

5.2.6.3. Rate of cooling

Freezing damage in plant cells is influenced by the speed of cooling. Slow cooling (~ 1 °C min^{-1}) favours the outflow of water from the cell and extracellular freezing. The intracellular milieu becomes increasingly concentrated. Above a certain cytoplasmic concentration, intracellular ice can no longer form, and the cells could be exposed to cryogenic temperatures. Cells that do not reach the required intracellular concentration can suffer fatal intracellular freezing if the temperature is lowered further (Mazur 1990). However, ultra-rapid cooling allows cells to move so quickly through the ice-forming temperature range (-2 °C to -80 °C) that either no ice crystals form or they do not grow to a harmful size (Pammenter and Berjak 2014). Thus, the intracellular solution is assumed to be in a non-crystalline glassy state and damages caused by intracellular ice can be avoided. The so-called intracellular vitrification can be further supported by partially drying of the tissue/cells or the use of cryoprotectants before freezing. However, sensitive material can only tolerate limited drying and the suitability of a cryoprotectants and the proper concentration must be empirically determined for each different plant material (see also section 5.2.5.6). Nonetheless, the faster the cooling and heating rates, the wider the range intracellular WC can be lowered in desiccation-sensitive material before freezing within a certain limitation to a lethal lower WC threshold (Wesley-Smith 2002). Some authors achieved ultra-fast cooling rates by using a nitrogen slush, in which liquid nitrogen is converted into a nitrogen slurry (Echlin 1992). Nitrogen slush has an extremely low temperature (-207 °C on average) and provides better thermal contact between the sample and the cryogen (Walters et al. 2008). Direct contact with the cryogen increases the cooling rates 4-fold by the reduction of the Leidenfrost effect, which creates a protective vapour barrier on the sample surface (Sansiñena et al. 2012; Santos et al. 2012). For example, excised embryonic axes of *Camellia sinensis* could survive a

combination of partial dehydration and rapid freezing in nitrogen slush at a WC range of 0.6 - 1.6 g H_2O g^{-1} DW. In comparison, the window of water range reduced dramatically to 0.4 to 0.44 g H_2O g^{-1} DW when cooling rates at 10 °C min^{-1} were used (Wesley-Smith et al. 1992). Excised embryonic axes from recalcitrant seeds of *Acer saccharinum* could survive the submersion in nitrogen slush (cooling rate: ~5,000 °C min^{-1}) even without further dehydration prior freezing (Wesley-Smith et al. 2015). The use of a nitrogen slush instead of LN may thus further increase the survival of wheat pollen and needs to be investigated in future experiments.

5.2.6.4. Thawing

After cryopreservation, the frozen tissues or cells must be thawed in such a way that ice crystallisation is avoided or at least reduced during heating. Cells can be damaged during thawing if intracellular ice crystals form at the crystallisation point, which become larger the longer the cells are at this point. Even in vitrified cells, ice crystals form as the temperature rises (Reinhoud et al. 2000). Many studies have shown that fast thawing rates reduce the formation of intracellular ice and cells survive more frequently (Nath and Anderson 1975; Bajaj 1995). Rapid heating rates of 400 °C min^{-1} can be achieved by direct immersion of the cryotube in a thermostatically controlled water bath at 35-40 °C (Wesley-Smith cited by Dumet et al. 2002).

In some studies, water, liquid MS medium, or other solutions were used as thawing solutions for embryonic axes (Wesley-Smith et al. 1992; Wesley-Smith et al. 2001). Encapsulated meristems from *Pelargonium x peltatum* immersed in a liquid 0.75 M sucrose medium at temperatures between 20 and 40 °C survived thawing with a viability of 66% after cryopreservation, while the viability of traditionally thawed meristems was 34% and 37 %, respectively. Moreover, at higher WCs (0.56 g H_2O g^{-1} DW), survival of meristems was found only after thawing with sucrose (30 to 54%). The high sucrose concentration in the thawing medium could contribute to the cold protection of the meristems. Previous studies indicated a direct role of sucrose in limiting ice crystal growth (Dumet et al. 2002). Thawing with sucrose could be particularly useful for cells that are highly sensitive to desiccation, such as the zygotic embryos of recalcitrant seeds and pollen.

Some studies have shown that a higher number of explants reconstitute normal cytoskeleton and develop normally after cryopreservation when calcium and magnesium cations are taken up by the cells from additive such as cryoprotective agents (CPA)-solution either before freezing (Mycock 1999) or after freezing as in the rewarming solution (Berjak et al. 1999). For example, a 1:1 solution of 1 µM calcium chloride dihydrate and 1 mM magnesium chloride

hexahydrate as thawing medium improved the proportion of normal plantlets of desiccation-sensitive embryonic axes of *Quercus robur* after cryopreservation (Berjak et al. 1999). Calcium and magnesium have important signalling and regulating functions in cells. They play a role in the assembly and stability of cyto- and nucleoskeletal elements and are possibly important for the normalisation of other intranuclear processes (Wolfe 1995).

5.2.6.5. Cryoprotection with CPA

Antifreeze agents or CPAs are chemicals and compound mixes that reduce freezing injury to cells during cooling and are therefore used as additives in cryopreservation (Meryman and Williams 1985). Glycerol was one of the first CPA used in the preservation of red blood cells (Lovelock 1953). Since then, many other natural and artificial CPAs have been tested. For example, this includes sorbitol, trehalose, ethanol, methanol, dimethylsulfoxide (DMSO), dextran and polyvinylpyrrolidine, and more (Meryman and Williams 1985). CPAs can be further divided into penetrating (small molecules that enter cells) and non-penetrating cryoprotectants (larger molecules that do not enter cells). Penetrating cryoprotectants such as DMSO and sugar alcohols (e.g. glycerol, ethylene glycol) regulate intracellular osmotic pressure. Thus, they lower the freezing point of the intracellular solution and osmotic damage can be reduced (Awan et al. 2020). Impermeable CPAs, on the other hand, have a dehydrating effect and remove water from the cells by osmosis, which ultimately reduces intracellular ice crystal formation (Muldrew et al. 2004). Increased salt concentration as a result of freezing was also shown to protect sensitive membranes (Meryman 1966) and alter membrane permeability properties as protection against excessive dehydration (Phelps et al. 1999); and/or reduce or delay ice crystal formation (Franks et al. 1977). Studies in pollen showed that external application of CPAs may have differential effects on post-thaw viability and germination. In pollen of *Cannabis sativa* (Gaudet et al. 2020) and date palm (*Phoenix dactylifera* L.) (Tisserat et al. 1983), application of CPA during cooling had a negative effect on pollen germination compared to frozen pollen without CPA. In carob (*Ceratonia siliqua* L.) pollen, vitality after thawing could be significantly increased by the use of CPAs (sorbitol, mannitol, sucrose or glucose) compared to untreated frozen controls (Custodio et al. 2006). Similarly, castor bean (*Ricinus communis* L.) pollen had a higher vitality when glycerol (Vargas et al. 2011b) or DMSO (Vargas et al. 2011a) were used as CPA. Furthermore, certain amino acids (e.g. proline, threonine, glutamine) can have positive effects at specific sites of cell membranes and can protect them from structural changes (Heber et al. 1971). For instance in maize, isolated sperm cells from pollen could survive more than 20 hours at room temperature when glutamine was

added to the isolation medium before cryopreservation (Roeckel and Dumas 1993). Also, heat-protective proteins can have a stabilizing effect on intracellular proteins. In pollen of *Paeonia lactiflora,* the heat shock protein 70 significantly improved post-cryo pollen viability when added before cryopreservation (Ren et al. 2019a). In conclusion, the effectiveness of cryoprotection depends on the kind of CPA used and its chemical properties, such as chemical nature, toxicity, molecular size, penetration ability, interaction of mixtures of compounds; and on the concentration and time of exposure of the cryoprotectant (Finkle et al. 1985). Furthermore, the effects of these factors on post-cryo viability differs between species, tissues or even cell types and, accordingly, the type and concentration of CPA must be empirically adjusted for each new explant that shall be cryopreserved. For wheat pollen, no report so far exists about the effect of using CPAs to reduce or avoid damages introduced by dehydration and low-temperature storage. Thus, further research is required to test the suitability of different CPAs and develop an effective cryopreservation protocol for wheat pollen.

5.2.7. Measuring ROS and scavenging of free radicals through antioxidants

The accumulation of ROS during dehydration and cryopreservation, the activity of the antioxidant system and indicators of damage can be investigated using various methods. ROS themselves are difficult to quantify because these molecules are highly reactive and therefore short-lived. Therefore, it is easier to measure the formation of by-products of oxidative damage or the antioxidant status of the cells. The ratio of oxidised to reduced low-molecular weight antioxidants gives an indication of the formation of ROS and the ability of cells to regulate oxidative stress. The identification of end products of ROS oxidation indicates possible damage and is a sign that cells have been unable to maintain the balance of ROS production and scavenging (Kaczmarczyk et al. 2012a). For example, an increase in DNA damage in cells can be measured using the comet assay (Collins 2014). Oxidation of proteins in cryopreserved cells can be determined by the amount of protein carbonylation (Len et al. 2019). Lipid peroxidation can be determined by an increase in 4-hydroxynonenal (HNE) and MDA (Len et al. 2019), which are indicators of oxidative stress in cryopreservation. High MDA or HNE concentrations correlate with reduced survival rates in, for example, rice suspension culture cells, somatic olive embryos, flax and blackberry shoot tips (Benson et al. 1992; Obert et al. 2005; Uchendu et al. 2010; Lynch et al. 2011). The reaction of biomolecules with free radicals can release volatile compounds such as methane, ethane, ethylene and pentane. The formation of these compounds can be measured using a non-destructive and non-invasive test method, volatile headspace

sampling, and can be used as an indicator of oxidative damage (Kaczmarczyk et al. 2012b). These measurements may help to identify the possible ROS-induced damages in wheat pollen.

Exogenous application of specific antioxidants in appropriate concentrations could support the remedy of ROS (Mathew et al. 2019) and may improve the efficiency of cryopreservation. In general, the addition of antioxidants prior to dehydration and cooling can reduce ROS production (Sun et al. 2020), eliminate intracellular ROS (Len et al. 2019), increase the activity of intracellular antioxidant enzymes (Azadi et al. 2017), regulate the synthesis of mitochondrial proteins (Banday et al. 2017), resist lipid peroxidation and DNA fragmentation (Yousefian et al. 2018). Addition of compounds with known antioxidant properties such as ascorbic acid (Vitamin C), tocochromanols, glutathione, mitochinon, carotenoids, coenzyme Q, quercetin, and melatonin are only few substances that have been reported to significantly reduce DNA damage in cryopreserved animal and plant cells (reviewed in Reed 2014; Len et al. 2019). Exogenous addition of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) can also lower the intracellular oxidative stress level and, thus, significantly improve viability in biomaterials after LN storage (Di et al. 2017). In pollen of *Paeonia* and *Magnolia*, the application of CAT and malonate dehydrogenase could significantly improve viability after LN storage (Jiang et al. 2019; Jia et al. 2018).

The priming of plant tissues is another way in which oxidative stress can be mitigated by inducing a higher activity of antioxidative mechanisms prior to the exposure of stress. For example, cold-hardening and high concentration sucrose preconditioning of mother plants results in endogenous changes in the plant tissues, which contribute to improvement of their freezing tolerance (Dumet et al. 2000; Stushnoff et al. 1997). In a study on garlic shoots (*Allium sativum*), it was reported that osmotic stress during pretreatment had a positive effect on survival after cryopreservation (Keller 2005). In raspberries (*Rubus idaeus* L.), exogenously supplied sucrose induced hardening that protected against osmotic stress or desiccation during cryopreservation (Palonen and Junttila 1999). Improvements in post-cryo survival rates through priming has also been illustrated, for example, by the use of agents such as melatonin in the seeds of maize and mung bean (*Vigna radiata* L.) (Janas and Posmyk 2013), in cryopreserved rose root (*Rhodiola crenulata)* callus (Zhao et al. 2011), and cryopreserved elm (*Ulnus americana* L.) shoot tips (Uchendu et al. 2013); by the use of ascorbic acid and vitamin E on cryopreserved *Rubus* shoot tips (Uchendu 2009), and cathodic water on desiccated and cryopreserved black monkey orange (*Strychnos gerrardii)* embryos (Berjak et al. 2011). These examples show that priming of the mother plant may reduce damages introduced through the

process of cryopreservation and increase the chance of post cryo-survival. In wheat, further research may reveal, if additions of sugars or cold hardening of the mother wheat plant before pollen collection may have a positive effect on the pollen's ability to survive cryopreservation.

6. A glance into future - How could the use of long-term stored wheat pollen be realised in the field?

Global population is predicted to increase to > 9 billion people by 2050 (Ahirwar et al. 2019). As mentioned earlier, agricultural production needs to be increased by up to +65% to meet the higher demands of human nutrition (van Dijk et al. 2021). This is an enormous challenge and may only be realised if advanced, automated, and computer-assisted technologies are adopted in modern agricultural production. The use of stored pollen for the fertilization of varieties with superior features to produce hybrids is expected to increase in cereal crops. This might be further improved if cereal pollen could be handled in an efficient, automated and controlled way.

6.1. Existing techniques to apply pollen on field scale

If wheat pollen could be kept stable and suspended and viable pollen could be applied onto receptive female plants in the field using sprayers that disperses a stream or droplets comprising the pollen (Arling et al. 2020, WO2020055647A1). Such a pollen dispenser was proposed by Cope and McAvoy in 2014 in a patent (Cope and McAvoy 2014, US20140223812 A1). It resembles a spray bottle with a temperature control unit attached to keep the pollen under controlled conditions. However, since this is a hand-held spraying device, pollen application onto female plants in the field still requires much effort and, thus, it is not feasible for large-scale hybrid-seed production. A similar yet automatic application of pollen in the field was proposed for maize pollen and presented in a patent in 2000. A man-driven apparatus was used to collect and purify the pollen from male maize plants of one field to apply them to female maize plants of another field. It was further proposed, that this apparatus could be used to prepare pollen to be stored in a genebank (Greaves et al. 2000). However, the authors did not describe in detail how the maize pollen was prepared for storage. This machine was developed for the use in maize breeding. Thus, if such an apparatus is to be invented for the use in autogamous plants like wheat, it requires some major adjustments in order to collect and purify the viable pollen.

6.2. The use of drones and unmanned flying imaging facilities for pollen application in the field

Yet, large areas of farmland make it very difficult and time-consuming to examine, identify the right flowering state for each plant and to prepare and apply the pollen manually. The whole process could be automated with minimal human interaction resulting in improved efficiency

by implementing unmanned aerial vehicle, also known as drones. Drones can be remote-controlled or programmed to follow precise flight routes in the field with accuracy in the cm-range. Drones are already used to assist farmers in the sowing e.g. in rough terrain (Reinecke and Prinsloo 2017), to examine soil and soil water before planting crops (Oleire-Oltmanns et al. 2012), for irrigation and to spray herbicides, insecticides and pesticides accurately in the affected area (Romero-Trigueros et al. 2017; Hoffmann et al. 2016). Furthermore, drones that are equipped with high resolution cameras with sufficient computing power and network connectivity could be highly useful to collect advanced crop image data for identification and analysis of useful crop traits at high spatial and temporal resolution and to facilitate crop monitoring in near real-time. Thus, smart image processing techniques may be used in future to identify the ideal timepoint for pollen application in the field. Furthermore, a drone could have a pollen dispersal device mounted to it releasing the pollen at exactly those female plants that are receptive for the pollen. Thus, it does not seem unlikely in future to use drones for pollen application. Additionally, drones could not only be used directly for delivery of the desired pollen but also for monitoring plant development and flowering time to estimate the best time point of pollen application. Specialised camera-technique and computer programs could be linked to the drones that can deliver the pollen at the best time point even for individual plants.

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Appendix

Supplemental Experiment:

Flowering process of a wheat spike

Introduction:

The flowering process in wheat is short and last only about 7 days (De Vries 1971). Flowering begins in the middle section of the spike and progresses up- and downwards along the spike (De Vries 1971). Knowing in which order a wheat spike flowers helps to find the optimal timepoint for pollen collection. However, so far, only few data exist about the order of flowering and, to best knowledge of the authors, the flowering process of a wheat spike has not been published in a photo- or videographic way. Thus, in this supplemental experiment I recorded the flowering process of a single wheat spike with the help of cameras in a time-lapse mode to investigate, I) how long the whole flowering period lasts, II) in which order individual florets flower, and III), how long the flowering of individual florets lasts.

Material and Method:

For the videographic recording of the process of flowering in wheat, one plant of variety 'Ferrum' was taken from the greenhouse one day before flowering started and placed in a dark room. Three studio lights were pointed towards the plant for optimal lighting during the recording. Two cameras were set up from two ankles and recording took place in time lapse mode (30 frames/sek). Details of photographic set up can be found in **Table X1**. **Figure X1** shows the spike that was used for recordings. Each individual spikelet in this spike was given a number to track the start and end of flowering (**Figure X1**). **Table X1** shows the numeration of each floret within the 25 spikelets. Although in some spikelets more than three flowers existed, the flowering could be tracked from the recordings only in the first three florets. For optimal growth, normal watering of the plant was kept as mentioned in Material and Methods in **Manuscript 1**.

Table X1 Photographic set up of time lapse recording of the flowering of a wheat spike

Camera 1	Camera 2	Studio lights
Nikon D750 Lense: AF Micro Nikkor 60mm 1:28 D ISO 100 SS 1/6 F5.6 distance from plant ca. 40cm	Panasonic Lumix G81 Lense: Olympus Digital M.Zuiko 12-40mm 1:2.8 ISO 200 SS 1/8 F3.2 distance from plant ca. 35 cm	left front: 5.4 light intensity, distance from plant ca. 1.10 to 1.20 m left back: 4.0 light intensity, distance from plant ca. 60-70 cm right back: 3.9 light intensity, distance from plant ca. 60 cm

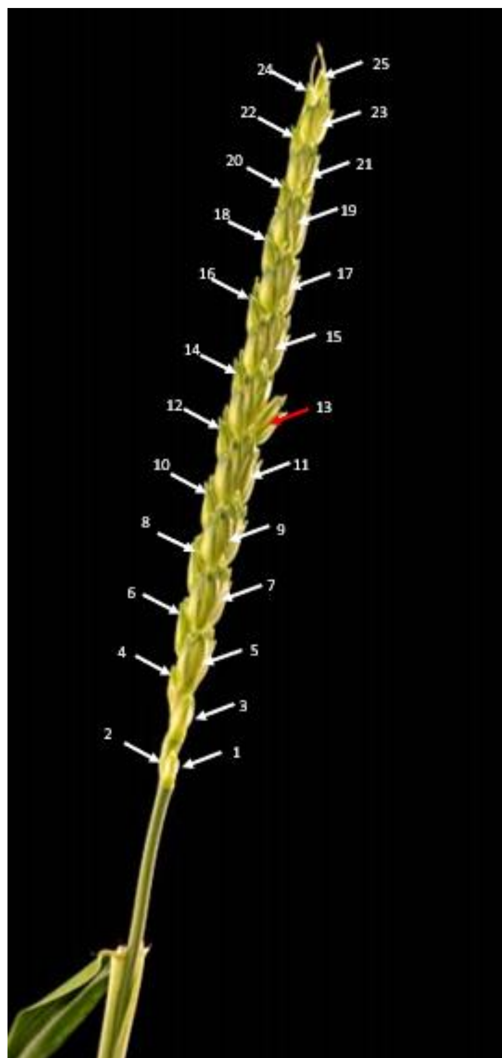

Figure X1: Spike of the variety 'Ferrum' used for videographic recording of the flowering process. Each spikelet is marked with an arrow and has been given a number starting at the base of the spike towards the tip. The first floret that started flowering in the spike is marked with a red arrow (13) and a detailed photo of it is given in Figure X2.

Table X2: Numeration of the individual florets in the different spikelet positions of the spike from Figure X1. Blue marked rows are showing spikelets that were oriented towars the camera whereas whit rows mark the spikelets that were on the opposite site of the camera and thus harder to track.

Spikelet position	Floret position		
	1	2	3
1	1.1	1.2	1.3
2	2.1	2.2	2.3
3	3.1	3.2	3.3
4	4.1	4.2	4.3
5	5.1	5.2	5.3
6	6.1	6.2	6.3
7	7.1	7.2	7.3
8	8.1	8.2	8.3
9	9.1	9.2	9.3

10	10.1	10.2	10.3
11	11.1	11.2	11.3
12	12.1	12.2	12.3
13	13.1	13.2	13.3
14	14.1	14.2	14.3
15	15.1	15.2	15.3
16	16.1	16.2	16.3
17	17.1	17.2	17.3
18	18.1	18.2	18.3
19	19.1	19.2	19.3
20	20.1	20.2	20.3
21	21.1	21.2	21.3
22	22.1	22.2	22.3
23	23.1	23.2	23.3
24	24.1	24.2	24.3
25	25.1	25.2	25.3

Result and Discussion:

The visible flowering of the whole spike (opening of palea and lemma, anther extrusion, closing of palea and lemma) lasted 66 h and 56 min. Flowering begun in the floret in position 1 in spikelet 13 (**Table X2, Figure X2**). **Table X3** shows the order in which the individual florets flowered, the time at which each floret begun and finished flowering, and the total time the floret opened. Additionally, it was calculated how much time passed between the flowering of the florets in the positions 1, 2 and 3 in an individual spikelet. On average flowering of the florets in position 1 lasted the longest with 90 min, followed by 68 min of florets in the second position, and 42 min of florets in the third position. The time between the flowering of florets in position 1 and 2 differed to a great extent from 3 h 5 min in spikelet 14 to more than 48 h in spikelet 11 and 12. There was no clear trend, whether florets from a spikelet in the middle of the spike finished flowering faster than florets that were positioned in spikelets of the base or tip of the spike. The time between the flowering of florets in position 2 and 3 was more uniform and lasted between 27 and 48 h. The variety ‘Ferrum’ was chosen, because plants in the greenhouse showed frequently opening of the paleas and lemmas and visible anther extrusion of florets during flowering. Nevertheless, the inner most florets may had a typical autogamous flowering inside a closed floret and, therefore, it is likely that some additional florets which are not listed in **Table X3** flowered during the recordings. The experiment is helpful to get an estimate of the flowering process in a wheat spike. Thus, I can conclude that the flowering usually starts at the floret of position 1 in the middle of a spike, continues through the other florets of position 1 in spikelets positioned towards the tip and base of a spike, and further continues with florets of position two again in one of the middle spikelets. The last florets that would flower are likely to be the innermost florets within the spikelets, and the florets at the base and top of a spike (**Table X3**). Furthermore, the time between flowering of florets in first position within a spikelet and second position tend to be shorter than the time between florets of the second and third position within a spikelet.

Figure X2: Begin of flowering in floret 1 of spikelet 13. Palea and lemma are opened and the anthers are pushed out. The rupture of the anther tip to release the pollen can be seen.

Video X1 Flowering process of a single wheat spike over a time period of 4 days.

Table X3: Flower process in a wheat spike. Data for date and time were taken from the time stamps in the time-lapse video recordings. Empty cells in the table mean that there was no visible flowering of the florets. Blue marked rows show spikelets that were oriented towards the camera, whereas white marked rows were on the opposite site of the camera and, thus, more difficult to track.

Spikelet position	Order of flowering in the floret positions			Floret	Start flowering [date:time]	Finish flowering [date:time]	Total time of flowering [min]	Time since start of timelapse [h:min:sec]	Floret	Start flowering [date:time]	Finish flowering [date:time]	Total time of flowering [min]	time since start of timelapse [h:min:sec]	Floret	Start flowering [date:time]	Finish flowering [date:time]	Total time of flowering [min]	Time since start of timelapse [h:min:sek]	Time between 1. to 2. floret [h:min:sec]	Time between 2. to 3. floret
	1	2	3																	
1				1.1					1.2					1.3						
2				2.1					2.2					2.3						
3				3.1					3.2					3.3						
4				4.1					4.2					4.3						
5	25	31		5.1	06.04.20 1:08:11	06.04.20 2:13:11	65	42:53:13	5.2	06.04.20 21:42:13	06.04.20 22:32:13	50	63:32:15	5.3					20:39:02	
6	16	30		6.1	05.04.20 18:23:11	05.04.20 20:43:11	140	36:08:13	6.2	06.04.20 15:57:13	06.04.20 16:52:13	55	57:47:15	6.3					21:39:02	
7	15	27	42	7.1	05.04.20 17:23:11	05.04.20 19:18:11	115	35:08:13	7.2	06.04.20 6:03:11	06.04.20 7:13:11	70	47:48:13	7.3	15:17:13	16:06:44	50	81:07:15	12:40:00	33:19:02
8	12	26	41	8.1	05.04.20 11:23:11	05.04.20 12:33:11	70	29:08:13	8.2	06.04.20 4:08:11	06.04.20 4:43:11	35	45:53:13	8.3	11:22:13	12:02:13	40	77:12:15	16:45:00	31:19:02
9	13	24	40	9.1	05.04.20 12:23:11	05.04.20 14:23:11	120	30:08:13	9.2	05.04.20 23:53:11	06.04.20 1:03:11	70	41:38:13	9.3	10:07:13	10:47:13	40	75:57:15	11:30:00	34:19:02
10	9	20	36	10.1	05.04.20 4:30:19	05.04.20 6:30:19	120	22:15:21	10.2	05.04.20 19:33:11	05.04.20 21:18:11	105	37:18:13	10.3	4:37:13	5:17:13	40	70:27:15	15:02:52	33:09:02
11	4	33		11.1	04.04.20 23:15:19	05.04.20 0:30:19	75	17:00:21	11.2	07.04.20 0:07:13	07.04.20 0:47:13	40	65:57:15	11.3					48:56:54	
12	5	34		12.1	04.04.20 23:35:19	05.04.20 0:50:19	75	17:20:21	12.2	07.04.20 0:07:13	07.04.20 0:47:13	40	65:57:15	12.3					48:36:54	
13	1	17	32	13.1	04.04.20 21:10:19	04.04.20 22:05:19	55	14:55:21	13.2	05.04.20 19:08:11	05.04.20 20:23:11	75	36:53:13	13.3	22:37:13	23:22:13	45	64:27:15	21:57:52	27:34:02
14	7	10	37	14.1	05.04.20 1:40:19	05.04.20 3:20:19	100	19:25:21	14.2	05.04.20 4:45:19	05.04.20 6:10:19	85	22:30:21	14.3	6:32:13	6:57:13	25	72:22:15	3:05:00	49:51:54
15	2	18	38	15.1	04.04.20 21:20:19	04.04.20 22:40:19	80	15:05:21	15.2	05.04.20 19:18:11	05.04.20 20:43:11	85	37:03:13	15.3	7:02:13	7:57:13	55	72:52:15	21:57:52	35:49:02

16	3	19		16.1	04.04.20 22:45:19	04.04.20 23:15:19	30	16:30:21	16.2	05.04.20 19:23:11	05.04.20 20:48:11	85	37:08:13	16.3					20:37:52	
17	6	23		17.1	05.04.20 1:35:19	05.04.20 3:15:19	100	19:20:21	17.2	05.04.20 23:08:11	06.04.20 0:48:11	100	40:53:13	17.3					21:32:52	
18	8	22		18.1	05.04.20 2:30:19	05.04.20 3:45:19	75	20:15:21	18.2	05.04.20 22:43:11	06.04.20 0:18:11	98	40:28:13	18.3					20:12:52	
19	11	28		19.1	05.04.20 7:05:19	05.04.20 8:45:19	100	24:50:21	19.2	06.04.20 10:22:13	06.04.20 11:02:13	40	52:12:15	19.3					27:21:54	
20	14	35		20.1	05.04.20 16:53:11	05.04.20 18:33:11	100	34:38:13	20.2	07.04.20 2:22:13	07.04.20 3:22:13	60	68:12:15	20.3					33:34:02	
21	21			21.1	05.04.20 22:38:11	06.04.20 1:53:11	195	40:23:13	21.2					21.3						
22	29			22.1	06.04.20 13:42:13	06.04.20 14:22:13	40	55:32:15	22.2					22.3						
23	39			23.1	07.04.20 8:37:13	07.04.20 9:37:13	60	74:27:15	23.2					23.3						
24				24.1					24.2					24.3						
25				25.1					25.2					25.3						

Acknowledgements

First and foremost, I want to express my sincere gratefulness to my supervisor PD Dr. Manuela Nagel. Her guidance and scientific advice were always an enrichment for the progress of my work, and her continued support motivated me to follow my path and not to give up even when things got difficult. I would like to express my appreciation to Prof. Dr. Traud Winkelmann for her support and advices in this thesis. I would like to offer my special thanks to Dr. Daniel Ballesteros who gave me excellent guidance during my time in Wakehurst. It was a pleasure to work with him and very valuable asset to one of the publications in this thesis. The advices given by Dr. Michael Melzer, Dr. Joachim Keller, and Dr. Twan Rutten have been of great help in the conduction of the microscopic experiments of this thesis. I also wish to acknowledge the help and advice provided by Dr. Hardy Rolletschek and Dr. Till Ischebeck which was great help in carrying out the metabolic parts of the thesis. My special thanks are extended to the current and former staff of the CSB group especially Angelika Senula, Marion Grübe and Claudia Köpnick. Many thanks also to previous students and other members of the group for stimulating scientific and non-scientific discussions. The support and assistance provided by the gardening team of IPK and Wakehurst as well as from the IPK administrative team was greatly appreciated. A big thanks goes to Prof. David Honys for his patience and support. Lastly but not least, I am forever grateful to my beloved Stefan who complements me in all ways and fills my life. Whenever I stand head down, he is there to turn me around again. Words can hardly describe how thankful I am to my Mama Regina and sisters Andrea and Janet for made me come to this stage. Without your endless support I would have never come so far in the first place. I would like to express my deep appreciation to all the support and joy I receive from some wonderful people who also belong to my family, Simon, Marie, Dirk, Lukas, and Detlev. I would also like to say a big thank you to all my friends Paride, Isa, Ben, Christian, Solmas, Saji, Sophie, Christoph, Atena, Ramin, Sara, and Kam for all the joyful moments we shared during my bachelor, master and PhD studies and which have significantly enriched my life. I also want to say big thank you for Vinod, Elnura, Natalia, Oliver, Zahra, Anke and Lucia, who also strongly supported me during my last big steps towards my PhD degree.

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Conference contributions

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