Trees (2018) 32:29–39
https://doi.org/10.1007/s00468-017-1606-7

ORIGINAL ARTICLE

Ultrastructural changes of pistachio (*Pistacia vera* L.) mature seeds and pollen in relation to desiccation

Elaheh Zamani Bahramabadi[1] · Parisa Jonoubi[1] · Farkhondeh Rezanejad[2]

Received: 1 March 2017 / Accepted: 14 August 2017 / Published online: 23 August 2017

Abstract

Key message **Pistachio seeds and pollen have desiccation tolerance and desiccation sensitivity ultrastructural characters at maturation, respectively. Seed cells filled with storage matter remain integrated, whereas some pollen show cell rupture.**

Abstract Maturation drying is a common trait of seeds and pollen. Orthodox seeds and pollen become tolerant to desiccation at maturation and remain their germinability in an inactive dry state for a period of time, whereas recalcitrant ones remain sensitive and must germinate immediately after maturation. This study investigated the moisture content, germinability and ultrastructure of pistachio (*Pistacia vera* L.) mature seeds and pollen in fresh and 24 h-desiccated states using microscopy methods. Seeds lost 90.5% water and remained 100% germinable demonstrating their orthodoxy. Fresh and desiccated cells of both cotyledon and ground meristem of root tip, mostly contained lipid and protein reserves. Large autophagic vacuoles in fresh cells contained autophagic bodies digesting organelles related to active metabolism and became converted to protein storage vacuoles at desiccated state. In ground meristem of root tip, most lipid bodies were tiered adjacent to plasma membrane probably with a secretory function and the cell walls were waved at desiccated state. Pollen lost 46.7% water along with 93% germinability by desiccation demonstrating its recalcitrance. After desiccation, 1.8% of pollen grains displayed intine degeneration, vegetative cell plasmolysis and detachment from intine, lobed vegetative cell nucleus, great vacuolation along with organelles destruction in both vegetative and generative cells and finally cell rupture. However, most pollen displayed normal structure indicating the recalcitrance behavior of pistachio pollen is not mainly related to cell structural damages but, lack of germinability through molecular damages that should be studied in details.

Keywords Orthodox · Pistachio · Pollen · Recalcitrant · Seed · Ultrastructure

Abbreviations

MC	Moisture content
LB	Lipid body
PB	Protein body
AV	Autophagic vacuole
PSV	Protein storage vacuole
VC	Vegetative cell
GC	Generative cell

Communicated by U. Luettge.

✉ Elaheh Zamani Bahramabadi
elaheh_zamani@yahoo.com

[1] Department of Plant Sciences, Faculty of Biological Sciences, Kharazmi University of Tehran, Southern Mofateh Street, Tehran, Iran

[2] Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Bistodobahman Street, Kerman, Iran

Introduction

In plants, two reproductive parts, namely seeds and pollen have some common traits so that after independence from parent plant, they may have to wait for a period of time in an inactive dry state until the conditions for germination are conducive ensuring the plant survival. Therefore, they

require mechanisms to maintain their internal stability in this developmentally programmed dry term and remain viable. Maturation drying resembles drought stress and induces the corresponding protective mechanisms such as accumulation of protective molecules, activation of antioxidant scavenging systems, repair mechanisms, modification in intracellular physical structure and many others (Radwan et al. 2014). However, these mechanisms are not possessed by all seed and pollen types. Orthodox seeds and pollen become tolerant to drying to specific low water levels at the end of maturation while recalcitrant ones remain sensitive to dehydration and must germinate immediately after maturation otherwise lose their viability soon after release (Franchi et al. 2011).

Following morphogenesis, developing seeds enter the phase of 'reserve accumulation' (Angelovici et al. 2010). Generally, a decrease in water content, degeneration of organelles related to metabolic activity, biogenesis of storage organelles that replace the vacuoles and cell wall invagination are common events during this phase and are necessary for acquisition of desiccation tolerance (Maia et al. 2016). By gradual accumulating food reserves in storage organelles, seeds are able to lose water without imposing severe dehydration stress (Walters 2015). These structural changes have been shown in orthodox seeds such as bean (*Phaseolus vulgaris*) (Farrant et al. 1997) and wheat (*Triticum aestivum*) (Golovina et al. 2001). In contrast, recalcitrant seeds that constitute a small percentage of all spermatophyta seeds (Franchi et al. 2011) remain highly vacuolated, metabolically active, and therefore, desiccation sensitive at maturation. After dehydration in such a situation, the plasma membrane is disrupted and detached from the cell wall due to excess plasmolysis and the cytoplasmic components experience extreme damage and total collapse of their structure (Maia et al. 2016). These events were demonstrated for example in *Avicennia marina* (Farrant et al. 1997) and *Trichilia emetica* (Kioko et al. 2006). Seeds that have better protection against damage that occurs under dry conditions survive longer; hence, seed longevity and storability are a manifestation of desiccation tolerance (Walters 2015).

Pollen has its own desiccation tolerance mechanisms. Before or during anther dehiscence, maturing pollen grains undergo some dehydration due to the disappearance of locular fluid. Orthodox pollen is released as partially dehydrated, whereas recalcitrant pollen is released as partially hydrated. Orthodox pollen is able to absorb or lose water depending on relative humidity and controls its shape and volume along with it during dispersal. This ability is contributed by reversible folding patterns of pollen wall, a mechanism known as harmomegathy. This kind of pollen regulates the turgor pressure and water displacement and withstands some dehydration, but recalcitrant pollen like that of grasses lacks such regulations and collapses immediately after water loss (Firon et al. 2012). Viability maintenance of pollen in the course of storage is directly related to dehydration tolerance (Franchi et al. 2011).

Pistachio (*Pistacia vera* L.) is a dioecious tree species mostly cultivated in warm temperate regions of the world (Yakubov et al. 2005; Kersten et al. 2017) producing desirable edible seeds which are easy to store. This species is wind pollinated with non-storable pollen that loses its germinability rapidly. Lack of pollen storability is a cultivation problem of pistachio. The aim of this research was to investigate the moisture content (MC), germinability and ultrastructure of pistachio mature seeds and pollen in fresh and desiccated stages to find the relation between these features with storability.

Materials and methods

Plant material

In a pistachio orchard located in Rafsanjan region (56°0′0″N 30°24′0″E) of Iran, fresh mature pollen was collected from ten male trees at pollination time in April 2014, while fresh mature seeds were collected from ten female trees of cv. Kaleghoochi at harvest time (165 days after anthesis) in September 2014. The materials were obtained from four sides of each tree and from different locations in the orchard. A portion of seeds and pollen was used fresh immediately in the orchard so that fresh seeds were obtained from ripped fruit and fresh pollen was obtained from just opened anthers. A portion of seeds and pollen was transferred immediately to the laboratory in plastic bags, and then desiccated for 24 h at room conditions (25 °C and 30% RH) and used thereafter to evaluate the storability of seeds and pollen. The average temperature and humidity during anthesis and pollination period (April 2014) (Chao and Parfitt 2003) and during seed maturation and harvest (135–165 days after anthesis, mid-August until mid-September 2014) (Shekari and Rezanejad 2012) are given in Table 1.

Moisture content determination

Ten fresh seeds or 1 g of very fresh pollen were weighted in the orchard, then desiccated as mentioned above and reweighted. Afterwards, they were completely dried at 100 °C for 24 h and reweighted. The MC of the seeds or pollen at either fresh or desiccated states was obtained from the difference between their weight and the complete dry weight and expressed as a percentage of the first weight at each state. The experiment was done in triplicate (Kalemba and Pukacka 2012).

Table 1 The regional average temperature and humidity where pistachio seeds and pollen were collected

Time	Average temperature and humidity
During anthesis and pollination	22.8 °C, 34%
During seed maturation and harvest	25.2 °C, 20%

Germination test

Thirty fresh and thirty desiccated seeds were soaked in water for 12 h and kept between moist papers at 5 °C. Germinated seeds with a radicle which emerged from 5 mm (Kalemba and Pukacka 2012) were counted weekly for 4 months. Germination medium for pollen was a semi-solid nutrient medium comprising 300 mg L^{-1} $CaCl_2$, 100 mg L^{-1} H_3BO_3, 120 g L^{-1} sucrose and 7 g L^{-1} agar (Cook and Walden 1965). Fresh and desiccated pollen samples were planted onto Petri dishes, kept for 3 h at 28 °C and then at 4 °C until the germinated pollen grains were counted using light microscopy (Olympus, Japan). A germinable pollen has a tube length of at least one diameter of the pollen grain (Cook and Walden 1965). Germination of seeds and pollen was expressed as percentage. The experiment was done in triplicate.

Light microscopy

From fresh and desiccated seeds, the cotyledons and embryonic axes were separated by hand and 2.5 × 2.5 mm^2 pieces of cotyledons and root tips were fixed in FAA (formaldehyde: acetic acid: 70% ethanol, 5:5:90, v/v/v). Fresh and desiccated pollen samples were poured into the same fixative solution. After 24 h fixation, all samples were washed severally with water, dehydrated in 30, 50, 70, 80, 100% ethanol series and embedded in paraffin. Sections of 5 µm of seed parts and 2 µm sections of pollen were cut by a rotary microtome (micro *Tec*, Germany) and stained by Congo red for detecting cellulosic cell walls (Sazci et al. 1986), Sudan black B for lipids, Lugol's iodine for starch, phloroglucinol for lignin (Johansen 1940), and double stained by Periodic acid-Schiff + Coomassie blue (PAS + Cb) for total polysaccharides and proteins, respectively (Schmidt et al. 2012). The sections were then observed by a light microscope (Olympus, Japan) and photographed. At least five different paraffin blocks were surveyed for each material. For cotyledon and embryonic axes, each block contained three samples while for pollen, each block contained numerous pollen grains.

Transmission electron microscopy

Fresh and desiccated cotyledons and root tips of 0.5 × 0.5 mm^2 pieces (in root tips, from periphery and 1 mm beneath the apex surface to obtain ground meristem) and also fresh and desiccated pollen samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 24 h, washed in the same buffer three times for 20 min, post-fixed in 1% osmium tetroxide in the same buffer for 2 h, washed as mentioned above, dehydrated in 30, 50, 70, 80, 100% ethanol series and embedded in araldite epoxy resin. Sections of 70 nm ultrathin were cut by ultramicrotome (Leica, Austria) with a diamond knife, placed on copper grids and stained by uranyl acetate and lead citrate. Sections were then observed and documented by a transmission electron microscope (LEO 912-AB, Alembic staff). Some 70 nm sections were mounted on glass slides, stained with Toluidine blue for metachromasia (O'Brien et al. 1964), observed by light microscope (Olympus, Japan) and photographed. At least three different resin blocks were surveyed for each material. For cotyledon and root tip, each block contained two samples while for pollen, each block contained numerous pollen grains.

Glass slides stained by Toluidine blue were used to determine the ratio of structurally damaged pollen grains to all grains. This ratio was expressed as a percentage.

Statistical analysis

MC and germination tests data are presented as mean of repeats ± standard error of means. Duncan multiple range test was performed using SAS software to compare the means at 0.05 significance level. The charts were plotted with Excel software.

Results

Moisture content and germination test

The MC of fresh and 24 h-desiccated mature seeds was 44.3 and 4.2%, respectively, showing 90.5% water loss (Fig. 1a) while the MC of fresh and 24 h-desiccated mature pollen was 15.4 and 8.2%, respectively, showing 46.7% water loss (Fig. 1b). The germinability of both seed samples was 100% (Fig. 2a) while the germinability of fresh pollen was 73.7% and decreased severely to 4.5% in 24 h, showing 93% loss in initial germinability (Fig. 2b).

Morphology, cytochemistry and ultrastructure of seeds

Mature Pistachio seeds of cv. Kaleghoochi are averagely 2.3 cm in length and 1.4 cm in width and light green to

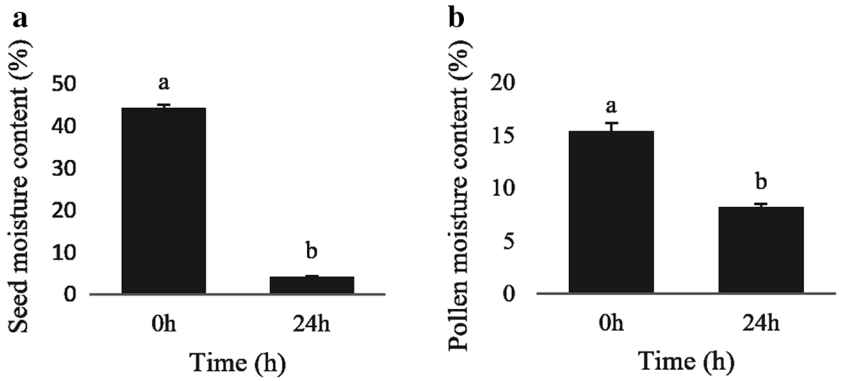

Fig. 1 MC of mature pistachio seeds and pollen before and after desiccation. **a** MC of fresh mature seeds was 44.3% and decreased to 4.2% after desiccation, **b** MC of fresh mature pollen was 15.4% and decreased to 8.2% after desiccation

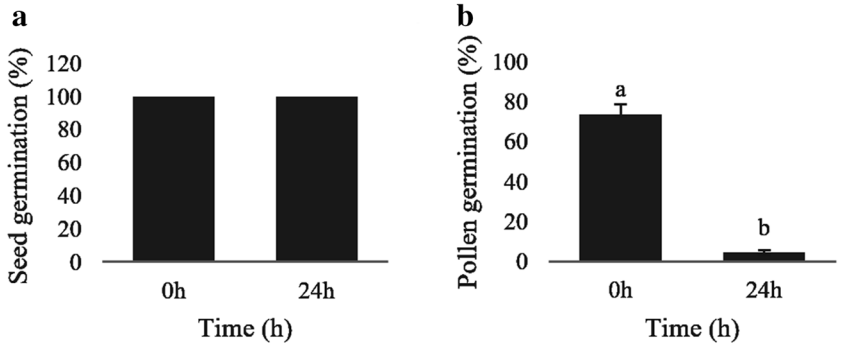

Fig. 2 Germinability of mature pistachio seeds and pollen before and after desiccation. **a** Germinability of seeds was 100% at both states. **b** Germinability of fresh pollen was 73.7% and decreased to 4.5% after desiccation

yellow and are covered by a red thin coat. The cotyledons are the storage part of the seeds (Fig. 3).

In cotyledons, the cells have cellulosic walls that are stained by Congo red (Fig. 4a, e) but not by phloroglucinol. Cell wall folding was rarely seen somewhere in desiccated state (Fig. 5f). Large intercellular spaces are evident (Fig. 5a, e). Lipid bodies (LBs) that were stained by Sudan black B (Fig. 4b, f) and appeared grey to light blue in metachromasia (Fig. 5a, e), filled a large portion of the cell volume at both states. Amyloplasts with starch grains appeared purple by Lugol (Fig. 4c, g) and white in metachromasia (Fig. 5e) and TEM micrographs (Fig. 5c, g). Protein reserves are highly abundant in desiccated compared to fresh cells and are distinguished in the two types. Protein bodies (PBs) are small—about 300 nm in diameter on average—uniformly electron-dense organelles and are observed only in fresh state (Fig. 5a, d), whereas protein storage vacuoles (PSVs) are large—about 3–4 μm in diameter on average—with protein deposits being amorphous (Fig. 5d, e, h) or as a uniform matrix with or without white inclusions (Fig. 5e, f, i). PSVs have higher abundance in desiccated state. In fresh cotyledons, large autophagic vacuoles (AVs) are evident which stain light brown by Lugol (Fig. 4c) and pink in PAS + Cb double-staining (Fig. 4d) with material deposition on the inner surface (Fig. 5a–c). Autophagic bodies, containing cytoplasmic organelles such as PBs, mitochondria, plastids and endoplasmic reticulum (ER), can be seen inside the AVs (Fig. 5b, c). PSV differentiation from AVs is recognizable in fresh state (Fig. 5a). AVs are absent in desiccated state. The nucleus is small and rarely visible in cotyledons (Fig. 5e).

Fig. 3 Morphology of pistschio seeds cv. Kaleghoochi. Cotyledons are the storage part of seeds having the embryonic axis in between. They are *light green* to *yellow* and a *red thin coat* covers them

In root tips, the ground meristem cells have cellulosic walls that are stained by Congo red (Fig. 6a, e) but not by phloroglucinol. The cell walls are obviously waved in desiccated state (Fig. 7f, h). The intercellular spaces are not as large as in cotyledons (Fig. 7a, e). LBs stained by Sudan black B (Fig. 6b, f), look grey in metachromasia (Fig. 7a, e) and more electron dense than cotyledonary LBs. They surround the PSVs and tier adjacent to plasma

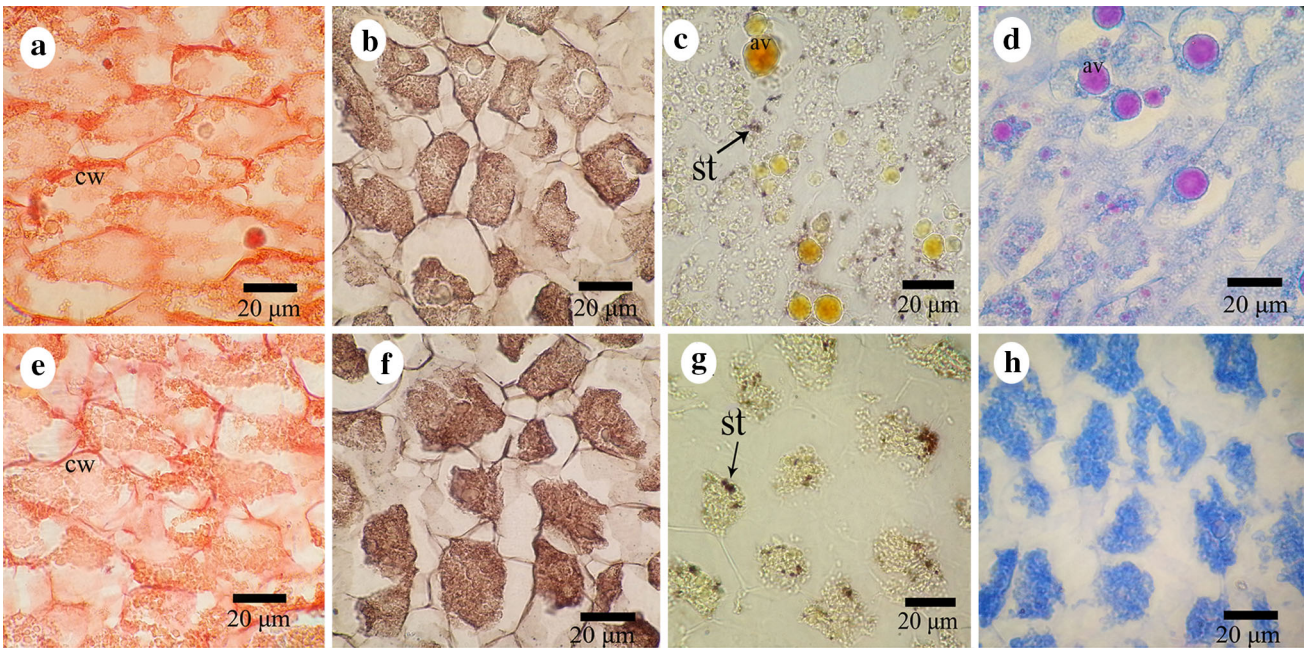

Fig. 4 Cytochemistry of mature fresh (**a**–**d**) and desiccated (**e**–**h**) pistachio cotyledons. **a**, **e** Cellulosic cell walls stained by *Congo red*. **b**, **f** Lipidic storage stained by *Sudan black*. **c**, **g** AVs look light brown and starch grains look *purple* by Lugol. **d**, **h** AVs look *pink* and protein storages look *blue* by PAS + Cb double staining. *cw* cell wall, *st* starch grain, *av* autophagic vacuole

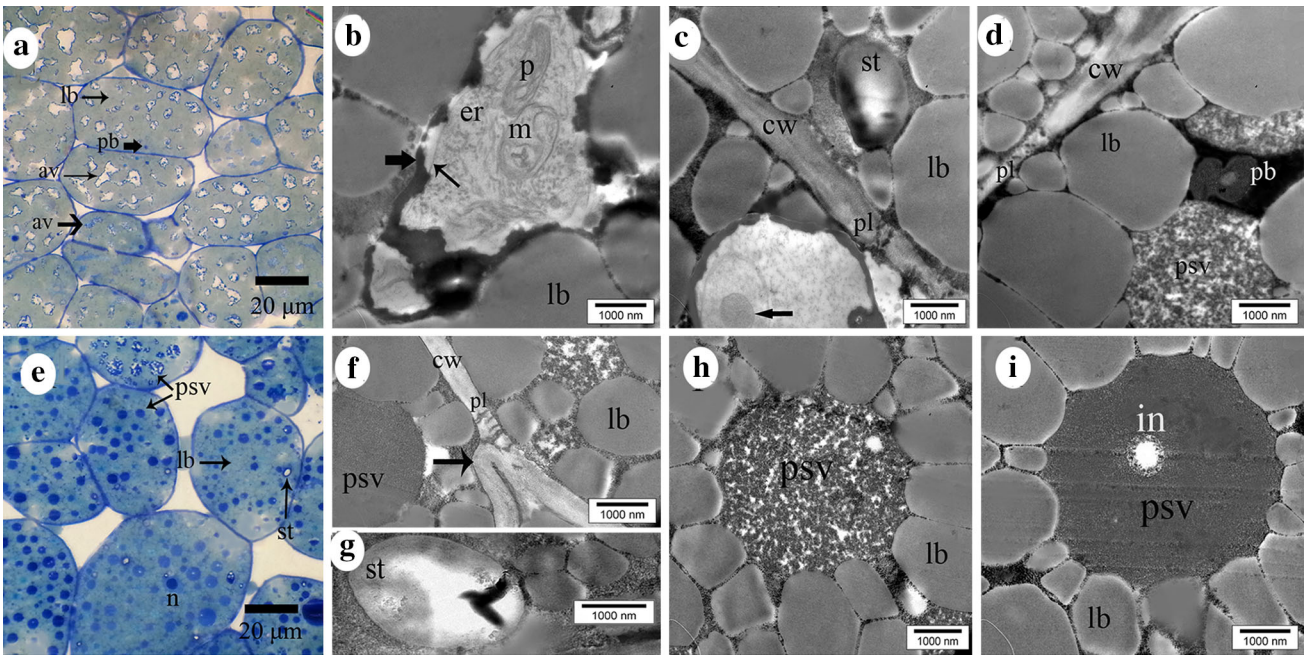

Fig. 5 Ultrastructure of mature fresh (**a**–**d**) and desiccated (**e**–**i**) pistachio cotyledons. **a**, **e** Toluidine *blue* metachromasia, lipid bodies look *grey* to *light blue*, fresh cotyledons contain PBs and AVs with some of them differentiating to PSVs (*lower arrow*), desiccated cotyledons contain fully differentiated PSVs. **b** TEM micrograph of an AV with deposits on the inner surface and containing an autophagic body digesting different organelles, *thick* and *thin arrows* refer to AV and autophagic body membranes, respectively. **c** Another AV with an autophagic body engulfing a probable PB (*arrow*). **d** A PSV with amorphous deposits and two PBs are shown. **f** A sign of cell wall folding in desiccated state. **g** A large starch grain. **h** A PSV with amorphous deposits. **i** A PSV with uniform deposits and a white inclusion. *cw* cell wall, *st* starch grain, *pb* protein body, *psv* protein storage vacuole, *av* autophagic vacuole, *lb* lipid body, *m* mitochondrion, *p* plastid, *er* endoplasmic reticulum, *pl* plasmodesma, *n* nucleus, *in* inclusion

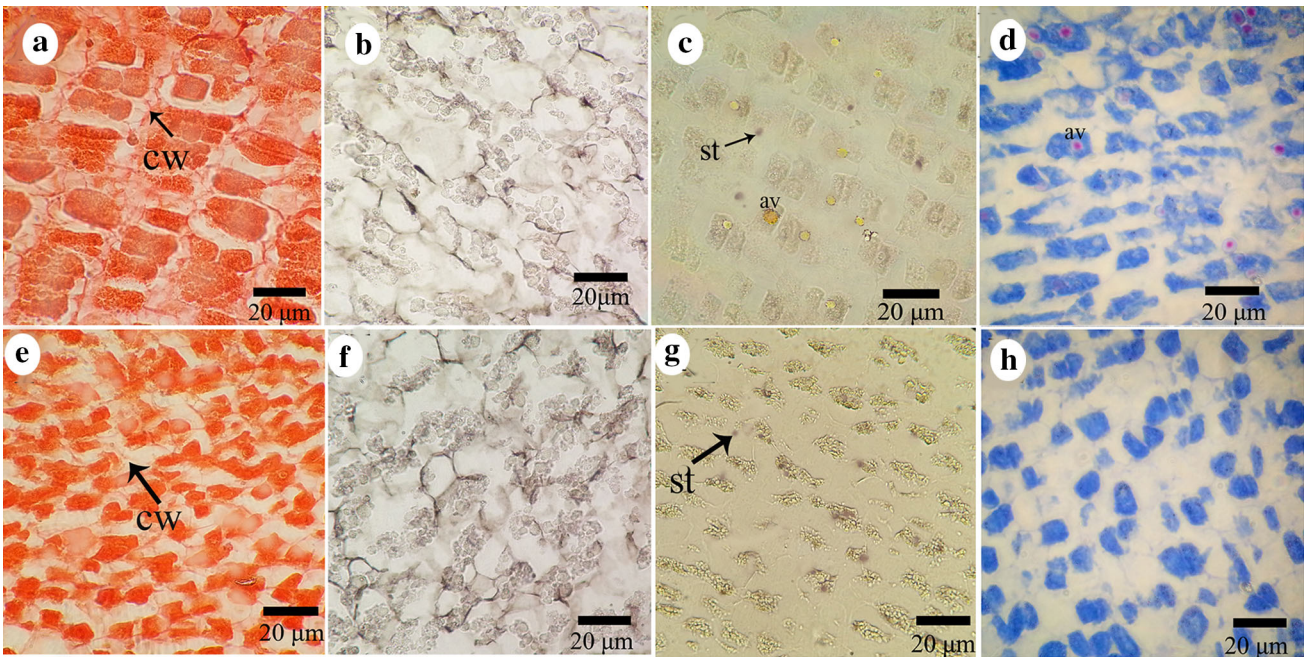

Fig. 6 Cytochemistry of mature fresh (**a**–**d**) and desiccated (**e**–**h**) root tip ground meristem cells of pistachio seeds. **a**, **e** Cellulosic cell walls stained by *Congo red*. **b**, **f** Lipidic storage stained by *Sudan black* B. **c**, **g** AVs look *light brown* and starch grains look *purple* by Lugol. **d**, **h** AVs look *pink* and protein storages look *blue* by PAS + Cb double staining. *cw* cell wall, *st* starch grain, *av* autophagic vacuole

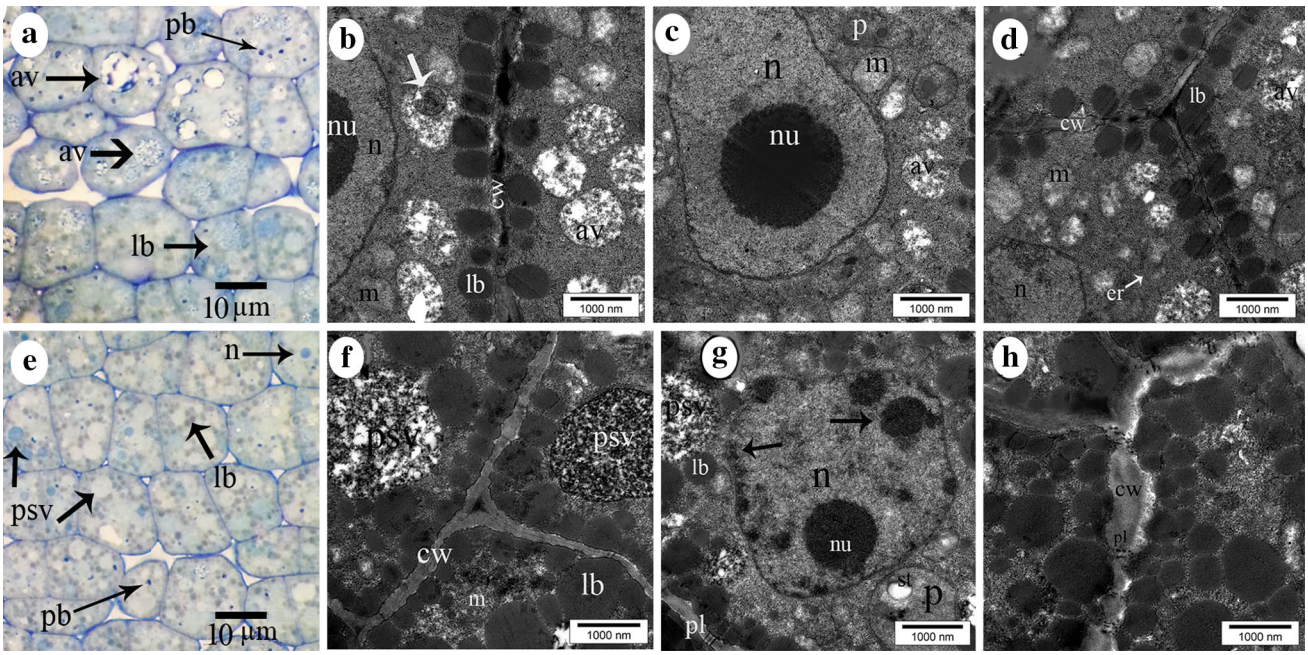

Fig. 7 Ultrastructure of mature fresh (**a**–**d**) and desiccated (**e**–**h**) root tip ground meristem cells of pistachio seeds. **a**, **e** Toluidine *blue* metachromasia, lipid bodies look *grey*, fresh cells contain AVs with some of them differentiating to PSVs (*thick arrow*), desiccated cells contain fully differentiated PSVs, small PBs and a nucleus with apparent nucleolus are visible. **b** TEM micrograph showing AVs, one contains an autophagic body containing cellular material (*white arrow*), LBs tier adjacent to plasma membrane. **c** Large euchromatic nucleus with apparent nucleolus and different organelles are visible. **d** Varied abundance of LBs adjacent to different sides of a cell. **f** PSVs with amorphous deposits and an acristate inactive mitochondrion are visible, LBs surround the PSVs, cell wall waving is visible. **g** A nucleus with heterochromatic parts (*arrows*), an amyloplast with starch grain is shown. **h** Intense cell wall waving in desiccated state. *cw* cell wall, *st* starch grain, *pb* protein body, *psv* protein storage vacuole, *av* autophagic vacuole, *lb* lipid body, *m* mitochondrion, *p* plastid, *er* endoplasmic reticulum, *pl* plasmodesma, *n* nucleus, *nu* nucleolus

membrane (Fig. 7b, d, f, h) with varied abundance along different sides of cells (Fig. 7d). Amyloplasts with starch grains look purple by Lugol (Fig. 6c, g) and are evident in TEM micrographs (Fig. 7g). Protein reserves look blue in PAS + Cb double-staining (Fig. 6d, h) and have two types, small PBs and large PSVs with amorphous deposition (Fig. 7a, e, f). AVs with different sizes are evident in fresh state appearing light brown by Lugol (Fig. 6c) and pink in PAS + Cb double-staining (Fig. 6d) with material deposition on the inner surface (Fig. 7a). Autophagic bodies, engulfing cytoplasmic components can be seen inside AVs (Fig. 7b white arrow). These vacuoles are differentiated to PSVs in desiccated state (Fig. 7e, f). Cytoplasmic components such as cristate mitochondria, plastids and ER are present in fresh state (Fig. 7b–d). Mitochondria with an inactive acristate appearance can also be seen in desiccated state (Fig. 7f). The nucleus is large and euchromatic in fresh but heterochromatic in desiccated states with an evident nucleolus (Fig. 7c, g).

Morphology, cytochemistry and ultrastructure of pollen

Pistachio pollen is porate, spheroidal and 28–30 μm in diameter. The exine includes tectum, columella and foot layer (Fig. 9a) and gets thin in the pore sites (Fig. 9b). Pollenkitt covers the exine and becomes blue in PAS + Cb double-staining and black by Sudan black B (Figs. 8c, d, 9a). The intine looks light red by Congo red (Fig. 8a) and pink in double-staining (Fig. 8c). It consists of a thin outer intine (exintine), with tubular electron dense inclusions, which thickens in the pore sites and a more homogenous inner intine (endintine) (Fig. 9a, b). VC cytoplasm possesses different organelles such as mitochondria and numerous amyloplasts. Amyloplasts become purple by Lugol (Fig. 8b). A large uniformly electron dense nucleus is visible (Figs. 8e, 9c). The GC is located close to VC nucleus and looks light purple to pink in metachromasia (Fig. 8e) with a large nucleus and apparent nucleolus (Fig. 9d). In some desiccated pollen, the inner intine is degraded and empty cavities can be seen all through the intine. VC plasmolysis and separation from intine is evident (Figs. 8f–h, 9e, f). VC and GC cytoplasms are highly vacuolated (Fig. 9g, h), VC nucleus is lobed and surrounded by vacuoles (Fig. 9g). Totally, 1.8% of desiccated pollen grains were structurally damaged in metachromasia.

Discussion

Mature pistachio seeds lost 90.5% of their MC after 24 h of desiccation, but no influence was seen on their 100% germinability, therefore, indicating a desiccation tolerant nature. Pistachio is a resistant species to dry climates (del Carmen et al. 2011) and is expected to have desiccation tolerant seeds. In addition to empirical observations, the viability maintenance of pistachio seeds after desiccation has been reported in the literature (Ozden-Tokatli et al. 2007).

Acquisition of seed desiccation tolerance commences before the onset of drying and is strongly increased along the stage of desiccation; therefore, transition from late 'reserve accumulation' phase to the completely desiccated state is associated with several metabolic and structural changes (Angelovici et al. 2010). Pistachio seed cells showed structural characteristics of this process such as autophagy of organelles related to metabolic activity (Fig. 5b), because metabolism dimming is a main tolerance requirement (Caccere et al. 2013). Macroautophagy in plants is performed by double-membrane autophagosomes that are autolysosomes as well (van Doorn and Papini 2013). Autophagosomes engulf cytoplasmic material destined for recycling and when reach an AV, their outer membrane fuses with the tonoplast to release the inner membrane—autophagic body—and the cargo it harbors to the AV lumen (Michaeli et al. 2015). This type of autophagy occurs during the late stages of seed development. Here, AVs reacted with both lugol and PAS that is attributed to phenolic glycosides (Geier 1980). In TEM micrographs, these vacuoles showed material deposition on the inner surface of tonoplast, which may be the result of fixation process (Herman and Larkins 1999). Pistachio seeds are a rich source of phenolic compounds (Tomaino et al. 2010) and glutaraldehyde fixation causes phenolic compounds to organize into electron-dense droplets (Martini et al. 2008). It has been demonstrated in pea cotyledons that AVs are succeeded by PSVs in a clear developmental sequence. For a short time, both types of vacuoles co-exist in the same cell (Hoh et al. 1995) as it can be seen in Figs. 5a and 7a. Similar observations were made in Arabidopsis and *Medicago truncatula* embryos (Frigerio et al. 2008). After complete conversion and division of AVs to PSVs, they only react with protein staining reagents (Figs. 4h, 6h).

Maturing plant seeds synthesize massive amounts of storage proteins within the ER and transport them to PSVs, where they are packed in highly condensed forms (Vitale and Hinz 2005). These proteins naturally possess an aggregative nature allowing efficient seed desiccation (Galili 2004). Budding of storage proteins from ER forms PBs whose internalization into vacuoles occurs by a non-lytic autophagosome type and much later, by the time of germination, the storage proteins in PSVs become degraded (van Doorn and Papini 2013). Figure 5c shows a probable PB trapped into an autophagic body inside an AV. PSVs may show an amorphous deposition of proteins—

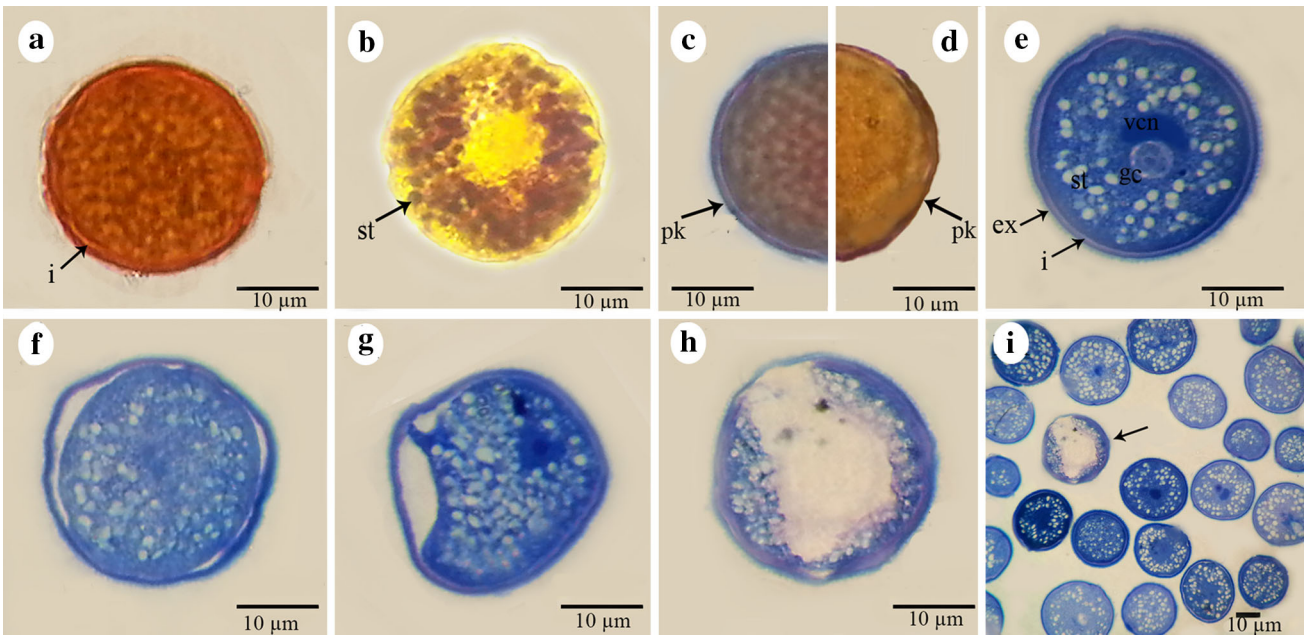

Fig. 8 Cytochemistry and structure of mature fresh (**a**–**e**) and desiccated (**f**–**i**) pistachio pollen. **a** Cellulosic intine looks *light red* by *Congo red*. **b** Amyloplasts with starch grains look *purple* by Lugol. **c** Pollenkitt proteins look *blue* by PAS + Cb double staining. **d** Pollenkitt lipids look *black* by *Sudan black* B. **e** Toluidine *blue* metachromasia of pollen, exine, intine, VC nucleus, GC and starch grains are visible. **f**, **g** Pollen wall separation from VC. **h** VC rupture. **i** The majority of pollen grains seem still intact after 24 h of desiccation, and among them, one is degrading (*arrow*). *ex* exine, *pk* pollenkitt, *i* intine, *st* starch, *gc* generative cell, *vcn* vegetative cell nucleus

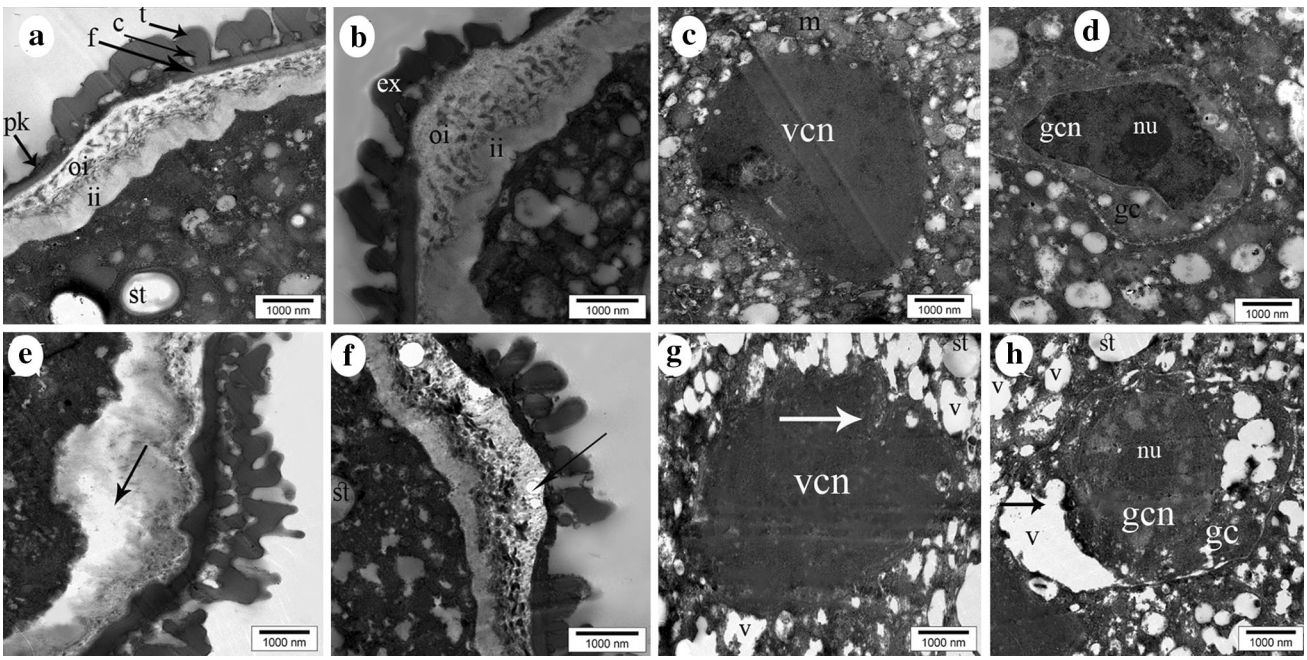

Fig. 9 Ultrastructure of mature fresh (**a**–**d**) and desiccated (**e**–**h**) pistachio pollen. **a** TEM micrograph of pollen wall, exine includes tectum, columella and footlayer, an electron-dense pollenkitt covers the footlayer, a thin outer intine (exintine) with tubular electron dense inclusions and a more homogenous inner intine (endintine) are shown. **b** Exine gets thin and outer intine thickens in the pore sites. **c** An intact VC nucleus surrounded by different cellular organelles. **d** A GC with large nucleus and nucleolus. **e** VC plasmolysis and intine degradation and separation from VC (*arrow*). **f** Empty cavities within intine (*arrow*). **g** VC nucleus is lobed (*white arrow*), VC cytoplasm is highly vacuolated. **h** GC is highly vacuolated, vesiculation from vacuole is evident (*arrow*). *ex* exine, *t* tectum, *c* columella, *f* footlayer, *pk* pollenkitt, *ii* inner intine, *oi* outer intine, *st* starch, *gc* generative cell, *gcn* generative cell nucleus, *vcn* vegetative cell nucleus, *nu* nucleolus, *v* vacuole

difficult to be distinguished from pre-existing vacuoles—or may be completely filled by a uniform protein matrix. White inclusions—globoids—may also be observed within the matrix (Fig. 5f, h, i). Globoids are membrane-bound compartments containing crystals of phytic acid and lytic agents that provide a ready source of digestive enzymes to initiate degradative processes early in germination (Jiang et al. 2001).

LBs were present at both fresh and desiccated states filling a high portion of cell volume in cotyledons but a lower portion in ground meristem of root tips compared to PSVs (Figs. 4b, f, 6b, f). They are ER-originated single phospholipid layered organelles enclosing triacylglycerides which serve as energy store for germination in mature seeds and also function in cell–cell signaling with their specific proteins (Van der Schoot et al. 2011). Their role in signaling could be the reason why they tier adjacent to plasma membrane as secretory organelles in ground meristem cells. They had varied abundance along different sides of the cells, probably indicating strong cell polarity in meristematic tissues (Fig. 7d). Amyloplasts exist but not as much as LBs and PSVs similar to some other species (Maia et al. 2016). According to Walters (2015), accumulation of storage matter is an essential component of orthodox seed development and provides protective benefits because it solidifies the fluid cytoplasm and regulates the cell shrinkage. The metabolic capacity of cell also reduces since less fluid volume is available. Here, not all the organelles were degenerated in the ground meristem cells but, some remained with an inactive appearance (Fig. 7f) because these cells require an active metabolism upon rehydration. Similar observation is reported in bean (*P. vulgaris*) root tips (Farrant et al. 1997).

The nucleus in cotyledon cells was diminished at both states. van Zanten et al. (2011) reported a significant decrease in the nuclei size of Arabidopsis cotyledons during seed maturation, accompanied by increased chromatin condensation and showed that the main reduction in nuclear size was established before major dehydration. In *P. vulgaris* seeds, studies showed that nuclei reversibly shrink toward the dormant phase (Kater 1927). Furthermore, desiccation of leaves of resurrection plant *Craterostigma plantagineum* led to a significant decrease in nuclear size, suggesting that this event is not restricted to seeds, but could represent a universal mechanism in acquisition of desiccation tolerance (van Zanten et al. 2011).

In ground meristem cells of root tip, however, the nucleus was so large at both states—as a meristematic cell indicator conferring rapid cell division after imbibition—but became mostly heterochromatic in desiccated state (Fig. 7g). van Zanten et al. (2011) showed that reduction in nuclear size and increased chromatin compaction in Arabidopsis seeds are independent processes of the seed developmental program. Here, in fresh ground meristem cells the only heterochromatic part was the nucleolus but it was much more inclusive in desiccated state. Accordingly, in Arabidopsis cotyledons, the rDNA sequences—constituting the nucleolus—were always condensed during seed maturation and after imbibition, whereas the other condensed domains were seen at low-MC states (van Zanten et al. 2011). Increased chromatin compaction is associated with decreased transcriptional activity in desiccating seeds through less accessibility of DNA for the transcription machinery (Fransz and de Jong 2011).

It is known that cell wall folding plays a vital role at the final stages of seed development in terms of shape coordination between protoplast and cell wall to maintain cell wall-plasma membrane association, thus preventing possible rupture of plasma membrane and mechanical damage to the tissue (Caccere et al. 2013) as it is reported in *Leucaena leucocephala* seeds (Maia et al. 2016). Nevertheless, in the cells of seeds that accumulate high content of storage matter, the cell size does not significantly change, thus they do not usually require such a strategy. In pistachio seeds, the cell wall is cellulosic and flexible enough to shrink. However, it was rarely folded in completely filled desiccated cotyledonary cells, but obviously waved in ground meristem cells which encompass less storage matter (Figs. 5f, 7f, h).

Insufficient pollination is one of the most important factors affecting the yield of field crops. Storing pistachio pollen for hand pollination purpose is inefficient due to its very short germinability time (Vaknin and Eisikowitch 2000). Here, mature pistachio pollen lost 46.7% of its MC after 24 h of desiccation along with 93% loss in its initial germinability indicating a desiccation sensitive nature. Rapid loss of MC and germinability of pistachio pollen have been reported frequently (Vaknin and Eisikowitch 2000; Acar and Kakani 2010). But at the same time, some research has demonstrated that if the stale non-germinable pistachio pollen is pre-hydrated before in vitro culture, it would display a level of germinability of about 55–60% (Vaknin and Eisikowitch 2000). These results suggest that inefficiency in storing pistachio pollen is related to loss of germinability of viable pollen.

It was shown in our previous work that pistachio pollen has 3–5 pores but no furrows (Hosseini et al. 2015). Lack of furrows is a principal recalcitrance indicant because this elongated aperture is critical for the reversible folding patterns of pollen wall imposed by changes in relative humidity during dispersal preventing the cells from collapsing (Firon et al. 2012). Protection of pollen from excess dehydration is somehow related to thick intine of carbohydrates (Franchi et al. 2011) and pollenkitt—a mixture of lipids, proteins, carbohydrates, carotenoids and flavonoids

on exine surface—but in wind pollinated plants the pollenkitt is absent or if present, exists in little amounts (Pacini and Hesse 2005). Here, the staining tests and TEM micrographs showed that only a small pollenkitt layer covers the foot layer surface (Figs. 8c, d, 9a) and although the intine is relatively thick, it is not sufficient to confer dehydration stability. Some (1.8%) of 24-h-desiccated pollen showed VC plasmolysis and separation from the intine due to the shape incoordination between pollen wall and VC, and finally the VC rupture. Generally, dehydration stress and decrease in turgor pressure result in plasmolysis of plant cells and, if persist, they can end in cytorrhysis, that is the permanent collapse of cell wall and protoplast (Vollenweider et al. 2015). Here, the intine texture revealed obvious destruction showing pollen wall cytorrhysis. The abundance of ROS produced during dehydration stress alters the membrane integrity and makes the cell wall more rigid (Sage et al. 2015). Nuclear lobulation in these pollen grains is attributed to cytoskeleton reorganization caused by dehydration stress which influences signaling systems that regulate cytoskeleton structure (Komis et al. 2002). In cells, nuclear shape is determined by nucleoskeleton and cytoskeleton organization (Webster et al. 2009). Moreover, both VC and GC showed high abundance of small vacuoles while the organelles were indistinguishable and only scattered remnants of cellular material could be observed (Fig. 9f–h). The vacuoles are structurally related to increased autophagic activity because the tonoplast showed invagination/vesiculation (Fig. 9h) denoting microautophagic processes (Bassham et al. 2006). These processes could contribute to raising the solute concentration and adjusting the osmotic potential. Symptoms such as cytorrhysis, plasmolysis or tonoplast invagination/vesiculation are indicative of disturbed osmotic homeostasis. In earlier stages, cells rearrange their cellular components, specifically through autophagy to slow down the degenerative processes. In later stages, cell content degeneration and disruption take over and lead to cell death (Vollenweider et al. 2015).

Some starch grains seemed intact in degenerated VC cytoplasm (Fig. 9f–h). Similarly, Franchi et al. (2011) observed that the cytoplasm of dead pollen is destroyed, while only large starch grains remain inside. These observations are contrary to the condition found in *A. thaliana* orthodox pollen where starch grains were missing after dispersal (Van Aelst et al. 1993). Orthodox pollen uses carbohydrate interconversion to increase turgor pressure that hinders excess water loss (Firon et al. 2012). The interconversion of carbohydrates seems to be reduced or even absent in recalcitrant pollen such as that of grasses, which have generally low sucrose content (Speranza et al. 1997).

Finally, as it is shown in (Fig. 8i), not all dehydrated pollen grains showed structural damage features and the rate of normal ones was obviously higher. Hence, the recalcitrance behavior of pistachio pollen may be mostly related to lack of germinability rather than structural damages. It appears that desiccation influences the pollen germinability through molecular damages that should be studied in the future.

Conclusion

Pistachio tree is adapted to harsh conditions and has orthodox seeds, unlike recalcitrant pollen. At maturation, the seeds showed ultrastructural characteristics of orthodoxy such as autophagy of organelles related to active metabolism and biogenesis of storage organelles. 1.8% of pollen grains had the signs of dying such as intine degeneration and VC rupture as the main ones, however, a higher rate of pollen grains were intact in appearance producing the consequence that the recalcitrance behavior of pistachio pollen is mostly the result of loss of germinability but not structural damage which is usually considered as the cause of pollen recalcitrance behavior. Investigating the causes of loss of germinability is of interest and will help improve the germinability duration of pollen.

This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

Author contribution statement EZB: planning, conducting experiments and preparing manuscript; PJ: participation in planning; FR: participation in preparing manuscript and providing lab equipment.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Acar I, Kakani VG (2010) The effects of temperature on in vitro pollen germination and pollen tube growth of *Pistacia* spp. Sci Hortic 125:569–572

Angelovici R, Galili G, Fernie AR, Fait A (2010) Seed desiccation: a bridge between maturation and germination. Trends Plant Sci 15(4):211–218

Bassham DC, Laporte M, Marty F, Moriyasu Y, Ohsumi Y, Olsen LJ, Yoshimoto K (2006) Autophagy in development and stress responses of plants. Autophagy 2:2–11

Caccere R, Teixeira S, Centeno D, Figueiredo-Ribeiro R, Braga M (2013) Metabolic and structural changes during early maturation of *Inga vera* seeds are consistent with the lack of a desiccation phase. J Plant Physiol 170:791–800

Chao CT, Parfitt DE (2003) Genetic analysis of phenological traits of pistachio (*Pistacia vera* L.). Euphytica 129:345–349

Cook FS, Walden DB (1965) The male gametophyte of Zea Mays L. II. In vitro germination. Can J Bot 43(7):779–786

del Carmen Gijon M, Gimenez C, Perez-Lopez D, Guerrero J, Couceiro JF, Moriana A (2011) Water relations of pistachio (*Pistacia vera* L.) as affected by phenological stages and water regimes. Sci Hortic 128:415–422

Farrant JM, Pammenter NW, Berjak P, Walters C (1997) Subcellular organization and metabolic activity during the development of seeds that attain different levels of desiccation tolerance. Seed Sci Res 7:135–144

Firon N, Nepi M, Pacini E (2012) Water status and associated processes mark critical stages in pollen development and functioning. Ann Bot 109(7):1201–1214

Franchi GG, Piotto B, Nepi M, Baskin CC, Baskin JM, Pacini E (2011) Pollen and seed desiccation tolerance in relation to degree of developmental arrest, dispersal, and survival. J Exp Bot 62(15):5267–5281

Fransz P, de Jong H (2011) From nucleosome to chromosome: a dynamic organization of genetic information. Plant J 66:4–17

Frigerio L, Hinz G, Robinson DG (2008) Multiple vacuoles in plant cells: rule or exception? Traffic 9(10):1564–1570

Galili G (2004) ER-derived compartments are formed by highly regulated processes and have special functions in plants. Plant Physiol 136(3):3411–3413

Geier T (1980) PAS-positive reaction of phenolic inclusions in plant cell vacuoles. Histochemistry 65:167–171

Golovina EA, Hoekstra FA, Van Aelst AC (2001) The competence to acquire desiccation tolerance is independent of seed morphological development. J Exp Bot 52(358):1015–1027

Herman EM, Larkins BA (1999) Protein storage bodies and vacuoles. Plant Cell 11:601–613

Hoh B, Hinz G, Jeong BK, Robinson DG (1995) Protein storage vacuoles form de novo during pea cotyledon development. J Cell Sci 108:299–310

Hosseini N, Zamani Bahramabadi E, Rezanejad F (2015) Study of morphological and anatomical traits of male flower, developmental stages of anther and pollen grain of pistachio (*Pistacia vera* L.). Iran. J Biol 28(1):116–125

Jiang L, Phillips TE, Hamm CA, Drozdowicz YM, Rea PA, Maeshima M, Rogers SW, Rogers J (2001) The protein storage vacuole: a unique compound organelle. J Cell Biol 155(6):991–1002

Johansen DA (1940) Plant microtechnique. McGraw-Hill, New York

Kalemba EM, Pukacka S (2012) Association of protective proteins with dehydration and desiccation of orthodox and recalcitrant category seeds of three *Acer* genus species. J Plant Growth Regul 31(3):351–362

Kater JM (1927) A cytological study of dormancy in the seed of *Phaseolus vulgaris*. Ann Bot (Lond) 41:629–641

Kersten B, Pakull B, Fladung M (2017) Genomics of sex determination in dioecious trees and woody plants. Trees Struct Funct. doi:10.1007/s00468-017-1525-7

Kioko JI, Berjak P, Pammenter NW (2006) Viability and ultrastructural responses of seeds and embryonic axes of *Trichilia emetic* to different dehydration and storage conditions. S Afr J Bot 72:167–176

Komis G, Apostolakos P, Galatis B (2002) Hyperosmotic stress-induced actin filament reorganization in leaf cells of *Chlorophyton comosum*. J Exp Bot 53(375):1699–1710

Maia J, Guimaraes CC, Da Silvia EAA, Faria JMR (2016) What can cell cycle and ultrastructure tell us about desiccation tolerance in *Leucaena leucocephala* germinating seeds? Biol Plant 60(2):319–328

Martini MH, Figueira A, Lenci CG, de Queiroz Tavares D (2008) Polyphenolic cells and their interrelation with cotyledon cells in seven species of *Theobroma* (Sterculiaceae). Revista Brasil Bot 31(3):425–431

Michaeli S, Galili G, Genschik P, Fernie AR, Avin-Wittenberg T (2015) Autophagy in plants—what's new on the menu? Trends Plant Sci. doi:10.1016/j.tplants.2015.10.008

O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373

Ozden-Tokatli Y, Ozudogru EA, Gumusel F, Lambardi M (2007) Cryopreservation of *Pistacia* spp. Seeds by dehydration and one-step freezing. Cryoletters 28(2):83–94

Pacini E, Hesse M (2005) Pollenkitt—its composition, forms and functions. Flora 200:399–415

Radwan A, Hara M, Kleinwächter M, Selmar D (2014) Dehydrin expression in seeds and maturation drying: a paradigm change. Plant Biol 16:853–855

Sage TL, Bagha S, Lundsgaard-Nielsen V, Branch HA (2015) The effect of high temperature stress on male and female reproduction in plants. Field Crops Res 182:30–42

Sazci A, Radford A, Erenler K (1986) Detection of cellulolytic funji by using Congo red as an indicator: a comparative study with the dinitrosalicyclic acid reagent method. J Appl Bacteriol 61:559–562

Schmidt EC, Pereira B, Pontes CLM, Santos R, Scherner F, Horta PA, Paula MR, Latini A, Maraschin M, Bouzon ZL (2012) Alterations in architecture and metabolism induced by ultraviolet radiation-B in the carragenophyte Chondracanthus teedei (Rhodophyta, Gigartinales). Protoplasma 249:353–367

Shekari M, Rezanejad F (2012) Comparison of pigments content, split rate and kernel dry weight of two pistachio cultivars (*Pistacia Vera* L.) during fruit development. Iran J Biol 25(2):295–302

Speranza A, Calzoni GL, Pacini E (1997) Occurrence of mono- or disaccharides and polysaccharide reserves in mature pollen grains. Sex Plant Reprod 10:110–115

Tomaino A, Martorana M, Arcoraci T, Monteleone D, Giovinazzo C, Saija A (2010) Antioxidant activity and phenolic profile of pistachio (*Pistacia vera* L., variety Bronte) seeds and skins. Biochimie 92:1115–1122

Vaknin Y, Eisikowitch D (2000) Effects of short-term storage on germinability of pistachio pollen. Plant Breed 119:347–350

Van Aelst AC, Pierson ES, Van Went JL, Cresti M (1993) Ultrastructural changes of *Arabidopsia thaliana* pollen during final maturation and rehydration. Zygote 1(02):173–179

Van der Schoot C, Paul LK, Paul SB, Rinne PLH (2011) Plant lipid bodies and cell-cell signaling. A new role for an old organelle? Plant Signal Behav 6(11):1732–1738

van Doorn WG, Papini A (2013) Ultrastructure of autophagy in plant cells. Autophagy 9(12):1922–1936

Van Zanten M, Koini MA, Geyer R, Liu Y, Brambilla V, Bartels D, Koornneef M, Fransz P, Soppe WJJ (2011) Seed maturation in *Arabidopsis thaliana* is characterized by nuclear size reduction and increased chromatin condensation. PNAS 108(50):20219–20224

Vitale A, Hinz G (2005) Sorting of proteins to storage vacuoles: how many mechanisms? Trends Plant Sci 10:316–323

Vollenweider P, Menard T, Arend M, Kuster TM, Gunthardt-Goerg MS (2015) Structural changes associated with drought stress symptoms in foliage of Central European oaks. Trees Struct Funct. doi:10.1007/s00468-015-1329-6

Walters C (2015) Orthodoxy, recalcitrance and in-between: describing variation in seed storage characteristics using threshold responses to water loss. Planta 242:397–406

Webster M, Witkin KL, Cohen-Fix O (2009) Sizing up the nucleus: nuclear shape, size and nuclear-envelope assembly. J Cell Sci 122:1477–1486

Yakubov B, Barazani O, Shachach A, Rowland LJ, Shoseyov O, Golan-Goldhirsh A (2005) Cloning and expression of a dehydrin-like protein from *Pistacia vera* L. Trees Struct Funct 19:224–230