Role of mucilage during achene germination and sprout growth of the endangered Tibetan medicinal herb *Mirabilis himalaica* (Nyctaginaceae) exposed to abiotic stresses

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Abstract

Aim

Mirabilis himalaica (Nyctaginaceae) is an endangered medicinal plant mainly distributed in the plateau region of northern Tibet, China. The outer surface of *M. himalaica* achenes is covered by a pectinaceous mucilaginous layer upon hydration. However, the role of the achene mucilage is poorly understood. In this study, we investigated the effects of mucilage on achene germination and sprout growth under abiotic stress to explain how *M. himalaica* survive the alpine environment.

Methods

We investigated the effect of mucilage on achenes germination by contrast the capacity of water absorption, dehydration and respiration of intact achene and the achene with mucilage removal. We performed abiotic stresses experiments including drought stress, salt stress, cold stress and high temperature stress, and quantified the effects of mucilage removal on achene germination rate, root and shoot lengths of seedlings.

Important Findings

Mucilage is extremely hydrophilic, and the mass of intact achenes can be 9-fold greater than that of demucilaged achenes. The removal of the mucilaginous layer did not significantly change final germination percentages under ideal conditions, but intact achenes (*i.e.*, with mucilage) took longer to germinate. The mucilage significantly decreased seed respiration rates by acting as a physical barrier that prevented oxygen diffusion. Germination rates, shoot and root growth of intact achenes were higher than those of demucilaged ones during exposures to cold, heat, osmotic, and salt stresses. Achene mucilage presumably plays an ecologically important role in the life cycle of *M. himalaica* by aiding the critical achene germination and early seedling growth in the stressful habitats of the plateau region of northern Tibet.

Keywords

Mirabilis himalaica, achenes, mucilage, germination, abiotic stresses

Introduction

Myxodiaspory refers to the release of hydrophilic mucilage from the seed coat or pericarp upon hydration. Over the past 150 years, myxodiaspory has been a common adaptation in at least 110 angiosperm families (Yang, 2012c). Mucilage is primarily composed of pectins and hemicelluloses, which are produced by the Golgi apparatus in developing epidermal cells of fruits or seeds of some species (Gorai et al. 2014; Western et al. 2000). The polysaccharides and acidic qualities of mucilages make them extremely hydrophilic (Deng et al. 2013; Kreitschitz et al. 2015; Paynel et al. 2013; Warrand et al. 2005 ; Thapliyal et al. 2008).

The structure, components, ecological roles, and production mechanism of mucilage have been well studied in the model plant *Arabidopsis thaliana* (Francoz et al. 2015; Western 2012; Haughn and Western 2012). Studies involving *A. thaliana* and other species have concluded that seed coat mucilages may have multiple roles, including: (1) ensure seeds mature sufficiently during fruit development to enable germination (Yang et al. 2010), (2) increase moisture levels, especially when seeds are exposed to drought or salt stresses (Western 2012), (3) serve as a storage of water that initiates or enhances germination and/or affects early seedling development (Huang and Gutterman 1999; Western 2012), (4) inhibit germination (*i.e.*, seed dormancy) by preventing the embryo from accessing oxygen, (5) improve germination by promoting embryo DNA maintenance and repair (*i.e.*, seed priming) (Huang et al. 2008; Yang et al. 2011), (6) enhance seed dispersal through epizoochory and/or endozoochory (Yang et al. 2012c), and (7) enrich the soil microbial community involved in biodegradative activities to promote early seedling growth (Yang et al. 2012b). Additionally, mucilage benefits seed germination and seedling establishment in extreme desert conditions during critical stages of plant growth and development (Yang, 2012c), which is ecologically significant (Gu et al. 2008; Huang et al. 2015; Sun et al. 2012; Yang et al. 2012a; Yang et al. 2012b; Yang et al. 2012c; Zhang et al. 2014). However, the precise roles of mucilages appear to be species dependent and environmental context (Western 2012；Bhatt et al., 2016).

Mirabilis himalaica (Nyctaginaceae) is mainly distributed in the arid-monsoon plateau region of northern Tibet, China. It is a perennial herb that occurs in the dry and hot valley regions 700–3000 m above sea level. Due to its wide distribution, the habitat of *Mirabilis himalaica* can be very heterogeneous. Frigid temperatures, dramatic daily temperature changes, and high-intensity solar radiation are common in alpine environments (Dai et al. 2015; Xu et al. 2014). Additionally, low temperatures, drought conditions and salinity are the dominant environmental stresses often experienced by *M. himalaica* plants during achene germination. *Mirabilis himalaica* roots have been used in Tibetan folk medicine for the treatment of many medical conditions, including edema, nephritis, renal calculi, arthrodynia, and uterine cancer (Lang et al. 2014). However, seedlings establishment is poor because

achene germination is particularly vulnerable to abiotic stress, such as low temperatures and drought conditions, which are the dominant environmental stresses experienced by *M. himalaica* plants during achene germination. Consequently, the population size decreased dramatically and become endangered. So, the availability of wild *M. himalaica* plants continues to decline. In 2009, *M. himalaica* was added to the list of endangered Tibetan medicinal plants. Luckly, one recent research indicated that there was no significant difference in the variability of wild *Mirabilis himalaica* and the cultivated ones from different districts. So, artificial culitivation is an effective way to protect the endangered wild *M. himalaica* (Lin et al., 2016).

The outer layer of the pericarp of *M. himalaica* achenes becomes mucilaginous when moistened. The roles of mucilages during the germination of achenes exposed to environmental stresses have not been characterized. We hypothesized that mucilage production in *M. himalaica* plants is important for germination and seedling establishment in alpine environments. Considering the stressful environmental conditions of *M. himalaica* habitats, we asked the following questions: (1) can mucilage aid the germination of *M. himalaica* achenes under salinity, osmotic, low and high temperature stresses? and (2) can mucilage promote early seedling growth under the former abiotic stress? A better understanding of germination requirements could inform *M. himalaica* conservation and rehabilitation programs. Our findings would also provide new evidences for understanding the ecological roles of seed mucilage .

Materials and methods

Plant materials

Fresh and mature *M. himalaica* achenes were collected from wild plants growing in Nyingchi prefecture of the Tibet Autonomous Region in southwestern China (29°35´N, 94°54´E; 3000 m a.s.l.). The *M. himalaica* habitat was dry and hot, with sandy soil and a cold arid-alpine climate. The mean annual precipitation (*i.e.*, snow and rainfall) was 350 mm, which was mainly received between June and September. The mean annual temperature was 8.7 °C. In order to reduce the errors, we picked out the black dry achenes with accordant maturity and plumpness (100 grain weight is 1.8 ± 0.10 g). Achenes were stored dry in a refrigerator in a closed cotton bag at 4 °C.

Achene morphology

The achene average weight was determined for four replicates of 1,000 achenes each. The length and width of 50 randomly chosen achenes from each replicate were measured to estimate achene size. Dried achenes were fully imbibed with 0.01% (w/v) ruthenium red solution (*i.e.*, a dye that stains acidic polysaccharides) and then double-distilled water (Western et al. 2000). Additionally, pericarps were removed from other randomly selected dried achenes. The pericarps and naked seeds were prehydrated in double-distilled water for 40 min, and then treated with 0.01% (w/v) ruthenium red solution. After samples became fully hydrated, they were mounted in water and analyzed.

Mucilage removal

To remove mucilage, dried achenes were immersed in water for 20–30 min. The hydrogel on the achene surface was carefully and rapidly rubbed with gauze several times

until the achenes stopped releasing hydrated mucilage upon exposure to water (*i.e.*, demucilaged achenes) (Yang et al. 2010).

Effects of mucilage on achene water absorption and dehydration

The water-absorbing properties of the mucilage were analyzed by measuring water absorption and dehydration rates for four replicates of 1 g samples of intact and demucilaged achenes. The achenes were immersed in sterile distilled water in Petri dishes. During imbibitions, the achenes were weighed quickly every 10 min for the first hour, and then every 30 min until the weight stabilized. Before weighing samples, filter paper was used to quickly and gently absorb water from the surface of achenes. After weighing, the achenes were quickly returned to the water. Samples were then dried at room temperature (23–25 °C, relative humidity of 11%-13%) and weighed every 2 h until seed weights stabilized.

Optimal germination temperature of demucilaged and intact achenes

The demucilaged and intact achene were surface sterilized in 75% ethanol for 2 min, washed with sterile deionized water three times, and air-dried before being used. Germination percentage for the demucilaged and intact achenes were determined for four replicates of 20 sterilized achenes. Samples were sown on two layers of filter paper (Whatman No. 1) in Petri dishes (9-cm diameter) containing 8 ml sterile distilled water. The dishes were sealed with laboratory film and incubated at 5, 10, 15, 20, 25, and 30 °C in darkness. Germination (*i.e.*, radicle emergence) was monitored every 24 h until no new radicles were observed for 3 consecutive days. Seedling and root lengths were measured after 15 days.

Effect of mucilage on achene respiration

Intact and demucilaged achenes were placed in separate Petri dishes (5-cm diameter) containing 2.0 ml sterile distilled water. Samples were incubated at 15 °C for 24, 48, and 72 h in darkness. The respiration rates of achenes were determined using a Yaxin-1151 biological oxygen monitor (Beijing, China) to measure oxygen consumption. Three replicates were analyzed.The respiratory rate of achenes（mmol O_2.g^{-1}DW.s^{-1}) was calculated by the following

formula: a×v/(M×DW×n), where *a* is dissipative oxygen concertration in each liter water per second ($mg.L^{-1}.s^{-1}$), *M* is the molecular weight of oxygen (32 $g.mol^{-1}$), *DW* is the dry weight of achenes (g), *v* is the volume of reactor (10 ml), *n* is the number of achenes (2 achenes).

Effect of salinity on germination of demucilaged and intact achenes

Intact and demucilaged achenes were incubated at 15 ℃ in darkness in sterile distilled water or 5, 10, 50, 100, and 200 mM NaCl solutions. The achenes were placed on two layers of filter paper discs saturated with sterile water in Petri dishes (9-cm diameter). Four replicates were used for each treatment, with 20 achenes and 8 ml solution per replicate. Laboratory film was used to seal the Petri dishes. Distilled water equal to the mean loss of water from dishes was added to each Petri plate every 2 days to maintain NaCl concentration near the target level throughout the germination period. Germination was recorded as protrusion of the radicle (1 mm) and monitored every 24 h until no new radicles were observed for 3 consecutive days. Germination was calculated after incubation for 9 days by the following formula: a/b ×100, where a is the number of achenes that germinated and b is the total number of of achenes. Lengths of seedlings from both intact and demucilaged achenes that had been growing for 15 d were measured to the nearest millimeter with a ruler (Yang et al., 2010).

Effect of osmotic potential on germination of demucilaged and intact achenes

Polyethylene glycol 6000 was used to simulate osmotic stress during the germination of intact and demucilaged achenes. Different concentrations of polyethylene glycol 6000 [i.e., 0, 2.5, 5, 10, and 20% (w/v)] were used to generate specific osmotic potentials (i.e., 0.0, −0.003, −0.027, −0.155, and −0.87 MPa, respectively). Twenty achenes were placed on two layers of filter paper discs in Petri dishes (9-cm diameter) containing 8 ml solutions. The Petri dishes were sealed with laboratory film and incubated at 15 ℃ in darkness. PEG solutions were renewed every 48 h under sterile conditions to ensure relatively constant osmotic potentials at each treatment. Germination rates and shoot and seedling lengths were determined.

Effect of freezing stress on germination of demucilaged and intact achenes

Four replicates of 20 demucilaged and intact achenes were placed on two layers of filter paper discs saturated with 8.0 ml sterile distilled water in Petri dishes (9-cm diameter). The dishes were sealed with laboratory film and incubated at 15 °C in darkness for 3 days. The Petri dishes were then transferred to an freezing temperature-controlled chamber (WD4005, Chongqing Yin He, China), the temperature of the chamber was lowered at a rate of 1.5 °C /h to −5, −10, −15, and −20 °C, and samples were then incubated in darkness at the freezing temperatures for 1 h. Then the Petri dishes were sealed with laboratory film and incubated at 15 °C in darkness. Germination rates were determined.

Effect of high temperature stress on germination of demucilaged and intact achenes

We placed four replicates of 20 demucilaged and intact achenes on two layers of filter paper discs saturated with 8 ml sterile distilled water in Petri dishes (9-cm diameter). Samples were incubated at 15 °C in darkness for 3 days. The achenes were then incubated in water baths at 40 or 50 °C for 2h, after that the Petri dishes were sealed with laboratory film and incubated at 15 °C in darkness. Germination rates were determined.

Data analysis

All data were expressed as mean ± s.e. Germination data were arcsine-transformed before being subjected to variance analysis and multiple comparisons to ensure homogeneity of variance. Tukey test (Honestly significant differences, HSD) was used for multiple comparisons to estimate significant ($P < 0.05$) differences between individual treatments. Means were compared at $P = 0.01$ and 0.05. Germination percentages were analyzed using SPSS (version 19.0) (IBM Corp., Armonk, NY, USA). All charts were plotted using Microsoft Excel.

Results

Morphological characteristics of achenes

The mature achenes were dark brown and oval (Fig. 1a, e), and length and width 0.18 ± 0.01 cm and 0.11 ± 0.01 cm, respectively. The mass of 1000 achenes was 18.12 ± 1.44 g.

After achenes were hydrated, a thick semi-transparent, gel-like mucilaginous coat was produced and surrounded the achene pericarp (Fig. 1b, f). Following ruthenium red staining, the mucilage appeared as a thick red layer on the exterior of achenes (Fig. 1c, g). These results indicated the hydrophilic mucilage released following hydration was produced by the pericarp, and not the seed coat.

Effects of mucilage on achene water absorption and dehydration

Water absorption by intact or demucilaged achenes was rapid during the first 10 min, and achenes were fully imbibed after 150 and 30 min, respectively (Fig. 2a). Intact achenes had a high ability to absorb water, and their mass (9.26 ± 0.21 g) had increased more than 9.3-time after 150 min. Whereas the hydrated demucilaged achenes (2.42 ± 0.01 g) experienced only 2.4-fold weight increase. The mass of intact achenes increased faster than that of demucilaged achenes (Fig.2a). The fully hydrated demucilaged achenes lost water and reached their original mass after 20 h, whereas the fully hydrated intact achenes lost water and did not reach their original mass until after 56 h (Fig.2 b).

Respiration of demucilaged and intact achenes

The respiratory rates of demucilaged achenes was higher than that of the intact achenes when incubated at 15 °C for 24, 48, and 72 h (Fig. 3). The difference between the intact and demucilaged achenes was especially noticeable after 48 h, at which time the respiration rate of demucilaged achenes was 2.33- fold that of intact achenes.

Germination and sprout growth of demucilaged and intact achenes under different temperatures

Intact and demucilaged achenes germinated at 5–30 °C, with an ideal temperature of 15 °C. The germination percentage of intact achenes was higher than that of demucilaged achenes at 5 and 10 °C ($P < 0.01$). However, the effect of mucilage removal was not observed at 15 °C and above (Fig.4a). Shoot and root lengths after a 15-day incubation also indicated 15 °C was the most suitable temperature for the growth of *M. himalaica* sprouts. Although incubation at 5, 10, or 30 °C resulted in the production of roots in germinated achenes, no shoots appeared. The removal of mucilage significantly inhibited shoot and root growth at 15 °C (Fig. 5a).

Germination percentage for intact and demucilaged *M. himalaica* achenes decreased with decreasing temperature (Fig. 4b). When exposed to -5 and −10 °C for 1 h, the germination percentage of intact achenes was significantly higher than that of demucilaged achenes ($P < 0.05$) (Fig. 4b). Furthermore, 8.3% intact achenes germinated after incubation at −10 °C for 1 h, whereas no demucilaged achenes germinated after −10 °C treatment for 1h (Fig. 4b). Both intact and demucilaged achenes did not germinate after −15 or −20 °C treatment.

Root and shoot lengths decreased with decreasing temperature. The roots and shoots of intact achenes were longer than those of demucilaged achenes at −5 °C ($P < 0.01$). The protection provided by mucilage resulted in a 79.3% increase in root length at −5 °C. Root and shoot growth was significantly inhibited at −10 °C (Fig. 5b), and roots and shoots didn't grow at −15 and -20 °C.

Results indicated that the germination percentage of intact achenes was higher ($P < 0.05$) than that of demucilaged achenes in the 40 °C treated group. In contrast, no difference was observed in the 50 °C treated group (Fig. 4c). Furthermore, root and shoot lengths of intact and demucilaged achenes significantly decreased at 40 °C and 50 °C, no difference was observed between intact and demucilaged achenes. Although both intact and demucilaged achenes germinated at 50 °C, shoots were lacking in demucilaged achenes (Fig. 5c).

Effect of osmotic potential on germination and sprout growth of demucilaged and intact achenes

After a 15-day incubation, germination percentages of demucilaged and intact achenes decreased with increasing osmotic potentials. Germination percentages were not significantly affected by the removal of mucilage at osmotic potentials above −0.155 MPa. In contrast, at −0.87 MPa, the germination percentage of intact achenes was 9-fold of the demucilaged achenes (Fig.6a).

When achenes were exposed to osmotic potentials of −0.155 MPa, root lengths of intact achenes were higher than those of demucilaged achenes ($P < 0.05$) (Fig. 6b).

Effect of salinity stress on germination and sprout growth of demucilaged and intact achenes

Achenes germination percentage decreased with increasing NaCl concentrations. Intact achenes germination was higher than that of demucilaged achenes in 50 mM NaCl ($P < 0.01$) (Fig. 7a). In 200 mM NaCl, some intact achenes were able to germinate (*i.e.*, 3.3%), while none of the demucilaged achenes germinated (Fig. 7a). Root and shoots lengths of germinated achenes were affected by NaCl, and the removal of mucilage also influenced the effects of

salinity stress on achenes in 10 and 50 mM) ($P < 0.05$). In 10 mM NaCl, the roots and shoots from intact achenes were longer than those from demucilaged achenes. The decrease in shoot length was greatest in 50 mM NaCl, and the shoots from intact achenes were 1.45-fold of that from the demucilaged achenes (Fig. 7b).

Discussion

Previous studies regarding myxospermous seeds of desert plants (Saez-Aguayo et al. 2014; Zaady et al. 1997) focused on structures and components, chemical and physical properties of seed mucilage, as well as ecological benefits under various stress conditions (Huang et al. 2000). In natural habitats, *M. himalaica* is subjected to inclement weather and multiple stresses, including cold, drought, and salinity, which inhibit seed germination. In the present study, intact *M. himalaica* achenes exhibited higher germination and sprout growth than demucilaged achenes under abiotic stress.

Mature intact achenes were able to produce mucilage in 10–30 min, resulting in a 9-fold weight increase after imbibition (Fig. 2a). Our findings suggest the mucilage layer surrounding *M. himalaica* achenes improves water absorption and preserves water in achenes for a relatively long time (Fig. 2), which is similar to some desert plants (Gutterman 1996, 1997, 1967; Yang et al. 2013). According to Huang and Gutterman (2000), the mucilage absorbs water, is degraded by soil microbes for nutrients, and dilutes soil salt concentrations, thereby creating suitable conditions for achene germination (Yang et al. 2010; Yang et al. 2012b). In *M. himalaica*, the effects of mucilage on water retention can contribute to enhanced germination and seedling survival in the environments of the Tibetan plateau regions.

Previous studies reported that mucilage inhibited *Henophyton deserti* seed germination under unfavorable conditions. This was believed to be because the mucilage formed a physical barrier that regulated the diffusion of water and oxygen to the inner tissue and

embryo of the seed (Gorai et al. 2014; Western 2012). We observed that the respiration rates of demucilaged achenes were significantly higher than those of intact achenes during incubations at 15 °C for 24, 48, and 72 h, indicating the mucilage blocked oxygen transport (Fig.3). However, mucilage removal did not affect the germination of *M. himalaica* achenes at 15 °C (Fig. 4a).

Temperature is a critical environmental factor that positively regulates achene germination. Differences among plant species regarding ideal temperatures for germination are considered to be ecologically significant (Gorai et al. 2013; Hu et al. 2015). The optimal temperature for germination is influenced by the mean annual temperature of the region from which the seeds originated (Windauer et al. 2012). Hu et al. (2015) reported that a large percentage of seeds from four Qinghai-Tibetan Plateau species germinated at 5 °C. This was in contrast to the seeds of four Alax Desert species, which did not germinate at 5 °C. In the present study, *M. himalaica* achenes exhibited low germinability at 25 °C, and the optimal temperature for germination was 15 °C (Fig. 4a). This reflects the adaptation of *M. himalaica* plants to the low temperatures of their natural habitats. The imbibed *M. himalaica* seeds can germinate at temperatures ranging from -5 °C to 50 °C (Fig. 4 b,c), indicating they are able to adapt to the diverse environmental conditions of the Tibetan plateau regions (Orsenigo et al. 2015).

In Tibet regions, even in the season when plants grow actively, *M. himalaica* often experiences temperatures below 0 °C. The germination percentage for intact *M. himalaica* achenes was higher than that of demucilaged achenes at 5 °C (Fig. 4a). Interestingly, we observed that the intact achenes were more tolerant to −10 °C than the demucilaged achenes (Fig. 4b). The roots and shoots from intact achenes were also longer than those of demucilaged achenes at −5 °C (Fig. 5b), suggesting the mucilaginous layer has an important role during achene germination. We hypothesized that the mucilage serves as a protective layer against freezing stress. Additionally, *M. himalaica* is mainly distributed in dry and hot valleys, which means it is likely exposed to heat stress conditions during the daytime. Our

findings revealed heat stress substantially inhibited the germination of *M. himalaica* achenes. We also determined that the germination percentage of intact achenes was higher than that of demucilaged achenes following treatment at 40 °C. The development of plants with enhanced heat stress tolerance is especially critical because of the current and anticipated global climate changes (Mittler et al. 2012; Wahid et al., 2007).

High temperatures cause heat stress, but also decrease soil moisture (Hu et al. 2015). In the dry and hot valleys where *M. himalaica* plants distributed, evaporation levels are 4-fold greater from the surface of water bodies than from saturated soils. The sandy soil where the *M. himalaica* settled in was poor in moisture-holding capacity. Thus, *M. himalaica* plants experience hydropenic stress conditions. When treated with polyethylene glycol, the intact *M. himalaica* achenes exhibited greater tolerance to low water potentials than the demucilaged achenes (Fig.6). This was consistent with previous studies that mucilage enhances seed germination under low water potential conditions (Gu et al. 2008; Zhang et al. 2014). The high germination percentages of intact *Artemisia sphaerocephala* achenes when available water is limited is likely at least partially because of the hydrophilic nature of mucilages (Huang and Gutterman 2000). Achene mucilage may also enable plants to adapt to the hydropenic environments encountered in the natural habitats of *M. himalaica*. Moreover, achene mucilage can aid germination in highly saline environments presumably playing an ecologically important role in the *A. sphaerocephala* life cycle (Yang et al. 2010). In the present study, the germination of intact achenes were significantly higher than those of demucilaged achenes in 50 mM NaCl solutions (Fig.7a). Additionally, when treated with 10 mM NaCl, root and shoot lengths showed significant differences between demucilaged and intact achenes (Fig.7b), indicating intact achenes are more tolerant to high salt conditions than demucilaged achenes. Mucilage may serve as a filter and/or sorbent to ensure intact achenes are in direct contact with relatively low concentrations of salt (Yang et al. 2010). This minimizes the inhibition of achene germination by exposure to salinity stress. Therefore, mucilage is beneficial for the germination of *M. himalaica* achenes under drought and salt stress conditions.

In conclusion, the present study indicate that *M. himalaica* achene mucilage is able to increase germination and early seedling growth during exposure to abiotic stresses. Our results provide new insights into the ecological significance of achene mucilage in plants growing in alpine habitats exposed to multiple environmental stresses. Our findings would also provide a foundation for effective way to protect endangered wild *M. himalaica* and the rational use of cultivated *M. himalaica*.

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Figure legends

Fig. 1 Morphology of *Mirabilis himalaica* achenes. (**a**) Dry achenes. (**b**) Fully hydrated achenes, surrounded by gelatinous mucilage. (**c**) Fully-hydrated achenes in water after ruthenium red staining. (**d**) Fully hydrated achenes without mucilage. (**e**) Achene seed (middle) and pericarps. (**f**) Imbibed seed and pericarps. (**g**) Fully hydrated pericarps stained with ruthenium red (upper), as well as a seed stained with ruthenium red (right) and an unstained seed (left)

Fig. 2 Imbibition and dehydration of intact or demucilaged achenes. (**a**) Absorption of water by dried intact or demucilaged achenes. (**b**) Dehydration process of the imbibed intact or demucilaged achenes. Values are presented as the mean ± standard deviation of four measurements.

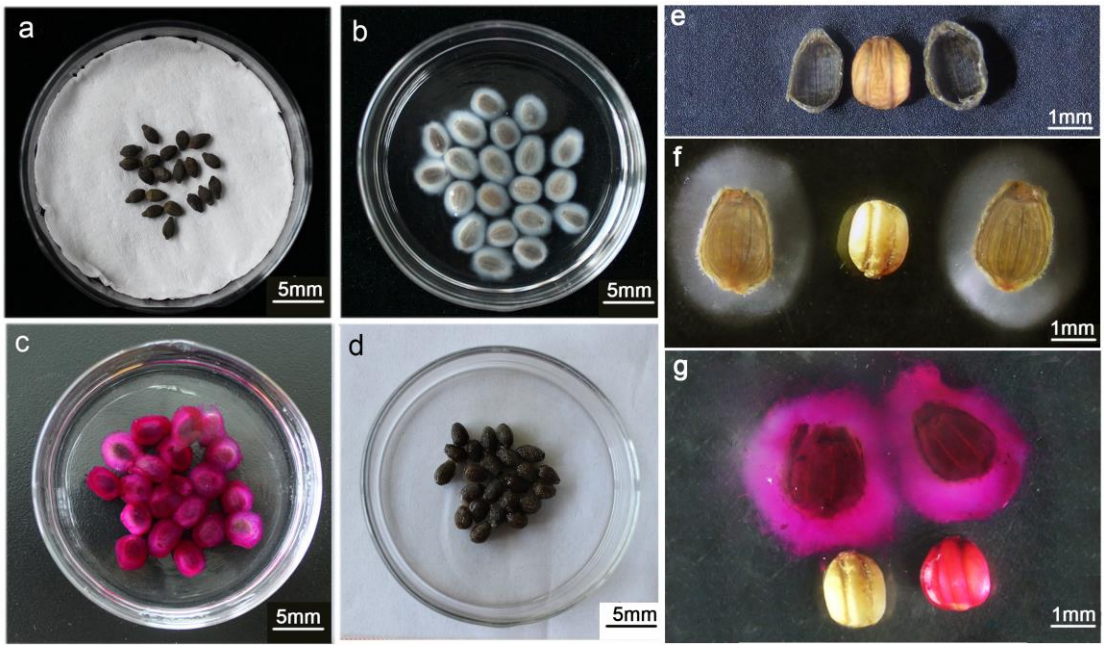
Fig. 3 Effect of mucilage removal on respiration of *M. himalaica* achenes incubated at 15 °C in darkness**.** Different uppercase letters indicate significant differences at the 0.01 probability level.

Fig. 4 **Effect of mucilage removal on germination of *M. himalaica* achenes under different temperatures.** (a) Germination at 5, 10, 15, 20, 25 and 30 °C, (b) imbibed achenes treated at -5, -10, -15 and -20 °C for 1h, then incubated at 15°C for 9 days, (c) imbibed achenes treated at 40 and 50 °C for 2 h, then incubated at 15°C for 9 days. Different uppercase letters indicate significant differences at the 0.01 probability level*,* and the lowercase letters denote significant differences at the 0.05 probability level.

Fig. 5 **Effect of temperature on sprout growth of intact and demucilaged *M. himalaica* achenes.** (a) Germinated at 5, 10, 15, 20, 25 and 30 °C for 15 days respectively, (b) imbibed achenes treated at,-5, -10 °C for 1h, then incubated at 15°C for 15 days, (c) imbibed achenes treated at 40 and 50 °C for 2 h, then incubated at 15°C for 15 days. Different uppercase letters indicate significant differences at the 0.01 probability level*,* and the lowercase letters denote significant differences at the 0.05 probability level.

Fig. 6 Germination and sprout growth of intact and demucilaged *M. himalaica* achenes under different osmotic conditions. (**a**) Germination percentages at 9 days, and (**b**) shoot and root lengths 15 days after incubation at 15 °C in darkness under different osmotic conditions. Different uppercase letters indicate significant differences at the 0.01 probability level, and the lowercase letters denote significant differences at the 0.05 probability level.

Fig. 7 Germination and sprout status of intact and demucilaged *M. himalaica* achenes under salinity stress conditions. (**a**) Germination percentages at 9 days, and (**b**) shoot and root lengths 15 days after incubation at 15 °C in darkness. Different uppercase letters indicate significant differences at the 0.01 probability level.



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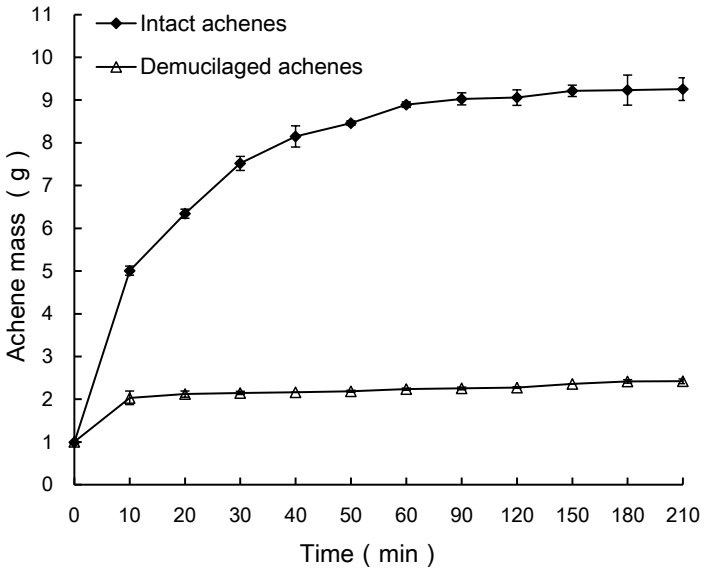
a


Intact achenes
Demucilaged achenes
Achene mass (g)
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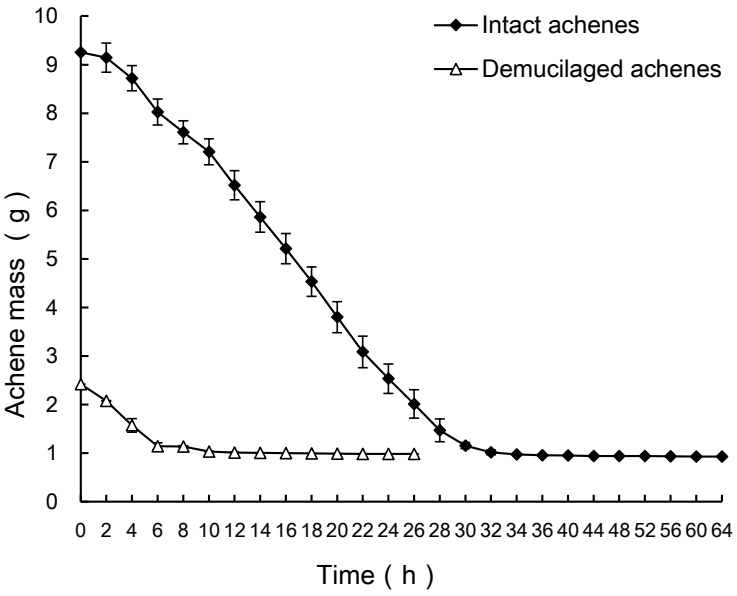
0 10 20 30 40 50 60 90 120 150 180 210
Time (min)

b


Intact achenes
Demucilaged achenes
Achene mass (g)
0 1 2 3 4 5 6 7 8 9 10
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 40 44 48 52 56 60 64
Time (h)

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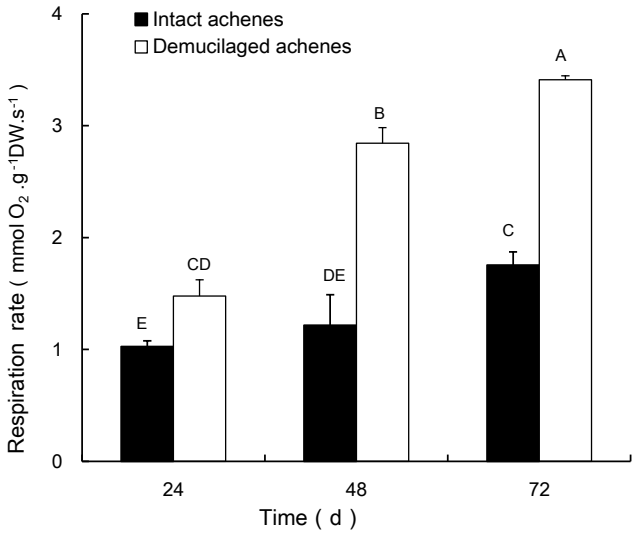

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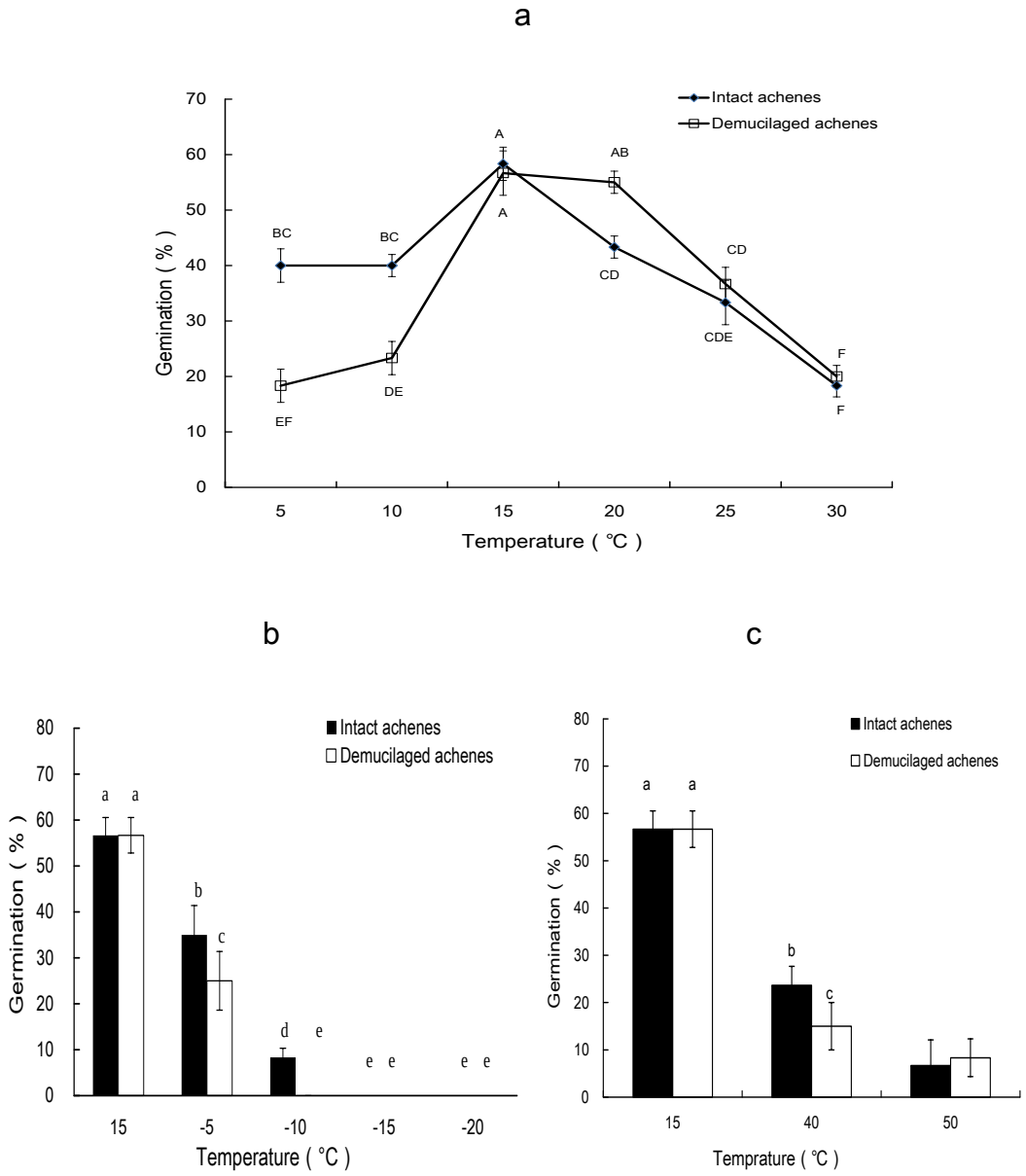

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indicate significant differences at the 0.01 probability level, and the lowercase letters denote significant differences at the 0.05 probability level.

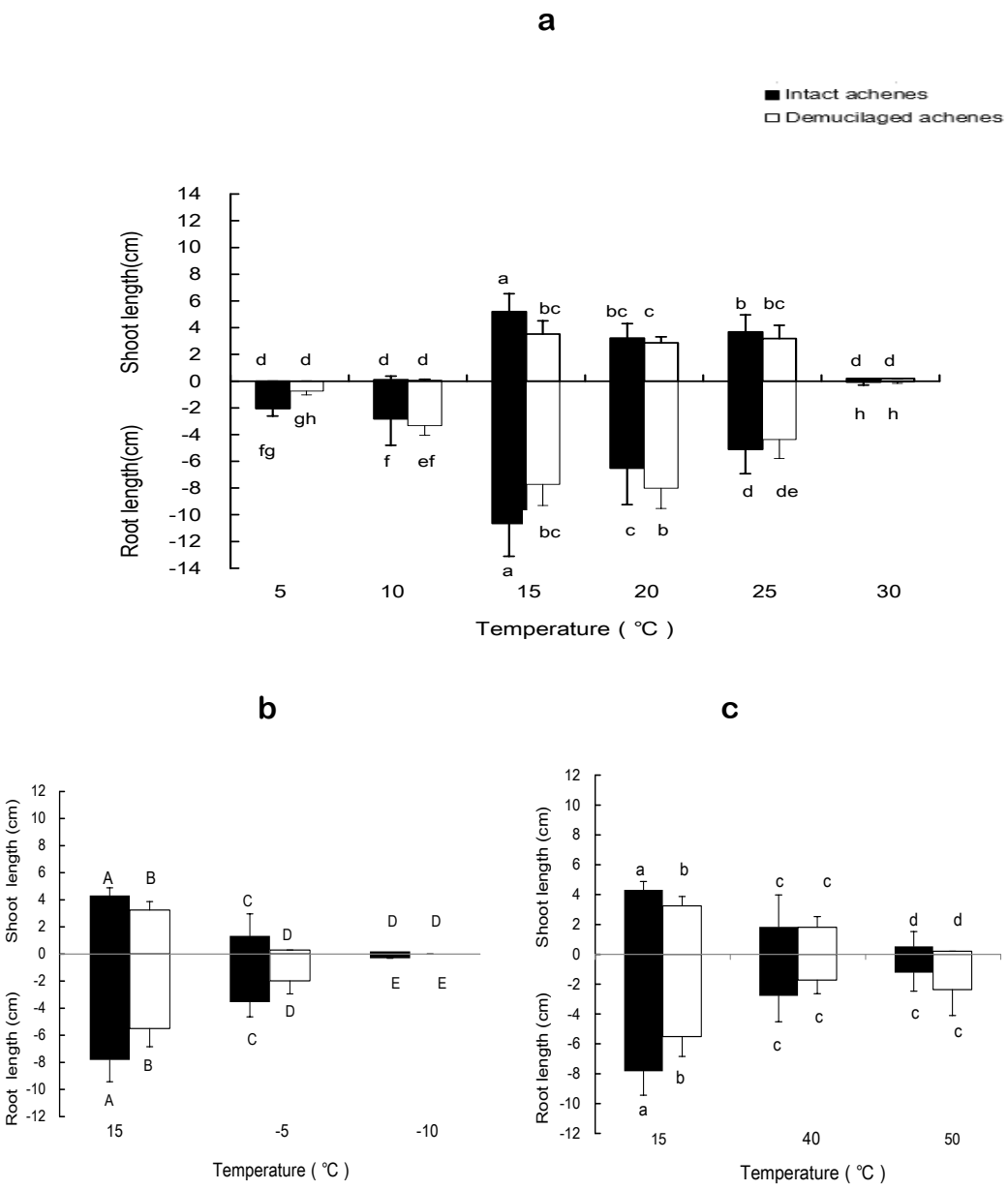

Fig. 5 **Effect of temperature on sprout growth of intact and demucilaged *M. himalaica* achenes.** (a) Germinated at 5, 10, 15, 20, 25 and 30 °C for 15 days respectively, (b) imbibed achenes treated at,-5, -10 °C for 1h, then incubated at 15°C for 15 days, (c) imbibed achenes treated at 40 and 50 °C for 2 h, then incubated at 15°C for 15 days. Different uppercase letters indicate significant differences at the 0.01 probability level, and the lowercase letters denote significant differences at the 0.05 probability level.

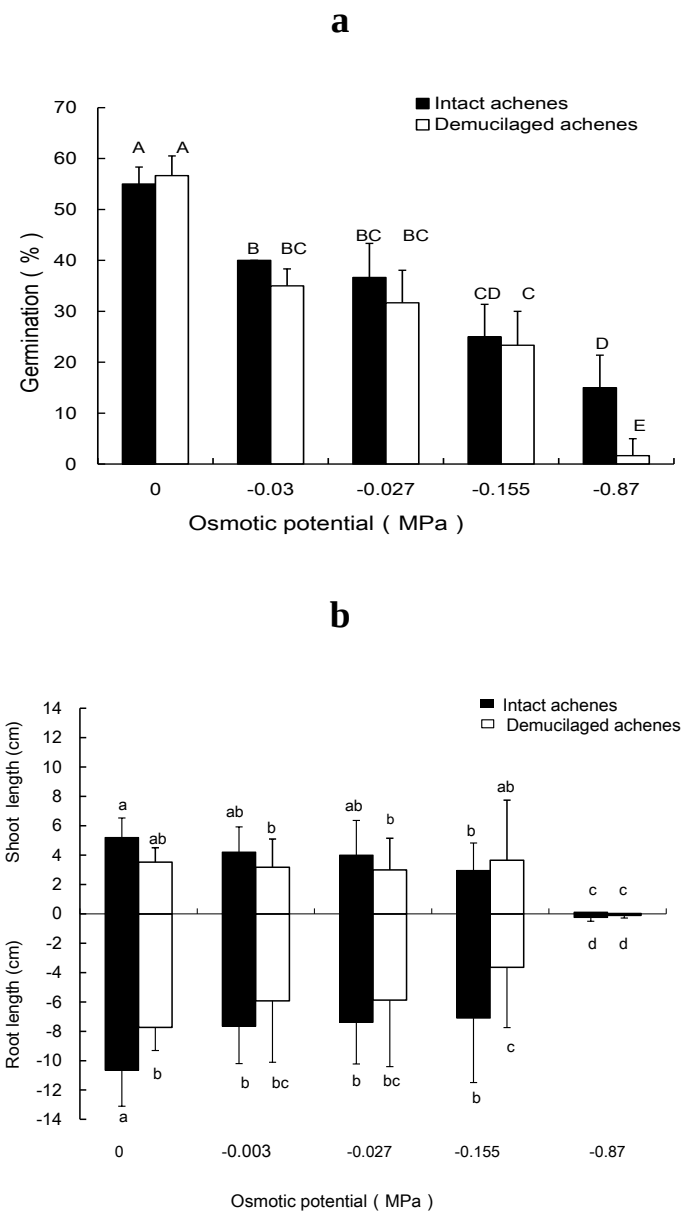

Fig. 6 Germination and sprout growth of intact and demucilaged *M. himalaica* achenes under different osmotic conditions. (**a**) Germination percentages at 9 days, and (**b**) shoot and root

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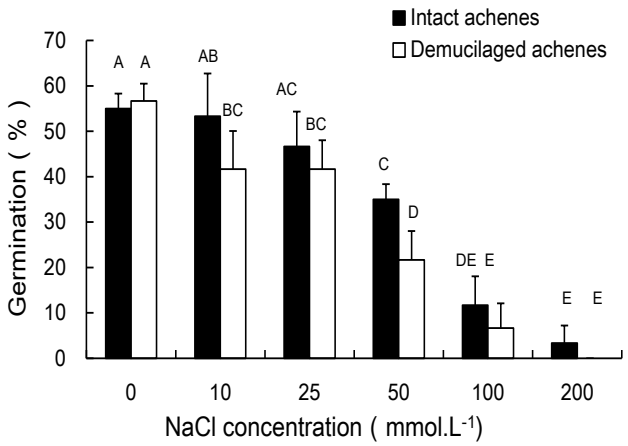
a



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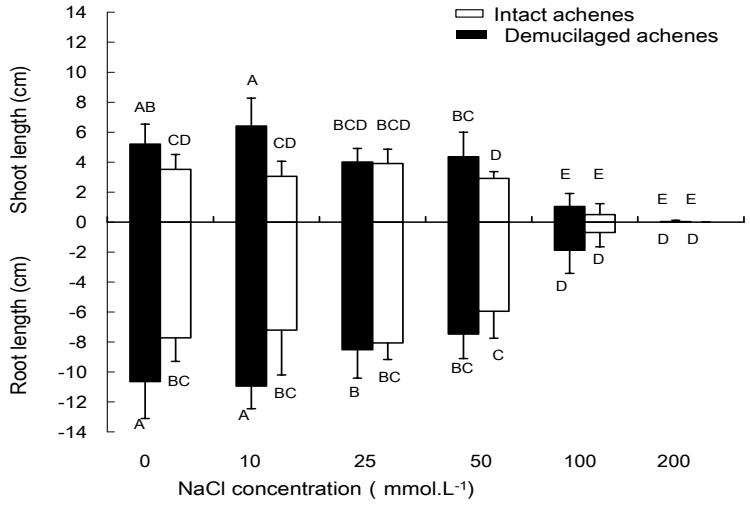

Fig. 7 Germination and sprout status of intact and demucilaged *M. himalaica* achenes under salinity stress conditions. (**a**) Germination percentages at 9 days, and (**b**) shoot and root lengths 15 days after incubation at 15 °C in darkness. Different uppercase letters indicate significant differences at the 0.01 probability level.