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Pollen exine spontaneously shedding in the angiosperm Annona montana (Annonaceae)

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Research Article

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Abstract

Background

It is generally considered that exine is vital for interactions with stigmas, and pollen exine shedding during pollination has never been reported in angiosperms. However, *Annona montana*, located at Annonaceae family in basal angiosperms, was observed spontaneously shedding exine in stigma exudates and any watery solution.

Results

We demonstrated the entire process of *A. montana* pollen exine shedding *in vivo* and *in vitro*, verified that water is the only necessary medium for *A. montana* pollen shedding, and exine shedding is an unavoidable step before pollen germination. Since pollen volume of *A. montana* did not change remarkably before and after hydration, it is undoubtedly that exine shedding in this species might be related with exine, rather than the intine-absorbing water.

Conclusions

Pollen shedding exine in *A. montana* exhibits a special and rare reproductive characteristic in angiosperms, which will update what we have previously recognized that exine is essential in reproductive processes.

Background

Intact pollen grains are crucial for pollen germination, and the exine is especially essential for reproductive processes, protection and maintenance of pollen grain viability during pollen dispersal, and controlling male-female interactions after pollination [1–5]. It is virtually impossible to obtain exines without modifying the sporopollenin, the major component in the exine [6–9]. However, we discovered that the tetrad pollen of *A. montana* spontaneously splits into single pollen grains in distilled water. Subsequently, the exine separates from the intine within 30 s (Fig. 1e), leaving the male gametophyte exposed and activated. The same exine shedding process also occurs on sticky stigmas, but takes more than 2 h (Fig. 1j).

Exine shedding was discovered and reported in certain gymnosperm groups, such as *Ginkgo biloba* [10], *Cupressus arizonica* [11], and *Ephedra americana* [12]. The isolated free exine after hydration had a common feature of the half-opened valves without any crushed coiled edges in *Ginkgo*, *Cupressus*, *Jutriperris*, and *Ephedra* [10, 12–15]. While, the mechanism behind the exine isolation was clarified as the intine absorbing water and expanding to double its volume, basing on *in vitro* experiments in *Cupressus arizonica* [16]. Obviously, it was contradicted to the isolated free exine after hydration without being

crashed in this species, and also to the illustrations in *Ginkgo biloba*, *Jutriperris cotritrrrttiis*, *Ephedra americana*, *E. foliata*, *E. rupestris*, *E. distachya*, and *E. fragilis* [10, 12–15].

A. montana appears a very similar exine shedding with that of gymnosperms. It implicates that in angiosperms, exine shedding as a special and rare reproductive characteristic might be latent rather than accidental. In this present study, we reported the entire process of *A. montana* pollen exine shedding both *in vivo* and *in vitro* studies, investigated the essential conditions for the occurrence of exine shedding, and verified that exine shedding is an unavoidable step before germination. This study will provide a clue for identifying potential species with pollen exine shedding in the family Annonaceae or even in basal angiosperms, and will also possibly further reveal the implications of exine shedding in both angiosperms and gymnosperms.

Results

The process of A. montana pollen shedding exine in vitro and in vivo

A. montana tetrad pollen aligns as clusters in matured anthers and spreads out into single tetrads when placed in distilled water (Fig. 1a-c). The single tetrads then spontaneously split into single pollen grains within about 10 s (Fig. 1d). The exines separate from the intine within 30 s without being crashed (Fig. 1e), leaving the activated male gametophyte covered only by the intine, which was stained in red with MTT (Fig. 1f). The diameter of pollen before and after hydration is approximately 100 μm without significant differences.

A. montana pollen shedding exine also occurs on the sticky stigmas prior to germination. Cluster of tetrad pollens are pollinated on the sticky stigma (Fig. 1g). After artificial pollination for 0.5 h, pollen tetrads are linked by pectin connecting threads (Fig. 1h) [17]. After artificial pollination for 1 h, the pollen tetrads slide down the stigma gap (Fig. 1i). The exine shedding process required more than 2 h (Fig. 1j). Additionally, it took 4 h for pollen tubes growing to about 150 μm (Fig. 1k). This feature maintains stability in samples collected consecutively from 2015 to 2021, located in Gangdong Province and Yunnan Province in China. Exine shedding in *A. montana* pollen appeared not only in the cross-pollination stigmas (Fig. 1g-k), but also on its own stigmas (Fig. 1l), and even on the stigmas with exudates from different families (e.g., *Lilium brownii* var. *viridulum*) (Fig. 1m). The investigations both on stigmas and water indicate that exine shedding is not dependent on stigmas, but is due to the pollen specificity.

The essential condition for A. montana pollen shedding exine

A. montana pollen grains were observed to shed exine in all aqueous solutions, and the rate of exine separation decreased as solution concentration increased. For example, the rate was more than 90% in NaCl solutions (concentration ranging from 1–25%) or in alcohol (concentration ranging from 10–70%). However, the rate was reduced to 60% in 30% glycerol, or even to 5% in 90% alcohol. In 100% alcohol or 100% glycerol where no water was contained, exine shedding could not occur (Fig. 2a). Absolutely, water is the only necessary medium for *A. montana* pollen shedding exine.

Exine shedding pre-germination is an unavoidable step in A. montana

In a nutrient medium with 5% sucrose and 0.3% agarose, it was observed that all the tetrad pollen could shed exines and approximately 47% of them germinated pollen tubes. With an increase in agarose (to 0.4% and 0.5%, respectively), exine separation still occurred completely, but pollen germination decreased (to 20% and 2%, respectively). When the agarose concentration increased to 0.6%, pollen did not germinate, due to the lack of sufficient water content in the medium, whereas exine shedding still appeared (Fig. 2b). It could be suggested that pollen shedding exine before germination on stigmas is an unavoidable step in *A. montana* fertilization.

Discussion

A. montana exine shedding is specific to pollen, but not dependent on stigmas, since it can appear on any stigma with exudates (including stigmas of its own and those of other species), as well as in any watery solution. In water or watery solutions, exine shedding occurs very quickly (usually within 30 s), and the free isolated exines are usually far from the completely exposed male gametophyte. However, pollen on stigmas needed more than 2 h to shed exine, and the free isolated exines were usually partly covered the naked male gametophyte, owing to the thick mucus of the stigma and poor fluidity.

In *Cupressus arizonica*, based on *in vitro* experiments, exine isolation was regarded as the intine-absorbing water and then expanding to double its volume. However, in *A. montana*, the pollen volume did not change remarkably before and after hydration, both *in vitro* and *vivo*. It exactly indicated that pollen shedding exine was not the result of the intine absorbing water and then swelling. On the other hand, the isolated free exine after hydration was considered as a regular aspect of the half-opened valves with coiled edges in *Cupressus arizonica, Jutriperris cotritrrrttiis*, or twisted in Ephedraceae (*Ephedra americana*, *E. foliata*, *E. rupestris*, *E. distachya*, and *E. fragilis*), which was did not appear crashed as that of *in vitro* experiments in Cupressaceae [13, 15, 16, 18]. Moreover, the free isolated exine is also without being crashed in *A. montana.* Obviously, for exine shedding both in gymnosperm and angiosperm, the exact mechanism might be related with exine, rather than the intine-absorbing water.

Exine shedding prior to germination was suggested in *Ginkgo biloba*, *Cupressus arizonica*, and *Ephedra americana* [10–12], in which Takas and Owens considered that pollen only with intine present deforming easily, and allowing it to squeeze through the micropyle [18]. The present study indicated that water is the only necessary medium for *A. montana* pollen shedding exine. In the medium where was lack of sufficient water content to support pollen germination, exine shedding could still appear with no difference from that of medium with sufficient water. Exine shedding happened prior to germination in *A. montana*, and is thereby an unavoidable step.

The male gametophyte of *A. montana* enveloped solely by the intine was fertile, proved by the MTT staining and the field yields in every year (Fig. 3). The exine seems not essential in *A. montana*, that presents a special and rare reproductive characteristic in angiosperms. As the largest family within Magnoliales, Annonaceae exhibits an extraordinary variety of morphological and structural features over

a long period of evolution [19, 20]. Probably, more species with exine shedding might exist in basal angiosperms, especially in Annonaceae, since four *Annona* species (*A. coriacea*, *A. crassiflora*, *A. dioica*, and *A. aurantiaca*) were reported to shed exine in water [21], but was not testified on stigmas. We speculate that with more exine shedding pollen presented, it will bring an update for the previous recognization of the necessity of exine for reproductive processes in angiosperms.

Conclusions

In the present study, we observed *Annona montana* pollen spontaneously shed exine in both any watery solution and sticky stigmas, exhibiting a special and rare reproductive characteristic in angiosperms. Water was proved to be the only necessary medium for *Annona montana* exine shedding, but pollen shedding exine was not the result of the intine absorbing water and then swelling. *Annona montana* exine shedding is an unavoidable step before germination, which will update what we have previously recognized the necessity of exine in reproductive processes.

Methods

Plant materials

Annona montana and *Lilium brownii* var. *viridulum* flowers were collected from the South China Botanical Garden in Guangdong and Xishuangbanna Tropical Botanical Garden in Yunnan Province, China.

Pollen exine shedding in vitro

The *A. montana* tetrad stage and mature pollen grains were placed in distilled water and stained with MTT solution. After approximately 30 min, the pollen grains were observed under a Leica-DM5500B light microscope (Leica, Germany). The percentage of pollen shedding exine was tested in the following media: (i) 1, 5, 10, 15, 20, and 25% NaCl; (ii) 30, 50, 70, 90, and 100% alcohol; (iii) 30, 50, 70, 90, and 100% glycerol.

Pollen-pistil interactions

Thirty flower buds were bagged prior to anthesis, and pollen from staminate-phase flowers was used for artificial pollination of pistillate-phase flowers. Pistils were collected 0, 1, 2, and 4 h after artificial pollination, and photographed using a Leica-DM5500B light microscope. *A. montana* pollen grains from staminate-phase flowers were pollinated by self-stigmas and cross-pollinated by *Lilium brownii* var. *viridulum* stigmas, collected 12 h after pollination, and the interaction between pollen and pistil was observed.

Cryo-scanning electron microscopy (cryo-SEM)

A. montana pollen grains from the dehiscent pollen sacs and pistils after artificial pollination for 0.5 h were collected. The block was mounted onto brass stubs using double-sided adhesive tape, and then was submerged in liquid nitrogen, transferred under vacuum to a fracturing chamber and fractured by a cold scalpel knife. The sample block was sublimated for 5 min at -70°C, sprayed with 10 ma for 60 s under high vacuum (PP3010T, QUORUM, UK), and observed at − 180°C using a 5 kV accelerating voltage (Regulus8100, JEOL, Japan).

In vitro pollen rehydration and germination

A. montana pollen grains were cultured in 3% sucrose semi-solid media with a graded agarose from 0.3 to 0.6% [22]. After 12 h of pollination, pollen shedding and germinating rates were observed and counted.

Declarations

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Authors' contributions

F.X.X. and B.X.L. wrote main manuscript text and B.X.L. prepared figures 1-3. All authors reviewed the manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

All methods were performed in accordance with the relevant guidelines of our Institute. The plant samples were obtained from South China Botanical Garden, Chinese Academy of Sciences, upon permission granted from South China Botanical Garden.

Consent for publication

Not application.

Competing interests

The authors declare that they have no competing interests.

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Figures

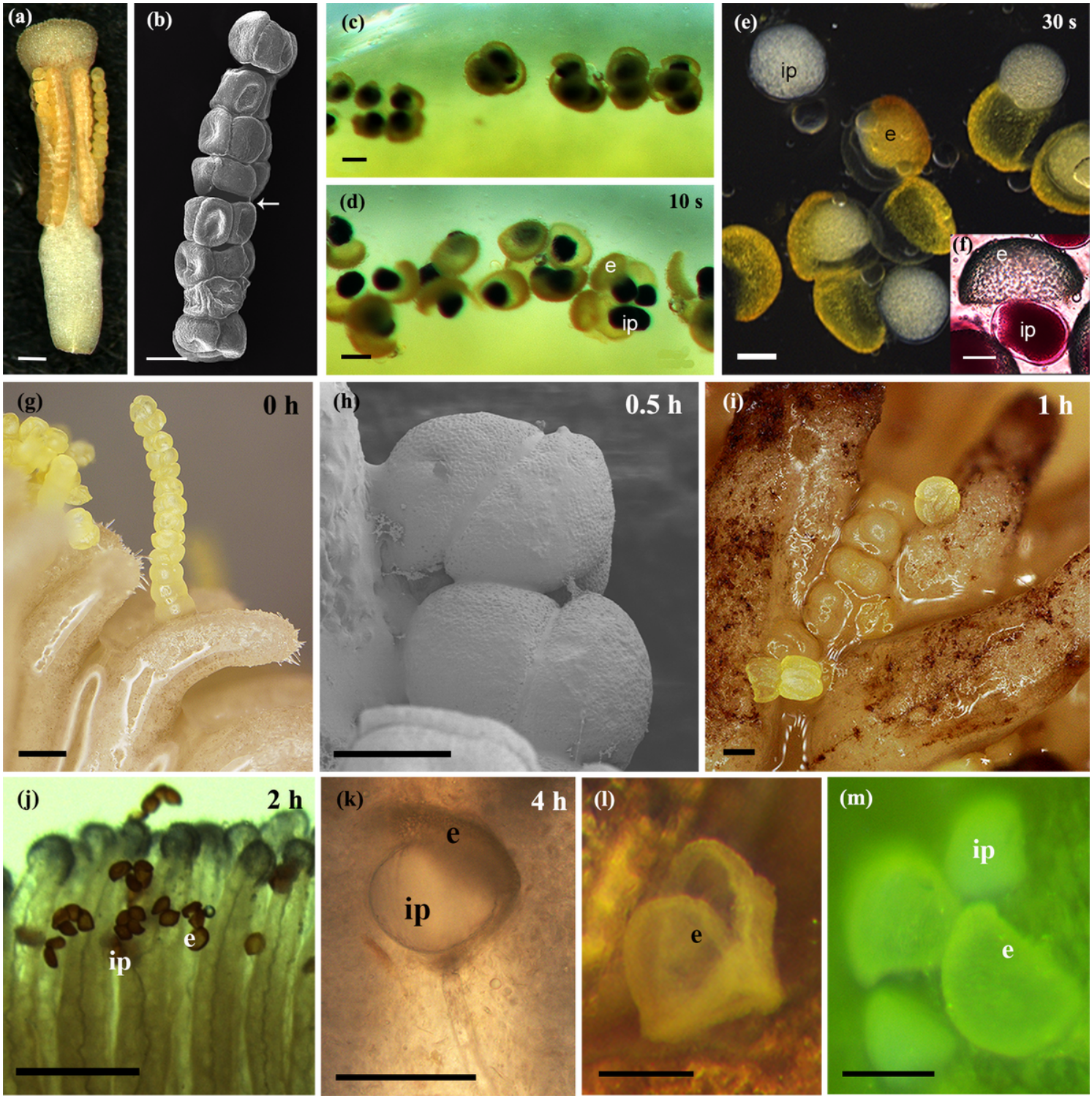

Figure 1

The progress of *A. montana* pollen shedding exine in distilled water and *in vivo*. **a** Stamen of *A. montana* from dorsal side. **b**Bunchy of *A. montana* pollen tetrads (arrow). **c** Bunchy of *A. montana* pollen tetrads disperse into individual tetrads in distilled water. **d** Pollen tetrads spread into single pollen grains and begin to isolate exine after about 10 s at distilled water. **e** Pollen grains shed exine in distilled water after about 30 s. **f** Isolated exine pollen are activated, stained by MTT. **g** Cluster of tetrad pollen on the stigma. **h** After artificial pollination for 0.5 h, pollen tetrads are linked by pectin connecting threads, and the exine

ornamentation is smooth. **i** The pollen tetrad located in the gap of stigmas after artificial pollination 1 h. **j** After artificial pollination for 2 h, pollen grains shed exine. **k** After artificial pollination for 4 h, exine was shed and pollen tubes germinated. **l** Pollen grains shed exine but did not germinate at self-pollination. **m** Pollen grains of *A. montana* shed exine but did not germinate in the stigma exudatesof *Lilium brownii* var. *viridulum* Baker. e = exine, ip = isoated exine pollen. *Scale bars* 500 μm (**a**), 100 μm (**b–f**, **h**,**i**, **k–m**), 250 μm (**g**), and 1000 μm (**j**)

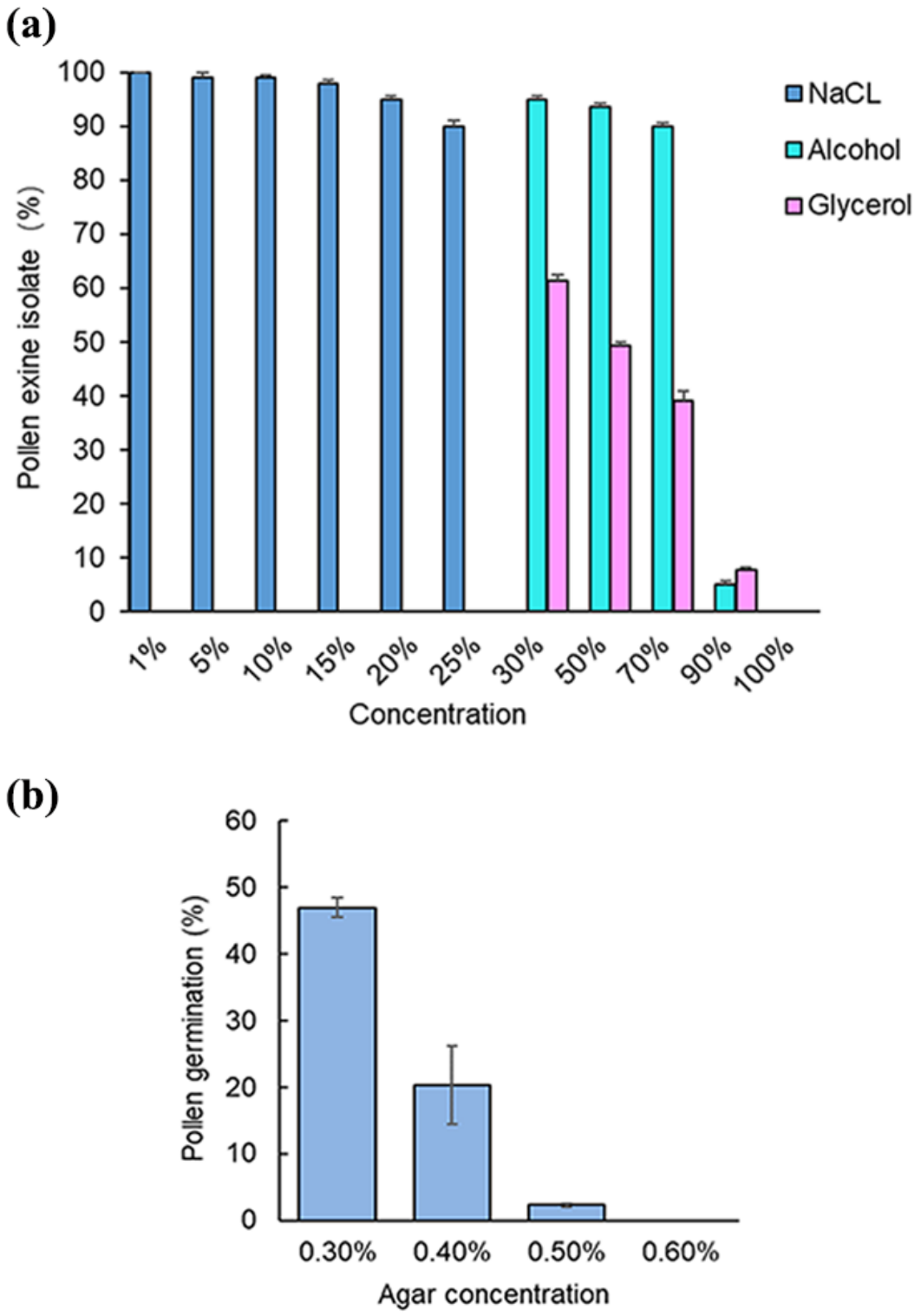

Figure 2

The essential conditions of *A. montana* pollen shedding exine and pollen germination in semi-solid media. **a** The percentage of pollen shedding exine in a series of concentration grades of NaCl, alcohol, and glycerol. **b** The rates of pollen germination in a graded agarose semi-solid media. Values are means ± SE, n = 3.

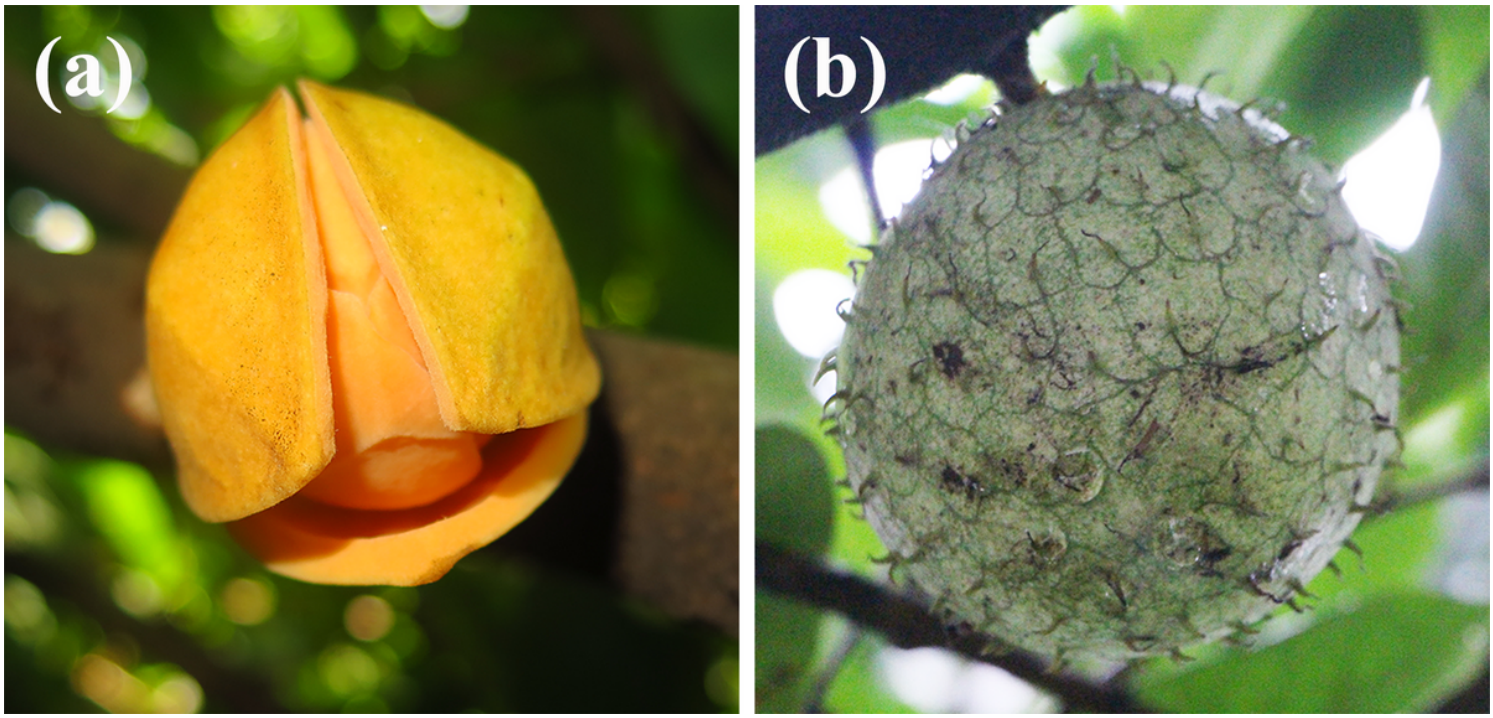

Figure 3

A. montana flower and fruit. **a** A flower at anthesis. **b** A fruit.

Supplementary Files

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- A.montanapollensheddingexineinLugosiodinesolution.mp4