*Plant Genetic Resources: Characterization and Utilization*

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# Seed set by artificial pollination and seed storage under cryogenic, freezer and dry conditions in the medicinal plant *Uncaria rhynchophylla*

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**Research Article**

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**Abstract**

*Uncaria rhynchophylla* (Rubiaceae), a woody liana with significant medicinal value, has been used as a traditional Japanese and Chinese medicine. While effective seed production is required for breeding and efficient seedling production, the physiology of sexual reproduction remains largely unknown in this species. Therefore, we first observed the flowering behaviour, and next attempted artificial pollination using flowering individuals in a greenhouse. In this study it became clear that this species sets seeds by allogamy, but not by autogamy. The obtained seeds showed about a 90% germination rate. We also examined seed desiccation tolerance and storage conditions which are important to preserve the genetic resources. Seeds of this species were found to have a characteristic of the orthodox type, having high desiccation tolerance. Seeds after 6 months of storage at +22, −20 and −160°C showed comparable germination rates to the seeds before storage.

## Introduction

Reproduction by seeds is one of the most effective and important manner both for maintaining natural populations and for producing seedlings in cultivation. Artificial pollination is essential for genetic analysis of inherited traits and improving traits of economically valuable plants, and storage of seeds with high germination viability is an important tool for preserving plant genetic resources (Mounce *et al*., 2017; O'Donnell and Sharrock, 2017; Kolomeitseva *et al*., 2022) and planned seedling production (Justice and Bass, 1978). Since plants exhibit a diverse array of flowering and pollination characteristics, protocols for artificial pollination often need to be developed for particular species. The storage of seeds at low temperatures, especially at subzero temperatures, can extend seed viability (Li and Prichard, 2009). Consequently, storing seeds at subzero temperatures, including at cryogenic temperatures, has been used extensively to store seeds for extended periods (Pritchard and Nadarajan, 2008; Popova *et al*., 2013). However, storing seeds at subzero temperatures requires that the storage behaviour of the seeds be characterized according to seed storage type, i.e., whether the seeds are orthodox, sub-orthodox or recalcitrant, based on their desiccation tolerance (Roberts, 1973; Bonner, 1990; Ellis *et al*., 1990; Chmielarz, 2010).

*Uncaria* spp. belonging to the family Rubiaceae are economically important liana plants due to their medicinal value. Cat's claw (*Uncaria tomentosa* [Willd. ex Schult.] DC.), which is native to the Amazon region, is widely used in the medicinal industry for its anti-inflammatory compounds (Honório *et al*., 2017). Gambier (*Uncaria gambir* [Hunter] Roxb) is native to tropical areas in southeastern Asia, and extracts of this species have been used in dyes, cosmetics and traditional medicines (Rizki *et al*., 2020). *Uncaria rhynchophylla* (Miq.) Miquel, which is distributed in the temperate and subtropical regions of Asia, has alkaloids, and exhibits hypotensive, sedative and antiarrhythmic effects (Kawazoe *et al*., 1988). *Uncaria rhynchophylla* has long been used as a traditional Chinese medicine and traditional Japanese medicine (Kampo medicine), and is one of original plant source of crude drug (online Supplementary Fig. S1(a), (b)), *Uncaria* hook, i.e., Chotoko in Japan and Gou-teng in China (Shi *et al*., 2012). In Japan, use of *Uncaria* hook has been confirmed from the Edo period (1600s) (Mikage and Endo, 2008), and usage has increased from 85,546 kg in 2008 to 272,450 kg in 2020 (Yamamoto *et al*., 2023). However, the species has not been cultivated in Japan, and all of the plant materials required to produce *Uncaria* hook are imported from China (Kawazoe *et al*., 1988; Yamamoto *et al*., 2023). In Japan, *U. rhynchophylla* is native to west of the Boso Peninsula (Chiba prefecture) in Honshu Island, Shikoku Island and Kyushu Island (Kawazoe *et al*., 1988). 'SEARCH SYSTEM of JAPANESE RED DATA'

shows that *U. rhynchophylla* has been classified as endangered species in some prefectures in Japan (Chiba, Tokyo, Kanagawa, Gifu, Kyoto, Hyogo and Shimane) (http://jpnrdb.com/database/species/search/#). Also in China, this species is now endangered in some regions because of extensive collection from wild population (Zhu *et al*., 2018). Therefore, for medicinal use, *U. rhynchophylla* should be cultivated rather than collected from its wild habitat. Propagation methods for cultivation of this species have been reported by seeds collected in wild habitat, cuttings and tissue culture (Kawazoe *et al*., 1988, 1990; Ishii *et al*., 2014). However, propagation by seeds collected in wild habitat is destress because of decrease of *U. rhynchophylla* natural habitats and difficulty to collect seeds from tall vines. Clonal propagation by cuttings is low proliferation rate, and tissue culture needs special equipment. Therefore, seed production at cultivation site is considered an effective propagation method, and artificially pollination is an essential tool for breeding in this species.

For cultivation test to produce crude drug, we have raised *U. rhynchophylla* test planting in Suzuka City, Mie prefecture and Kami City, Kochi prefectures. In Suzuka site where clonal plants propagated by tissue culture from single genotype was planted in 2017, it was found that *U. rhynchophylla* produced flowers early-summer after 4 years of planting, however no seeds were produced. In Kami site where 25 genotypes were planted in 2018, and after one year plant parts had cut every year at about 70 cm high from ground level in order to evaluate annual growth of vine and yield of crude drug (i.e., branches with hooks), *U. rhynchophylla* had no flowers at all (online Supplementary Fig. S1(c), (d)). At these two test sites, though we obtained some information necessary for cultivation of this species, such as its growth, yield, and medicinal ingredients, we were unable to obtain any knowledge regarding its sexual reproduction characteristics and seed production. It is known that this species has spherical inflorescences in early summer and diurnal insects and nocturnal moths are pollinators of *U. rhynchophylla* (Funamoto and Sugiura, 2016). Germination test of seeds collected from national habitant showed that the seed of this species was photoblastic (Kawazoe *et al*., 1990). However, many of sexual reproduction physiology of *U. rhynchophylla* remain unclear.

In this study, we first observed the flowering behaviour of *U. rhynchophylla* grown in a greenhouse in order to obtain basic knowledge about the physiology of sexual reproduction. Next, we attempted artificial pollination using flowering individuals in a greenhouse. Using the obtained seeds, the storage behaviour of *U. rhynchophylla* seeds was examined under cryogenic, freezer and dry condition. Our research results here are the first knowledge about seed production and seed storage of this medical value plant, and can be used for preserving genetic resources, breeding and seedling production.

## Materials and methods

### Plant materials

*Uncaria rhynchophylla* (Miq.) Miquel specimens growing in a greenhouse at the Forest Bio-Research Center, Forestry and Forest Products Research Institute in Hitachi, Ibaraki, Japan (36°41′31″N, 140°41′27″E) were used for experiments on seed production by artificial pollination (online Supplementary Fig. S1(e)). *Uncaria rhynchophylla* that were collected in Chiba, Kochi, Fukuoka, Miyazaki and Kagoshima prefectures, Japan, in 2014 and 2015 were clonally propagated via tissue culture (Ishii *et al*., 2014). Plants propagated *in vitro* via tissue culture were transferred to plastic pots and then grown at ambient temperature in a greenhouse. In 2021, developing flowers were confirmed in eight specimens of five genotypes and these were used for the experiments in this study.

### Observation of flowering behaviour and artificial pollination

*Uncaria rhynchophylla* is a hermaphrodite plant that produces head inflorescences (or capitulum). To clarify the timing of pollen sample collection, the process of anthesis (flowering behaviour) was observed. Anthesis in the greenhouse specimens occurred in the early summer of 2021, and on June 11, flowers were collected and observed under a light microscope (BX51, Olympus, Tokyo, Japan). Photographs of the flowering process were taken.

Flowering was observed from the early evening to night-time on 11 July 2021. Artificial pollination was performed immediately after flowering at about 19:00 to 20:00. Since a unique flowering behaviour was observed in which pollen was attached to the stigmas of the same flower before anthesis, pollen grains were observed on the stigmas before artificial pollination; this phenomenon is called secondary pollen presentation (Howell *et al*., 1993). Therefore, for artificial pollination, pollens were removed from the stigmas of flowers on the mother plants using a paint brush, and the flowers were pollinated by artificial contact with pollen on elongated styles collected from father specimens which were different genotypes with mother plants. To clarify whether the seeds were produced from self-pollination (autogamy), inflorescences were covered with paper bags before flowering and isolated to prevent pollination from other specimens.

### Examination of seed storage behaviours

Mature fruit containing germinable seeds were collected from October to November (Kawazoe *et al*., 1988, 1990) when the fruit was green in colour with a slightly brown tip. Collected fruit was dried to equilibrium in a drying room for 1 week at a relative humidity of 8–15% and a temperature of +22°C. After drying, the dry fruit were placed in cryotubes and stored for 6 months at −20°C in a freezer (MDF-U539, Sanyo Electric Co., Ltd., Osaka, Japan) and −160°C in LN vapour in a cryopreservation tank (DR-100LM7, Taiyo Nippon Sanso Co., Tokyo, Japan). After freezer (−20°C) and cryogenic (−160°C) storage, the cryotubes were transferred to the drying room (+22°C) for 1 h. Similarly, seeds from the fruit were also stored in the drying room for six months (+22°C storage). For germination of stored seeds, 10–30 seeds per fruit were isolated and then germinated in a plastic dish (90 × 15 mm) containing 1% agar media and incubated at 25°C under a photoperiod of 16L:8D. Seeds that were isolated from dry fruit that were not subjected to storage measures, and fresh undried fruit (control), were germinated in a similar manner. The number of normal seedlings, i.e., seedlings that developed cotyledons and roots, was determined, and the percentage of normal seedlings to the total number of seeds was calculated as the germination rate of each treatment. Germination rates were determined using 250 seeds obtained from 10 fruit for the −20°C freezer-storage, 230 seeds from 9 fruit for the −160°C cryogenic-storage and 306 seeds from 10 fruit for +22°C storage. In dry treatment, 175 seeds obtained from seven fruit dried without storage were used, and 140 seeds obtained from eight fresh fruit were used in the control.

Due to the very small seed size of *U. rhynchophylla*, the fruit's moisture content (MC) was determined before and after one week drying. The mass of 10 fruit before and after drying was measured

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to determine MC. The fruit were then placed in an oven at 105°C for 1 day, and the mass of the oven-dried fruit was measured again (Endoh *et al*., 2021). The mean MC (%) was determined using eight replicates. The MC of fruit after dry storage for 6 months was also determined.

### Statistical analysis

Differences in germination rates among treatment classes were compared using a generalized linear mixed model (GLMM) with a binomial distribution in R version 3.2.5 (R Core Team, 2016). For the explanatory variables, the treatment class (i.e., control, dry, +22, −20, −160°C) was set as the fixed-effect factor, while the seed lot was set as the random-effect factor. The treatment × seed-lot was included as an interaction term. The statistical significance of the difference among treatment classes was assessed by using a likelihood-ratio test via analysis of deviance.

## Results

### Flowering behaviour

Inflorescence buds of this species developed in early spring from the axil and tip of newly elongated shoots from branches with hooks that had developed in the previous year (online Supplementary Fig. S2). In 2021, the specimens in the greenhouse of Hitachi City started growing from mid to late April, followed by flower development in early June. To optimize the timing of artificial pollination, flowering behaviours were observed in *U. rhynchophylla* specimens reared in a greenhouse (Fig. 1(a)–(i)). Based on its flower structure, the species has small complete flowers (i.e., hermaphrodite) having a set of male (stamen) and female (pistil) organ. Flowering occurred for approximately 1 h from the evening to night-time (Fig. 1(a)–(c), online Supplementary Fig. S3). Before flowering (Fig. 1(a)), stigmas were surrounded by anthers bearing pollen within the flowers (Fig. 1(d)–(f)). Once anthesis had begun, pollen was observed on the stigma (Fig. 1(b), (g), (h)). After the completion of style elongation (Fig. 1(c)), the stigma that emerged had pollen grains attached to the surface (Fig. 1(i)).

### Germination of seeds produced by artificial pollination

Artificial pollination was performed within 1 h after the completion of style elongation. Fruit developed gradually from summer to autumn, and took 4–5 months to mature. Fruit developed in flowers that were artificially pollinated (Fig. 1(j)), but not in some flowers (Fig. 1(k)); conversely, no fruit developed in any of the flowers that were not artificially pollinated (Fig. 1(l)). In the fruit that did develop (Fig. 1(m)–(o)), a single fruit contained 51.5 ± 8.0 seeds (mean ± SD, $n = 10$ fruit).

Seeds were obtained by artificial pollination (Fig. 2(a)). After placing the seeds removed from mature fruit on agar media and incubating at 25°C under a 16L:8D photoperiod, the seeds showed germination ability (Fig. 2(b)). The seeds started developing roots approximately 2 weeks after sowing, germinating within four weeks. The mean germination rate ± SD of these control seeds was 90.0 ± 10.2% ($n = 8$) (Fig. 2(c)).

### Seed storage behaviour

The seeds in the fruit were dried to equilibrium at 8–15% RH. Drying of the fruit, which contained approximately 50 seeds, for 1 week decreased the mean MC ± SD ($n = 8$) of the fruit on a fresh weight basis from 73.4 ± 1.1% before drying to 4.7 ± 1.2% after drying. The MC of dried fruit placed in a drying room for approximately 6 months was 4.6 ± 1.0%.

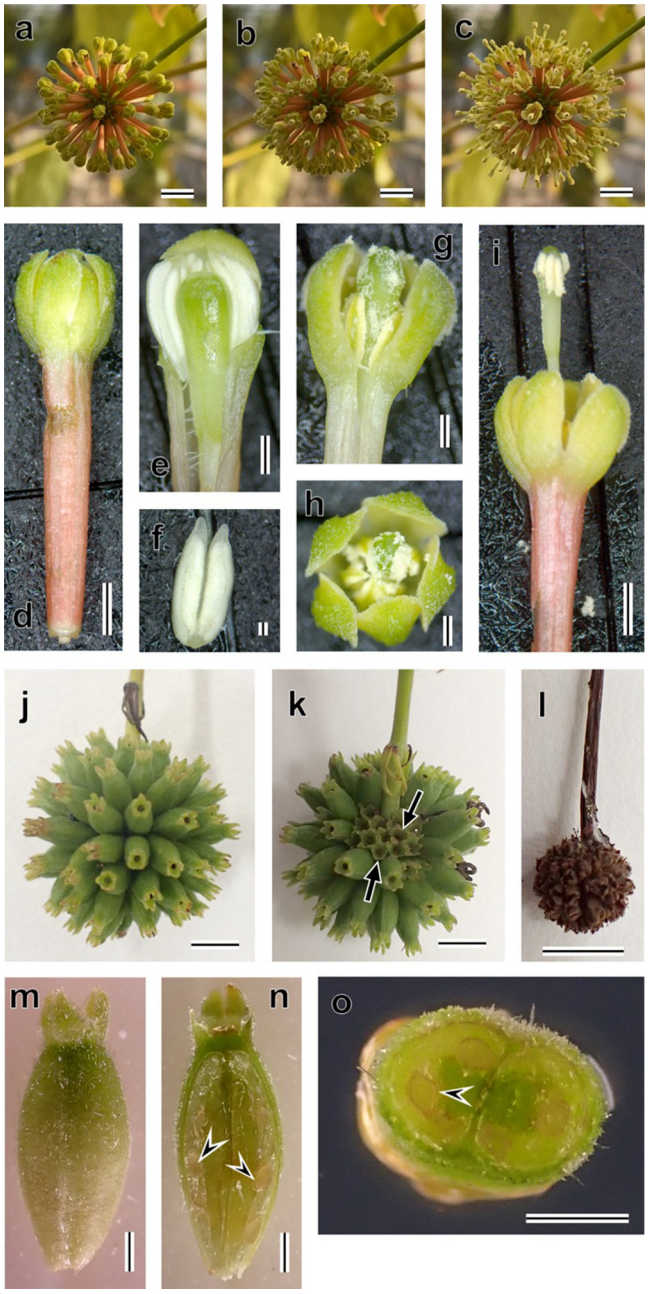


**Figure 1.** Flowering behaviour (anthesis) (a-i) and development of fruit in the inflorescence (j–o) of *Uncaria rhynchophylla*. (a) Inflorescence before flowering at 16:05 on 11 June 2021. (b) Appearance of pollen attached to the stigma at 17:50. (c) Inflorescence completed flowering at 18:20. (d) Appearance of a single flower on the inflorescence before onset of flowering. (e) Longitudinal plane of the flower before onset of flowering. The stigmas surface was smooth. Stamens surrounding the stigmas. (f) Anther bearing pollen grains inside before flowering. (g and h) Flower at the onset of anthesis. Pollen grains were attached to stigmas within the same flower. (i) Flower with elongated style. Pollen was observed on stigmas after completion of anthesis. (j and k) Fruit developed after artificial pollination. Some flowers did not produce fruit (arrows), possibly due to the failure of pollination (k). (l) Inflorescence not subjected to artificial pollination. No fruit and no seeds developed from the inflorescence without artificial pollination. (m-o) Appearance (m), longitudinal section (n) and transverse section (o) of a single fruit produced by artificial pollination. Approximately 50 seeds were contained in a single fruit. Arrowheads indicate seeds in the fruit. Bars (d, i, m, n and o) = 1 mm. Bars (e, g and h) = 0.5 mm. Bar (f) = 0.1 mm. Bars (a, b, c, j, k and l) = 5 mm.

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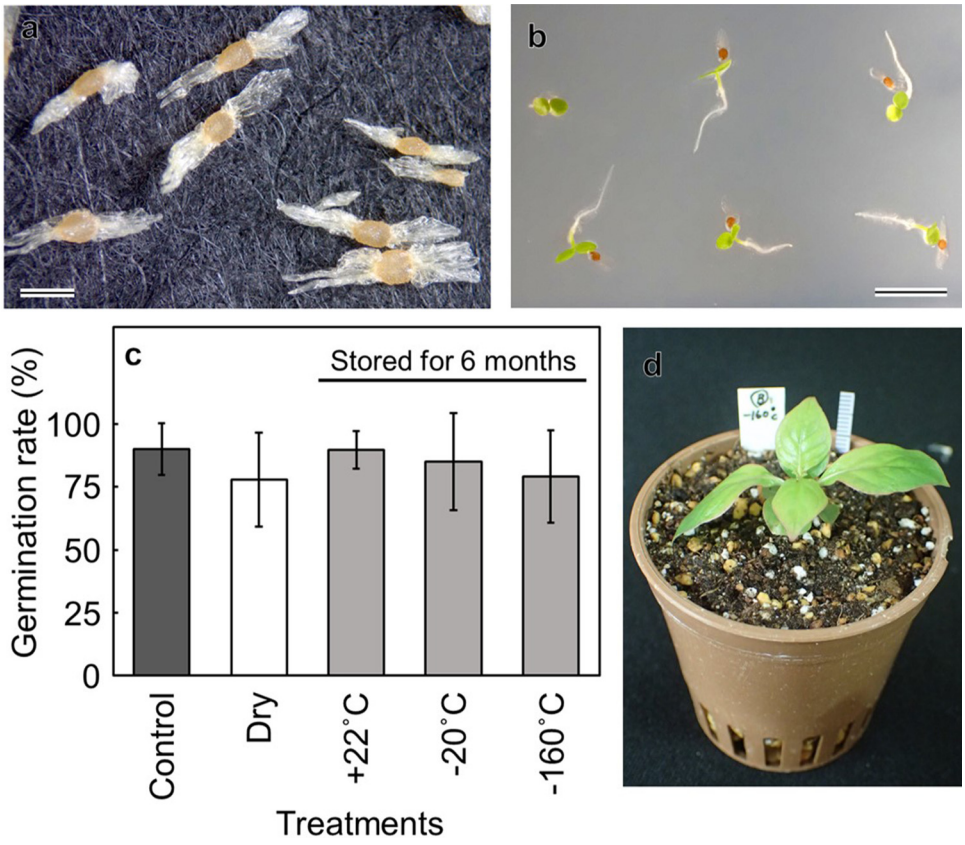


**Figure 2.** Germination of seeds of *Uncaria rhynchophylla.* (a) Artificially pollinated seeds. The seeds were tiny and had wings on both sides. (b) Germination of the seeds. Normal seedlings grew within four weeks after sowing. The seeds were germinated on 1% agar medium with incubation at 25°C under a photoperiod of 16L:8D. (c) Mean germination rates ± SD were shown. Freezer- (−20°C) ($n = 10$ fruit, total of 250 seeds), cryogenically (−160°C) ($n = 9$ fruit, total of 230 seeds) and dry-stored seeds (+22°C) ($n = 10$ fruit, total of 306 seeds) showed germination rates that were comparable with the control (undried) ($n = 8$ fruit, total of 140 seeds) and dry seeds before storage ($n = 7$ fruit, total of 175 seeds). No significant effect of treatment on germination rate was observed ($\chi^2 = 2.574$; $P = 0.631$). (d) Seeds stored for six months germinated and developed into normal seedlings within eight weeks after sowing. Bar (a) = 1 mm. Bar (b) = 5 mm.

The germination rates (mean ± SD) for fresh control seeds, dry seeds not subjected to storage, seeds stored at + 22°C, seeds stored at −22°C, and seeds stored at −160°C were 90.0 ± 10.2% ($n = 8$), 78.0 ± 18.6% ($n = 7$), 89.7 ± 7.4% ($n = 10$), 85.0 ± 19.3% ($n = 10$), and 79.3 ± 18.4% ($n = 9$), respectively (Fig. 2(c)). In the GLMM analysis, no significant difference was observed in germination rates among the five treatments ($\chi^2 = 2.574$; $P = 0.631$), indicating that seeds had a similar germination ability in these treatments without reducing germination rates. After germination, normal seedlings were obtained from seeds subjected to freezer, cryogenic and dry storage; the growth of saplings produced from these stored seeds was comparable with seedlings obtained from the control and dry seeds (Fig. 2(d)), and no abnormal or injured seedlings were observed in this experiment.

## Discussion

Different plant breeding protocols and tools have been established for a variety of other plants, and artificial pollination is one such tool. Given the considerable medicinal value of *U. rhynchophylla* (Kawazoe *et al.*, 1988; Yamamoto *et al.*, 2023), compelling production of this species requires developing and optimizing breeding protocols. Seeds collected in wild habitat, cuttings, and tissue culture (Kawazoe *et al.*, 1988, 1990; Ishii *et al.*, 2014) have been reported previously to propagate *U. rhynchophylla* (Kawazoe *et al.*, 1988, 1990), but artificial pollination remains to be developed as propagation and breeding technique. In this study, seed set of *U. rhynchophylla* using the potted plants growing in green house was examined through artificial pollination. Also, since seed storage is useful for conserving plant genetic materials and planned seedling production, seed storage behaviour was clarified using artificially pollinated seeds of this species (Smith *et al.*, 2003; O'Donnell and Sharrock, 2017). Pollination was performed immediately after flowering by placing the pollen of male plants onto the stigmas of female plants. Since artificial pollination was effectively used to produce seeds having germination ability, seed production was considered to be successful. Experiments on seed storage behaviour revealed that *U. rhynchophylla* has orthodox seeds (Roberts, 1973; Bonner, 1990; Ellis *et al.*, 1990) and that the seeds germinated after storage for six months at −20 and −160°C following drying treatment. In addition, *U. rhynchophylla* seeds maintained germinability after storing the seeds in a dry state for six months. It is revealed that artificial pollination is feasible for producing *U. rhynchophylla* seeds and that long-term storage of the obtained seeds can be performed at subzero temperatures.

As shown in online Supplementary Fig. S2, inflorescences were initiated at the axil and tip of the branch with hook, i.e., the medicinal part. Therefore, no flowers will be produced if all these branches are cut off to harvest the medicinal parts, as in the

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Kami test planting site (online Supplementary Fig. S1(c), (d)). Flowering behaviour was observed to clarify the process of artificial pollination. Flowering took approximately 1 h and occurred from evening to night in early summer. Pollen attached to the stigma of the same flower was observed when flowering began. The findings showed that *U. rhynchophylla* shows secondary pollen presentation, which is known among members of the Rubiaceae (Howell *et al*., 1993). Pollen attached to the stigma of the same flower immediately before anthesis has also been reported in gambier (*Uncaria gambir*) (Hayati *et al*., 2020), which is known to employ both cross pollination (allogamy) and self-pollination systems (Zainal *et al*., 2020). In gambier self-pollination, seed production was only observed after geitonogamous pollination with a low fruit set rate that was approximately one-fourth that of allogamy, and not after autogamic pollination (Rizki *et al*., 2020; Zainal *et al*., 2020). In this study, *U. rhynchophylla* seeds were only produced from flowers subjected to artificial pollination. No seeds were obtained from inflorescences without artificial pollination, suggesting that autogamy does not occur in *U. rhynchophylla*. Although, the specimens in the present study did not produce self-pollinated seeds, it is possible that self-pollinated seeds can be obtained using pollens from different flowers within the same specimen, i.e., by geitonogamic pollination, as reported in gambier (Rizki *et al*., 2020; Zainal *et al*., 2020). Monoclonal cultivation, i.e., planting only a single genotype, will set no seed or a fewer seed in *U. rhynchophylla* because of no autogenic pollination as shown in this study, or possibly low late of geitonogamous pollination as in related species, *U. gambir* (Rizki *et al*., 2020; Zainal *et al*., 2020).

Since seed storage is an integral part of preserving plant genetic resources and planned seedling production, the seed storage behaviour of *U. rhynchophylla* which has decreased in particular regions and categorized as endangered species was clarified in this study. Clarification of seed storage behaviour involves assessing the desiccation tolerance of seeds (Roberts, 1973; Bonner, 1990; Ellis *et al*., 1990; Popova *et al*., 2013). Dry seeds of orthodox seeds have a high desiccation tolerance and maintain germination rates for extended periods after freezer and cryogenic storage (Chmielarz, 2010; Endoh *et al*., 2021). In *U. rhynchophylla*, the seeds maintained germinability after being dried to equilibrium at low humidity of 8–15% RH. The germination rates of seeds that had been stored in a dry state for six months were comparable to seeds before drying, indicating that the seeds had a desiccation tolerance. Further, dry seeds germinated after cryogenic and freezer storage for six months, and the rates were comparable to those of seeds that were not stored and dried. The desiccation tolerance and storage behaviours observed in *U. rhynchophylla* seeds were typical of orthodox seeds, and it is considered that freezer and cryogenic storage is feasible using dry seeds.

In conclusion, we clarified the artificial pollination and seed storage requirements of the medicinal plant *U. rhynchophylla* for the first time. These findings will be helpful for seedling production, genetic improvement, and genetic resource preservation in *U. rhynchophylla* and possibly other *Uncaria* spp. plants, which have been used all over the world as traditional herbal medicine. *Uncaria rhynchophylla* reproduces sexually by allogamy, as shown in this study, and is well known to proliferate by asexual reproduction, i.e., root sucker, as we have recognized in its natural habitat and test planting site. Therefore, this species is considered to have the ability to maintain or increase genetic diversity through sexual reproduction in its habitat, and to expand its ramet through asexual reproduction.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S1479262124000571

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**Authors' contributions.** All authors contributed to the conception and design. KE, KK and TT performed material preparation and data collection. All authors performed data analysis. The first draft of the manuscript was written by KE, KK and TT, and all authors commented on previous versions of the manuscript. All of the authors read and approved the final manuscript.

**Competing interests.** The authors have no relevant financial or non-financial interests to disclose.

**Data availability.** All data sets generated in this study are included in this published article and its supplementary information files.

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