Embryo position of *Castanopsis sclerophylla* (Fagaceae) seeds with recalcitrant storage behavior differs from *Quercus* genus, but response to desiccation shows no difference

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Embryo position of *Castanopsis sclerophylla* (Fagaceae) seeds with recalcitrant storage behavior differs from *Quercus* genus, but response to desiccation shows no difference

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Running title: Desiccation-sensitivity in *Castanopsis*

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Abstract

In many -but not all- recalcitrant *Quercus* seeds, water loss during drying only occurs through the scar. Consequently, the embryo axis sitting on the opposite side of the scar is well protected from desiccation. However, whether such a mechanism is common throughout Fagaceae species is unexplored. Similarly, little is known about the desiccation response of other Fagaceae genera. Germination and desiccation tolerance of *Castanopsis sclerophylla* (Fagaceae) were studied by drying the seeds with silica gel. Fresh seeds had a moisture content (MC) of 36% and germinated to 92.5% when incubated at 15/20°C. Drying seeds to 22.5 and 20.7% MC decreased germination to 27 and 5%, respectively; indicating that seeds are recalcitrant. X-ray computed tomography and photomicrographs showed that the embryo axis of *C. sclerophylla* is located just below the scar. Regardless of drying occurring either through only scar or the whole pericarp, embryo drying was deemed unavoidable. Nevertheless, germination percentage during drying showed exceptional similarities with the other Fagaceae species.

Keywords: *Quercus*; pericarp; embryo position; morphology; recalcitrant.

Introduction

Based on their ability to withstand drying, two broad groups of seeds have been recognised by Roberts (1973). One group includes nearly 90% of the known plant species with seeds that can survive drying to the low moisture content (MC), c. 3-10% on a fresh weight basis (f. wb), thus they are desiccation-tolerant, i.e. orthodox (Wyse and Dickie, 2016). Another group of species produces seeds that lose viability if dried to the MC range of 20-30% f.wb, therefore they are referred to as desiccation-sensitive or recalcitrant (Roberts, 1973). The seeds of desiccation-sensitive species are not amenable to low temperature storage e.g. -20°C in seed bank and storing them at higher temperatures in the range of 0-10 °C does not preserve viability for more than 1 year (Berjak and Pammenter, 2008).

The family Fagaceae is widely distributed in the temperate forests and includes nine genera with approximately c. 1000 species. Information on seed storage behavior and germination ecology of Fagaceae seeds mostly is restricted to the largest genus *Quercus*, which includes c. 400 species (Bonner, 1996; Xia et al., 2012a). These studies suggest that most *Quercus* seeds are large, they do not withstand drying to MC less than 15-20% f. wb and are recalcitrant. However, the presence of desiccation tolerance has been observed in other genera within the Fagaceae, e.g. *Fagus* (Suszka and Zieta, 1977; SID, 2020). Surprisingly, little is known about the seed storage behavior of species in other genera within Fagaceae, making it an important family to study the response of seeds of desiccation. *Castanopsis* is the second largest genus in Fagaceae, mainly distributed in the subtropical and temperate region of Asia, and it often grows with other genera of Fagaceae such as *Quercus* to form a pure "oak forest" (Huang et al., 1999). *Castanopsis* includes approximately 120 species, and there are 63 species in China, of which 30 are endemic (Huang et al., 1999; Wu et al., 2014). Current knowledge about seed storage behavior of *Castanopsis* species is unclear. For example, Kew's seed information database (SID,

2020) reports seed storage behavior of 10 *Castanopsis* species, yet 6 of them are labelled as uncertain and 4 species (1 orthodox and 3 recalcitrant) are doubtfully assigned.

Such confusion is further complicated by previous studies lacking desiccation experiments or failing to report the MC. Schopmeyer (1974), for example, reported that only 5% of the seeds of *Castanopsis chryophylla* Dougl. were viable following hermetic air-dry storage at 5°C for 5 years, but the MC of the seeds was not mentioned. Similarly, Campbell (1980) showed that seeds of *C. indica* Roxb, *C. purpurella* Miq. and *C. tribuloides* J.E.Sm. from Nepal can only maintain viability for 2 months in moist storage at room temperature but did not provide any information about viability following desiccation. Tian and Tang (2010) found that fresh seeds of *C. fissa* with 42.7% MC had 100 % germination. However, drying seeds to 25.3% MC reduced the germination to 17%. Further desiccation to 17.3% MC within 72 hours resulted in complete seed death. However the quick drying rate of *C. fissa* seeds is in contrast to that of other Fagaceae species reported by various authors (Xia et al., 2012b; Li et al., 2018). In general, the silica gel and *Quercus* seeds are mixed and sealed in airtight containers, in a 1:1 to 5:1 ratio by weight (Xia et al., 2012a; Xia et al., 2012b; Li et al., 2018). Seeds dried in silica gel require about 20 days to reach approximately 20% MC. Recently, Basrudin et al. (2019) reported that the seeds *C. buruana* Miq. dried in the sunlight after washing reduced viability. Neither the MC measurements nor any desiccation experiments were performed. Apparently, there are no other studies on seed desiccation-sensitivity of *Castanopsis*.

Seed anatomy plays an important role in controlling water loss of Fagaceae species. The pericarp of Fagaceae seeds was sub-divided into three regions (Kaul, 1985; Xia et al., 2012b): (1) the apex; (2) the scar; and (3) the main pericarp (the remaining area between the apex and the scar region). Using nine *Quercus* species, Xia et al. (2012b) reported that the water loss occurs (only) through the scar during

desiccation in many -but not all- species, and the main pericarp being impermeable to water, protects the embryo axis by not allowing the water to leave seeds. In most *Quercus* seeds, the embryo axis is located on the opposite side of the scar (Bonner and Vozzo, 1987; Finch-Savage and Clay, 1994). Water loss occurs only through the scar and will dry the cotyledons and keep the embryo in a hydrated state for a long-time (Finch-Savage and Blake, 1994). Consequently, this mechanism could protect the seeds under ecological drying due to drought. As such, embryo axis desiccation is avoided until the whole seed cotyledons dries out. Therefore, the embryo position in *Quercus* aids in survival of seeds during extreme drying conditions. Chen et al. (2000) showed longitudinal section diagrams of the representative seeds/fruits of five genera of Fagaceae, including *Castanea mollissima* BL., *Castanopsis faberi* Hance, *Cyclobalanopsis pachyloma* (Seemen) Schottky, *Fagus longipetiolata* Seem., *Lithocarpus glaber* (Thunb.) Nakai and *Quercus aliena* var. *acutiserrata* Maximowicz ex Wenzig. The embryo axes of these six species are located at the apex region. As such, it can be expected that they are protected against drying stress during desiccation in the field. However, whether a similar seed anatomy is found across other species of Fagaceae is currently unknown and worth considering for further investigation.

Castanopsis sclerophylla is distributed in southern and eastern subtropical forests of China and is one of the dominant species of evergreen broad-leaved forest (Huang et al., 1999). It is an important economic tree providing valuable timber for construction and water-soil maintenance. Further, the seeds of *C. sclerophylla* are rich in starch, and consumed as food (Xin et al., 2007). However, the population is shrinking with poor establishment of new trees and expansion of agricultural land area adds further pressure for regeneration. Clearly, we know little about the physiological and morphological changes during desiccation and nothing is known about the desiccation tolerance ability of seeds of this species.

X-ray computed tomography (CT) is a non-invasive approach that provides 3-D visualization and can quantify biological structures (Foucat et al., 1993; Dell'Aquila, 2007; ISTA, 2020). In *Syagrus flexuosa* fruits, Stuppy et al. (2003) used high-resolution X-ray computed tomography (HRCT) to clearly distinguish all morphologically relevant parts and organs such as the endocarp with fibrovascular bundles and endosperm with embryo. Kunishima et al. (2020) used a laboratory-based X-ray microscope to study the 3D-structure of pansy (*Viola × wittrockiana*) seeds without any pretreatment. Here, *C. sclerophylla* endemic to China was selected as a species of the genus *Castanopsis* and the first objective of this study was to define the germination characteristics and drying pattern of *C. sclerophylla* seeds. In particular, we wanted to investigate seed desiccation tolerance. The second objective was to use CT to scan the whole seed without any treatment, to view the internal structure of seeds and better understand the implications of the morphological structure of seeds for drying. This study provides the description of the anatomical features of *C. sclerophylla* seeds for the first time through CT and anatomical observation, so as to improve the lack of anatomical studies on seeds of *Castanopsis* species.

Materials and Methods

Fully matured fruits (acorns; but hereafter referred to as seeds) of *C. sclerophylla* were collected which was the natural dispersal time during the last week of November 2018 from 11 trees in Thousand Island Lakes (TIL) of Zhejiang province, China (29°22" – 29°50" N, 118°34" – 119°15" E). Previous field trips showed only a handful number of trees were capable of bearing fruits, therefore only a minimum amount of seeds were collected so that the natural regeneration process is unaltered. The seed collection region of TIL has a temperate climate with a mean annual temperature of 17.6 °C between 2008 and

2017, ranging from 5.4 °C in January to 29 °C in July. The study site receives an annual rainfall of 1637.46 mm, with peak rainfall occurring between March and June. After collection, the seeds were placed in plastic bags containing holes and brought to the University of Shanghai for Science and Technology, Shanghai, China, within three days. After arrival, any insect infested seeds were removed by visual inspection. Seeds were surface sterilized by soaking in sodium hypochlorite (NaOCl) solution for 30 minutes and washed in sterile water thrice. The seeds were stored at 4 °C until used in the experiments in plastic bags with holes for gas exchange. Seeds were used in the subsequent experiments within 5 days after collection.

The average weight of 100 seeds was determined by measuring the weight of three replicates of 100 seeds using a standard balance (0.001 g). MC was determined by drying three replicates of five seeds at 103°C for 17 hours in a standard oven drying method, following the methods recommended by the International Seed Testing Association (ISTA, 2020). All MC are expressed on a f. wb.

Given limited seed availability, germination tests were conducted using four replicates of 10 seeds. Many previous studies have used similar, if not, lower number of seeds for germination assessment, due to the recalcitrant nature of Fagaceae species (Pritchard, 1991; Xia et al., 2012a; Xia et al., 2012b; Xia et al., 2015). All the germination tests in this study employed incubating seeds on 1% agar-water in closed plastic boxes with small holes (l × b × w : 154 × 95 × 45 mm) at 15/20°C (12 hours dark / 12 hours light of 60 μ mol m⁻²s⁻¹ light provided by cool tungsten filament bulbs available in the germination chamber). Light was supplied only during the warm phase. Seed germination was defined as radicle emergence by at least 2mm and counted weekly until no germination for three consecutive weeks.

Our preliminary trials showed that seeds of *C. sclerophylla* dried slowly and lost viability at around 25% MC. Therefore, to properly assess the seed responses to desiccation, a total of 275 seeds were

grouped into five sets each containing 55 randomly selected seeds and dried separately using freshly regenerated silica gel (2-4 mesh) in a 5:1 ratio by weight. Seeds and silica gel were mixed, sealed in five different airtight polythene boxes ($l \times b \times w$: $170 \times 115 \times 45$ mm) and placed at room temperature (22 ± 1 °C). After 3, 5, 7, 14 and 21 days, one set of seeds (i.e. one box) each were opened and three replicates of five seeds (15 in total) were used to determine the MC of the seeds. The remaining seeds (four replicates of 10) were used for germination as mentioned above and seeds were photographed at different germination stages with a camera (EOS 60D, Canon, Japan). Germination percentage following drying to various MC were arcsine transformed before analysis and tested for statistical significance ($P < 0.05$) using one-way ANOVA with Duncan test in SPSS, version 21.0.

To determine the morphology, full and cross-sections of the seeds were photographed and the whole seeds were scanned using Industrial Computer Tomography (XTH 225 ICT, Nikon, Japan). The seeds were placed on the CT sample table and moved to the appropriate position. After scanning several times and to improve the clarity of the images, the parameters of CT scan were set as: tube voltage 90 kV, tube current 90 μ A, exposure time 500 ms, and the number of pictures taken 2000 times. Using the built-in image processing software (CT Pro 3D, Nikon, Japan), the imported scanned images were reconstructed in three dimensions (3D) to obtain the CT image of the seed, including the front view, the top view and the side view.

Results

The average 100-seed weight was 134.1 ± 6.8 g. Freshly collected seeds had a MC of $36.2 \pm 1.9\%$ and $92.5 \pm 0.4\%$ germination (Fig. 1). After a 7-day drying period, seed MC decreased to 22.5% and germination decreased significantly ($P < 0.05$) to 27.5% . The viability of seeds dried to 20.7% MC (seeds

dried for 14 days) was 5% and further drying to lower MC resulted in complete loss of viability (Fig. 1). The sharp decline in germination when seeds MC was reduced to c. 20% f. wb. indicates that the seeds are desiccation-sensitive.

The anatomical structure and germination stages of *C. sclerophylla* seed are shown in Figs. 2,3. The pericarp of *C. sclerophylla* is similar to other Fagaceae seeds, such as *Quercus* (Xia et al., 2012b) with three distinguishable regions including (1) the apex; (2) the scar; and (3) main pericarp (Figs. 2,3a). The embryo axis is clearly on the opposite side of the apex and sits just below the scar region (Fig. 2). When the seeds germinate, the embryo axis elongates and grows thicker initially before protruding through the scar, then elongating into cotyledonary petiole and radicle (see Figs. 2,3).

Discussion

Desiccation-sensitive nature of the seeds appears to be a common trait in Fagaceae species across genus. Compared with other desiccation-sensitive seeds of Fagaceae species, e.g. *Q. robur* (Finchsavage, 1992), *Q. coccifera* L., *Q. pubescens* Willd. and *Q. pedunculiflora* K. Koch (Ganatsas and Tsakalimi, 2013), *C. sclerophylla* seeds had a similar desiccation response, showing that the percent germination of seeds decreased with a decline in MC. The seeds lost their viability completely when the MC of the seeds dropped to less than 20% in 2-3 weeks. In particular, the result is consistent with drying rates reported for *Quercus* seeds, where a relatively slower drying rate has been observed (Xia et al., 2012a). Clearly, the seeds of *C. sclerophylla* required 15-25 days to reach MC level of 20%, which is lethal to seeds when dried in silica gel. However, Tian and Tang (2010) reported that *C. fissa* and *Q. fabri* seeds can be dried to approximately 20% MC within 72 hours, which is in marked contrast with our results. This is not surprising, as we found in our earlier work (Li et al., 2018) that *Q. fabri* seeds required a longer drying period of at least 20 days to reach approximately 20% when dried in a similar ratio of

silica gel used in the present study, which is in agreement with Xia et al. (2012b). The contradicting result on the drying rate could be attributed to the differences in the drying method. Whether or not the seeds were dried in a sealed environment and the amount of silica gel used was not provided by Tian and Tang (2010). It must be noted, however, that the silica gel provided a very dry environment that can be only created *ex-situ*, the natural drying rates might be slower and depend on the environmental relative humidity and temperature.

Xia et al. (2012b) found that water loss during seeds desiccation of *Quercus* species occurs only through the scar instead of the main pericarp or the apex in some species including *Q. nuttallii*, *Q. suber* and *Q. palustris*. Xia et al. (2012a) showed that the MC of *Q. franchetii* only reached 23% after 164 days of drying in silica gel. For this species, a comparatively small scar (ca. 6% of the pericarp area) might be the mechanistic basis of limited water loss through the pericarp (Xia et al., 2012b). For *Q. suber*, Eduardo and Belén (2000) studied the morphological and micro-morphological differences of the scar and main pericarp. The main pericarp provides a protective barrier that decreases the rate of water loss, and it mainly consists of a layer of epidermal wax, a thick cuticle covering the epidermal layer and a single layer of palisade cells. However, the scar lacks this protective structure, which consists of a vascular strand presumably facilitating moisture loss. Thus, this feature was reported to have ecological advantage, because the embryos of *Quercus* seeds cannot reach lower water content during brief exposure to drought conditions in natural environment.

While investigating the route of water loss in *C. sclerophylla* might be arguably an important area worth exploring, we argue the results of photomicrographs and CT scan suggest otherwise. In all the Fagaceae species, the positioning of embryo on the apex side of the acorns tends to be a common feature (Chen et al. 2000). As such it can be expected that the embryo axes of these species are protected against

desiccation. However, in *C. sclerophylla*, the embryo axis is clearly on the opposite side of the apex and sits just below the scar region (Fig. 2). This is in marked contrast with the *Quercus* embryo axis, which is located on the apex side and far away from the scar (Finch-Savage and Blake, 1994; Xia et al., 2012b). Thus, during desiccation regardless whether water loss only occurs through scar or intact pericarp, the embryo axis of *C. sclerophylla* seeds will be the first part within the seeds to lose water due to its position and continuous drying may kill the seeds. However, our drying results indicate that *C. sclerophylla* seeds lose viability only when the MC drops to c. 20% f.wb.

There appears to be a strong difference in the germination pattern between *Quercus* and *C. sclerophylla* seeds. Notably, in *C. sclerophylla* seeds, the radicle protrudes through the scar during germination (see Figs. 2,3), while for *Quercus* seeds, the radicle protrudes through the apex region (Bonner and Vozzo, 1987; Löf et al., 2019). It is possible to suggest that this difference could be due to the position of the embryo axis between these two genera. However, this requires detailed investigation given that seeds of many *Quercus* species have epicotyl physiological dormancy (Farmer Jr, 1977; Baskin and Baskin, 2014).

In conclusion, like most *Quercus* species, seeds of *C. sclerophylla* are desiccation-sensitive and lose viability only when the MC drops to c. 20% f. wb. Drying rate is similar to those reported for *Quercus*, indicating high similarity between the genus. For Fagaceae seeds, studies have been restricted to pericarp anatomy and water loss rate on different regions of the seed coat (Eduardo and Belén, 2000; Xia et al., 2012b). Through CT and other observations, we found that position of the embryo axis in *C. sclerophylla* is different from that of *Quercus* or other genera reported thus far within Fagaceae. Despite this, the seeds showed a similar pattern of desiccation-sensitivity during drying, indicating that the route of water loss may be less important in *C. sclerophylla* and other species with the same anatomical

characteristics. However, whether this particular position of seeds embryo axis is common within *Castanopsis* genus has not been thoroughly studied and should form a basis for further study.

Declarations

The authors declare no human subjects were involved in this study. Neither any endangered nor any species that are threatened were studied. Funding authorities have no role in experimental design, writing of manuscript or submission. Authors maintain sole responsibility for the paper.

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

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342 **Figure legends**

343 Figure 1. Effect of silica gel drying on germination of *Castanopsis sclerophylla* seeds. Different lower-
344 case letters indicate significant difference in germination between seeds dried to various moisture
345 contents ($P < 0.05$). Error bars represent standard deviation of the mean.

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347 Figure 2. Photomicrographs and CT images of *Castanopsis sclerophylla* seeds (a) half cotyledon with
348 embryo axis; (b) growth of the embryo axis; (c) top view; (d) front view. SC, seed coat; COT, cotyledons;
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352 Figure 3. Photographs of *Castanopsis sclerophylla* seeds at different stagess of germination (a) whole
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Figure 1. Effect of silica gel drying on germination of *Castanopsis sclerophylla* seeds. Different lower-case letters indicate significant difference in germination between seeds dried to various moisture contents ($P < 0.05$). Error bars represent standard deviation of the mean.

254x180mm (300 x 300 DPI)

Figure 2. Photomicrographs and CT images of *Castanopsis sclerophylla* seeds (a) half cotyledon with embryo axis; (b) growth of the embryo axis; (c) top view; (d) front view. SC, seed coat; COT, cotyledons; EMB, embryo axis.

691x691mm (72 x 72 DPI)

Figure 3. Photographs of *Castanopsis sclerophylla* seeds at different stages of germination (a) whole seed; (b) 0h; (c) 48h; (d) 96h; (e) 144h; (f) 240h. SC, seed coat; SR, scar; AP, apex; R, radicle, COT, cotyledons; S, stem; CP, cotyledonary petiole.

391x258mm (72 x 72 DPI)