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Relationship between seed moisture content and acquisition of impermeability in *Nelumbo nucifera* (Nelumbonaceae)

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ABSTRACT

Seeds of *Nelumbo nucifera* do not imbibe water, and thus have physical dormancy (PY). However, a proportion of seeds are permeable to water, and so we hypothesized that variation in moisture content is a reason for the development of both permeable and impermeable seeds. The permeable proportion of seeds present in a lot collected from Suzhou, China, was separated using an imbibition test. The permeable proportion had an average moisture content of 15.6 %, compared with 8.5 % for impermeable seeds. Drying permeable seeds above silica gel to 10 % and 8 % f. wb., resulted in 77 and 100 % impermeable seeds, respectively, compared with no impermeable seeds at 15 % moisture content. Dried to 10 % moisture content, and incubated above water in an airtight container, 46 % of the seeds reverse impermeability. Permeable seeds with 15 % moisture content maintained above $LiCl^2$ (RH=70 %) did not develop impermeability after three months of storage. The seeds dried to 6 % moisture content and stored above water in an airtight container showed no increase in moisture. Based on these results, we conclude that there is a strong relationship between moisture content and the onset of impermeability in this species.

Keywords: dormancy reversal, maternal environment, maturation drying, moisture content, physical dormancy

Introduction

Seed/fruit coats of many species belonging to several - but not all- genera of 18 angiosperm families become impermeable to water during maturation drying, i.e. they have physical dormancy (PY) (Baskin & Baskin 2014). The impermeable nature of the seed/fruit coats is due to the palisade layer present in the seed coat preventing water reaching internal structures (Baskin *et al*. 2000). Numerous studies on seed development have shown that the transition from a permeable to impermeable seed coat coincides with the decline in moisture content during the maturation drying phase of seed development (Jaganathan 2016). Indeed, it has been observed in a few species that the seed coat becomes impermeable only when the moisture content of the seeds falls to a specific threshold level (Hyde 1954; Gladstones 1958; Egley 1979; Chinnasamy & Bal 2003; Hay *et al*. 2010; Gama-Arachchige *et al*. 2011; Gresta *et al*. 2011). Thus, the number of seeds with impermeable seed coats produced by plants may vary between sites or within years based on the moisture content reached during maturation drying, which is affected by the environmental conditions including temperature and relative humidity. By producing a mixture of both permeable and impermeable seeds, a species can spread the germination across several growing seasons, maximizing the ability to colonize in natural environment.

The most common perspective is that PY is an irreversible trait, meaning that the seed coat once it

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becomes impermeable cannot cycle back to permeable state; unless the seed coat is ruptured or specific structure present in the seed coat called as 'water gap' (e.g. lens in Fabaceae) opens allowing the water to hydrate the internal structures (Baskin & Baskin 2014). However, there are some studies describing the reversibility of an impermeable seed coat to permeable state without breaking dormancy. For example, in *Trifolium ambiguum*, seeds with 12.2 % moisture content were impermeable to water, but the subsequent exposure of seeds to higher relative humidity increased the moisture content of the seeds, thus reversed PY (Hay *et al*. 2010). Jaganathan (2016) suggested that during maturation drying, during the continuous decrease in moisture content seeds will reach the moisture level for the onset of impermeability in those species that develop impermeable seed coats. Seeds at this moisture content are in a transition state (i.e. shallow dormancy); thus the subsequent maintenance of impermeability or loss depends on the relative humidity to which these seeds are present. If the external humidity is high, the seeds may become permeable again, whereas under continued drying the seed coat is permanently sealed, thus the seeds develop 'absolute dormancy'. However, the importance of moisture content in the development of an impermeable seed coat and the possibility of dormancy reversal have not been rigorously investigated.

It is reasonable to hypothesize that drying plays an important role in PY development, irrespective of life-forms and environment where the plants grow because drying exerts a pressure on moisture content decline and species with a known history of developing impermeable seed coats, would become impermeable after enough drying (Jaganathan *et al*. 2017). However, this generalization is somewhat counterintuitive because most of the species investigated hitherto are seeds of Fabaceae and one species in Geraniaceae. In order to advance our understanding of this knowledge gap, we chose to study the acquisition of dormancy in *Nelumbo nucifera*, which is an aquatic herbaceous perennial plant belonging to the family Nelumbonaceae (Tian *et al*. 2009). Although this species originated in the eastern part of Asia and the northern part of Australia, it has been cultivated for centuries in many countries including China and India, leading to a wide geographical distribution (Masuda *et al*. 2006). The species can reproduce both by seeds and rhizomes, which is also cultivated for food. The Nelumbonaceae is a basal eudicot small family comprising only two species of Nelumbo, both have PY (Gama-Arachchige *et al*. 2013). Thus our intention here is not to document the PY in this species, rather the specific goals of this study were to (1) identify if there is a critical moisture range at which the seed coat become impermeable and (2) understand if the permeable proportion of seeds were not as dry as impermeable counterparts resulting in a mixture of permeable and impermeable seeds in the lot.

Materials and methods

Seed collection

Seeds of *Nelumbo nucifera* Gaertn. were collected from a lake full of adult plants growing in Suzhou, China (31°3'N 120°6'E) in May 2015. The average annual temperature of the collection site was 15-17 °C. The warmest months are July to August and lowest temperature occurs during January. The average rainfall is over 1000 mm. After collection, the seeds were shipped to University of Shanghai for Science and Technology, Shanghai, China. Seeds were stored at room temperature (approximately 20 °C and 50-60 % RH) in jute bags until used in the laboratory experiments. All the experiments began within three days of seed collection.

Seed weight and moisture content

The average weight of 100 seeds (five replicates) was measured using a digital balance by randomly picking seeds from the lot and the average values are presented. Seed moisture content of the seeds on receipt was determined gravimetrically by drying three replicates of 15 seeds in a 103 °C oven for 17 h. Moisture content of the three replicates is expressed as an average of percentage of fresh weight (f. wb.).

Separation of permeable and impermeable seeds

Since the materials collected contained a mixture of permeable and impermeable seeds as observed in preliminary imbibition tests, the permeable proportion of seeds was separated from impermeable counterparts in the lot by placing seeds in sandwich boxes on wet filter paper and kept under laboratory conditions (~20-22 ºC). The seeds that were permeable began to swell within 12 hours and these seeds were handpicked and allowed to dry under laboratory conditions by spreading them on a bench. After 24 hours of bench drying, the moisture contents of the seeds were determined as described above.

Identifying specific moisture content inducing impermeability

To test the hypothesis that the permeable proportion of seeds were not as dry as impermeable counterparts, the moisture content of the permeable and impermeable groups was tested as described above. In order to understand the relationship between moisture content and seed coat impermeability, aliquots of permeable seeds were dried over silica gel (4:1 to seed) in an air tight container. The silica gel was replenished every 12 hours. Five replicates of 25 seeds were removed after every 8 h up until 40 hours, four of which were used for imbibition test and the remaining 25 seeds were tested for moisture content in five replicates of 5 as described above. In addition, four replicates of fifty permeable seeds were held above a saturated solution ($LiCl_2$) which generated a relative humidity of 70 % to determine whether these would become impermeable during storage. These seeds were tested for permeability after three months of storage.

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A further sample of permeable seeds dried above silica gel for 32 hours (by which time the moisture content dropped to just below 10 % and seeds became impermeable) were subsequently moved into air tight jars and held over water, and incubated at 20 °C for 1 week. At the end of storage time, the moisture content of the seeds and ability of the seeds to imbibe were determined. In parallel experiments, permeable seeds dried above silica gel for 24 hours were subsequently placed in wet medium to absorb water. After 24 hours in a wet medium, the seeds were either dried to lower moisture level by placing them over silica gel in an airtight container or the drying and wetting was repeated (for 12 hours with moisture content reaching 27 %) 10 times and then dried to lower moisture level (12 %). All the experiments were conducted using four replicates of 25 randomly chosen seeds for imbibition and three replicates of five seeds for moisture content determination.

To determine the effects of high ambient humidity on moisture increase, permeable seeds of *N. nucifera* dried to ~6 % moisture (112 hours above silica gel) were incubated above water in an airtight container. The moisture content of the seeds was determined daily for one week using three replicates of ten seeds at each sampling point, as described above.

Data analysis

Data were tested for statistical significance using one-way analysis of variance (ANOVA) in SPSS, version 21.0. We used LSD post-hoc test to determine the difference between groups. Whenever needed, data were arcsine transformed to improve the normality, but original values are reported.

Results

Seed characteristics

The seeds of *N. nucifera* at the time of collection were pale to dark black in color. The average weight of 100 seed weight was 113.27 ± 0.9 g. The moisture content of the seeds at the time of collection was 11.1 ± 0.7 %. On average, 73 % of the seeds were impermeable to water.

Separation of permeable and impermeable seeds

The initial lot contained both permeable and impermeable seeds. In order to separate them, we conducted an imbibition test. During imbibition the permeable seeds became swollen and were handpicked. The moisture content of these seeds after 12 hours imbibition was 25.8 ± 1.6 %. These seeds were bench dried for 24 hours at laboratory conditions and the moisture content after drying was 15.6 ± 0.9 %. In contrast, the moisture content of the impermeable seeds was 8.5 ± 0.5 %.

Identifying specific moisture content inducing impermeability

Permeable seeds of *N. nucifera* with a moisture content of around 15 % f. wb. dried above silica gel slowly lost moisture, reaching 8 % after 40 hours (Fig. 1). However, it took almost 72 hours for the seeds to reach 5.9 % moisture content (data not shown). Seeds developed impermeability when the moisture content dropped to 10 %. At 10 % moisture content, 77 ± 3.4 % of the seeds failed to imbibe water. When the seeds with 8 % moisture content were tested for permeability, none of them imbibed water (Fig. 1).

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After incubating impermeable seeds with 10 % moisture content above water in an airtight container, 46 ± 6.1 % of the seeds became permeable, whilst 54 ± 6.1 % remained impermeable (Tab. 1). No impermeable seed dried to 8 % MC and incubated above water became permeable (Tab. 1). Seeds subjected to continuous wet-dry cycles with the moisture content maintained above 10 % did not become

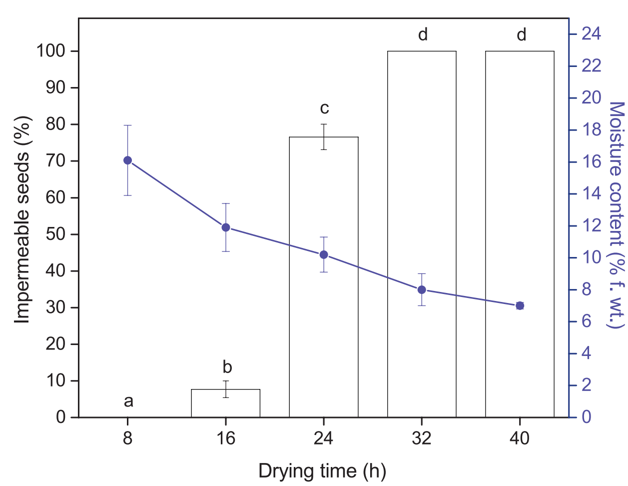

Figure 1. The proportion of impermeable *Nelumbo nucifera* seeds at different moisture contents. Different letters indicate a statistically significant difference in the number of impermeable seeds at the different moisture contents. Error bars indicate the standard deviation of the mean.

Table 1. Effect of different treatments on development of different proportion of impermeable and permeable *Nelumbo nucifera* seeds.

Treatment	Impermeable seeds (%)	Permeable seeds (%)
Impermeable seeds (10 % MC) incubated above water for one week	54 ± 6.1	46 ± 6.1
Impermeable seeds (8 % MC) incubated above water for one week	100	0
Seeds allowed to wet-dry above 10 % MC	0	100
Seeds allowed to wet-dry above 10 % MC and dried to 8 % MC	100	0
Permeable seeds (16 % MC) incubated above $LiCl_2$ (RH=70 %) for three months	0	100

impermeable. (Tab. 1). Drying the seeds that had already undergone 10 wet-dry cycles to 8 % resulted in all the seed becoming impermeable (Tab. 1).

Seeds with permeable seed coats held above a $LiCl_2$ solution retained permeability during three months of storage. At the end of storage, drying to a 10 % moisture content range resulted in impermeability developing in 82 ± 3.6 % of the seeds. Further drying to 8 % moisture content resulted in 100 % impermeable seeds.

When the permeable seeds dried to 6 % moisture content were incubated above water, there was a small increase in the moisture content during the one-week of storage, but this was not statistically significant (P >0.05; Fig. 2).

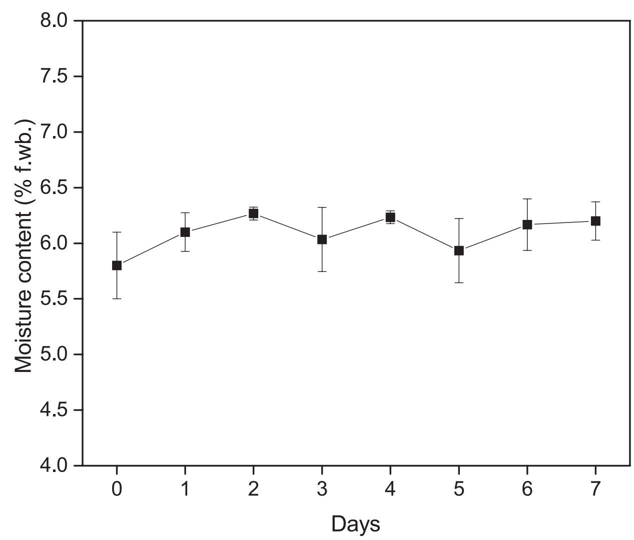

Figure 2. The moisture content of seeds dried to 5.9 % and incubated above water for one week. The measurements were made on three replicates of 10 seeds at daily interval. Data presented are the means ± s.d of the three replicates for each day.

Discussion

Many studies have recognized that seed coats become impermeable during the maturation drying phase of development, during which the seeds began to lose moisture content to the level that could onset impermeability, e.g. 15 % in *Peltophorum pterocarpum* (Mai-Hong *et al*. 2003), 12 % in *Gleditsia triacanthos* (Geneve 2009), *G. aquatica* (Geneve 2009), *Gymnocladus dioicus* (Geneve 2009), *Lupinus arboreus* (Hyde 1954), *Trifolium ambiguum* (Hay *et al*. 2010), *T. pretense* (Hyde 1954) and *T. repens* (Hyde 1954), 11 % in *L. digitatus* (Gladstones 1958) and *Geranium carolinianum* (Gama-Arachchige *et al*. 2011). However, the reason why some seeds develop impermeable coats, while others maturing on the same plant produce permeable seed coats remains unclear. In our study, we found that impermeable seeds had lower moisture content compared with permeable counterparts. Drying permeable seeds with 15 % moisture content above silica gel to 10 % moisture content resulted in them becoming impermeable, indicating that the permeable proportion of seeds in the lot had not dried to the level at which impermeability was induced (Fig. 1). There are numerous explanations for the insufficient drying of seeds in the field including (1) maternal environment having higher relative humidity during seed development (D'hondt *et al*. 2010; Hudson *et al*. 2015); (2) position of seeds in inflorescence possibly under leaf cover preventing direct exposure to sunlight resulting in inadequate drying (Taylor & Palmer 1979; Hay *et al*. 2010); (3) age of mother-plant (Baskin & Baskin 2014) and (4) maturation stage at which the seeds were collected (Egley 1979; Baskin *et al*. 2004; Jaganathan & Liu 2014). Whatever the case, our results unequivocally illustrate that seeds of *N. nucifera* become impermeable only when moisture content drops to 10 %. This moisture content as the threshold point for the development of impermeability is close to the levels reported in other species (see introduction). The results of the present and previous studies suggest a strong relationship between moisture content and the development of impermeability in seeds.

Color of the coat reflected the maturity status of the seeds, as also observed in other physical dormant species (Hay *et al*. 2010). In general, seeds with dark black colored coat were impermeable compared with pale black color group which were mostly impermeable. Drying the pale colored group above silica gel resulted in seed coat color change, which coincided with the induction of impermeability.

Our studies on *N. nucifera* further show that the impermeability of seeds at 10% moisture content can be reversed when the seeds are exposed to high ambient relative humidity. This exposure ultimately increases the moisture content of the seeds. This supports the previous findings that impermeability can be reversed after seeds became impermeable as reported (Hay *et al*. 2010) in Trifolium ambiguum and (Gladstones 1958) in *Lupinus digitatus*. However, the moisture content reported in those species and in N. nucifera at which the dormancy can be reversed varies, indicating that there might be species-specific moisture thresholds at which reversal occurs. Furthermore, our finding of dormancy reversal in *N. nucifera* stems from the experiment that impermeable seeds at 10 % moisture content were exposed to 100 % relative humidity. This condition does not occur in natural environments, suggesting the reversal mechanisms reported here and in previous studies might be more of an empirical occurrence. In addition, we observed that the seeds of *N. nucifera* dried to 8 % moisture content followed by exposure to high relative humidity environment did not show a reverse in impermeability. Interestingly, not all the seeds equilibrated to 10 % moisture content and subsequently incubated at higher relative humidity developed impermeable seed coat (Tab. 1). This can be explained by seed-to-seed variation in moisture content (also see Hay *et al*. 2010). We presume the seeds that did not reverse impermeability had been dried to lower levels. The standard deviation of the data suggests that there was seed-to-seed variation (Fig. 1, Tab. 1).

There appears to be at least two limitations with this study that merits some discussion. First, the moisture content determination of permeable and impermeable proportion of seeds. There is no easy way to separate permeable and impermeable proportion of seeds, unless the seeds are kept on moist substrate and allowed to imbibe. Under such conditions, only permeable seeds would swell and this method has been used previously to separate permeable and impermeable proportions (Paulsen *et al*. 2013). Because permeable seeds can only be separated after they become hydrated, there is no reasonable estimate of the original moisture content of permeable seeds. However, we believe the impermeability in these permeable seeds is tightly controlled by the moisture content, thus we rule out both permeable and impermeable proportions had same moisture content at the time of collection. Indeed, in a preliminary experiment we found that seeds with moisture content 11.1 ± 0.7 % containing 73 % and 24 % permeable and impermeable seeds respectively were dried to 9 %, all of them became impermeable. As such, this supports the fact that hydration to the level used in this study (12 hours on wet substrate) does not change anything in the seed coat leading to impermeability. Second, seed-to-seed variation in moisture content can be determined accurately if the moisture content of the individual seed was established gravimetrically (Hay *et al*. 2010). However, given the destructive nature of this method, determining permeability of individual seeds after moisture content estimation is not feasible. Further, it is often a desirable practice to extrapolate the moisture content of seeds present in the lot from the estimates made on random proportion of sub-samples. Thus, the experimental procedures we used here tend to be the most practically suitable approach, despite the inability to predict the accurate moisture content to its decimal level at which seeds of *N. nucifera* become impermeable.

Hyde (1954) suggested that the hilum present in the seeds of *Trifolium repens*, *T. pratense* and *Lupinus arboreus* acts as a 'one-way' valve for the diffusion of water from internal structures of seeds. This means that after the seeds become impermeable they can only lose more water from inside the seed by diffusion to an external environment at a lower relative humidity. In contrast, water vapor present in higher relative humidity external environment does not diffuse through hilum and enter internal structures of seeds. Thus, despite higher humidity environment, the dry seeds would remain dry and these seeds come to equilibrium with the lowest external humidity they are exposed to and remain at that equilibrium even if exposed to a higher relative humidity environment. The failure of water absorption in seeds dried to 6 % moisture range and then incubated above water (RH=100 %) for one week suggests a similar mechanism is likely operating in *N. nucifera* seeds. The small increase in moisture content to 6.2 % compared with 5.9 % at the time of incubation might be due to the water accumulating in seed coats (Fig. 1). Further, this result partly elucidates the reason why no seeds dried to lower moisture content reverse impermeability. More future studies are required on the anatomy and structural changes of seed coat during permeable to impermeable transformation.

When the permeable seeds of *N. nucifera* at a moisture of around 15 % were kept in a higher humidity environment these seeds do not develop impermeability even after three months of storage. This is consistent with the results reported previously in *Ornithopus compressus* (Barrett-Lennard & Gladstones 1964) and *Phaseolus vulgaris* (Hopkins *et al*. 1947). D'hondt *et al*. (2010) reported that plants of *Trifolium repens* matured in higher humidity region produced more permeable seeds compared with less number of permeable seeds at low humidity environment, suggesting the importance of relative humidity in drying seeds, thus the permeability. In addition, our data showed that as long as the moisture content remains above 10 %, the seeds of *N. nucifera* did not induce coat impermeability even after repeated imbibition and drying. However, if the moisture content drops to 10 % the seed coat becomes impermeable. Thus, the number of impermeable seeds produced by plants may vary based on the environmental conditions prevailing during maturation drying and further the permeable seeds can become impermeable after dispersed from mother plants when the environmental conditions are dry. One adaptive significance of this variation in seed morphs produced by plants may be to spread the germination over many years with permeable seeds germinating soon after germination, whilst impermeable seeds delay germination to latter years.

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