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SHORT COMMUNICATION

# Scanning electron microscopy reveals contrasting effects of liquid nitrogen on seeds of legumes *Neonotonia wightii*, *Phaseolus vulgaris* and *Tamarindus indica*

**Yanier Acosta[1] · Barbarita Companioni[2] · Doris Escalante[1] · Byron E. Zevallos–Bravo[3] · Lisbet Pérez-Bonachea[1] · Pawel Chmielarz[4] · Elliosha Hajari[5] · Christoph Neinhuis[6] · Michael Melzer[7] · José Carlos Lorenzo[1]**

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## Abstract

Cryopreservation remains the technology of choice for the long-term preservation of plant germplasm. The current contribution reports on the response of seeds of *N. wightii*, *P. vulgaris* and *T. indica* to cryopreservation in terms of plantlet survival post cryostorage as well as examination of the external morphology of seed coats using scanning electron microscopy (SEM). Survival was determined in Petri dishes in the laboratory as well as in the soil. The results showed differential responses in seeds of the three tested species. In the case of *P. vulgaris*, exposure to liquid nitrogen (LN) did not adversely affect seedling emergence or characteristics of the seed coat. For *N. wightii* and *T. indica*, cracks in the seed coat that were apparent in control seeds, appeared more frequently following exposure to LN. In the case of the former species, this observation did not yield adverse consequences and seed germination rate did actually increase from 5.8 to 85.9% after LN treatment. However, in the case of *T. indica*, the initial growth rate of seedlings was delayed relative to the control although the germination rate was improved. It is postulated that seeds of *T. indica* possibly incurred additional damage to other seed components which might have led to delayed recovery.

**Keywords** Germination · Plantlet emergence · Seed coat · Testa break · Testa cracks

## Introduction

LN is used for the cryopreservation of plant and animal samples due to its ability to maintain stable, ultra-low temperatures. In this context, LN poses several advantages for the stable storage of plant germplasm (Chmielarz 2009, 2010). Seed viability is maintained in LN as the formation of harmful ice crystals within seeds is minimized (given that the hydration status of cells is sufficiently low). Preservation in LN also delays the natural process of seed aging. Exposure to LN temperatures results in a significant reduction in seed metabolic processes, and as a consequence, aging is delayed. This allows for seeds to be stored for extended periods of time without viability loss (Doijode 2012). Seeds are also protected against pathogens and pests when stored in LN, since they are not only physically excluded from exposure to such pathogens in the natural environment but also the growth of microorganisms is inhibited at ultra-low temperatures (Panis et al. 2020). Storage in LN also facilitates simple maintenance and transport of germplasm, as simple equipment (LN freezers) allows for storage of large quantities of

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✉ José Carlos Lorenzo
lorenzojosecarlos68@gmail.com

1 Department for Plant Breeding and Conservation of Genetic Resources, Bioplant Center, University of Ciego de Ávila Máximo Gómez Báez, Ciego de Ávila, Cuba

2 Universidad Autónoma Agraria Antonio Narro, Coahuila, México

3 Universidad Estatal del Sur de Manabí (UNESUM), Jipijapa, Ecuador

4 Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland

5 Plant Improvement, Agricultural Research Council-Tropical and Subtropical Crops, Private Bag X11208, Nelspruit 1200, South Africa

6 Faculty of Biology, Technische Universität Dresden, 01062 Dresden, Germany

7 Leibniz Institute of Plant Genetics and Crop Plant Research, IPK, Gatersleben, Germany

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seed without the need for complicated equipment or large cold rooms. Furthermore, seeds can be readily transported in sealed containers with LN for ease of movement between locations while maintaining cryogenic temperatures (Pence 2011).

It is critical to ensure that seeds are at the correct hydration status (water content) before immersion into the cryogen. If the water content of seeds is too high, the result will be the rapid formation of ice crystals at ultra-low temperatures which may cause lethal mechanical and structural damage to cells and organelles ultimately leading to viability loss and eventually death (Pammenter and Berjak 2013). Conversely, if seeds are excessively dehydrated either during the preparative stages prior to exposure to the cryogen or if cooling rates are too slow and exposed for an extended period of time (leading to freeze-induced dehydration), this can also lead to viability loss (Naderi et al. 2017).

Proper handling and an understanding of the specific requirements of each type of seed (orthodox, intermediate or recalcitrant) are essential to ensure reliable results. Since early cryopreservation studies, much insight has been gained as to how plants respond to the stresses imposed by exposure to ultra low temperatures. One such development noted by our research group has been the categorization of species based on the time taken for seeds to germinate after exposure to LN temperatures relative to control plants.

In this context, three categories of seed responses have been described. The first category comprises of species in which cryopreservation appears to promote seedling germination and subsequent growth as for example in *Neonotonia wightii* (Acosta et al. 2020). The second category includes species that show no apparent stimulatory or inhibitory response to LN treatment, for example, *Phaseolus vulgaris* (Cejas et al. 2012). In the third category species are found that exhibit moderately delayed germination and emergence, as in the case of *Tamarindus indica* (Villalobos-Olivera et al. 2023). This study investigated the extent of damage to the seed coat, germination, conversion into plants and early growth of three important legumes (neonotonia, common bean and tamarind) after exposure to LN.

## Materials and methods

### Seed collection and storage before exposure to LN

Seeds of *Neonotonia wightii* Wigth and Am (cv. Tinaroo), *Phaseolus vulgaris* L. (cv. ICA Pijao) and wild *Tamarindus indica* L. were collected in Ciego de Avila, Cuba (Fig. 1). Seeds were handled according to the recommendations outlined in the manual for seed management in germplasm banks (Rao et al. 2007). The seed moisture contents at harvest (fresh weight basis) were 7.8% for *N. wightii*, 12.3% for *P. vulgaris* and 6.8% for *T. indica*. To measure moisture contents, samples of seeds were weighted at harvest and after drying at 67°C until reaching constant weight. All seeds were stored in hermetically sealed glass containers at 5°C (Baskin and Baskin 2014) until use.

### Seed treatments

The collected seeds of each species (n = 500 true-to-type seeds) were separated into two batches with the first maintained at 4°C (control) and the second exposed to LN. Seeds of both treatments (250 for each species) were placed within tightly closed centrifuge tubes (50 mL) and immersed into LN. After 24 h of immersion in LN, the tubes were removed from the cryogen and allowed to thaw at room temperature (Cardoso et al. 2000).

### Evaluations performed

The following indicators were evaluated in both treatments: scanning electron microscopic (SEM) evaluation of seed coats; percentage of germination in Petri dishes

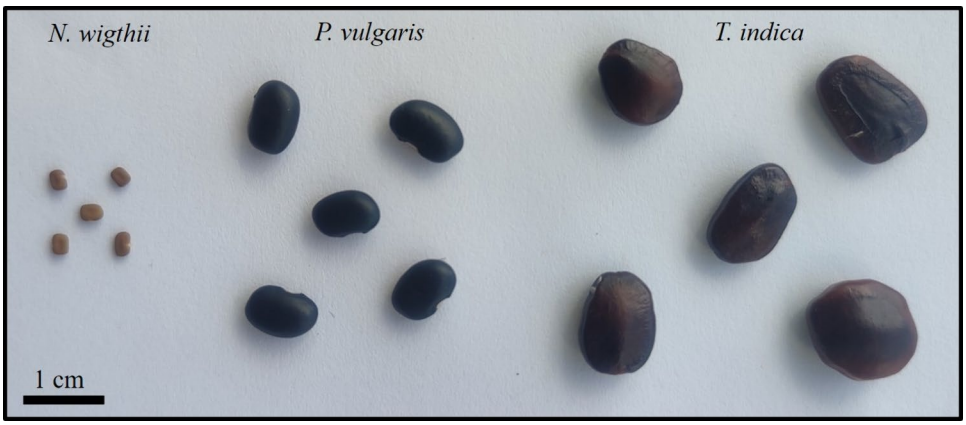


**Fig. 1** Phenotypes of seeds compared

following 14 days of growth; and percentage of plantlet emergence from the soil up to 14 days.

For SEM of seed coats, the seeds of both treatments were air dried and mounted on aluminum stubs with double-sided adhesive carbon pads. Subsequently, the specimens were sputtered with gold (EMITECH K550 Sputter Coater) and examined using a Zeiss Supra 40 VP SEM (Zeiss Germany). The seed coats of 20 cryo-stored and control seeds were then assessed for cracks/breaks and photomicrographs were captured. To quantify the length of cracks and breaks in the seed coat, ImageJ (1.5e) software was used. Seventeen pictures (0.23 mm x 0.15 mm; 0.0345 $mm^2$) were randomly studied per treatment.

For the germination trial in Petri dishes, 100 seeds (4 replicates of 25 seeds each) were placed on filter paper moistened with 5 ml distilled water in a pre-germination chamber (Model, R-TOP-D) with a controlled environment (30 ± 2ºC, 80% relative humidity, in the dark). The water was replenished every 2 days. Germination was recorded at 14 d of growth (radicle ≥ 2 mm).

In a parallel experiment, cryopreserved and non-cryopreserved (control) seeds were placed in pots with 18 g of a mixture containing ferralytic-red soil and humus (1:1, v:v; 33 ± 1 °C; 12/12-h photoperiod; PPFD: 800 µmol $m^{-2}$ $s^{-1}$ at 12:00 PM). Four replicates of 5 seeds each were studied. Pots were irrigated with 25 ml of water daily. Plantlet emergence from soil was recorded up to 14 d of growth.

## Data analysis

The statistical program SPSS (version 20.0) was used to perform two-way ANOVA, Tukey, and *t* tests (completely randomized experiments; $p \leq 0.05$). Normal distribution and homogeneity of variances were also demonstrated according to Kolmogorov–Smirnov (α=0.05) and Levene (α=0.05) tests, respectively.

## Results and discussion

Seeds of *N. wightii*, *P. vulgaris* and *T. indica* were immersed in LN and thereafter the gross external morphology of the seed coats was examined and germination efficiencies of the three species determined. All three species displayed varying seed sizes with *N. wightii* < *P. vulgaris* < *T. indica* (Fig. 1). The SEM analysis revealed characteristic seed coats morphologies in the three species. In the control (non-cryopreserved) seeds of *N. wightii* exhibited a seed coat with numerous ridges and some cracks with an average size of 449.5 µm$^2$ (Fig. 2A) while *T. indica* had a smooth testa with cracks of 208.3 µm$^2$ (Fig. 2E). In contrast, *P. vulgaris* had a smooth seed coat without obvious cracks (Fig. 2C). Following exposure to LN, seeds of *P. vulgaris* remained without cracks (Fig. 2D) while the crack size in the seed coat of *N. wightii* and *T. indica* increased considerably (2 234.9 µm$^2$ and 4 950.0 µm$^2$, respectively) (Fig. 3).

Exposure of seeds to LN had an effect on germination (Petri dishes) in the laboratory only in *N. wightii* and *T.*

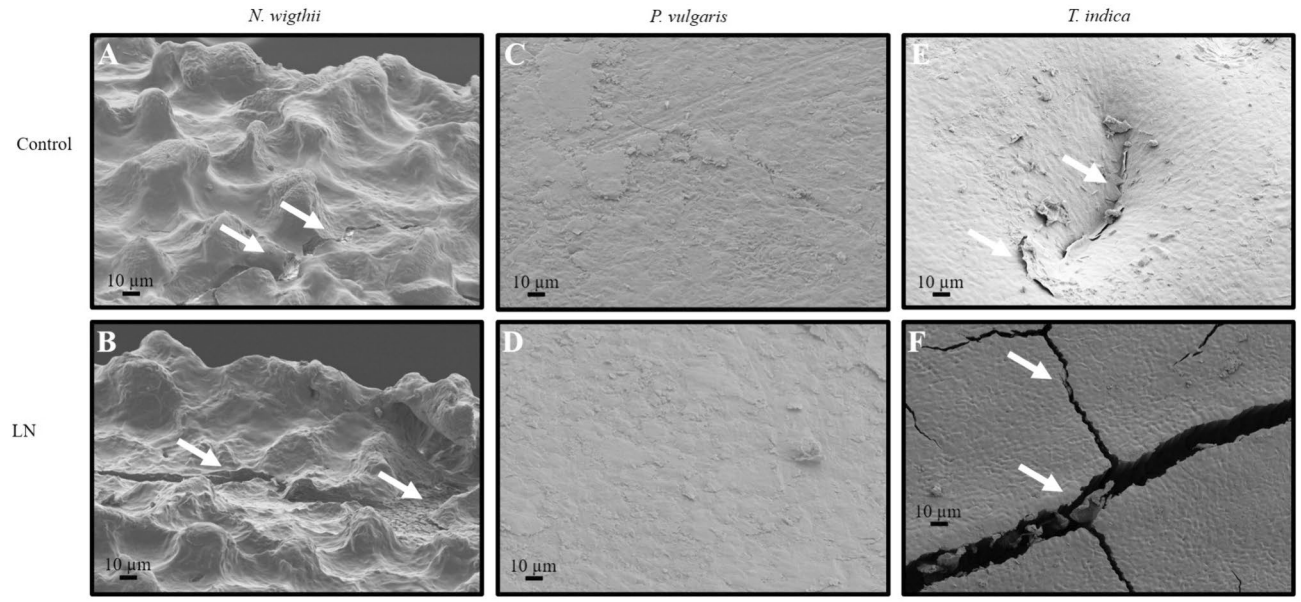


**Fig. 2** Effects of seed exposure to liquid nitrogen (LN) on formation of eracks and breaks (white arrows) in the seed coats. Area of each seed coat evaluated with SEM = 3456.72 µm.$^2$

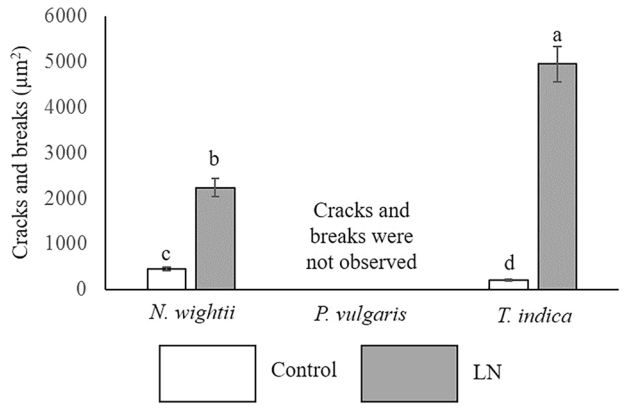


**Fig. 3** Effects of seed exposure to liquid nitrogen (LN) on formation of cracks and breaks. Area of each seed coat evaluated with SEM = 3456.72µm$^2$. Results with same *letter* are not statistically different (Two-Way ANOVA, Tukey, $p > 0.005$). Vertical bars represent ± SE

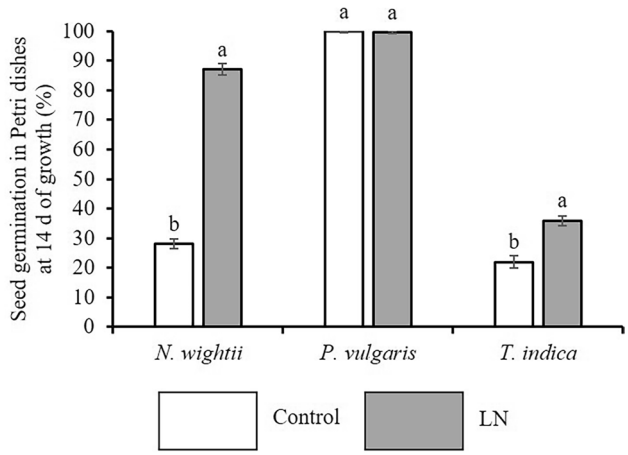


**Fig. 4** Effects of seed exposure to liquid nitrogen (LN) on germination in Petri dishes. In each plant species, results with the same *letter* are not statistically different (t-test, $p > 0.005$). Vertical bars represent ± SE

*indica* where germination was promoted following cryo-exposure in both species (three fold higher in the former and 1.5 fold higher in the latter). Seed germination in *P. vulgaris* was not affected by exposure to LN (100% germination, Fig. 4). When plant development was assessed in the soil, it was apparent that LN exposure did not have an adverse effect on plant height in both *N. wightii* and *P. vulgaris* following 5 d of growth as both control and cryopreserved plants displayed similar heights (Fig. 5A, B—approximately 6 cm and 25 cm, respectively; Fig. 6A). In contrast, LN exposure appeared to delay the emergence of the first true leaves in *T. indica* (Figs. 5C, 6A) as LN treated plants were significantly shorter (7.3 cm) than control plants (9.9 cm). Similar to the Petri dish trial, LN promoted the emergence of *N. wightii* plants in soil from 5.8% (control) to 85.9% (Fig. 6B), however in contrast to the results observed in the Petri dish trial, emergence in *T. indica* seeds was lower after exposure to LN (82.7%) than in the control (98.9%, Fig. 6B). For *P. vulgaris*, LN did not affect emergence of seeds (approximately 95%, Fig. 6B). These results are in agreement with our previous observations (Cejas et al. 2012; Acosta et al. 2020; Villalobos-Olivera et al. 2023).

Collectively, the results indicate contrasting responses of the three species to LN exposure with potentially varying mechanisms at play for each species. As we have reported in our previous studies, LN can have a promoter or inhibitive effect on seedling emergence. Seeds of *P. vulgaris* tolerate exposure to LN with no apparent adverse effects either anatomically (in terms of damage to the testa—Fig. 2C, D) or in terms of survival (Fig. 4). In the case of *N. wightii*, LN appears to increase the size of cracks in the testa, ultimately leading to improved germination relative to non-cryostored seeds (Fig. 2 A,B and 4. It is postulated that the fissures formed in the testa after exposure to LN, might lead to improved imbibition and/or dormancy breaking resulting in higher germination rates. While seeds of *T. indica* also showed larger cracks in the testa after LN treatment (Fig. 2E, F), the observed cracks were approximately 24 times bigger than in the control seeds (4 950.0 µm$^2$ (LN) / 208.3 µm$^2$ (control)). There appeared to be some impact on the ability of seeds to recover. Unlike *N. wightii,* which showed improved germination following cryo-exposure, the results for *T. indica* were somewhat contradictory showing higher germination in cryo-stored seeds than in the control in Petri dishes but the reverse trend was observed after sawing in soil. The varying conditions in these two experiments might have contributed to the results obtained (i.e., the Petri dish study was conducted in the dark while the soil experiment was in the light after germination). Over many decades of research, it has been shown that recovery from LN temperatures involves a delicate balance between the damage caused by ultra-low temperature stress (physical damage caused by ice crystals as well as oxidative damage), combined with the ability of recovery systems (e.g. anti-oxidants) to overcome damage. It is possible that in *T. indica* seeds, while physical fissures in the testa might have enabled or improved imbibition, cell elongation, and subsequent germination (Fig. 3), the associated biochemical and physiological changes (in cotyledons and embryo axes) might have necessitated the switching on of recovery systems, which redirected resources towards ameliorating the damage incurred. This was apparent by the delayed emergence of the first true leaves in LN treated seeds (Fig. 5C). Furthermore, oxidative and other damage accumulates in the light and this could account for the varying results observed in the Petri dish and soil studies.

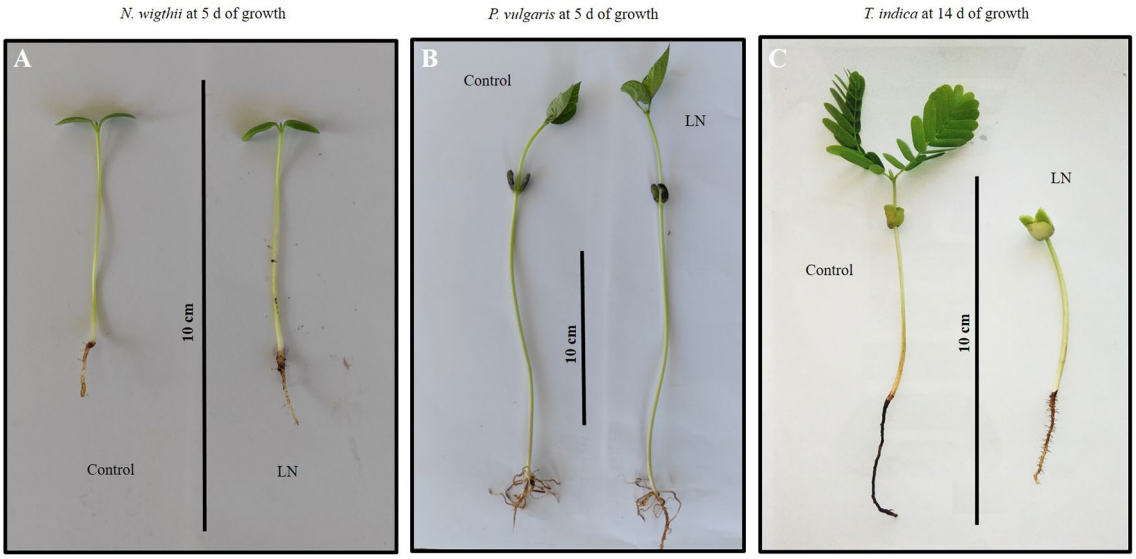


**Fig.5** Effects of seed exposure to liquid nitrogen (LN) on early plant growth in soil

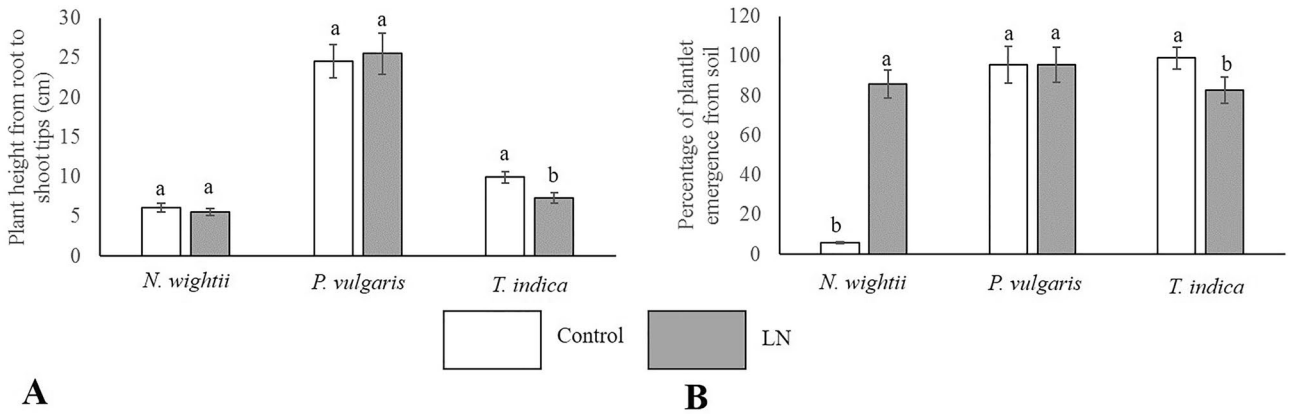


**Fig.6** Effects of seed exposure to liquid nitrogen (LN) on early plant height (**A**) and emergence from soil (**B**). In each plant species, results with the same *letter* are not statistically different (t-test, $p > 0.005$). Vertical bars represent ± SE. Data of *N.wightii* and *P. vulgaris* resprent 5 d of growth. Data of *T. indica* represent 14 d of growth

In a previous study, we reported on the effect of LN on *Tamarindus* (Villalobos-Olivera et al. 2023). We also examined the levels of chlorophylls (a, b), malondialdehyde, other aldehydes, phenolics, proteins and activities of superoxide dismutase and peroxidases in seeds, stems, leaves and roots, however, the cotyledonary tissues and embryo axes were not individually considered. These two anatomical structures seem to have a role in the response to LN.

The SEM showed extensive damage to the tamarind seed coat, which may have reached the deeper cell layers and perhaps even the cotyledons. After emergence of the radicle, the greatest mobilization of reserves from the storage tissues occurs to guarantee the growth and development of the new plant (Bewley et al. 2013). However, disorders at the metabolic level in cryopreserved seeds caused a delay in the initial growth of the seedlings (Benson and Bremner 2004; Funnekotter et al. 2017; Arora 2018) and specifically, also in *T. indica* (Villalobos-Olivera et al. 2023). Therefore, the stress imposed by LN on *T. indica* seeds, together with mechanical damage to the testa and the internal tissues of the seeds, could cause challenges in for the mobilization of the stored reserves necessary for initial growth of the seedlings. In the present study, from a physiological perspective of the germination and initial growth of the seedlings, the cracks observed in the testa were favorable in *N wightii* (species with physical dormancy), they had no apparent effect in *P. vulgaris*, but had a negative effect in *T. indica*.

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**Author contributions** YA, BC, DE, BEZB, LPB, PC, EH, CN, MM and JCL designed the research; YA, DE, LPB and JCL conducted the experiment; YA, BC, BEZB, PC, EH, CN, MM and JCL analyzed the data and wrote the paper; and JCL had primary responsibility for the final content. All authors have read and approved the final manuscript.

**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** Authors do not have any conflict of interests.

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