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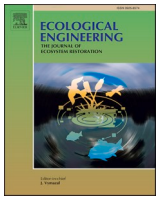

Short communication

Short-term seed storage of two Mediterranean shrubs used in restoration: Simple procedures to reduce seed deterioration

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ABSTRACT

Seed-based revegetation is fundamental in global restoration efforts. When seed storage is mandatory, knowledge of storage conditions that reduce ageing is essential for effective conservation of viable seeds. However, inappropriate storage is still frequent, particularly in local restoration projects involving native species.

We investigated how different storage conditions (temperature and relative humidity) affected seed viability of the Mediterranean shrubs *Arbutus unedo* and *Myrtus communis*. We aimed to identify which conditions reduced seed deterioration and to provide practitioners with practical storage guidelines to overcome the natural fluctuations in seed production of these two species.

Seeds equilibrated at 15%, 30% and 60% relative humidity (RH) and sealed in airtight containers were stored at 5 °C or ambient temperature over one year. Additional seed lots were kept under ambient conditions, according to the usual local practice (open storage). Germination tests were performed prior to and after storage.

Significant seed deterioration (80–100%) occurred in seeds equilibrated at 60% RH and stored at ambient temperature, and in seeds kept in open storage. The remaining treatments maintained high seed viability.

Short seed longevity under ambient conditions was demonstrated for both species. To store seeds over one year, practitioners may either (i) equilibrate seeds up to 60% RH and store them in a refrigerator (5 °C), or (ii) equilibrate seeds up to 30% RH (preferably 15% RH for *A. unedo*) and store them at ambient temperature. All seeds should be sealed into airtight containers immediately after equilibrium. The effectiveness of the 5 °C-60% RH treatment demonstrated that procedures less demanding than international standards for short-term storage can still ensure seed viability.

While contributing to the understanding of species responses to storage conditions, our findings can be applied to both species by local practitioners across the Mediterranean. Moreover, our approach can be adapted and extended to other restoration projects and native species, supporting specific improvements to seed storage protocols, thereby enhancing the cost-effectiveness and the capacity of restoration efforts.

1. Introduction

The demand for native seeds to restore plant diversity and function in degraded land increased in the past decade (Broadhurst et al., 2015; Kildisheva et al., 2016; Pedrini and Dixon, 2020), and was further intensified in the framework of the United Nations Decade on Ecosystem Restoration (2021−2030).

Limited availability of seeds and/or seedlings, poor seed quality and low rates of seedling establishment are main constraints to restoration with native species, irrespective of the project scale (Broadhurst et al., 2015; Cañadas et al., 2015; Kildisheva et al., 2016). Dedicated seed production areas and seed storage to meet restoration requirements have been advocated to overcome limitations in seed supply and quality (Merritt and Dixon, 2011).

Several initiatives to conserve native species in seed banks (e.g., Millennium Seed Bank Partnership, European Native Seed Conservation Network) apply international standards guiding the collection, testing and storage of seeds (e.g., ENSCONET, 2009; FAO, 2014). As seeds age and lose viability during storage (Ellis and Roberts, 1980), these standards aim to maximize longevity in the long-term (10–100 years or more; Hong and Ellis, 1996). Orthodox (desiccation tolerant) seeds are generally dried at 15 °C and 15% relative humidity (3–7% moisture

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content) and stored at −18 °C to −20 °C (FAO, 2014).

Building upon seed conservation practice, pioneer restoration seed banks were established and gain prominence, promoting cooperation and technology transfer between academia, conservation seed banks, industry, and government bodies (León-Lobos et al., 2012; Turner et al., 2022; White et al., 2023). However, the current equipment, standards and procedures derived from conservation seed banks are often difficult or impossible to apply in restoration contexts (Merritt and Dixon, 2011; Pedrini and Dixon, 2020; Turner et al., 2022), and many practitioners still request support to use local native species (Nunes et al., 2016). So, while the use of seeds from local provenance is advocated, ensuring their appropriate storage poses a challenge for restoration projects relying on practitioners' infrastructures and resources. Seed companies, farmers and local practitioners, often driven by economic and resource limitations, persist in storing seeds under uncontrolled or rudimentary conditions, with consequent loss of valuable genetic material and resources (Nagel and Börner, 2010; Merritt and Dixon, 2011; Morgan and Salmon, 2019).

Conventional seed bank standards are currently recommended for ecological restoration, but while those focus on long-term storage, short- or medium-term (3–18 months and 18 months to 5–6 years, respectively; Hong and Ellis, 1996) storage may be sufficient for restoration purposes (De Vitis et al., 2020). While guidelines in Australia have already been adjusted to storage length and restoration purpose (Merritt et al., 2021), comparable efforts in Europe are still pending.

Although it is essential to understand seed lifespan changes under a wide range of storage conditions to predict seed longevity (Colville and Pritchard, 2019), and a growing body of literature reports species-specific seed lifespans and decay rates under storage (Probert et al., 2009; Nagel and Börner, 2010; Merritt et al., 2014), such information is still lacking for many native species. Therefore, it is crucial to test their seed viability under storage conditions tailored to the technical capacity and objectives of local facilities.

We investigated the effect of different storage conditions (air temperature and relative humidity) on the seed germination of two Mediterranean shrubs, *Arbutus unedo* (strawberry tree) and *Myrtus communis* (common myrtle). Seeds of both species are collected locally and germinated in a local nursery for subsequent revegetation of a quarry in SW Portugal. There, previous seed storage attempts yielded low or nil germination. We expected to provide easy-to-apply guidelines for affordable and successful seed storage over one year and thereby contribute to a more efficient seed use in restoration.

2. Methods

The study was performed at the SECIL-Outão quarry in Serra da Arrábida, southwest Portugal (38°30′05"N, 8°56′19"W). The climate is Mediterranean, with hot and dry summers; mean annual temperature is 16 °C and mean annual rainfall is 716 mm (IPMA, 2023). Post-exploited quarry areas are revegetated with native shrubs grown in the local nursery (Correia et al., 2001) using seeds collected in the surrounding vegetation.

2.1. Species and seed collection

Two native evergreen sclerophyllous shrubs producing fleshy fruits and used in quarry revegetation were studied: *Arbutus unedo* L. (Ericaceae) and *Myrtus communis* L. (Myrtaceae). Both are likely to exhibit orthodox seed storage behaviour (SER, INSR, RBGK, Seed Information Database (SID), 2023). To overcome the annual fluctuations in the natural fruit crops, the nursery staff stores seeds in a storeroom, at ambient temperature and humidity. Following poor or nil germination of these seeds, the quarry company sought advise to ensure seed viability over one year.

For this study, fruits were collected from the usual collecting sites at the time of natural dispersal, in November 2012. Seeds were extracted through sieves under running water, air-dried at room temperature for one week (ENSCONET, 2009), and subsequently used in the experiment.

2.2. Seed storage experiment

With a fully factorial experiment, we quantified the effects of storage temperature and relative humidity (RH) over one year on seed germination. The selected temperature and RH levels ranged from rudimentary storage to recommended best practice seed banking conditions (De Vitis et al., 2020): ambient temperature in the storeroom (Tamb) and 5 °C, and 60%, 30% and 15% RH. Six treatments (experimental storage environments) were applied: 5 °C-15%, Tamb-15%, 5 °C-30%, Tamb-30%, 5 °C-60% and Tamb-60%.

Seeds were first equilibrated at each of the three RH environments: in a drying incubator (S600 PLH, Aralab, Lisbon) set to 15 °C and 15% RH, and in two sealed boxes containing solutions of lithium chloride (30% and 60% RH, respectively; Hay et al., 2008) placed in a drying room set to 17 °C. In each case, 12 batches of 25 seeds per species were used. Seed equilibrium relative humidity (eRH) was monitored with a hygrometer (HC2-AW, Rotronic Instruments, UK) until equilibrium was reached at the specific RH environment. After 42 days, each batch of seeds was sealed into an airtight glass container. Then, 6 containers of each RH were stored in the storeroom, in the dark, at each of the two temperatures: 5 °C (in a refrigerator) or uncontrolled ambient temperature (Tamb). It is worth noting that eRH at the temperature of storage is expected to differ slightly from the eRH prior to storage due to the relationships between RH and temperature (Pritchard and Dickie, 2003).

To determine seed moisture content (mc) after storage, another group of seeds (50 per species and per treatment) was also equilibrated with the three tested RH environments and stored following the procedures described above. Seeds were weighted before and after being dried at 103 °C for 17 h (ISTA, 2003). Results are expressed in percentage, on a fresh weight basis.

Four additional batches of 25 seeds per species were packed in paper bags and stored in an open shelf in the same storeroom. This treatment (Open storage) represented the usual nursery practice, whereby seeds were exposed to ambient RH and temperature. Temperature and RH of the storeroom were recorded daily (at 8 am, 12 am and 17 pm) and five days a week during the study (Fig. 1).

2.3. Seed germination

One year after storage, seeds from each treatment were retrieved and tested for germination. All tests were carried out on 90 mm diameter

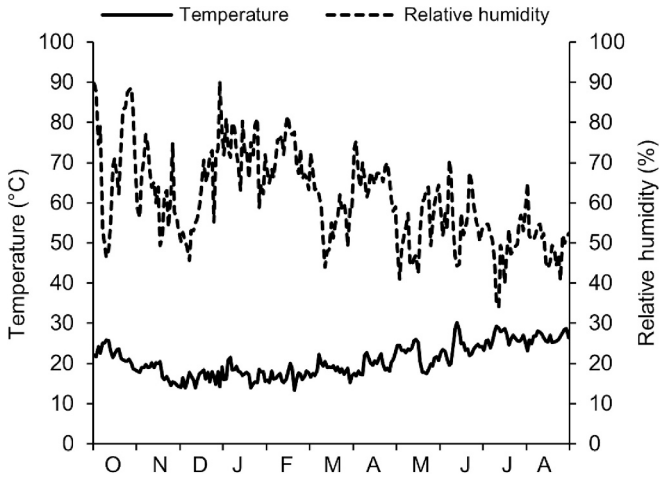

Fig. 1. Mean daily temperature (°C) and relative humidity (%) at the nursery storeroom over the study period (2012−2013). Values were recorded five days a week and three times a day from 8 am to 5 pm.

Petri dishes containing 1% agar; each replicate (25 seeds) was sown on one Petri dish and placed in an incubator (Fitoclima S600, Aralab, Lisbon) at 15 °C with a 12 h/12 h photoperiod. Seeds were placed in an airtight container at 100% RH for 24 h before sowing, to avoid imbibition damage to dry seeds. Germination conditions and pre-treatments (cold stratification: 56 days at 5 °C for *A. unedo*) were retrieved from a seed bank database (Jardim Botânico, Universidade de Lisboa).

Germination was recorded every two days over 50 and 65 days (for *A. unedo* and *M. communis,* respectively), until no additional germination was observed for three weeks. Radicle emergence was the criterion to score a seed as germinated. Cut tests were performed on non-germinated seeds at the end of the incubation period to determine the number of empty or infested seeds. Germination percentage was calculated as the number of germinated seeds / (number of initial seeds − (number of empty seeds + number of infested seeds)) × 100.

Seed germination shortly after collection (i.e., prior to storage) was also determined using the procedures described above.

2.4. Data analysis

Data were analysed in R version 4.0.2 (R Core Team, 2020). The effects of RH and storage temperature on seed germination were tested using a generalized linear model (GLM) with a logit link function and a binomial error (nlme package, Pinheiro et al., 2020). The GLM included RH and temperature as fixed factors and proportion of seeds germinated in each Petri dish as the response variable (coded as number of germinated seeds and (number of seeds sown − number of empty seeds)). Full models including the two main factors and the interaction were fitted for each species. When overdispersion was detected, quasibinomial error distribution was used to fit the models. Chi-square or F tests were used to evaluate if selected predictors explained a significant fraction of the deviance (car package, Fox and Weisberg, 2019). We made post hoc comparisons between storage treatments within species using the glht command (multcomp package, Hothorn et al., 2008). The single step method for *P*-value adjustment was used for pair-wise comparisons between germination of each experimental environment and germination prior to storage, and between each experimental environment and Open storage.

3. Results

Mean temperature and relative humidity in the storeroom during the study period were 20.7 °C and 61.1%, respectively (Fig. 1). Temperature ranged between 5.3 °C (December) and 38.2 °C (June), while RH varied between 18% (July) and 99% (October). High temperature and humidity prevailed, with approximately 60% of the daily records with mean temperatures ≥25 °C or mean RH ≥60%.

Seed moisture content (mc) in the experimental environments ranged from 4.3 to 11.2% (Table 1). Within species, seeds equilibrated at 60% RH exhibited 1.5 times higher mc than those equilibrated at 30% RH.

Germination was strongly affected by the storage environment, and the two species exhibited similar responses (Fig. 2, Table 2). The effect of RH depended on storage temperature: seeds stored at ambient temperature and the highest RH (Tamb-60%) exhibited a 4- (*A. unedo*) to 12-fold (*M. communis*) decrease in germination, compared to the other treatments. Conversely, germination did not differ significantly among RH environments for seeds stored at 5 °C.

Maximum germination percentage of seeds stored under experimental environments was similar to germination before storage except for the Tamb-60% treatment (only 8–20% of pre-storage germination); in *A. unedo*, however, germination also decreased significantly in the Tamb-30% treatment (Fig. 2).

Germination of seeds stored in the experimental environments was always significantly higher than of those in Open storage, except in the Tamb-60% treatment of *M. communis* (Fig. 2). After one year at ambient conditions in the storeroom, all seeds of *A. unedo* decayed, and germination dropped 10-fold in *M. communis*. The cut tests confirmed deterioration of non-germinated seeds (data not shown).

Table 1
Seed moisture content (%) of *Arbutus unedo* and *Myrtus communis* after one year of storage under different relative humidity (15, 30 and 60%) and temperature (5 °C and Tamb) environments. Seeds were equilibrated at three relative humidity environments (15, 30 and 60% RH) and subsequently stored at 5 °C and ambient temperature (Tamb) in the nursery storeroom.

RH	Temperature	*A. unedo*	*M. communis*
15%	5 °C	4.7	4.7
	Tamb	4.5	4.3
30%	5 °C	5.6	7.1
	Tamb	5.8	7.1
60%	5 °C	8.1	10.8
	Tamb	8.3	11.2

4. Discussion

Germination of seeds of *A. unedo* and *M. communis* stored in airtight containers over one year remained consistently high in all experimental environments except in the treatment with the highest moisture content stored at ambient temperature (Tamb-60%), where a sharp decrease was observed. The effectiveness of the 5 °C-60% RH treatment demonstrated that procedures less demanding than international standards for short-term storage (FAO, 2014; De Vitis et al., 2020) can be equally effective in preserving seed viability of both species over one year. Conversely, seasonal high air temperature and recurrently high RH were confirmed to accelerate seed deterioration in the Open storage treatment, the usual procedure in the nursery. This finding highlights a short seed lifespan for the studied species under ambient conditions.

It is well-established that reducing seed water content and/or temperature increases the longevity of orthodox seeds, with evident advantages for long-term conservation (Ellis and Roberts, 1980). Therefore, it was expected that drying and/or cooling conditions would extend seed lifespan compared to the uncontrolled conditions. International standards for short-term seed storage (FAO, 2014), which generally suit restoration purposes (De Vitis et al., 2020), recommend drying at 10–25% RH and storage at 0–10 °C or under ambient temperature ("as cool and stable temperatures as possible but not >25 °C"). Despite the undeniable advantages of applying these standards, economic and resource limitations still lead to inadequate seed storage (Nagel and Börner, 2010; Merritt and Dixon, 2011; Morgan and Salmon, 2019). Although in such cases it can be argued that any degree of drying and/or cooling is advantageous to reduce seed deterioration, selecting their best levels becomes particularly significant when intending to enhance seed longevity while minimizing the associated effort and resource investment. We aimed to determine such optimal levels tailored to specific target species, storage duration and local resources and facilities. According to our findings, to maintain seed viability of *A. unedo* and *M. communis* over one year the practitioner was advised as follows: (i) if a refrigerator is available, equilibrate the seeds of these species up to 60% RH and seal them into airtight containers for cold storage (5 °C, household refrigerator); (ii) without a refrigerator, equilibrate the seeds up to 30% RH (preferably 15% RH in *A. unedo*, as a slight reduction in viability might occur) and seal them into airtight containers to be stored at ambient temperature. These procedures are less stringent than the abovementioned international standards but still ensured seed viability over one year, under higher RH (60%) and ambient temperature (often ≥25 °C and up to a maximum of 38 °C) than the upper limits of those standards. Since storage temperature is easier to control than seed moisture content, cold storage seems the most favourable procedure; in this case, air-drying seeds is an option provided that ambient RH is suitable. Practitioners should ensure that the appropriate seed moisture content is achieved, and that relatively cool

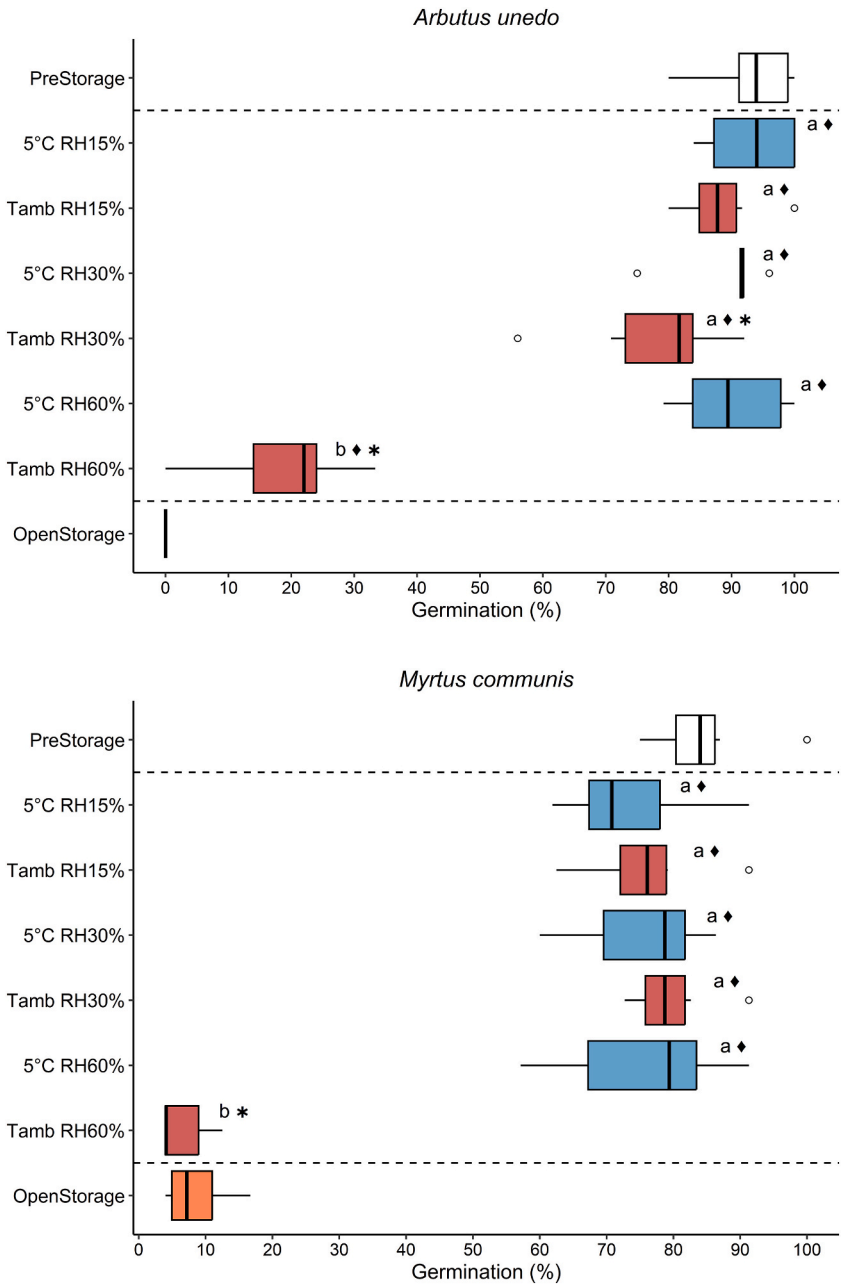

Fig. 2. Seed germination (%, $n = 6$) in *A. unedo* and *M. communis* after one year under different storage environments. Results for experimental environments (between the two dashed lines) according to the relative humidity under which seeds were equilibrated (15, 30 and 60% RH) and to the storage temperature (5 °C and ambient temperature at the quarry nursery storeroom, Tamb). Results for Open storage (the usual local practice: seeds stored at ambient temperature and RH in the nursery storeroom) and Pre storage germination are also presented. Boxplots show the median and 25th–75th percentiles; horizontal solid lines outside the box are minimum and maximum values; open circles indicate outliers. Different letters indicate significant differences between experimental storage environments ($p < 0.05$). Significant differences between experimental environments and germination prior to storage are indicated by *, and those between experimental environments and Open storage are indicated by ◆.

and stable air temperatures are maintained during ambient storage. Additionally, they should adopt the best practices in collecting and cleaning (Pedrini and Dixon, 2020), as fundamental factors to maximize seed quality, and therefore, longevity.

To the best of our knowledge, this is the first report of a short seed lifespan for the studied species under ambient storage, except for anecdotical evidence of a shorter seed lifespan in *A. unedo* than in *M. communis* at room temperature (García-Fayos et al., 2001). The reports of seed longevity under ambient or unconventional storage conditions are scarce (but see Nagel and Börner (2010) and Colville and Pritchard (2019)), although they are valuable to guide storage decisions and determine species lifespan category. The Myrtaceae is consistently recognized as a plant family encompassing species with long-lived seeds (Probert et al., 2009; Merritt et al., 2014). Recording short-lived seeds of *M. communis* emphasizes the variability in seed lifespans among species and urges caution when inferring similar seed longevities within a plant family. Adding to broad phylogenetic patterns, intrinsic seed longevity also correlates with climate of origin and seed characteristics (Probert et al., 2009; Merritt et al., 2014). Moreover, intra-specific variation in seed lifespan is also caused by provenance, genetic variation and effects of harvesting and processing on seed quality (Probert et al., 2007; Nagel and Börner, 2010). Multiple seed lots of each species should therefore be

Table 2
Generalized Linear Models (binomial error, logit link) fitted to the germination data (coded as sown − germinated) of each species after one year storage under different relative humidity (15, 30 and 60%) and temperature (5 °C and Tamb) environments. A full factorial model with temperature, RH and the interaction term as explanatory variables (fixed factors) was fitted for each species. The intercept corresponds to the germination of seeds equilibrated at 15% RH and stored at 5 °C.

Species	Parameter	Effect	s.e.	t	p
A. unedo	Intercept	2.625	0.447	5.876	< 0.001
	30%	−0.465	0.581	−0.800	0.430
	60%	−0.412	0.589	−0.699	0.490
	Tamb	−0.583	0.568	−1.025	0.313
	30%-Tamb	−0.328	0.731	−0.449	0.656
	60%-Tamb	−3.067	0.743	−4.127	< 0.001
M. communis	Intercept	1.041	0.194	5.372	< 0.001
	30%	0.067	0.275	0.242	0.808
	60%	0.114	0.276	0.414	0.679
	Tamb	0.162	0.284	0.571	0.568
	30%-Tamb	0.107	0.404	0.264	0.792
	60%-Tamb	−4.011	0.488	−8.219	< 0.001

assessed to account for these effects on seed longevity under the investigated storage conditions.

5. Conclusions

This study identified short-term storage conditions that maximized seed viability for *A. unedo* and *M. communis* while minimizing the associated effort and resources. The results showed that seeds of both species can be stored over one year with little viability loss using less stringent procedures (ambient seasonal temperature ≥ 25 °C or 60% RH) than current globally accepted standards (FAO, 2014; De Vitis et al., 2020). While this practice can be extended to other restoration projects involving the same species, it is important to recognise potential effects of the seed lots on seed longevity. Our results also indicate short seed lifespan under ambient conditions for the two species. Taken together, these findings contribute to the understanding of species responses to storage conditions, with implications for optimization of species performance and management of seed banks targeting wild species. Our approach is a valuable tool to enhance the capacity of local restoration seed banks across the Mediterranean with low-cost technology and minor changes in seed processing. Informed protocols will underpin restoration practices, reducing the impact of seed collection on source populations and increasing cost-effectiveness.

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CRediT authorship contribution statement

Adelaide Clemente: Conceptualization, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Catarina A. Costa:** Data curation, Investigation, Writing – review & editing. **Graça Oliveira:** Formal analysis, Supervision, Writing – review & editing. **Otília Correia:** Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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