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In vivo and *in vitro* propagation of bay leaves (*Laurus nobilis*) in Northern Morocco

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Abstract

This study explored seed germination and propagation through cutting in *Laurus nobilis* L. with a focus on *in vitro* and *in vivo* regeneration techniques for multiplication and sustainable conservation of diversity in the species. Seed germination was achieved through pre-soaking for 7 days, followed by cold stratification at -4 °C for 50 days. The results showed that seed imbibition greatly increased germination rates (65%), however, germination in sand (14%), and peat (12%) was less successful. Imbibed seeds had a greater seedling survival rate (40%) in peat attaining better plant height. The *in vitro* germination of wild Moroccan *L. nobilis* was accomplished by scarification of kernels with sulphuric acid (34 N). Seedlings from whole fruits had the best survival rate (33.33%), followed by kernels (25%). Micropropagation of twigs using MS media with kinetin and indole-3-acetic acid proved efficient, providing useful methods for the domestication and conservation of *L. nobilis*.

Key words: Bay leaves, *Laurus nobilis* L., conservation, *in vitro* and *in vivo* culture, Morocco

Introduction

During the last decade, the domestication of medicinal and aromatic compounds has attracted worldwide attention for their reliability in agricultural economies and their ability to produce bioactive compounds, including secondary metabolites and engineered biomolecules. However, knowledge about the domestication of spontaneous aromatic and medicinal plants in Morocco has been very recent, as domestication has led to the generation of plants with high added value. Indeed, a high-quality product is a sustainable way to satisfy the increased global demand in local and international markets (Hinane *et al.*, 2020).

Among these plants, *Laurus nobilis* L. (Bay leaves) is a beautiful medicinal and aromatic plant and an important model plant of the Lauraceae family. All the plant parts (leaves, shoots, flowers, and fruits) have been used since ancient times in the Mediterranean region, including Morocco, for medicinal-aromatic plants with special scents, spice crops, and ornamental, ecological and economic purposes (Cavusoglu and Bozkurt, 2020; Cavusoglu, 2021).

Bay leaf is an important aromatic dioecious tree in the family Laureaceae that can be found growing wild or cultivated in the Mediterranean region (Ordoudi *et al.*, 2022). A special characteristic of this plant is that the leaves contain essential oil with their abundant of monoterpenes and sesquiterpenes that have been increasingly acknowledged over the years as providing an essential contribution to the food and pharmaceutical industries (Yahyaa *et al.*, 2015; Paparella *et al.*, 2022).

Due to its many bioactive chemical substances isolated, *L. nobilis* is widely used for medicinal and culinary purposes (Mrabet *et al.*, 2024). Bay leaves are commonly used in Moroccan cuisine and traditional medicine. They are also employed to treat chronic diseases associated with oxidative stress, likely due to their strong antibacterial (Taroq *et al.*, 2019) and immunomodulatory effects (El Faqer *et al.*, 2023). As a result, bay leaf extracts and essential oil have a wide range of applications across various fields.

This technology enables rapid mass propagation and enhances the sustainability of the species (Benkaddour *et al.*, 2022). As a result, some studies utilize micropropagation from stem cuttings, with seedlings offering a viable solution for the protection, conservation, and domestication of *L. nobilis* (Royandazagh, 2019; Cavusoglu and Bozkurt, 2020; Cavusoglu, 2021). Additionally, tissue culture techniques, such as somatic embryogenesis from young leaves or seeds, provide promising opportunities for the propagation and conservation of this plant (Nadarjan and Pritchard, 2014; Al-Gabbiesh *et al.*, 2015).

Despite its economic, environmental, and ethnomedicinal importance, this famous plant in Morocco, especially in the Rif region, is disappearing due to several factors, such as climate change (Akyol *et al.*, 2023), drought conditions, overgrazing, and uncontrolled harvesting from farmers. The examples of Talassemtane National Park (Western Rif of Morocco) Redouan *et al.* (2020) and Moulay Abdeslam commune speak that. Moreover, this plant is common in its wild form and is widespread in limestone rock gardens in the mountains, mainly in the western Rif. Indeed, developing an efficient and quick protocol for *In vitro*

plant culture and tissue culture of *L. nobilis* must be carried out to protect natural habitats in our country.

Although *L. nobilis* is a significant plant species, there has been limited effort and insufficient knowledge regarding its conservation in Morocco and globally using both *in vivo* and *in vitro* techniques. Therefore, it is crucial to develop an efficient and rapid protocol for preserving this natural resource and to contribute to global efforts supporting plant tissue culture through *in vitro* methods. The aim of this study was to establish an effective protocol for the *in vivo* seed germination and *in vitro* culture of *L. nobilis* sourced from the Northern region of Morocco.

Materials and methods

Plant materials: The ripe black fruits of wild *L. nobilis* (LN) were gathered from female trees in northern Morocco, specifically from Tazia and the commune Tangier-Tetouan, N 35°21.652', W005°33.739', altitude 731m (Fig. 1). The fruits were chosen based on their dark pericarp color, indicating ripeness. To prevent spoilage during transportation, the fruits were promptly placed in securely sealed plastic bags. After harvesting, the plant materials were transported to the lab at the University of Abdelmalek Esaadi, Tetouan, Morocco. Additionally, the fruits were manually cleaned to remove any foreign matter, then dried and stored at 4ºC until required for germination studies. It's important to note that all the fruits used in this study were fully ripe.

***In vivo* germination**: Germination assays were conducted for the purpose of developing a protocol for *in vivo* seed germination which can be both efficient and reproducible. The assays studied germination under an array of conditions, including clinical cotton, peat, and sand.

Fig. 1. Female *L. nobilis* plant selected for the study.

Seed germination in clinical cotton: The seeds were soaked for 7 days to facilitate subsequent manual coat removal as well as to promote embryo expansion and germination; additionally, sterilized distilled water was changed daily to protect zygote embryos against bacterial contamination. The sterilized seeds were then germinated in flasks and placed in an air-conditioned culture chamber at 21.12±1°C and 52% relative humidity. Lighting was provided for 18 hours per day using 11-watt compact fluorescent lamps (Philips) with a light intensity of 2000 lux.

Germination of kernels in peat and sand: The seeds were kept in plastic bags and refrigerated at -4°C in the dark for fifty days to replicate cold stratification. Cold stratification treatment was intended to break dormancy and promote germination. To remove surface contaminants, the *L. nobilis* seeds were first rinsed for five min under sterile distilled water. The seeds were then grown in sand (N=50) and sterilized peat (N=50). The seedling survival rate of kernels was calculated using the equation below:

$$\text{Percentage germination} = \frac{\text{Total germinated seeds}}{\text{Total seeds sown}} \times 100$$

Seed germination on peat: The seeds were soaked in water for 10 days at room temperature and the water was changed every day to avoid bacterial contamination then, planted in peat-filled plastic pots, with either one seed per pot or three seeds per pot. The plants were watered daily with potable water by either spraying or sprinkling. Additionally, the plantlets were immediately covered with plastic bags to maintain high humidity. The potted plants were transferred to a well-lit chamber with a light intensity of 2000 lux that maintained 24±2ºC to grow. After four-month periods of germination, the survival and germination percentages were calculated using the classical equation. During the *in vitro* growth of *L.nobilis*, the number of buds, number of plants, number of leaves and leaf size were measured.

Fruit treatment with acid: Mature black fruits were treated with 34 N sulfuric acid for one hour as scarification treatment. The samples were rinsed gently three times with sterile distilled water for 5 minutes each time. The sterile kernels were soaked in sterile water for 48 h in 250 mL conical flasks placed on a rotary shaker at 13 rpm. The water was changed daily during the soaking period.

Seed scarification: The seeds were treated with 34N concentrated sulfuric acid for one hour and 30 minutes. After that, they were rinsed three times with sterile distilled water for 5 minutes each time. Following the rinsing, the sterile seeds were soaked for 7 days in sterile distilled water in 250 mL conical flasks that were placed on a rotary shaker set at 13 rpm. Furthermore, the sterile distilled water was changed daily to prevent the growth of bacteria and fungi.

Scarified seed germination: The soaked kernels of *L. nobilis* that were scarified using 34 N sulfuric acid for one hour and soaked 7 days were placed in 200 mL flasks containing 50 mL of MS media. These flasks were then transferred to an air-conditioned culture chamber.

Endocarp removed seed germination: Under a laminar flow hood, the endocarp of each seed was removed using a scalpel while avoiding touching the embryos.

***In vitro* hernel germination:** The *L. nobilis* kernels were sterilized for 30 minutes and then soaked. Each soaked kernel was placed in a separate 200 mL flask containing 50 mL of micro and macro nutrients (Murashige and Skoog, 1962). The media was sterilized in an autoclave at 121°C for 20 minutes. The flasks were placed in an culture chamber under the same conditions as mentioned above.

***In vitro* regeneration using seedlings from germinating seeds:** Shoots were taken from *L. nobilis* seedlings germinated *in vitro*, and their surfaces were sterilized using two methods under a laminar flow hood.

Two steps were used in sterilizing explants. The explants were first surface-disinfected by 30 seconds of 70% ethanol immersion. Depending on the treatment, the explants were then treated with mercury chloride ($HgCl_2$) at either 0.2% (w/v) for a more rigorous sterilization or 0.01% (w/v) for a less intense treatment. After this step, the explants were rinsed thoroughly with sterile distilled water to remove any residual mercury chloride. They were then subjected to a 14% (w/v) calcium hypochlorite ($CaClO_2$) solution with a few drops of Tween 80 for 25 minutes, which further enhanced sterilization. Finally, the explants underwent three consecutive rinses in distilled water, each lasting 5 minutes, to eliminate any chemical residues, thus preparing them for subsequent experimental procedures.

The sterilized shoots were cut into 2-3 cm segments, each containing at least two axillary buds, and these segments were used as explants. Pyrex glass test tubes (18 x180 mm) with one explant per tube were filled with 15 mL of agar culture medium, which contained 0.7% bacteriological agar type E, macroelements, microelements, and vitamins from MS. Additionally, the medium included 100 mg/L myo-inositol, 30 g/L sucrose, and 2 mg/L Kin + 0.5 mg/L indole-3-acetic acid. The pH of the media was adjusted to 5.6-5.8 using one mol/L NaOH (1 N). The culture medium was sterilized by autoclaving for 20 minutes at 121°C. The explants (nodes with axillary buds) were placed in the culture medium and it was conducted under aseptic conditions in a sterile horizontal laminar flow hood. The plants were placed in a culture chamber at 21.12±1°C and 52% RH with 18 hours of light per day at 4000 lux. After 3 months of growth, the following parameters were assessed:

Statistical analysis: The experiment was based on individual value of the mean length, the mean number, and the mean length roots. Data analysis was presented as mean ± SD or DMRT post hoc comparision using IBM SPSS v.20 software.

Results

***In vivo germination of** L. nobilis*

Seed germination on cotton: Seeds of *L. nobilis* were germinated on cotton and after 15 days, the first radical appeared. This method exhibited the highest average germination percentage (65%) with an average plant height of 3.63±3.054 cm (Fig. 2).

Germination in sand: The findings of our investigation showed that the germination percentage was 14% in sand but with reduced

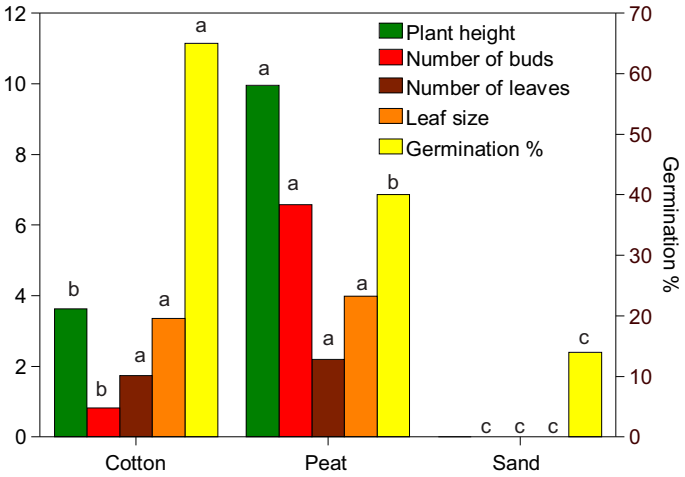

Fig. 2. Seed germination and seedling characteristics in cotton, peat, and sand. Different letters indicate significant differences at P=0.05 based on Duncan's Multiple Range Test (DMRT).

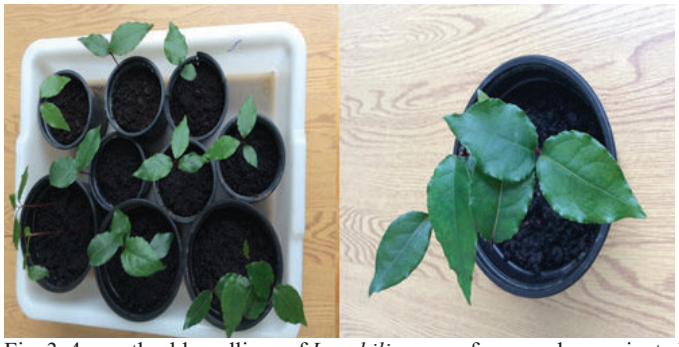

Fig. 3. 4-month-old seedlings of *L. nobilis* grown from seeds germinated on peat

effectiveness and seedling survival rate of seedlings after 50 days of cold stratification (-4 °C) was negligible(Fig. 2).

Germination in peat: With less percentage of seeds effectively sprouting in peat, the results show that kernel germination in peat was less effective than in cotton. But once germination took place, seedlings grown in peat clearly grew and established themselves far better than in cotton and sand. Better development and improved shoot growth from peat-grown seedlings suggested that, although peat may not encourage as high a germination rate, it provides better conditions for seedling establishment and early growth than cotton and sand (Fig. 2, 3).

Young plant propagation through cuttings: Cuttings from young *L. nobilis* plants, grown under controlled conditions (temperature of 25±1°C, 18-hour photoperiod, and light intensity of 4000 lux), showed promising growth results. The average plant height reached 8.35 cm, with each plant producing about 6.04 buds. On average, 1.29 new plants were generated per cutting, indicating moderate propagation success. The cuttings developed an average of 2.71 leaves, with a mean leaf size of 3.40 cm. These results suggest that the propagation conditions effectively promoted growth, although some variation in development was observed among cuttings (Table 1).

In vitro seed germination after scarification with 34N sulfuric acid

Soaking for 48 hours: After treating the kernels with 34N sulfuric acid for an hour and soaking them in water for 48 hours, none of the seeds had germinated on MS media.

Germination in epicarp removed seeds: Study was also carried

Table 1. The characteristics of cutting plants produced from young plants of *L. nobilis* L.

Parameter	Mean±SD
Plant height	8.350±3.484
Number of buds	6.035±2.898
Number of plants	1.286±1.138
Number of leaves	2.714±1.204
Leaf size	3.403±1.529

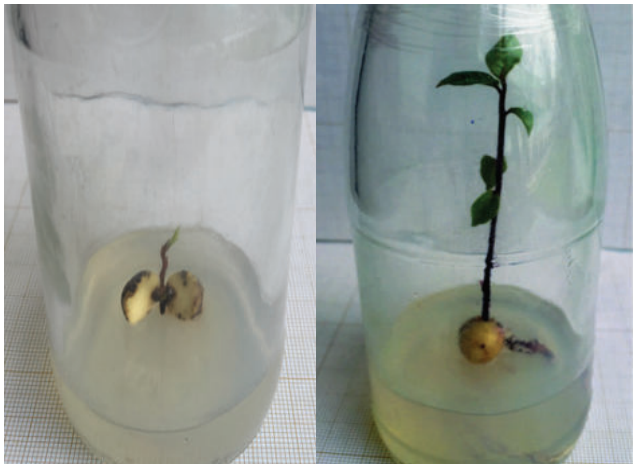

Fig. 4. Three-month-old seedlings from *In vitro* seed germination on MS media after treatment of whole fruit with 34 N sulfuric acid for one hour

out on the germination of seeds without a pericarp. After 50 days of sowing, it was discovered that no seed had germinated on MS media.

***In vitro* seed germination in whole-fruit treatment with sulfuric acid:** When the entire fruit was treated with sulfuric acid for an hour, the seeds that germinated in nutrient solution (MS medium) showed the highest seedling survival rate (33.33%) compared to kernels that were sterilized (0%) under the same conditions (Fig. 4).

***In vitro* seed germination after sulfuric acid treatment and followed by imbibation in water:** *In vitro* seed germination after treatment of seeds with sulfuric acid for 30 minutes and imbibed in water for 10 days was studied. After seven days of soaking,

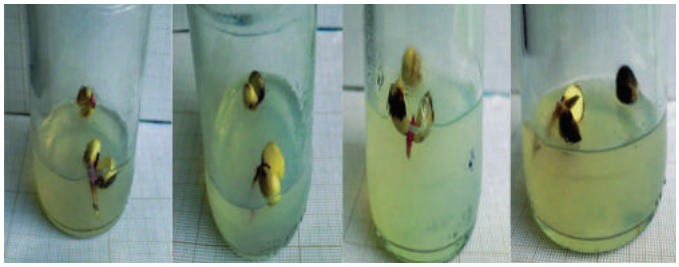

Fig. 5. *In vitro* seed germination after treating kernels with sulfuric acid for 30 minutes for scarification

Fig. 6. *In vitro* 30-day-old plants on MS media after 30 minutes of the kernels scarification with 34 N sulfuric acid.

Fig. 7. Six-month-old plants resulting from germinating seeds scarified+ in sulfuric acid for 30 minutes.

the sterilized seeds exhibited a 25% germination rate in an sterile environment. The formation of the first radicle was observed after 15 days in MS medium, marking the beginning of root development. Plant growth progressed, with the emergence of the first leaf at 30 days (Fig. 5). The most successful regeneration, characterized by well-developed root hairs and a branched

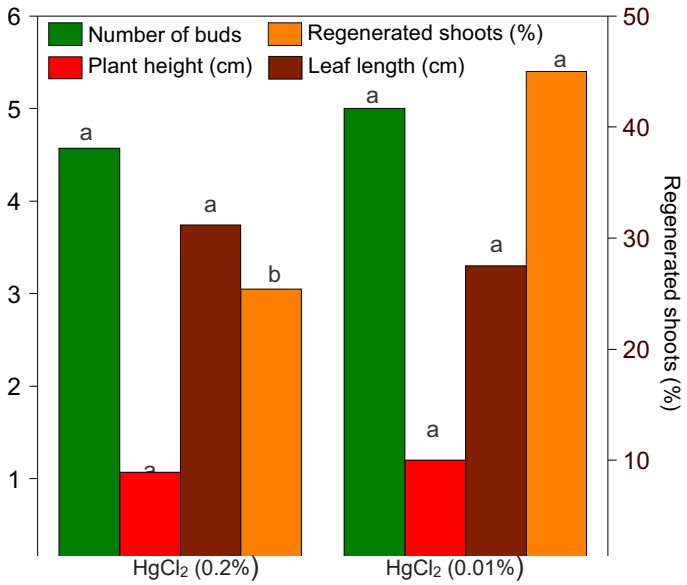

Fig. 8. Response of sterilized apices cultured using different concentration of $HgCl_2$ on MS+2 ml of kinetin+0.5 ml of AIA medium

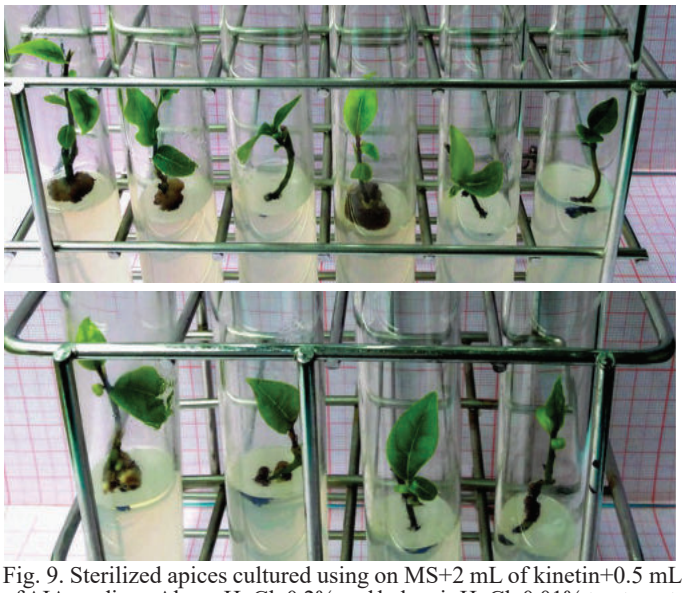

Fig. 9. Sterilized apices cultured using on MS+2 mL of kinetin+0.5 mL of AIA medium. Above $HgCl_2$ 0.2% and below is $HgCl_2$ 0.01% treatment.

structure, was achieved after 180 days of culture in the same MS medium.

***In vitro* regeneration using seedlings from germinated seed:** The study explored two sterilization concetration of $HgCl_2$ to facilitate the rapid *in vitro* micropropagation of twigs from *L. nobilis* seedlings. The results demonstrated that both techniques were effective with better results in 0.01% $HgCl_2$ treatment (Fig. 8).

Discussion

Numerous ethnobotanical studies highlight the medicinal uses of *L. nobilis* fruits for ailments like inflammation and arthritis (Awada *et al.*, 2023). However, this demand has led to overexploitation in Morocco, where around 20 tons of bay leaves are harvested annually, threatening its populations in the Middle Atlas, Eastern Rif, and Western Rif. Effective measures to curb uncontrolled harvesting and deforestation, alongside comprehensive conservation strategies, are crucial (Labiad *et al.*, 2020; El Baghazaoui *et al.*, 2024).

Morocco's fragile ecosystems face severe threats from human activities, yet research on the conservation of *L. nobilis* is limited. While Ettaqy *et al.* (2023) noted the critical status of *L. azorica*, the value chain for *L. nobilis* remains informal and dominated by small-scale harvesters. Concerted efforts in conservation, propagation, and large-scale cultivation using both traditional and modern methods are essential to address its scarcity.

This study introduces innovative approaches to the propagation of *L. nobilis*, advocating the integration of both *in vivo* and *in vitro* methods, such as cold stratification, micropropagation, and sterilization techniques, to enhance germination success. Notably, the research reports a promising 65% success rate for *in vivo* germination, alongside a 33.33% survival rate for *in vitro* propagation from sterilized whole fruits. These findings signify substantial progress in addressing the long-standing germination challenges of *L. nobilis*.

In vitro germination, as elucidated in this study, offers a distinct advantage, reducing the time to germination to just three months while minimizing the risk of failure. In contrast, *in vivo* methods, though efficient, reached a maximum germinability of only 40% after four months. Despite these advances, *in vivo* protocols remain more effective for large-scale applications, with germination rates as high as 65%. While *in vivo* protocols boast higher overall efficiency, they are less time-efficient, necessitating further refinement and standardization of *in vitro* conditions to maximize germination rates and shorten germination periods. This aligns with existing literature, which asserts that, though propagation via seeds or cuttings is viable, it remains laborious and unpredictable in terms of uniformity (Nadeem *et al.*, 2018). Additionally, the growing demand for *L. nobilis* in medicinal, agro-food, and cosmetic industries cannot be met through traditional methods alone, echoing findings from studies in other regions (Ertekin and Çorbacı, 2018).

Pre-sowing treatments, notably those involving gibberellic acid and cold stratification, have been shown to significantly improve germination rates by breaking seed dormancy, a key factor in seed viability (Nautiyal *et al.*, 2023). Our research reveals that the pericarp is a primary cause of seed dormancy. When the pericarp was surgically removed, and seeds were pre-soaked for 48 hours to seven days, germination rates soared to 65% in cotton, 40% in peat, and 33.33% and 25% in MS medium, respectively. These results corroborate prior studies, affirming that pericarp removal and pre-soaking dramatically enhance both the germination rate and uniformity (Ertekin & Çorbacı, 2018; Royandazagh, 2019; Cavusoglu & Bozkurt, 2020; Cavusoglu, 2021).

Several studies have explored the resilience of *L. nobilis* to environmental factors (Ben Ayed *et al.*, 2018, 2023), as well as its ability to withstand extreme conditions (Lamarque *et al.*, 2018; Pashtetsky *et al.*, 2018; Pasha *et al.*, 2019; Trifilò *et al.*, 2021; Aykol *et al.*, 2023; Ishimwe & Deligöz, 2024). However, a better understanding of seed dormancy and key abiotic factors—such as temperature, light, pH, water potential, salinity, darkness, and burial depth—will be crucial for developing effective propagation protocols.

In vitro germination experiments further revealed that the sterilization of different materials (whole fruits and kernels) and sterilization times significantly impacted germination rates. While no previous data for *L. nobilis* exists in this domain, future research should prioritize optimizing sterilization protocols. Insights from studies on other species have proven invaluable in developing effective protocols for aseptic seed germination (Šoch *et al.*, 2023; Trajković *et al.*, 2019).

Micropropagation trials with wild Moroccan *L. nobilis* have demonstrated that the decontamination protocols used were highly effective, yielding low contamination rates from bacterial and fungal sources. However, further optimization is necessary for *in vitro* propagation, with critical factors such as mineral composition, growth regulators, and culture media all playing pivotal roles (Sharma *et al.*, 2023). In this study, MS media combined with kinetin led to superior outcomes in terms of both plantlet percentage and size. This is consistent with prior research showing that nutrient-rich media significantly accelerated seedling growth (Cavusoglu & Bozkurt, 2020; Cavusoglu, 2021).

Scarification using sulfuric acid is another technique highlighted in this study for its efficacy in breaking dormancy in hard-coated seeds, facilitating water uptake and promoting germination. Typically, a 95% sulfuric acid solution was used for 5-10 minutes, although excessive exposure can damage the seed structure, as shown in recent studies (Carbnera *et al.*, 2022). Our research confirmed that a one-hour exposure to sulfuric acid completely inhibited bay seed germination. However, Junior *et al.* (2018) found that immersion in sulfuric acid for 60 minutes accelerated germination in *Colubrina glandulosa*, and Ardiarini *et al.* (2021) demonstrated that oven-heating at 55°C was the most effective scarification technique for long bean seeds.

This study demonstrated that integrating both *in vivo* and *in vitro* propagation methods effectively enhanced germination success in *L. nobilis*. *In vivo* methods achieved a 65% germination rate, while *in vitro* protocols reduced germination time to three months with a 33.33% success rate. Key techniques, including pericarp removal, pre-sowing treatments (cold stratification), and optimized sterilization, significantly improved germination rates, addressing dormancy challenges. Additionally, nutrient-rich MS media with kinetin in micropropagation showed superior plantlet development, highlighting viable options for sustainable cultivation of *L. nobilis* and conservation of genetic resources.

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