UNIVERSITÀ DEGLI STUDI
DI MODENA E REGGIO EMILIA

Dottorato di ricerca in
__ Models and Methods for Material and Environmental Sciences__

Ciclo XXXVIII
Academic Year: 2024/2025

Preserving Plant Biodiversity in Emilia-Romagna with a Multidisciplinary Conservation Approach: Orchidaceae as a Case Study

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Abstract in Italian

CONSERVAZIONE DELLA BIODIVERSITÀ VEGETALE IN EMILIA-ROMAGNA ATTRAVERSO UN APPROCCIO MULTIDISCIPLINARE: LE ORCHIDACEAE COME CASO DI STUDIO

La perdita di biodiversità, determinata da fattori quali il degrado degli habitat e i cambiamenti climatici, rappresenta una minaccia significativa per gli ecosistemi globali, in particolare per i gruppi vegetali ecologicamente specializzati, tra cui le Orchidaceae. La ricerca sviluppata in questo progetto di dottorato ha portato alla definizione di un quadro integrato e multidisciplinare per la conservazione della flora regionale dell'Emilia-Romagna (Italia settentrionale), utilizzando le orchidee come sistema modello. Il progetto integra strategie di conservazione ex situ e indagini in situ con l'obiettivo di garantire la tutela della flora regionale e di rafforzarne la resilienza a lungo termine.

La componente ex situ ha portato alla creazione della Banca dei Semi delle Orchidee UNIMORE, che attualmente conserva 50 accessioni rappresentative di 38 taxa, secondo i protocolli internazionali e con una banca dati dedicata all'interno del sistema UNIMORE Seedbank. I risultati hanno confermato l'efficacia della conservazione ottimizzata a −20 °C, con una vitalità dei semi pari al 59% e tassi di germinazione del 63%, dimostrando la validità del metodo. Al contrario, i dati comparativi hanno evidenziato un rapido deterioramento dei campioni conservati a +5 °C per lunghi periodi, suggerendo che tali condizioni possano essere utilizzate solo per una conservazione a breve termine e per sperimentazioni future. L'adattamento con successo del protocollo alla specie *Allium angulosum* ne conferma ulteriormente la rilevanza regionale, fungendo da introduzione alla conservazione di altre specie della flora locale.

Le indagini in situ hanno riguardato la distribuzione delle orchidee, la diversità degli habitat e le interazioni ecologiche. I rilievi di campo condotti tra il 2023 e il 2025 hanno rilevato popolazioni di *Ophrys apifera*, *Himantoglossum adriaticum* e *Anacamptis pyramidalis*, anche in contesti urbani, evidenziando la notevole resilienza di queste specie. L'isolamento e l'identificazione molecolare dei funghi micorrizici sottolineano il ruolo cruciale delle associazioni fungine nella germinazione e nello sviluppo delle piante di orchidee terrestri spontanee.

È stato inoltre sviluppato un quadro filogenetico comprendente 61 orchidee native italiane, utilizzando il megatree GBOTB (tramite il pacchetto R V.PhyloMaker), che ha rivelato un raggruppamento evolutivo del rischio di estinzione. Le specie minacciate, come *O. spitzelii*, mostrano una stretta parentela evolutiva (Smith et al., 2022), mentre taxa rari come *Malaxis monophyllos e Liparis loeselii* rappresentano un patrimonio evolutivo unico (Jones et al., 2021).

La presente tesi propone un modello di conservazione integrato che unisce dati ecologici, molecolari (come l'identificazione dei simbionti fungini) e filogenetici. L'integrazione tra strategie *ex situ* e indagini *in situ* ha portato alla definizione di una metodologia replicabile per la tutela della biodiversità regionale, contribuendo al rafforzamento delle basi scientifiche delle politiche di conservazione nella regione Emilia-Romagna e ponendo le basi per studi futuri in altre aree geografiche.

Parole Chiave: Orchidaceae – Biodiversità – Conservazione – Filogenesi – Micorrize

Abstract in English

PRESERVING PLANT BIODIVERSITY IN EMILIA-ROMAGNA WITH A MULTIDISCIPLINARY CONSERVATION APPROACH: ORCHIDACEAE AS A CASE STUDY

Biodiversity loss, driven by factors such as habitat degradation and climate change, poses a significant threat to global ecosystems, particularly among ecologically specialized plant groups, including the Orchidaceae. The research undertaken in this PhD project constitutes the development of an integrated, multidisciplinary framework for plant conservation in the Emilia-Romagna Region (Northern Italy), with the use of orchids as a model system.

The project integrates *ex situ* strategies and in-situ investigations with an objective to ensure the preservation of regional flora and enhance long-term resilience. The ex-situ component established the UNIMORE Orchid Seed Bank, which now conserves 50 accessions representing 38 taxa, in accordance to international protocols and encoded with a specific database within UNIMORE Seedbank. The findings confirmed the effectiveness of optimized storage at −20 °C, with seed viability (59%) and germination rates (63%) demonstrating the efficacy of this method. In contrast, comparative data demonstrated the rapid deterioration of samples stored at +5 °C for a long period of time therefore it could be used for short term storage to uncover future conservation processes. The protocol's successful adaptation to *Allium angulosum* further reinforces its regional relevance this project was done as an introduction to another flora within the seedbank.

Complementary *in situ* investigations were conducted to assess orchid distribution, habitat diversity, and ecological interactions. Field surveys conducted between 2023 and 2025 revealed the presence of viable populations of *Ophrys apifera, Himantoglossum adriaticum,* and *Anacamptis pyramidalis*, even within urban landscapes. This finding serves to underscore the resilience of these species. Mycorrhizal isolations and ongoing molecular identification of symbionts highlight the crucial role of fungal partnerships in germination and establishment.

A phylogenetic framework encompassing 61 native Italian orchids was developed using the GBOTB megatree (via V.PhyloMaker), revealing an evolutionary clustering of extinction risk. Endangered taxa such as *O. spitzelii* have been shown to share close evolutionary ancestry (Smith et al., 2022), while rare lineages like *Malaxis monophyllos* and *Liparis loeselii* have been found to embody unique evolutionary heritage (Jones et al., 2021).

The present thesis establishes a comprehensive conservation model integrating ecological, molecular (as in the fungal identification), and phylogenetic data. The integration of ex-situ and investigation of in-situ approaches has resulted in the establishment of a replicable methodology for the preservation of regional biodiversity. This contributes to the strengthening of the scientific foundation for conservation policy in Emilia-Romagna region as a start and other regions for future studies.

Key Words: Orchidaceae, Biodiversity, Conservation, Phylogeny, Mycorrhiza

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Thesis Summary

Orchids represent one of the most diverse and ecologically specialized plant families, yet they are disproportionately threatened due to habitat fragmentation, land-use change, climate instability, and obligate biotic interactions with pollinators and mycorrhizal fungi. These vulnerabilities make orchids both high-priority conservation targets and effective bioindicators of ecosystem integrity. While *in-situ* conservation remains central to biodiversity protection, accelerating environmental change has underscored the need for complementary ex situ strategies capable of safeguarding genetic resources, supporting restoration, and enabling long-term research.

Orchid seeds are biologically distinctive due to their minute size, lack of endosperm, and reliance on post-germination symbioses. These traits make them particularly sensitive to ageing processes and complicate the application of standard seed-banking protocols.

Chapter 2: Establishing an Orchid Seedbank in Emilia-Romagna: The UNIMORE Orchid Seedbank

Chapter 2 documents the establishment and initial evaluation of the UNIMORE Orchid Seedbank, which serves as a regional ex situ conservation infrastructure for terrestrial orchids in Emilia-Romagna, primarily in the provinces of Modena and Reggio Emilia in northern Italy.

Seed banking is widely recognized as the most efficient and cost-effective method of conserving plant genetic diversity. However, orchid seeds present challenges due to their powdery texture, variable tolerance to drying, and species-specific physiological responses to storage.

Despite Italy's exceptional orchid diversity, there is currently no coordinated national framework for orchid seed banking. Chapter 2 addresses this gap by presenting the design, implementation and validation of a standardized, regional orchid seed bank integrated within national biodiversity initiatives, this project was also within the National Biodiversity Future Centre (NBFC).

The chapter pursues three interrelated objectives:

- To establish a traceable, standardized orchid seed bank that aligns with international conservation protocols.
- To rationalize historical orchid seed collections and assess their viability and germinability under different storage regimes.
- Generate a new dataset of ethically collected seeds (2023–2025) linked to the monitoring of wild orchid populations in Modena and Reggio Emilia.
- Together, these objectives establish the UNIMORE Orchid Seedbank as a conservation safety net and research infrastructure that supports regional and national biodiversity strategies.

Seedbank Design and Data Infrastructure

One of the chapter's key contributions is the development of a rigorous passport data system that ensures traceability, reproducibility and interoperability. Each accession was assigned a unique code to distinguish between active (short-term, 5 °C) and base (long-term, −20 °C) collections. The database included taxonomic identification, GPS coordinates, a description of the habitat, the phenological stage, estimates of population size, Natura 2000 status and CITES frameworks.

Rationalization of historical collections

The initial rationalization process addressed 138 preexisting orchid seed collections that had been stored under heterogeneous conditions. Duplicate samples, defined as those with identical species, location, collection year and storage conditions, were merged, resulting in 130 unique accessions. This process reduced redundancy, improved data clarity and optimized the use of limited seed material.

Viability and germinability testing revealed stark contrasts between storage regimes. Seeds stored at 5 °C showed almost complete loss of viability (mean <0.5%) and minimal germination, suggesting advanced physiological ageing. In contrast, seeds stored at −20 °C retained partial but meaningful viability (up to ~16%) and germination capacity, including protocorm formation in some taxa, even after ten years. These results confirm that refrigeration is not suitable for the long-term conservation of orchid seeds, whereas subzero storage provides an effective, not indefinite, preservation strategy.

Importantly, the chapter emphasizes that non-viable seeds still have scientific value as sources of DNA for molecular identification and population genetic analyses, thus reinforcing the multifunctional role of historical collections.

Field monitoring and seed collection (2023–2025)

Fieldwork conducted between 2023 and 2025 centered on the provinces of Modena and Reggio Emilia, covering calcareous grasslands, woodland edges, semi-natural meadows, urban parks, cemeteries and private gardens. Fourteen sites were monitored annually with repeated visits.

Seed collection followed strict ethical guidelines to minimize ecological impact. Capsules were harvested shortly before dehiscence from multiple individuals per population, in order to preserve natural seed dispersal and maximize genetic diversity. A total of 54 new accessions representing 22 species were collected, with an increasing focus on urban and peri-urban habitats in the latter years of the study.

Field observations revealed significant variability in population size and reproductive success, frequently associated with management practices. For instance, reduced mowing and physical protection in urban parks were associated with the enhanced persistence of *Himantoglossum adriaticum*, whereas declining capsule production was observed in overgrown or unmanaged sites, as seen with *Ophrys apifera*. These findings reinforce the importance of site level management for orchid conservation.

Seed processing, storage and contamination control

All newly collected seeds were cleaned and dried over silica gel to reach a relative humidity of around 12%. They were then stored in airtight glass vials at −20 °C. Aseptic handling within laminar-flow cabinets and strict sterilization protocols resulted in exceptionally low contamination levels (less than 2% for nearly all accessions), confirming the effectiveness of the processing workflow.

Viability and Germinability of Newly Collected Seeds

Viability assessment using the tetrazolium chloride (TTC) test revealed substantial variability between species among newly collected samples, with values ranging from 0% to 88% (mean ~59%). Genera such as *Anacamptis, Gymnadenia* and *Himantoglossum* consistently exhibited high viability, whereas some *Epipactis and Orchis* accessions showed reduced or absent staining, likely due to premature harvesting or inherent physiological differences.

Methodological refinements, including controlled rehydration, sucrose pre-incubation and optimized scarification, significantly improved staining clarity and interpretability. This highlights the importance of adapting protocols for orchid seeds.

Germination trials on BM-1 medium demonstrated strong correspondence with TTC results. Germination ranged from 0% to 94% (mean ~63%), with many accessions reaching advanced developmental stages and forming protocorms after 20–22 weeks.

These results confirm that storage at −20 °C effectively preserves short- to medium-term physiological integrity, and that TTC testing reliably predicts vitro performance when properly optimized.

Integrated interpretation and conservation significance

Chapter 2 shows that the UNIMORE Orchid Seedbank has evolved from a conceptual initiative into a functional, data-driven conservation infrastructure in just three years. Seedbank's strengths lie in its integration of field ecology, laboratory testing and seedbank management. This provides empirical evidence on how storage temperature, seed quality and species biology interact to determine conservation outcomes.

The Seedbank now fulfils a dual role:

1. Genetic safeguard: preserving regional orchid diversity in the face of ongoing habitat loss and fragmentation.
2. A research platform that supports studies on seed longevity, germination biology, symbiosis and conservation planning.

By linking *ex-situ* collections to detailed ecological and spatial data, the UNIMORE Orchid Seedbank connects laboratory-based conservation with *in-situ* management and national biodiversity policy. As such, it provides a scalable model for the conservation of regional orchids in Mediterranean landscapes and makes a significant contribution to Italy's commitments under European and global biodiversity frameworks.

Chapter 3: Effectiveness of Storage Temperature to Maintain Germinability and Viability of rare and common Orchidaceae species

Chapter 3 provides a comprehensive multi-decadal evaluation of orchid seed longevity under three ex situ storage regimes: refrigerated storage at 5 °C, deep freeze storage at −20 °C and ambient herbarium conditions at ~20 °C. It addresses a critical and under-explored question in plant conservation biology: how does storage temperature interact with seed age and physiology to determine long term viability and germinability in Orchidaceae?

The chapter is structured around three clearly defined experimental objectives:

1. Assessing whether long-term refrigerated storage (5 °C) can maintain seed viability and germinability over periods exceeding ten years.
2. To quantify the effectiveness of −20 °C frozen storage across multiple seed ages (1–10+ years).
3. Evaluate whether herbarium-stored seeds retain any residual viability that could support conservation or genetic applications.

The central hypothesis that lower storage temperatures and shorter storage durations yield higher viability and germinability, with herbarium conditions performing poorest, is explicit, testable and well aligned with contemporary seed conservation theory.

Experimental Design:

The study analyses:

- 79 long-term refrigerated accessions (5 °C), some of which have been stored for almost 30 years.
- 103 frozen accessions (-20 °C), which span multiple collection years.
- 11 herbarium accessions, which were sourced from the University of Michigan Herbarium.

All seed-bank samples were dried prior to storage and then sealed in airtight containers with silica gel. They were also handled according to established conservation protocols. This ensures that any observed differences can be attributed primarily to storage temperature and time rather than to the effects of moisture.

Seed viability was assessed using tetrazolium chloride (TTC) staining, while functional capacity was evaluated via asymbiotic in vitro germination on BM-1 medium, in accordance with recognized orchid propagation standards. The methodological decision to exclude herbarium seeds from germination trials is justified given the scarcity of seeds, their advanced age, and the ethical constraints on destructive sampling.

Results: Refrigerated storage (5 °C)

The results for refrigerated storage are unambiguous and biologically significant. Of the 79 accessions, TTC viability was effectively 0% in both 2023 and 2025, with the minor exception of one *Anacamptis pyramidalis* accession showing 8% viability after protocol refinement, which is adding a step in rehydration by incubating the seeds with 10% sucrose solution for 24 hrs. at 40 °C in a water bath.

Despite the apparent loss of metabolic activity, functional germination was only observed in two species (*Anacamptis pyramidalis and Gymnadenia conopsea*) and only in accessions stored between 2013 and 2018. Initially, germination rates were moderate (up to 48%), but they declined measurably over two years, demonstrating ongoing deterioration.

The discrepancy between TTC viability and germination reveals a significant physiological insight: the loss of detectable enzymatic reducibility occurs before the loss of developmental competence in certain orchid seeds. These findings reinforce the limitations of TTC assays for dust-like seeds that have been documented and highlight the need to interpret viability metrics cautiously.

From a conservation standpoint, the results demonstrate that storing seeds at 5 °C is inadequate for preserving long-term metabolic viability, but it could be used as a short- or medium-term 'active collection' strategy for propagating particularly resilient species. Furthermore, it is important to note that even non-germinating seeds retain value as genetic material for molecular studies.

Results: Frozen storage at −20 °C

In contrast, frozen storage at this temperature proved consistently effective across taxa and timeframes. Germination percentages typically ranged from 50 to 85 per cent, with TTC viability values closely matching these outcomes. Even the oldest accessions (11–12 years old) maintained high functional integrity.

The close correspondence between TTC staining and germination under frozen conditions indicates preserved membrane integrity, enzymatic activity and overall embryo stability. Minor declines in older seed lots were modest and did not compromise conservation utility.

These results are mechanistically aligned with established seed ageing theory: deep freezing suppresses oxidative damage, lipid peroxidation and molecular mobility, particularly in desiccation-tolerant seeds, such as orchids. The absence of ice crystal damage further supports the suitability of storing at −20 °C when moisture content is highly controlled.

Results: Herbarium storage (at ~20 °C)

The herbarium analysis revealed pronounced species-specific outcomes. Of all the taxa examined, only *Epipactis helleborine* retained measurable viability after long-term ambient storage, with TTC values ranging from 1–14% across samples aged up to 29 years.

All other genera examined (*Spiranthes, Corallorhiza* and *Liparis*) exhibited complete loss of viability, confirming that ambient herbarium conditions are generally unsuitable for preserving orchid seeds.

The persistence of viability in *E. helleborine* is biologically significant and likely reflects its exceptional ecological plasticity, genetic diversity and stress tolerance. This finding supports the broader conclusion that seed longevity in orchids is strongly species-dependent and influenced by evolutionary life-history traits. Although herbarium storage is clearly ineffective for physiological conservation, this chapter rightly emphasizes its value for genetic archiving, given the demonstrated stability of DNA in historical specimens.

Chapter 3 makes a clear, evidence-based contribution to orchid conservation science. It demonstrates that:

- Deep-freezing storage (−20 °C) is essential for long-term conservation
- Refrigerated storage (5 °C) has limited, short-term utility
- Herbarium storage is unsuitable for seed viability but valuable for genetic resources

The chapter supports a tiered ex situ conservation strategy, integrating subzero preservation, active propagation collections, and herbarium archiving.

Chapter 4: Integrated Ecological Monitoring and Conservation Interventions of Urban Orchid Populations

Chapter 4 presents the results of a three-year study investigating the ecology, conservation and reproductive biology of terrestrial orchids in urban and semi-natural environments in Modena and Reggio Emilia area of northern Italy. The study integrates population monitoring, spatial mapping, pollination ecology and root-associated fungal analysis to evaluate the potential of urban green spaces to support rare and specialized orchid species, with a particular focus on *Himantoglossum adriaticum* and *Ophrys apifera*. The study emphasizes the importance of ecologically managed urban habitats in sustaining healthy orchid populations and illustrates the efficacy of specific conservation initiatives.

Despite often being associated with habitat degradation and biodiversity loss, urban areas can harbor pockets of ecological value where specialized plant species persist. Terrestrial orchids are sensitive indicators of habitat quality due to their complex ecological requirements, such as

pollinator interactions and dependence on specific mycorrhizal fungi. Urban green spaces, such as managed parks, cemeteries, hedgerow margins and semi-natural reserves, can therefore function as refuges for these species, provided ecological management practices minimize disturbance and maintain suitable microhabitats.

This study aimed to:

1. address knowledge gaps in the monitoring of orchids,
2. their pollination reproductive ecology
3. and their mycorrhizal associations particularly in urban environments.

Monitoring and Conservation:

Over three years, systematic surveys were conducted in urban parks, semi-natural green spaces and nature reserves to document the presence, abundance and spatial distribution of orchid populations. Monitoring coincided with key phenological phases from early spring to summer, enabling the identification of individuals, the recording of flowering events, and the evaluation of population trends. The geographic coordinates of the orchids were recorded for spatial mapping purposes, and the threats posed by mowing, trampling and habitat disturbance were assessed at each site.

Targeted conservation interventions were then implemented to mitigate these threats. Protective barriers and exclusion zones were established around vulnerable plants or clusters to prevent mechanical damage during routine maintenance. Additionally, ex situ seed collection was undertaken for species of conservation interest to ensure long-term preservation and enable potential future reinforcement or restoration measures. These in situ and ex situ strategies were designed to enhance population persistence and reproductive success in urban contexts.

Pollination Ecology:

Studies of the pollination ecology of *H. adriaticum* were conducted across two contrasting habitats: the urban park Parco San Lazzaro and the semi-natural Salse di Nirano reserve. At different stages of flowering, observations were conducted using video recording and direct insect capture to identify pollinators and determine their roles. Bagging inflorescences controlled for self-pollination, while unbagged flowers were monitored to assess insect-mediated cross-pollination. The data revealed a diverse range of visitors, with bees _ particularly those belonging to the Halictidae and Andrenidae families _ serving as the primary pollinators. Other insects, including hoverflies, fungus gnats, ants and moths, acted as secondary visitors, contributing variably to pollen transfer. Fertilization success correlated strongly with bee visitation in open urban habitats. In contrast, structurally complex semi-natural sites exhibited lower fruit set despite similar visitation rates. This highlights the importance of habitat openness and spatial arrangement for effective pollination.

Mycorrhizal Association:

Analyses of root-associated fungi were conducted to assess the presence and diversity of mycorrhizal and endophytic fungi in *H. adriaticum* and *O. apifera*. Root samples were collected during flowering, surface-sterilized and cultured on malt extract agar. The emerging fungi were purified and their DNA extracted for sequencing of the internal transcribed spacer (ITS) region to identify taxa. The results revealed diverse assemblages dominated by disturbance-tolerant

ascomycetes, including *Fusarium, Diaporthe/Phomopsis, Dicyma* and *Chaetomium.* Classical orchid mycorrhizal fungi were not detected using culture based methods; however, the persistence of orchid populations suggests that urban adapted fungal communities may sustain essential physiological and ecological processes. These endophytic fungi can influence nutrient acquisition, host physiology and interactions with other soil organisms. Depending on the environmental context, they may complement or compete with classical mycorrhizal partners.

Discussion:

The study revealed significant variations in orchid population trends between urban and semi-natural habitats. In urban parks, populations of *O. apifera* and *H. adriaticum* exhibited higher density and more stable growth patterns than populations in semi-natural sites, which were smaller and more dispersed. For instance, the number of *H. adriaticum* individuals at Parco San Lazzaro increased from one in 2019 to twenty in 2025, thanks to protective measures and reduced mowing. Conversely, semi-natural reserves exhibited lower reproductive output, likely due to habitat complexity and microclimatic conditions limiting pollinator efficiency. Similarly, populations of *O. apifera* in Parco della Resistenza showed decreasing capsule production over three years, reflecting the impact of habitat shading and reduced pollinator access.

Overall, the findings emphasize the vital role of urban habitat management in orchid conservation. Measures that maintain habitat openness, reduce disturbance and support pollinator access can significantly enhance reproductive success and population persistence. Furthermore, urban soil harboring compatible fungal endophytes may compensate for the absence of classical mycorrhizal fungi, enabling orchids to thrive in altered human environments. Integrating demographic monitoring, pollination biology and fungal ecology provide a comprehensive understanding of the factors influencing orchid survival and emphasizes the potential of urban green spaces to meaningfully contribute to biodiversity conservation.

In conclusion, this study shows that ecologically managed urban parks can be valuable refuges for rare and specialized terrestrial orchids. Key to promoting population growth and reproductive success are protective interventions, careful habitat management and attention to belowground symbiotic relationships. The results provide a framework for urban biodiversity conservation and demonstrate that cities can play an active role in preserving species traditionally considered vulnerable to human disturbance. By bridging gaps in population ecology, pollination biology and fungal symbioses, the study provides practical insights into conserving orchids in urban and peri-urban landscapes, thereby supporting the broader goal of integrating biodiversity preservation into human-dominated environments.

Chapter 5: Does Phylogenetic Relatedness Predict Conservation Status in Italian Orchids?

Understanding extinction risk within an evolutionary framework is increasingly recognized as essential for the effective conservation of biodiversity. Traditionally, species-based assessments, such as those employed in the IUCN Red List, primarily evaluate extinction risk through demographic trends, population size and geographic range. These approaches are valuable, by providing an overlook on how threatened species are distributed across the tree of life. Consequently, conservation planning may fail to recognize situations in which the loss of a few species results in significant erosion of evolutionary history.

This chapter takes a phylogenetic approach to evaluate whether the conservation status of Italian orchids is evolutionarily structured. Specifically, it examines whether threatened species cluster within evolutionary lineages and whether phylogenetically distinct taxa are disproportionately at risk. This provides a basis for integrating evolutionary history into orchid conservation strategies.

Objectives and Research Questions

The primary aim of this study was to assess whether phylogenetic relatedness can predict conservation status in native Italian orchids.

Species selection and conservation data:

The analysis included 61 native Italian orchid species, which represented a broad taxonomic and ecological spectrum ranging from widespread generalists to narrow endemics. The selection of species was limited by the availability of reliable occurrence data and conservation assessments. Taxonomic standardization was applied to ensure consistency across datasets.

Conservation status was assigned using the IUCN Red List categories. The species were classified as Least Concern (LC), Near Threatened (NT), Vulnerable (VU), Endangered (EN), Critically Endangered (CR) or Data Deficient (DD). While most of the assessed species fall within the LC category, several taxa are listed as NT or EN, and others remain insufficiently evaluated. This highlights the persistent knowledge gaps in Italian orchid conservation.

Phylogenetic reconstruction and analytical approach:

A species-level phylogeny was constructed using the R package V.PhyloMaker, integrating the Italian orchid species list into the extended GBOTB megatree of vascular plants by Brown and Smith 2018 and Zanne et al.,2014 This approach enabled all target species to be included, even those lacking molecular sequence data, by grafting taxa onto well-supported higher-level nodes based on accepted taxonomy.

The resulting phylogeny retained branch lengths and hierarchical relationships suitable for comparative analysis. Conservation status categories were mapped onto the tree to visualize and assess the phylogenetic distribution of extinction risk. Clustering patterns, lineage isolation and the correspondence between evolutionary distinctness and threat level were then examined qualitatively and comparatively.

Phylogenetic Distribution of Conservation Status

Mapping IUCN categories onto the phylogeny revealed clear, non-random patterns of extinction risk. Species classified as 'Least Concern' dominated the tree and were concentrated within species-rich, rapidly diversifying clades, such as Ophrys, Serapias and Anacamptis. These groups generally comprise widespread taxa with broader ecological tolerances and greater geographic ranges.

In contrast, Near Threatened and Endangered species were unevenly distributed and frequently associated with phylogenetically isolated or weakly diversified lineages. Several endangered (EN) taxa, such as *Gennaria diphylla, Nigritella widderi, Platanthera chlorantha* and *Orchis patens subsp. brevicornis*, occupied long branches. This indicates that extinction risk is concentrated in evolutionary distinct lineages rather than spread evenly across the orchid phylogeny.

Lineage-level case studies

The close phylogenetic proximity of *Orchis patens subsp. brevicornis* and *Orchis spitzelii subsp. spitzelii* within the Orchis clade illustrates how extinction risk can cluster within evolutionarily coherent lineages. Both species have limited distributions and specific habitat requirements, resulting in low population densities and making them highly susceptible to habitat fragmentation and climate change. Their cooccurrence within a vulnerable lineage suggests that they face shared ecological constraints that limit their adaptive potential.

Similarly, *Nigritella widderi* and *Nigritella corneliana* form a distinct, long-branched alpine lineage. *N. widderi* is classified as Endangered, while *N. corneliana* is classified as Data Deficient. Their phylogenetic isolation, combined with their narrow elevational ranges and sensitivity to climate warming, indicates that this lineage faces a high risk of disproportionate evolutionary loss under future climate scenarios.

Gennaria diphylla is another striking example of evolutionary isolation. Its distinct phylogenetic position, combined with its restricted Mediterranean distribution and fragmented habitats, suggests latent vulnerability that may not yet be fully captured by formal threat categories. While its reproductive system may buffer against pollinator instability, small population size and habitat loss remain critical risks.

In contrast, closely related species can exhibit divergent conservation trajectories. The butterfly orchids *Platanthera bifolia* and *P. chlorantha* form a tight phylogenetic pair yet differ markedly in habitat specificity and vulnerability. This demonstrates that, while phylogeny cannot predict risk deterministically, it can provide a probabilistic framework for identifying lineages where ecological traits interact with anthropogenic pressures.

This chapter demonstrates that phylogenetic relatedness provides meaningful predictive insight into the conservation status of Italian orchids. By revealing lineage-level patterns of vulnerability, it highlights the importance of evolutionary perspectives in conservation biology. Beginning orchid conservation planning with phylogenetics offers a powerful strategy for anticipating biodiversity loss and preserving both functional and evolutionary diversity in a rapidly changing world.

Chapter 6: Conservation and Reintroduction of *Allium angulosum L.* in Emilia-Romagna: Assessing Ex Situ and In Situ Strategies for Reinforcement in Parco della Resistenza

To increase the conservation capacity within UNIMORE Seedbank, addition of new threatened species leads to this study.

Allium angulosum is a bulbous perennial plant found across Central and Eastern Europe. It typically grows in wet meadows, floodplain grasslands and irrigated lowland pastures. In Italy, however, its distribution is highly localized, with historical populations concentrated in the north of the country. While not listed in the Italian national Red List, the species is classified as Endangered in Emilia-Romagna due to restricted habitat availability, a declining population size and increasing anthropogenic pressure.

The extinction of the Tagliati di Albareto population in the early 2000s marked significant regional biodiversity loss. Therefore, the rediscovery of around 50 individuals in 2020 in the urban park Parco della Resistenza in Modena represents a critical opportunity for conservation and restoration. This rediscovery has prompted the present study, which aims to evaluate the feasibility of reinforcing and reintroducing *A. angulosum* through integrated *ex-situ* and *in-situ* conservation strategies.

Objectives of the study:

The primary objective of this study was to evaluate the germination potential and initial growth of *Allium angulosum* seeds under different conservation strategies.

1. Ex-situ conservation, including seed banking and controlled laboratory germination; and in situ conservation through direct sowing in Parco della Resistenza under semi-natural conditions.
2. In-situ conservation through direct sowing in the semi-natural conditions of Parco della Resistenza.

The study sought to quantify germination rates. It also sought to evaluate environmental constraints. And it sought to identify management factors affecting seedling survival. This is so it can inform future restoration initiatives in Emilia-Romagna.

Materials and methods:

A total of 800 seeds, collected from the rediscovered population, were divided into four experimental groups. Two hundred of these were dried and stored in the UNIMORE Seedbank in accordance with standardized desiccation protocols for long-term conservation. The remaining 600 seeds were used for germination trials.

Controlled laboratory germination involved sowing 200 seeds in a perlite–substrate mixture under stable temperatures (initially 20 °C, later 25 °C) and regulated photoperiods. Germination was monitored for 162 days, with radicle emergence serving as the germination criterion. Germination dynamics were quantified using the Germination Index (GI), which considers both speed and uniformity.

Field trials were conducted in the Parco della Resistenza, where 200 seeds were sown directly into unamended soil in November 2023. Two sowing densities were tested: low- and high-intensity cultivation. Plots received no irrigation or fertilization, relying entirely on natural rainfall. Germination and seedling persistence were monitored until spring 2024.

Results:

Laboratory germination:

Under controlled conditions*, A. angulosum* seeds exhibited delayed but sustained germination. Initial germination occurred 92 days after sowing, which is consistent with physiological dormancy. By May 2024, 80 out of 200 seeds (40%) had germinated, yielding a final germination index (GI) of 0.93, indicating relatively uniform germination once dormancy had been overcome.

Germination rates increased markedly in April, suggesting a cumulative response to stable moisture and temperature conditions. Similar lag phases have been reported in other Allium species, such as *A. cepa and A. schoenoprasum*, particularly when dormancy-breaking treatments are absent. These findings confirm that *A. angulosum* seeds are viable but require extended periods of favorable conditions to germinate.

Field Germination and Seedling Survival:

Field results contrasted sharply with laboratory outcomes. In the low-intensity plot, 15% of seeds germinated, whereas only 1% germinated in the high-intensity plot, highlighting strong density-dependent effects. However, all seedlings were lost following periods of intense rainfall that caused prolonged waterlogging.

Excess soil moisture likely reduced oxygen availability, leading to hypoxic stress, root damage and increased susceptibility to microbial decay processes well documented in monocot seedlings. These conditions effectively eliminated early establishment, despite the initial success of germination in low-density plots.

Conclusions:

This study provides an integrated assessment of seed-based reinforcement strategies for *Allium angulosum* in Emilia-Romagna. While seeds are viable and capable of germination, the findings demonstrate that successful establishment is highly dependent on environmental control. Laboratory trials confirmed moderate germination potential, whereas field trials revealed extreme vulnerability to waterlogging and density stress.

Therefore, long-term conservation of *A. angulosum* will require a combination of *ex-situ* propagation and carefully managed reintroduction efforts tailored to the species' ecological requirements. Beyond its regional significance, this work offers broader insights into the conservation of rare, moisture-sensitive geophytes in an era of increasingly unpredictable climatic conditions.

Chapter 1: Introduction

1.1. Biodiversity and Conservation Strategies: *in-Situ and ex-Situ* approaches

The diversity of life forms found on Earth. Biodiversity includes variety as well as diversity at genetic and specific levels of the ecosystem. This variety is essential for the ecosystem services that support wellbeing by helping with processes like nutrient cycling and pollination while also regulating climate and purifying air, water and soil (MEA 2005). Despite its importance both in terms of value and practical benefits to humans, some of these services are in danger due to human activities such as habitat destruction and climate change (IPBE 2019). In many countries, like Italy are facing same patterns: areas such as meadows and wetlands that are semi- natural, along with forest boundaries, which are usually abundant in orchids and other unique plant species, are notably at risk, due to alterations in land use according to the European Environment Agency report from 2020. Italy stands out as a biodiversity hub in Europe hosting more than 7.600 plant species with a significant number of them being unique to the region, as noted by Conti and colleagues in 2005. The intricate relationship between the influences from the Mediterranean and alpine regions in Italy's landscapes and climate variations plays a significant role in its biodiversity richness. However, factors, like intensified agriculture practices, expanding city areas, unpredictable climate changes, and occurrence of invasive alien species are posing growing challenges to the preservation of its variety of species (Orsenigo et al. 2018).

Biology conservation came about to tackle the decline in biodiversity by blending principles with real world initiatives. Today's conservation plans include both on-site methods, which strive to safeguard species within their environments, and off-site approaches, that prioritize protecting species beyond their habitats. Preserving species in their settings through on-site conservation is widely regarded as the most effective method, since it maintains ecological relationships and evolutionary developments, within their natural context (Primack, 2006). In Italy's conservation efforts, for habitat and species protection, rely heavily on the creation of protected areas like parks and Natura 2000 sites. The goal is to not only preserve biodiversity but also to use these spaces as natural testing grounds for monitoring ecosystems and restoring balance (Blasi et al., 2011). However, making these initiatives successful involves management of habitats, strict enforcement of rules and active involvement from local communities. Conservation of orchids presents a challenge because they rely on specific mycorrhizal fungi and pollinators for survival. Habitat fragmentations and changes in how lands are managed can easily disrupt these relationships (Rasmussen & Rasmussens, 2009). In places like Emilia Romagna region there is an illustration point; old school methods of tending grasslands such as allowing animals to graze or cutting grass in the season play a pivotal role in preserving meadows abundant with orchids (Pierce et al. 2011; Pezzi et al. 2025). Without care strategies in place even protected conservation sites can experience a decline in biodiversity because of progression and competition among species.

Conservation methods done outside habitats are crucial, for endangered or locally extinct species as they offer a valuable backup plan to protect genetic material and plant species through seedbanks and botanical gardens. For plants like orchids and other small-scale species that are hard to reproduce in the wild, due to their needs and reproduction process complexity *ex-situ* conservation methods play an important role. In Italy there are various organizations, like the Rete Italiana Banche del Germoplasma (RIBES) as regional botanical gardens such as those in

Bologna, Rome, Catania, and Palermo are actively involved in conservation efforts. The conservation efforts frequently partner with universities and non-governmental organizations to reintroduce species into environments (Dumont, V. IUCN/SSC Orchid Specialist Group; Seyler, 2017).

Additionally, crucial action *ex-situ* conservation efforts serve as valuable tools for education and scientific exploration. It allows researchers to deepen more about physiological aspects and genetic profiles of species, for example, while studying the responses to various stressors, beyond natural environments. However, these initiatives work best when combined with *in-situ* preservation efforts and are supported by ecological observation to guarantee that offsite populations maintain genetically sustainable and ecologically significant (Heywood et al. 2005; Dumont, V. IUCN/SSC Orchid Specialist Group).

To sum up, preserving biodiversity among transformations necessitates a comprehensive strategy integrating both *on-site* and off-site conservation approaches, that are crucial to safeguard biological diversity effectively (Swarts, 2007). Preserving habitats and restoring ecosystems are components of biodiversity conservation efforts while off site methods provide a safety measure for endangered species (Khanna, 2025). In regions of high biodiversity, like Italy, policies and community participation are vital to secure the existence of biological legacy for the upcoming generations.

1.2. Orchid Biology in Italy: Distribution, Morphology, Seed Traits, Growth & Adaptation

The Orchidaceae family serves as the orchid family name while it stands as one of the largest and diverse flowering plant families with between 25,000 to 30,000 species across 800 genera worldwide (Chase et al., 2015; Govaerts et al., 2021). The Orchidaceae family exists in all ecosystems except polar ice and extreme deserts because it succeeds in tropical rainforests and alpine meadows (Dressler, 1993). The worldwide distribution of orchids as dominant plant groups branches from their ability to adapt to various ecosystems. Italian orchid species and subspecies number 236 according to GIROS (2024) while they belong to 27 genera and include 87 endemic species. The exceptional biodiversity in Italy results from its position as a transition area between Central Europe and the Mediterranean region. The combination of diverse climate zones and mountainous terrain within Italy supports an abundant orchid species diversity (Orsenigo et al., 2018). The Apennine mountains function as ecological corridors which enable montane and lowland species to interact and sometimes produce hybrids thus maintaining genetic diversity and enhancing ecological resilience. The ecological significance of Italian orchids becomes more evident through recent botanical surveys and national red lists because they demonstrate high levels of endemism while being susceptible to environmental changes and habitat destruction (Pezzetta et al., 2018).

Orchids in Italy thrive across multiple habitat types including sunny coastal dunes and diverse lowland meadows and deep montane forests and harsh alpine grasslands (Antonio, 2016). Puglia maintains its position as a major orchid biodiversity center which supports more than 113 documented species with an abundance of *Ophrys* and *Serapias* species (GIROS, 2024). The Emilia-Romagna region, which serves as the basis for this thesis, hosts over 60 taxonomic

species across its diverse environments of calcareous grasslands, woodland edges and abandoned terraced fields throughout Parma, Reggio Emilia, Modena, Bologna, and Ferrara (Brancaleoni et al. 2024, della Romagna, N. D. S. N. Segnalazioni floristiche n. 127-138.). Four significant species among the region's native flora include *Anacamptis morio* and *Orchis anthropophora* together with *Himantoglossum adriaticum* and *Platanthera chlorantha*, as they are very abundant and well distributed among this region. The combination of environmental factors, including elevation along with microclimatic variations, various soil pH, and historical land use practices, determines the preferred habitats for these species. Particularly, the highest number of orchid species occurs in traditional grasslands which receive maintenance through mowing and described as Habitat 6210 in Annex 1 of Habitat Directive EU (1992). These dynamic ecological environments sustain species that exist both widely and in limited geographic areas, while certain species maintain strong attachments to sites or microhabitats. The disruption of delicate ecosystems due to changes in land use necessitates both habitat management and extended monitoring practices for their preservation (Fay, 2018).

Orchids gained widespread recognition because of their extraordinary pollination-related morphological adaptations. Various plant species developed complex floral arrangements through the process of pollinator attraction through deception or reward and mimicry (Tremblay et al., 2005). The *Ophrys* genus in Italy uses deceptive methods to impersonate female insects while producing pheromones which attract male pollinators. The *Serapias* and *Anacamptis* genera use tubular or helmet-shaped flowers as shelter instead of nectar provision. The story extends beneath the surface where most orchids need mycorrhizal fungi to germinate and grow. Symbiotic relationships play an essential role in Mediterranean soils which lack nutrients because they enable water and nutrient uptake during seedling development and adulthood (Rasmussen & Rasmussen, 2009).

The orchid achieves evolutionary success through its special reproductive strategy which is most evident in seed biology. The seeds of orchids reach sizes between 200–400 μm yet lack endosperm so they must use symbiotic fungi (Tulasnellaceae, Ceratobasidiaceae and Sebacinaceae families) to start germination (Arditti & Ghani, 2000). The dependence of orchids on fungi has developed precise evolutionary relationships. Research findings on Italian plant species *Orchis patens* and *Ophrys panormitana* demonstrate that seed germination success depends on the compatibility between seed and fungal partner (Shefferson et al., 2020; Selosse et al. 2025). The propagation studies involving symbiotic and asymbiotic methods have shown positive outcomes for several species, which helps in conservation initiatives and supports potential reestablishment programs (Rasmussen & Rasmussen, 2009; Sgarbi et al. 2007, McKendrick et al. 2000). Seed coat features in different orchid genera affect water absorption and gas exchange and dormancy behavior through variations in seed coat ornamentation and thickness (Prutsch et al., 2000).

The unpredictable environments of Italian orchids have led to the development of diverse ecological survival strategies. Plants with underground survival structures known as geophytes endure harsh times by forming underground organs before they become dormant during hot and dry Mediterranean summers (Yasemin et al., 2024, Trail, 2018). These species protect their limited germination success by producing countless dust-like seeds that wind easily carries.

Orchids survive environmental challenges through vegetative persistence and clonal growth while maintaining their populations even when their habitats decline or climate conditions become adverse. The persistence of orchids throughout Italy's fragmented landscapes depends on these traits but ongoing threats from habitat destruction, illegal collection, and climate change thus requires immediate conservation measures (Orsenigo et al., 2018).

1.3. Seedbanks and Their Role in Orchid Conservation

Seedbanks function as fundamental tools for plant conservation by storing genetic material for extended periods while serving as essential backup systems during biodiversity decline. The conservation of rare endemic or threatened species benefits from seedbanks, because of their natural populations decreasing, habitat destruction, climate change, and anthropogenic disturbances (Li & Pritchard, 2009). Seedbanking enables the maintenance of viable seeds in controlled storage facilities which protects genetic diversity and facilitates future restoration research as well as propagation activities. Seedbanks represent opportunities for orchid conservation because of their small size - dust-like seeds - difficult germination requirements, as well as limited habitat range (Seaton et al., 2013).

Some flowering plants produce seeds that measure less than 1 mm in length and lack endosperm yet requiring specific mycorrhizal fungi to start their germination and initial development (Rasmussen & Rasmussen, 2009). The special nature of orchid biology creates challenges for both seed collection and storage operations. Research shows that orthodox seeds can survive decades at low temperatures, but orchid seeds demonstrate storage behavior variability based on moisture content and lipid composition and species-specific tolerances (Pritchard et al., 2004). The proper optimization of desiccation to low relative humidity and cryogenic storage in liquid nitrogen has allowed researchers to prove that orchid seeds can survive for prolonged periods. The advancement of seed storage methods has allowed seedbanks throughout Europe to include native orchid species within their collection holdings.

Seedbanks across Europe, including Italian institutions, now actively prioritize the conservation of orchid seeds. Italy coordinates seed conservation activities through the Rete Italiana Banche del Germoplasma (RIBES) which unites botanical gardens and universities across Italy devoted to conservation activities. Multiple regional initiatives have focused on seed collection and storage for endangered orchid species, including *Ophrys panormitana and Anacamptis longicornu* and *Serapias neglecta* which are either endemic or have limited geographical distribution (Pierce et al. 2011; Pezzi et al. 2025). These seed collections function as genetic resources which support both ecological restoration efforts, reintroduction programs, and *ex-situ* cultivation methods. The storage capabilities of seedbanks enable scientists to conduct experimental research about germination biology along with storage properties and symbiotic interactions in controlled environments, which guides *in-situ* conservation strategies (Seaton & Pritchard, 2003).

The technical obstacles in orchid seed biology do not diminish the essential importance of seedbanks in their conservation efforts. The combination of collaborative networks with advanced

storage technologies and habitat restoration approaches enables seedbanks to strengthen orchid population resilience for achieving plant biodiversity conservation in our changing world.

1.4. Rationalization Process

Seedbank management is an important approach for the continuity of a seedbank, important elements of management at both the seedbank and the collection level are analyzed, options for more efficient and cost-effective management are discussed, and genetic and economic implications are inferred. This will hopefully lead to rationalization of seedbank operations under a range of economic conditions, taking account of various government policies and other important factors (Engels, 2003).

Ex-situ Conservation of different types of orchid collections has increased enormously in number and size during the past years. These collections are maintained under widely different conditions, depending on national and international policy frameworks, institutional environments, available expertise, facilities and budgets, and on the extent of national and international collaboration (Guerrant et al 2014).

Thus, rationalization stands for the reorganization of a desired resource, which is orchid collections within previously collected samples at the University of Modena and Reggio Emilia in Italy. For more clarifications about rationalization, here are some reasons why Rationalization is an important process in management.

A simple definition for rationalization accessions is eliminating unwanted accessions and either eliminating or combining duplicate accessions. Thus, curators are urged to decrease their costs and this will happen by rationalizing their collections. Maintenance of large collections is questionable if they are used so little. An alternative is to rationalize, reducing the size of collections and the cost of their maintenance and increasing use of what remains. Conventionally in the past, the term rationalization has been applied only to the elimination of unwanted accessions from the entire collection (Adams ,1997).

When considering whether to rationalize or not, a genebank manager must assess the scientific and financial costs and benefits of both forms of rationalization. The primary prerequisite for effective rationalization is that it must be possible to assign a value to each accession easily, accurately and cheaply. In other words, after rationalization is done, there will be a step to sort or arrange the accessions, according to their added value that could be later used in propagation or breeding programs as an example. This step is called Prioritization.

Within UNIMORE Collections of orchid seeds, and under the topic of Establishing a seedbank, Rationalization was done to previously collected samples. This rationalization step is summed up by the combining or eliminating duplicates, and this type of rational is done to minimize the resulting loss of genetic diversity of the desired collections, and to determine which of the accessions are true duplicates. For the last of these reasons, it is inevitable that a major loss of genetic integrity will occur if duplicates are defined purely based on their measured genetic similarity. Arguably, the risk of unacceptable loss of genetic integrity caused by combining two accessions may be small if they are known to be geographical as well as biological duplicates. If they have the same origin, then failing to detect a difference between them for the measured traits

may indicate that they have not undergone genetic changes since separation. Eventually, the rationalization of the conservation collection will involve decisions based on incorrect info. To avoid such errors, keep the eliminated accessions in achieve instead of disposing them. Therefore, any damage that could happen to the collection caused by rationalization is then fixed and repaired (Engels 2003).

To achieve a successful rationalization, the comparison between the two orchid collections will rely on rationalizing through passport data or the presented available data. The presented data of the collected samples is the exact geographical location of the samples (Longitude and Latitude), accession name (unique code used for the collected sample), species name (scientific name), country of origin, acquisition date (date of collection).

The objective of the work is to Identify the duplicated accessions of orchid samples collected, to improve the efficiency of their management. This is accomplished by: A) Analyzing the presented available data for orchid collections in the database, first by sorting the samples according to the scientific name and year of collection. B) analyzing the resultant data using geographical references and the locations of the samples as the second level.

1.5. Germinability and Viability Tests

Understanding seed germinability and viability is fundamental to the effective functioning of seedbanks, especially when dealing with species of conservation concern such as orchids. Since the ultimate purpose of seed conservation is the eventual recovery or restoration of viable populations, it is essential to assess whether stored seeds can germinate and develop into healthy seedlings. This is particularly critical for orchids, whose seeds lack endosperm and rely heavily on mycorrhizal fungi to germinate under natural conditions (Arditti & Ghani, 2000). While seed viability refers to the physiological state of being alive and capability of development, germinability reflects a seed's potential to initiate and complete germination under given conditions (Bewley et al., 2013). Accurate testing of both traits ensures that seed lots in long-term storage conditions remain functional and allows regular monitoring of deterioration over time.

In orchid conservation, germination tests are often conducted using either asymbiotic (nutrient medium-based) or symbiotic (fungus-inoculated) methods. Asymbiotic protocols are preferred for germinability monitoring in seedbanks, as they provide controlled, replicable environments for evaluating germinability without the variability introduced by fungal partners (Seaton et al., 2013). Media such as modified Knudson C and Murashige and Skoog (MS) formulations are widely used, often with added growth regulators to promote protocorm development, and BM1 showed the best results (Sgarbi et al, 2007). These methods are especially useful for Mediterranean orchids, including *Anacamptis*, *Serapias*, *Ophrys*, and *Himantoglossum* where seed response to storage and germination conditions can be highly species-specific (Shefferson et al., 2020; Sgarbi et al, 2007). However, due to the limited nutrient reserves in orchid seeds, successful germination often depends on minute environmental factors such as temperature, light, and the developmental maturity of seeds at harvest.

Viability assessments in orchid seedbanks are commonly carried out using tetrazolium (TZ) staining, a rapid biochemical assay that detects living tissues by their ability to reduce colorless

tetrazolium salts into red formazan pigments (Seaton et al., 2018). This test allows for viability estimation even in seeds that are highly dormant or reluctant to germinate in vitro. Nevertheless, interpreting TZ test results for orchids requires significant expertise, as the tiny embryos are often difficult to visualize and stain patterns may vary across species (Pritchard et al., 2004).

Therefore, standardizing germination and viability testing protocols, particularly for taxa of high conservation priority, remains a key objective in both national and international seedbanking strategies.

1.6. Urbanization as a Threat to Wild Orchid Diversity

Urbanization functions as a major worldwide force behind biodiversity loss because it modifies ecosystems and breaks up habitats and introduces various ecological pressures that primarily harm delicate orchid species. Wild orchid populations encounter major threats to their existence because cities expand into rural and natural areas through habitat destruction and soil sailing, combined with pollution, altered hydrology and invasive species introduction (McKinney, 2006). The changes disrupt essential ecological relationships which orchids need to survive because they disrupt their symbiotic relations with particular mycorrhizal fungi and pollinators (Rasmussen & Rasmussen, 2009). The sensitive survival requirements of orchids operating in Mediterranean and temperate ecosystems become threatened by minimal human disturbances.

The expansion of peri-urban zones across northern and central Italy represented a primary threat which jeopardizes habitats that are rich in orchids, particularly semi-natural grasslands as well as forest edges and wetland margins (Orsenigo et al., 2018). The combination of urban expansion and infrastructure development with traditional land abandonment practices has caused Emilia-Romagna to witness decreased orchid species populations according to Ferrari et al. (1993). The resulting pressures transform landscapes into uniform spaces which diminish the critical heterogeneity required by orchids. The combination of artificial light and noise pollution affect pollinator activities which subsequently decrease both pollination success and reproductive output in orchids, that rely on insects for pollination (Knop et al., 2017). Open meadows that were once species-rich have been replaced by road paving, compacted soil and manicured green spaces, which destroy the essential microhabitats required for orchid seed germination and seedling growth.

The native plant species found in urban areas tend to be generalist and non-native plants which compete with native orchids for resources and transform soil and microbial environments (Aronson et al., 2014). The essential mycorrhizal networks which support orchid seed germination and nutrient acquisition become highly susceptible to fragmentation and pollution because heavy metals along with road salts and chemical runoff inhibit fungal growth and function (Lauber et al., 2008). The breakdown of plant-fungus-pollinator interactions result in loss of living organisms biodiversity, in addition to reduction in genetic diversity along with decreased ecosystem resilience.

The challenges require conservation efforts in urban and peri-urban landscapes to establish protected habitats while restoring ecosystems through green infrastructure, aimed to replicate natural ecological processes. The planning of improving urban biodiversity should include orchid-

rich sites within their frameworks while implementing protective measures for ecological corridors and supporting traditional land-use practices when appropriate. European regions have started testing these approaches which show promise for balancing urban development with wild orchid preservation, as an example NBFC Project.

1.7. Highlight on *Himantoglossum adriaticum* and Its Status in Emilia-Romagna

Himantoglossum adriaticum is considered a rare orchid species in the Emilia-Romagna region. For this reason, and because it served as the focal species for both the pollination study and the fungal isolation analyses (the latter conducted together with *Ophrys apifera*), this section focuses primarily on this species.

Himantoglossum adriaticum H. Baumann, commonly known as the Adriatic lizard orchid, is a stately and distinctive terrestrial orchid endemic to the southern-central European region, including Italy. This long-lived perennial reaches an impressive 50–100 cm in height and features paired underground tubers—one sustaining current growth and the other serving as a nutrient reserve for the following season (Bódis et al., 2019; Bojňanský & Fargašová, 2007). The flowering stem, which emerges between May and July, bears a cylindrical spike of 25–40 hermaphroditic flowers characterized by a spiraling, strap-like labellum—an evolutionary signature that gave rise to its genus name (himas meaning strap and glossa meaning tongue) (American Orchid Society, 2021; Bódis et al., 2019).

The lizard orchid's flowers emit a mild scent and offer no nectar reward. Instead, their unusual shape attracts various insect pollinators—including bees, flies, and beetles—through mimicry and sheltering cues (Bódis et al., 2019). Following pollination, the plant develops a fissure-opening capsule containing dust-like seeds that rely entirely on mycorrhizal fungi for germination (Arditti & Ghani, 2000; Rasmussen & Rasmussen, 2009). Ecologically, *H. adriaticum* is a calcicole, preferring well-drained, calcareous substrates found in dry grasslands, woodland margins, rocky screes, and occasionally successional roadside areas, from sea level up to about 800 m and occasionally higher (Bódis et al., 2019; Bodis et al., 2018).

Within its range—including Italy, Slovenia, Croatia, Austria, Czechia, Slovakia, Hungary, and Bosnia-Herzegovina—*H. adriaticum* occupies fragmented populations and is considered rare or endangered in several countries (Bódis et al., 2019; Dostalova et al., 2011). In Hungary, Austria, and Czechia, it is listed as Endangered (EN) or Critically Endangered (CR); it is classified as Vulnerable (VU) in Slovenia and Near Threatened (NT) in Croatia, while the global IUCN Red List categorizes it as Least Concern (LC) due to its wide—but disjunct—distribution (Bódis et al., 2019; Dostalova et al., 2011; IUCN, 2013).

In Italy, the species was first described by Baumann in 1978 (Bódis et al., 2019). It is protected under Annex II of the EU Habitats Directive and is designated as Least Concern nationally, though included in regional conservation lists and monitored across Natura 2000 sites (Dostalova et al., 2011; Regione Emilia-Romagna, 2022).

Field observations have identified populations in dry calcareous grasslands, clearings, and mosaic habitats across Piacenza, Reggio Emilia, Modena, Forlì-Cesena, and Bologna provinces. However, these habitats face pressure from land abandonment, invasive species (*Robinia pseudoacacia*), intensive mowing, and wild boar rooting (Del Vecchio et al., 2019). Without active management, semi-natural grasslands undergo ecological succession, reducing the light and soil conditions favorable for *H. adriaticum.*

Despite its specialized biology, *H. adriaticum* responds positively to conservation interventions. Moreover, the species benefits from traditional mowing practices that maintain habitat openness, prevent shrub encroachment, and support orchid population stability (Bódis et al., 2019). Conservation strategies in Emilia-Romagna include Natura 2000 site management plans, invasive species control, orchid monitoring, and *ex-situ* seed storage programs coordinated through regional botanical networks (L.R.2/1977, in Regione Emilia Romagna 2025).

In summary, *H. adriaticum* exemplifies the delicate balance between biological specialization and environmental vulnerability. Its conservation in Emilia-Romagna relies on an integrated approach that combines ecological research, active land stewardship, and regional planning under EU directives. With appropriate investment in monitoring and habitat management, this remarkable orchid has strong potential for long-term persistence in the region.

1.8. Pollination Strategies in Orchids

Orchids display remarkable evolutionary pollination techniques through various complex methods that span from authentic nectar rewards to sophisticated deceptive approaches. Orchids depend on animal pollinators for reproduction because 97% of their species do so (Darwin, 1877; Dressler, 1993) which led them to develop pollinia and fused reproductive column structures for efficient pollen transfer (Darwin, 1877; Govaerts et al., 2021). Different floral rewards could be recognized among orchids, the most common of which is nutritional, with food rewards, mainly nectar, more rarely oils or pollen. A particular type of reward consists in floral fragrance attracting male of Euglossine bees. Finally, some bees or wasps could have waxes or resins from orchid flowers for building the nest (see Tremblay et al., 2005 for a wide review). Research shows deceptive pollination methods dominate among orchids because about one-third of species - including many Mediterranean genera - practice deceptive tactics without providing any rewards (Jersáková et al., 2006; Fantinato et al., 2017). Among these ones, we can recognize orchids with flowers appearing nectariferous, or very similar to female individuals of a insect species, so resulting sexually deceptive (Nilsson, 1992). Food-deceptive orchids were traditionally believed to have developed specialized phenotypes through flower mimicry or scent duplication to deceive specific pollinators (Fantinato et al., 2017). The research conducted by Fantinato et al. (2017) presents evidence that contradicts this established perspective. They performed groundbreaking research on five widespread food-deceptive orchids — *Anacamptis morio, A. pyramidalis, Himantoglossum adriaticum, Orchis purpurea,* and *O. simia* — to examine both predicted pollinator guilds based on floral characteristics and real-world observations throughout Northeast Italy. The researchers discovered no meaningful distinction between projected pollinator groups and actual pollinator groups which indicates these orchids operate through general rather than specialized pollinator attraction.

The evidence discovered extends our comprehension of deceptive pollination methods. Research in southern Italy supports generalized pollination strategies because food-deceptive species draw different pollinator groups including bees, flies and beetles, according to Fantinato et al. (2017), and Johnson & Schiestl (2016). The early flowering pattern of generalized deceptive orchids allows them to draw naïve pollinators seeking food sources before rewarding flowers become available (Fantinato et al., 2017).

Food-deceptive orchids successfully pollinate despite their lack of rewards because they appeal to multiple pollinators. Floral visual cues such as shape and color and pseudo-copulatory structures act as close-range attractors, yet scent functions as the primary long-distance attractant according to Fantinato et al. (2017) and Jersáková et al. (2006). The strategies have been proven to influence pollinator conduct in controlled studies since flower size reduction or scent elimination lowers both pollinator visits and seed production rates (Sletvold and Ågren, 2014). Although generalized deception protects them from pollinator scarcity the plants still face challenges from competing rewarding plants as well as habitat degradation and climate change (Jersáková et al., 2006; Cozzolino & Widmer, 2005).

The emphasis on food-deceptive orchids in this subsection reflects the focus of the pollination study, which was conducted exclusively on *Himantoglossum adriaticum*. According to the literature, this species is classified as a food-deceptive orchid; therefore, the discussion primarily addresses pollination mechanisms associated with this strategy.

1.9. Phylogenetics and Conservation Status

The process of understanding orchid conservation status requires information that goes beyond basic diversity data. The conservation field now widely accepts phylogenetic methods because these methods display both evolutionary uniqueness and historical depth of different plant lineages. This specific perspective holds great importance for orchids because they exhibit both exceptional diversity and specialized characteristics. A phylogenetic framework used for conservation status mapping enables scientists to identify specific lineages which possess exceptional evolutionary value thus supporting targeted conservation initiatives.

Through V.PhyloMaker and similar tools scientists have made major progress in constructing extensive phylogenetic trees. Through integration of Italian orchid species with the GBOTB extended megatree researchers can now study the evolutionary relationships of dozens of species at once (Jin & Qian, 2019, 2022) using frameworks by Smith and Brown (2018). The IUCN Red List categories of 61 native Italian orchids received phylogenetic mapping through this analysis. The phylogenetic patterns indicated that Least Concern species cluster in stable evolutionary branches which may possess traits like generalist pollination systems and wide ecological tolerances. Species that receive Near Threatened or Endangered designations group together in specific sections of the tree that contain shared vulnerabilities such as limited habitat specialization or dependence on pollinators.

The distribution of extinction risk observed in the orchid phylogeny matches findings from Pati Vitt et al. (2023) who studied evolutionary distinctness and rarity (EDR) across thousands of orchid species globally. Their research showed that extinction risk does not distribute uniformly across

evolutionary lineages because it tends to focus on unique evolutionary groups that currently lack conservation assessments or *ex-situ* measures. The protection of only visibly threatened species would result in overlooking evolutionarily valuable lineages according to the current research findings about Italian orchids. Through the application of phylogenetic conservation metrics scientists can detect these high-priority taxa before local field observations reveal their declines.

The current national and regional conservation programs for Italian orchids face ongoing gaps in their coverage. According to Lussu et al. (2023) several terrestrial orchids exist mainly in specialized habitats including mature forests and primary grasslands but their representation within the Natura 2000 network remains insufficient. A significant number of Italian orchid species remain unassessed by the IUCN which creates uncertainties regarding their conservation status. Without thorough assessments and phylogenetic understanding, conservation policy will likely become reactive because it focuses on declining species rather than evolutionary vulnerable ones.

The integration of phylogenetic data within national biodiversity strategies enhances both taxonomic conservation and ecological systems' long-term stability. The Apennines and Mediterranean islands represent evolutionary reservoirs because they contain high phylogenetic endemism in their regions. These areas should receive priority status because they contain ancient evolutionary branches even though they do not appear to support many species. Implementing this framework for conservation requires overcoming various operational obstacles. The conservation planning for Italian endemic orchids lacks direct alignment with evolutionary metrics and most of these species remain without species-level genetic phylogenetic data. The integration of phylogenetics with spatial and ecological datasets presents an exciting direction for future development (Givnish et al. 2015).

The application of phylogenetics in orchid conservation creates a broader perspective of biodiversity by showing patterns that standard approaches fail to detect. Conservation efforts should protect both the most charismatic species and the irreplaceable ones by using extinction risk and evolutionary distinctness as planning criteria. The conservation of Italian orchids must adopt a new approach that balances ecological necessities with evolutionary significance as the environment continues to deteriorate and climate patterns change.

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Chapter 2: Establishing an Orchid Seedbank in Emilia-Romagna: UNIMORE Orchid Seedbank

Abstract

Orchids (Orchidaceae) represent a particularly diverse and ecologically specialized group of angiosperms, yet they face a disproportionate risk of extinction due to a combination of factors including habitat loss, climatic shifts and obligate symbioses. This highlights the strategic necessity of rigorous *ex-situ* conservation measures.

This chapter documents the design, implementation and first evaluation of the UNIMORE Orchid Seedbank as a *ex-situ* conservation platform for terrestrial orchids in Emilia-Romagna (Italy) at the University of Modena and Reggio Emilia. The study integrates a series of procedures, including field monitoring, the creation of passport data, controlled dehydration and sub-zero storage. These procedures are aligned with international standards for seed banking. Field collections (2023–2025) were conducted in Modena and Reggio Emilia, encompassing grasslands, woodland margins, dry meadows, urban and peri-urban parks, yielding 54 newly accessioned seed lots (e.g., *Anacamptis*, *Himantoglossum*, *Gymnadenia*, *Ophrys*, *Orchis*, *Serapias*, *Limodorum* and *Dactylorhiza* species). Each accession was georeferenced and documented with respect to its habitat and phenology. Each sample was then desiccated over silica gel to a controlled equilibrium RH, processed aseptically, and stored at −20 °C in glass vials.

Quality assessments conducted immediately post-drying showed moderate-to-high viability by TTC staining (range 0–88%; mean ≈ 59%), and substantial germinability on BM-1 medium (range 0–94%; mean ≈ 63%). The findings indicated a strong correlation between viability and germinability, suggesting that TTC can serve as a reliable predictor of *in-vitro* performance. A rationalization of very old collected samples revealed that long-term storage at 5 °C is unsuitable for orchid seeds (near zero viability; maximum ≈ 2%), whereas −20 °C retained partial functionality and, in a subset, protocorm formation after extended incubation.

Collectively, these outcomes demonstrate the feasibility and effectiveness of a standards-aligned, regional orchid seedbank, delivering traceable data, reproducible quality metrics, and biologically meaningful patterns across *taxa* and habitats. The seedbank now functions in two capacities: firstly, as a genetic safety net (for the previously collected not discarded samples), and secondly, as a research infrastructure. In this capacity, it supports conservation planning (including Natura 2000 sites), guides storage policy, and enables future work on symbiotic germination and targeted reintroduction.

Keywords: Orchidaceae; *ex-situ* conservation; Emilia-Romagna; seed viability; germination; biodiversity management; UNIMORE Orchid Seedbank

2.1 Introduction

Biodiversity loss has been identified as one of the most pressing environmental challenges of the twenty-first century. Current assessments estimate that nearly one million species are threatened with extinction, largely because of land-use conversion, climate change, pollution and biological invasions (Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services [IPBES], 2019, 2024). These trends pose a threat to the ecosystem services that underpin human life, including nutrient cycling, pollination, climate regulation and water purification (Millennium Ecosystem Assessment [MEA], 2005). It is evident that biodiversity plays a pivotal role in ensuring ecological resilience and fostering human well-being. Consequently, the development of effective conservation strategies has emerged as a global priority within the Convention on Biological Diversity (CBD) and the European Union (EU) Biodiversity Strategy for 2030 (European Commission, 2023).

As discipline, conservation biology integrates ecological theory with practical management, in order to address the issue of biodiversity decline. It is evident that the cornerstone of ecosystem protection remains traditional *in-situ* conservation, which involves the protection of species within their natural habitats. In this context, the European Natura 2000 network, that incorporates a multitude of regional sites that have been designated for *in-situ* conservation of wild plants, plays a very important role. It complements the actions carried out by protected areas, ecological restoration areas, and national and regional parks, in the *in-situ* conservation of biodiversity (Evans, 2012; Gaston et al., 2008). However, the increase in environmental change has demonstrated the need for complementary *ex-situ* approaches that safeguard genetic material outside natural environments (Guerrant et al., 2004; FAO, 2021). *Ex-situ* conservation techniques have become essential to help stop population decline because they support *in-situ* conservation strategies. Among *ex-situ* practices, seed banks represent a globally approved method for preserving plant genetic resources during extended periods. In fact, they operate as biodiversity storage facilities by collecting seeds then maintaining them in controlled storage conditions. Seed banking is the most efficient, cost-effective and long-term method for conserving plant genetic resources (FAO, 2014; Li & Pritchard, 2009; Merritt & Dixon, 2011), functioning as living archives, facilitating reintroduction practices, ecological restoration and research on endangered species. This role of seedbanks as a genetic insurance policy against extinction is of particular significance. Furthermore, they are aligned with the objectives of the Global Strategy for Plant Conservation and the Sustainable Development Goals.

Orchids belong to the family **Orchidaceae** Juss. (Order **Asparagales** Link), which represents one of the most developed and diverse plant groups with 30,000-35,000 accepted species worldwide (Chase et al., 2015; The Plant List, 2025). Terrestrial orchid species exist in almost all ecosystems, despite their remarkable morphological diversity, specific pollination requirements and relations with endophytes, as well as their ecological sensitivity (Dressler, 1993). Specific associations between orchids and mycorrhizal fungi and pollinators make them highly sensitive to habitat modifications and climate shifts and land use changes (Swarts & Dixon, 2009; Gaskett et al., 2014). The sensitivity of orchids positions them as important botanical assets as well as bioindicator species for evaluating ecosystems health (Cozzolino & Widmer, 2005).

The orchid species found in Europe represent a diverse group that faces growing threats to their existence. Europe is home to approximately 500 native orchid species, yet nearly one-third of these species are at risk of conservation (Rossi et al., 2022). The Mediterranean Basin, designated as one of the planet's 36 biodiversity hotspots, is characterized by a particularly elevated level of orchid richness and comprises more than 250–300 species of wild orchid species. Italy alone contains more than 7,600 vascular plants, of which over 130 are classified as orchids, and approximately 87 are endemic to the country (Conti et al., 2005; Delforge, 2006). The nation's location between the Alpine and Mediterranean biogeographic zones creates steep ecological gradients that sustain this exceptional diversity. The Emilia-Romagna Region, in northern Italy, maintains an important part of this biodiversity wealth. The combination of lowland plains and northern Apennine foothills creates various terrestrial orchid habitats across this area: a mosaic of calcareous grasslands, forest margins, wetlands, and montane meadows supports over 60 orchid *taxa* (Regione Emilia-Romagna, 2022; Giardini, 2000). The combination of changing land use patterns, agricultural intensification and traditional practice abandonment, together with climate changes, has resulted in significant population reductions of orchids throughout the Region (Sgrò et al., 2011; Pirondini, 2012). Recent floristic surveys reveal that nearly 40% of historically documented orchid localities in the region have declined or vanished (Rossi et al., 2022). A considerable proportion of extant populations are sustained as diminutive, isolated components, which are susceptible to genetic erosion and local extinction. Some species are easily observed to be quite common, such as *Orchis purpurea*, *Ophrys sphegodes* and *Anacamptis pyramidalis;* they frequently survive today in isolated areas without official protection or sufficient ecological management. The public lacks understanding about orchid conservation efforts and conservation planning tends to be both fragmented and delayed in its responses. However, shifts in land use, encompassing such factors as intensification of agriculture, abandonment of traditional mowing areas, urban expansion and infrastructural development, have precipitated a pervasive phenomenon of habitat fragmentation (Banks-Leite et al., 2020).

The process of conservation of orchid seeds can entail specific technical problems. In fact, orchid seeds display characteristics of dust-like appearance, being without endosperm and cotyledon: furthermore, show different reactions to drying and freezing processes (Seaton et al., 2010; Arditti & Ernst, 1984). In nature, the process of seed germination requires very particular mycorrhizal fungi relationships, which could result in difficulties during both viability tests and propagation methods (Rasmussen, 1995; Baldrian et al., 2012). Recent research indicate that effective protocols enable the successful collection and desiccation of orchid seeds followed by testing and deep-freezing storage for extended conservation (Pritchard et al., 2004; Seaton et al., 2010; Pirondini & Sgarbi, 2014). Validated protocols include those developed at the Millennium Seed Bank (Royal Botanic Gardens, Kew), or by the Orchid Seed Stores for Sustainable Use (OSSSU) and the Australian Orchid Conservation Program (Way, 2003; Swarts & Dixon, 2009). Further indication for an effective seed collection and storage of native wild plants, including orchids, has been developed through networks like RIBES (Rete Italiana Banche del Germoplasma) and ENSCONET (European Native Seed Conservation Network).

A coordinated Italian framework for orchid seed conservation does not currently exist. Some botanical gardens together with academic laboratories have started their own local projects, for example “Tuscia Germplasm Bank - BGT” at the University of Tuscia (Viterbo, Italy), which applies OSSSU protocols for orchid seed conservation. The Emilia-Romagna Region, such as all other Italian Region, have orchid populations but there is no established system for a long-term conservation of their seeds through seedbanks.

Recent years have seen the establishment of the National Biodiversity Future Centre (NBFC) in Italy (https://www.nbfc.it/). Funded under the NextGenerationEU initiative as part of the National

Recovery and Resilience Plan (PNRR), the NBFC is the first national research and innovation hub devoted entirely to biodiversity. NBFC has established a national, multidisciplinary network comprising over 2,000 researchers from universities, research institutions, companies, and other stakeholders. Its mission encompasses the monitoring, conservation, restoration, and valorization of biodiversity across marine, terrestrial, wetland, and urban ecosystems. The NBFC is conceived as a mechanism to provide decision makers, territorial administrations and society at large with scientific knowledge and technological tools for the effective protection of biodiversity and ecosystem management. The present project is embedded within the overarching mission of NBFC and thus the Orchid Seed Bank initiative can address regional conservation issues, whilst also contributing to the national commitment to preserving and valorizing Italy's plant biodiversity in a scientifically robust and socially relevant way.

This part is considered as a general opening of the main aims of my study.

2.2 Aim of this study

The project is firstly aimed at organizing an existing orchid seed collection, verifying seed viability and germinability maintenance. Secondly, surveys to check wild orchid populations in Modena and Reggio Emilia territories have been carried out. In addition, to increase orchid seed collection, using proper ethical methods by not affecting wildlife, has been a third goal, alongside the scientific evaluation of newly collected samples.

The Seedbank relies on regional expertise in floristic and ecological studies as demonstrated by Pirondini (Pirondini, 2012), who documented the native orchid distribution, studied orchid population decline and conservation needs in Emilia-Romagna. His research established a foundation for this research project, which proved that appropriate and integrated conservation initiatives are necessary and need immediate development.

This chapter will present and evaluate the establishment process of the UNIMORE Orchid Seedbank.

2.3 Materials and Methods

2.3.1 Plant Material

One hundred eighty-eight samples of orchid seeds have been stored at the Laboratory of Environmental and Applied Botany - EAB Lab. – UNIMORE, Reggio Emilia; of these, fifty samples have been conserved at -20 ˚C. In the present study, all these accessions have been examined and selected to proceed with rationalization as a first step and then testing viability and germinability. All these accessions are of terrestrial (only three are of tropical – epiphytic orchids), divided mainly into 18 genera. Plants names reported in the List (Table 2.1) are those of the original accessions.

Table 2.1: Orchid species within UNIMORE - EAB-Lab.

Family	**Genus**	**Species**
Orchidaceae	Aceras	*Aceras* sp.
	Anacamptis	*Anacamptis laxiflora*
		Anacamptis papilionacea
		Anacamptis purpurea
		Anacamptis pyramidalis
	Barlia	*Barlia robertiana*
	Dactylorhiza	*Dactylorhiza fuchsii*
		Dactylorhiza incarnata
		Dactylorhiza insularis
		Dactylorhiza maculata sub. *fuschii*
		Dactylorhiza sambucina
	Epidendrum	*Epidendrum* sp. *(from Costa Rica)*
		Epidendrum prismatocarpum
	Epipactis	*Epipactis helleborine*
		Epipactis microphylla
		Epipactis palustris
	Gennaria	*Gennaria diphylla*
	Goodyera	*Goodyera repens*

	Gymnadenia	*Gymnadenia conopsea*
	Himantoglossum	*Himantoglossum* sp.
		Himantoglossum adriaticum
	Limodorum	*Limodorum abortivum*
	Listera	*Listera* sp.
	Neotinea	*Neotinea maculata*
	Ophrys	*Ophrys* sp.
		Ophrys sp.
		Ophrys apifera
		Ophrys bertolonii
		Ophrys crabronifera
		Ophrys fusca
		Ophrys garganica
		Ophrys sphegodes
		Ophrys tyrrena
	Orchis	*Orchis fragrans*
		Orchis italica
		Orchis morio
		Orchis papilionacea
		Orchis pauciflora
		Orchis sp.
		Orchis tridentata
	Phragmipedium	*Phragmipedium schlimii*
	Platanthera	*Platanthera bifolia*
		Platanthera sp.
	Serapias	*Serapias lingua*
		Serapias neglecta

		Serapias parviflora
		Serapias vomeracea
	Spiranthes	*Spiranthes spiralis*

2.3.2 Creating Passport data for seedbank

As a pipeline for establishing a new seedbank and a main step in seedbank management is passport data creation.

Passport data regarded all the newly collected samples (2023- 2025) and all previously found samples in EAB Lab. – UNIMORE, at which a new code has been attributed.

As a first step in creating passport-data, a new specific code has been realized for each sample within the seedbank. Thus, all the samples will have an abbreviation of SB AC and a unique number, where the symbol SB stands for Seedbank and AC for active collection; this code is for the samples stored at 5°C for maximum a year, used for yearly propagation and for scientific studies such as germinability and viability testing. For those samples stored at -20 °C, the code will be SB BC followed by a unique number and SB stands for Seedbank and BC stands for Base Collection; this collection is for long-term conservation and used for maintenance the viability and germinability of these seeds for years.

Comprehensive passport data were created to ensure traceability, reproducibility, and scientific integrity in accordance with ENSCONET (2009) and FAO Genebanks Standards (FAO, 2021). Each record was assigned a unique accession code, which was linked to its geographic coordinates, collection locality and date of acquisition. The following metadata fields were also included: species and genus identification, collector name, habitat description, population size estimated and phenological stage at collection. Geospatial references were derived from GPS waypoints (latitude and longitude), while Natura 2000 site codes were added when collections originated within designated conservation areas.

During fieldwork, ecological parameters such as habitat type and estimated population size were documented to support future viability analyses and reintroduction planning. Data pertaining to the processing of seeds, including the drying method (silica gel desiccation), the target equilibrium relative humidity (eRH), the type of packaging and the storage temperature of −20 °C, were integrated into the database, in order to comply with long-term seed conservation protocols (Pritchard et al., 2004; Merritt & Dixon, 2011). The incorporation of designated fields for CITES status, permit or ABS reference, and duplication flag has been implemented to ensure adherence to international biodiversity and access-and-benefit-sharing frameworks (Buck et al, 2011). This standardized passport dataset constitutes the foundation of the UNIMORE Orchid Seedbank information system, facilitating both internal quality control and interoperability with European ex-situ conservation networks. Annex 1 shows a detailed section of this database.

2.3.3 Data Processing and Visualization

The data collected in the passport data were compiled in Microsoft Excel files. The geographic coordinates were subjected to a process of verification and correction, employing the World Geodetic System 1984 (WGS84) reference frame. The process includes data cleansing, the identification of missing coordinate values and the implementation of taxonomic spelling, in

accordance with the World Checklist of Selected Plant Families (Govaerts et al., 2021). Spatial visualization was conducted utilizing QGIS version 3.40.11. Point layers were created using the latitude and longitude fields of the cleaned dataset, projected in EPSG:4326.

Descriptive statistics and visual summaries were generated in R version 4.4.2 (R Core Team, 2024) using the ggplot2 package (Wickham, 2016). This included habitat type representation, species richness per site, and seed viability distributions. The formatting of the graphs was conducted in accordance with the APA publication guidelines.

This workflow was implemented to ensure data reproducibility and consistency between field records, digital archives, and analytical outputs.

2.3.4 Rationalization of Existing Collections

A rational management is an important approach for the continuity of a seedbank. The previous collection of orchid seeds consisted of a heterogeneous set of samples from different years and localities; moreover, they were maintained under different conditions, depending on the aim of collecting at that time.

In this case, samples of same species, of same geographical location, having the same date of collection and stored at the same temperature, have been considered duplicate (Fig. 2.1).

Samples that underwent the rationalization process are 138, belonging to the collection of orchid seeds stored at 5 °C, while 50 other samples belonged to that of the seeds stored at -20 °C.

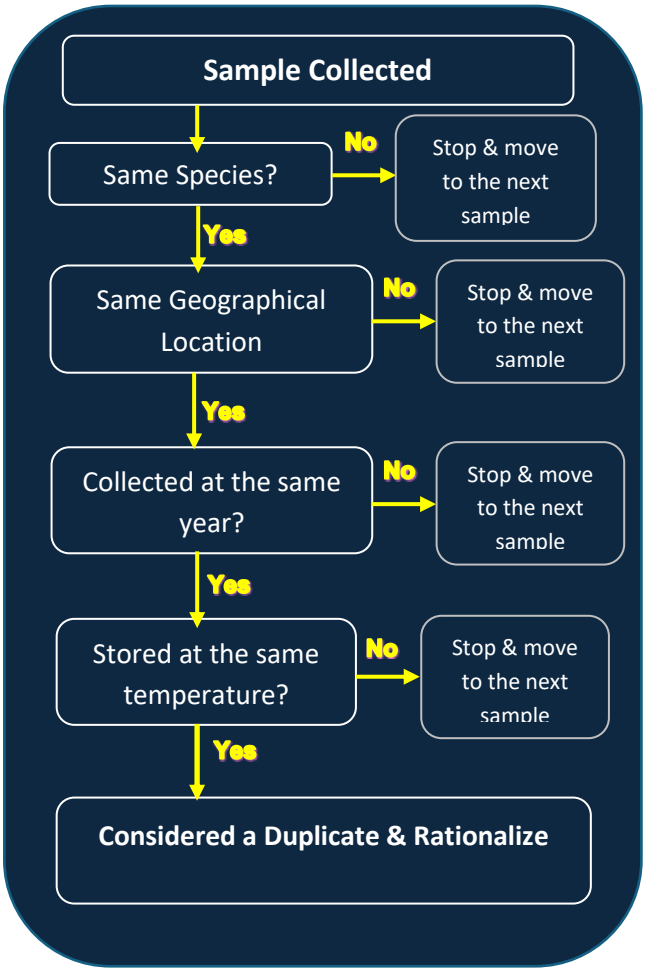


Figure 2.1: workflow for rationalization process done for the samples collected previously.

2.3.5 Field Monitoring in Emilia-Romagna - Study Area and Context

Within this framework, our sampling focuses on two provinces, Modena and Reggio Emilia. These territories have many different environments where orchids grow, for example calcareous grasslands, abandoned fields, wood, woodland margins, wetlands, coastal dunes and natural and semi-natural meadows, creating different habitats with microclimatic and edaphic variance favorable for orchid diversity.

In 2023, 2024, 2025 sampling activity was carried out in 14 different sites, well known to be rich in orchid species. The localities of these sites are: Cento Croci, Gainazzo, Malandrone, Casona, Serramazzoni, Rio Benedello, Salse di Nirano and "Parco della Resistenza" and other green areas in the city of Modena, all these in province of Modena. In province of Reggio Emilia two sites were monitored and used for sampling activities: "Parco San Lazzaro" and a semi natural area along Via Ca' Corghi (Scandiano). In addition, some samples were from Trieste (private garden), Friuli-Venezia Giulia and Mantova. Detailed information on orchid species and sampling sites are shown in Annex II.

Monitoring activity has been possible thanks to a collaboration with floristic experts and botanists. Each site was visited and then orchid populations labeled, reporting that a university trial was underway. Furthermore, the geographical location was recorded using a GPS (Garmin Dakota™ 20). Data of geographic distribution of orchid populations studied are shown in Figure 2.6 (Results section). Many of these locations have been monitored every year.

2.3.6 Field Sampling and Seed Collection

Field sampling was conducted between 2023 and 2025. The plants were identified during preliminary monitoring and followed throughout the flowering period, until the ripening of the fruits. Each plant was labeled with a tag that has a specific code, using the coding system mentioned earlier for later recognition. Only plants growing in Parco S. Lazzaro needed to be preserved from periodic mowing, so they were protected with a plastic net placed around them (Annex II) and labeled as "Don't cut - UNIMORE project". Most seed samples were obtained between late June

and August, though early flowering species (e.g., *Ophrys sphegodes, Orchis purpurea*) required earlier collection from late May until mid-June. Each accession corresponded to a unique waypoint recorded using GPS (precision ± 3 m) in WGS84 decimal coordinates (this format is used to be able later to plot the maps using QGIS). For each site, field notes included habitat description, population size estimates, microtopography and sometimes associated vegetation. Photographic documentation was made for every collection event to support identification. All information is recorded in a specific folder called Field Book (see Annex II).

Since the fruits of orchids are capsules, dry fruits which open for dispersal when ripe, they were taken from the plants shortly before dehiscence; this is to avoid the loss of large quantity of seeds. The capsules were taken from different individuals so as not to compromise spontaneous dissemination in the natural environment and for increase the gene pool of seeds to be conserved. Attention was paid to ensure that the seeds were mature and physiologically suitable for long-term storage period. Capsules were stored in paper envelopes, labeled with the species name, date and locality and transferred to the laboratory. Here, the samples were put in a glass container with silica gel and dried keeping them for one week to reach RH of 12% before processing. Check Annex IV to understand how to collect ripe seeds.

A total of 54 samples were collected from 2023 to 2025, belonging to 22 species: *Anacamptis coriophora, Anacamptis pyramidalis, Anacamptis berica, Barlia robertiana, Cephalathera longifolia, Dactylorhiza fuchsii, Dactylorhiza maculata, Epipactis helleborine, Gymnadenia conopsea, Gymnadenia odoratissima, Himantoglossum adriaticum, Himantoglossum hircinum, Limodorum abortivum, Neottia ovata, Ophrys apifera, Ophrys bertolonii, Ophrys sphegodes, Orchis morio, Orchis papilionacea, Orchis purpurea, Orchis simia,* and *Serapias vomeracea.* Some photos of these species are in Annex III.

In 2023, 29 samples were collected from natural sites, protected areas and urban areas, while in 2024, only12 samples were collected in Urban areas. Finally, in 2025, 13 samples were collected from urban areas, simi-natural habitats, protected area and private gardens. All detailed information is shown in Annex I.

After collecting, the seeds were cleaned using sieves with mesh size of 1, 0.5 and 0.25 mm, put in a paper envelope in a glass container deprived from air containing silica gel in its lower part, to reach 12% RH, maintaining it at room temperature for two weeks. This value of RH is usually applied in agreement with the Orchid Seedbank Manual of Millennium Seedbank in UK. 5 mg of seeds in each sample were taken out for germinability and viability testing. The remaining seeds were weighed, put in 4 ml glass vials and stored at T = -20 ± 1°C for conservation within UNIMORE Seedbank.

For each collected sample all the passport data were inserted in the main database for the orchid collections, and the data is divided as elaborated in Data Processing and Visualization section.

2.3.7 Viability tests

The assessment of seed viability was conducted utilizing the tetrazolium chloride (TTC) assay, a biochemical method that has been extensively employed in the field of orchid conservation for the detection of living cells (Ligrone et al., 2012; Seaton et al., 2013).

Seeds were firstly scarified using 5%NaOCl solution with few drops of Tween 20. Each species had different scarification times; they varied among a minimum scarification time of 2 minutes, 36

seconds for *Limodorum abortivum* samples until 6 minutes, 40 secs for *Orchis morio* samples - maximum scarification time. Exposure time varied among species mainly depending on seed coat characteristics, in addition to seed coat characteristics and harvest.

To know the success of scarification phase, bleaching out of seeds was observed under a stereo microscope (Leica M205C): seed integuments became white - light yellow from an initial dark color. Following this step, seeds were rinsed 3 times with water, 3-5 mins/time.

Prior to staining, seeds were subjected to a 24-hour rehydration period in distilled water, followed by 24-hour incubation at room temperature where the seeds were dipped in a 10% sucrose solution, then rinsed with distilled water. Finally, seeds were submersed in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) solution buffered at pH 7.3 with phosphate buffer. Samples were subjected to incubation in conditions of darkness at a temperature of 40 °C for a period of 48 hours, in accordance with modified protocols previously established by Seaton et al. (2013) and Pritchard et al. (2004).

Following the incubation period, the seeds were rinsed with water to remove TTC solution and observed under a stereo microscope. Embryos that exhibited distinct red or pink coloration were designated as viable. Embryos that were colorless or only partially stained were deemed non-viable; the number of seeds without embryos (empty seeds) was not considered in the count (Fig. 2.2). For each accession, a minimum of 200 seeds were analyzed, and viability was expressed as the percentage of seeds with stained embryos out of the total examined.

To complement the viability assessment, germination trials were then performed on selected species to evaluate seed vitality under controlled laboratory conditions.

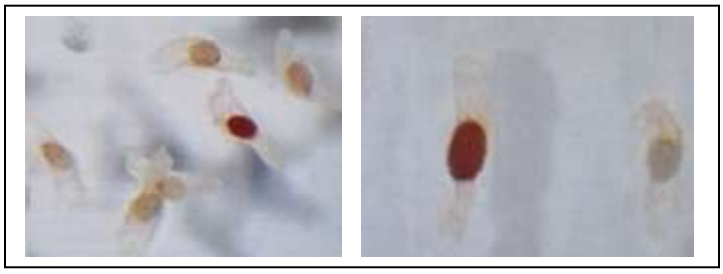


Figure 2.2 showing the red coloration of the viable embryos after TTC test; it allows to distinguish viable seeds from non-viable ones, these last with pale embryos (seeds of Anacamptis pyramidalys)

2.3.8 Contamination Prevention

Knowing the microscopic size of orchid seeds and their susceptibility to microbial colonization, contamination control was a key consideration throughout all stages of seed processing. As germination test was performed in an *in vitro* system, the handling of seeds was conducted exclusively in aseptic conditions, under a laminar-flow cabinet. Internal surfaces were periodically subjected to UV-irradiation for a duration of overnight and before use were cleaned with 70% ethanol.

2.3.9 Germination tests

Germination tests started usually at the end of July of the 3 years. Seeds were surface sterilized using a 5% sodium hypochlorite (NaOCl) solution containing a few drops of Tween 20. In addition to its primary function of surface sterilization, NaOCl may also contribute to a scarification of the seed coat, facilitating subsequent staining or germination. Following this step, seeds were rinsed

with sterilized water for 3 times, 3-5 mins/time. An *in vitro* culture medium, BM-1 Terrestrial orchid Medium (Phytotechnology laboratories®, USA) was used; it is a Basic Medium modified according to Van Waes and Debergh (1986) that contains only organic nitrogen, 0.5 g/l casein enzymatic hydrolysate and 0.1 g/l l-glutamine. The pH of the medium was adjusted to 6.3 ± 0.1 before autoclaving at 121 °C and 1 atm., for 30 min. The medium was supplemented with 0.6% plant agar (Plant Tissue Culture grade, Duchefa) and 0.1% activated charcoal (Plant Tissue Culture Tested, PhytoTechnology laboratories® USA) before autoclaving. Approximately, 1mg of seeds were sown under sterilized conditions in each petri dish (3 cm in diameter), containing 10 ml of BM-1 medium. 5 replicate dishes were prepared by accession of seeds. Then, petri dishes were sealed with parafilm, placed in dark conditions (drawer like container fig. 2.3c), and incubate at 25 ± 1 °C.

The process of seed germination was recorded according to embryonic development, divided to four stages, Early Stage “Stage 1”, no germination, Pre-germination Stage, “Stage 2” where the embryo swell to fill the seed coat, Germination Stage, “Stage 3” where the embryo emerge from the seed coat forming star like shape, and finally, Protocorm stage, “Stage 4” the embryo discharge from the seed coat forming a protocorm (fig. 2.3).

Figure 2.3: (a) Orchid with full green capsules, (b) collected dust like seeds, (c) petri dishes stored in dark for the germination test (drawer like container), (d) empty seed, (e) seed with embryo, (f) Stage 1 Early Stage seed with embryo, (g) Stage 2 Pre-Germination stage, (h) Stage3 Germination stage, (i) Protocorm stage, (j) small plantlets are formed

2.4 Results and Discussion

2.4.1. Collection Summary and Data Elaboration

The UNIMORE collection captures a representative cross-section of the region's orchid diversity, comprising 18 genera and 38 taxa. They are listed in Table 2.2 where on the right, the names of orchid species are updated in agreement with Biagioli and De Simoni (2024).

Most of these genera are dominant in semi-natural grasslands and woodland margins, which are typical of the low-to-mid-elevation landscapes of the provinces of Modena and Reggio Emilia.

Considering the samples previously found at UNIMORE and those collected between 2023 and 2025, all samples have been assigned the new code. The figure below illustrates the new coding system (Fig. 2.4).

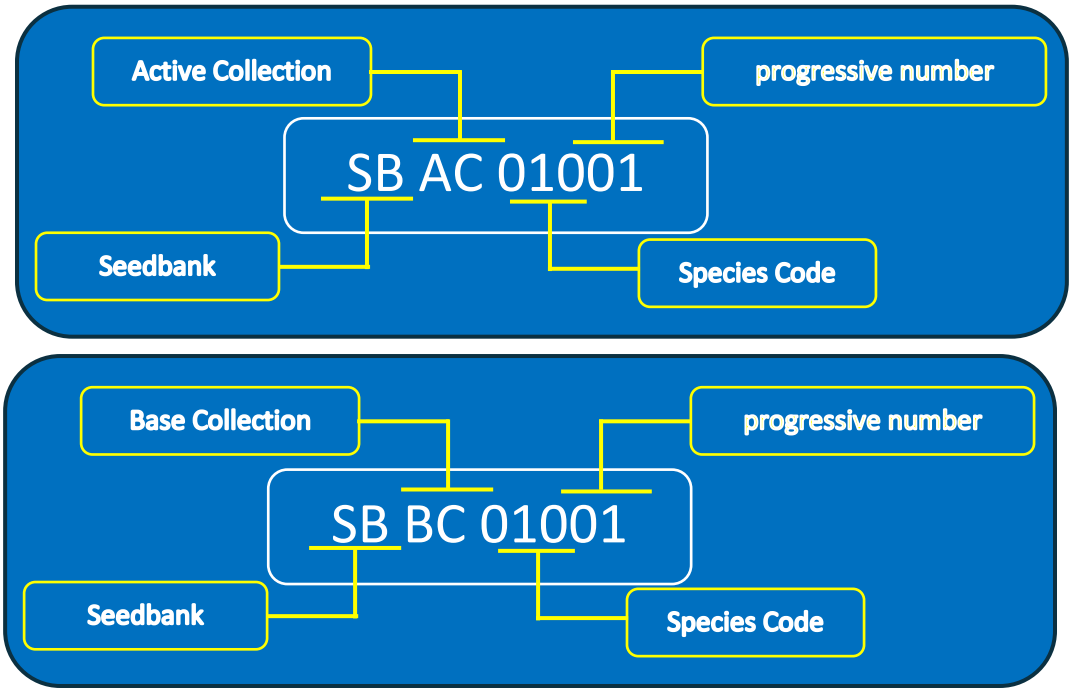


Figure 2.4: Coding criterium

Coding was the first step in Seedbank management, while the second step is creating a database for each accession, new and old collections. A new editable database called Passport Data and Field book was done, using Microsoft Excell, shown in Annex I.

The field missions were designed to maximize ecological and geographical representation rather than numerical abundance. Around 60% of all accessions originated from grassland habitats, 25% from woodland edges and 15% from ruderal or urban fringe sites. This distribution reflects the regional pattern of terrestrial orchid occurrence as documented in previous floristic inventories (Biagioli and De Simoni, 2024).

Most of the populations sampled in 2023-2025 were small to medium in size, typically ranging from 20 to 150 individuals per site. This reflects the fragmented nature of orchid populations in the Region. Those located within Natura 2000 sites, such as IT4040007 Salse di Nirano and IT4030016 Scandiano (RE), tended to exhibit higher individual abundance and less evidence of habitat degradation than populations collected from unprotected areas. Around 28% (15 accessions) of the total sites originated from Natura 2000 areas, highlighting the complementary role of *ex-situ* storage in supporting the management of protected areas.

The species richness within the dataset is high relative to the region's known diversity. The genus *Ophrys* accounts for four distinct species in the collected samples (details are in Annex I), while nine different are recorded in the Region. Rarer species such as *Serapias neglecta* and *Himantoglossum adriaticum* were collected from only one or two sites, highlighting their restricted local distribution and increased conservation value.

The establishment of 50 documented accessions within three years is a major step towards building a coordinated orchid conservation platform in UNIMORE. The dataset captures the ecological diversity of the region's orchid flora, ranging from lowland rich in *Anacamptis* plants to mountain marshes with *Dactylorhiza* species. This lays the groundwork for the viability, germination and storage analyses discussed in the following sections. Annex I reports all data of the database generated by UNIMORE Orchid Seedbank.

Table 2.2: Table showing the UNIMORE Orchid seedbank - EAB-Lab updated species name according to Biagioli and De Simoni (2024)

Family	**Genus**	**Species**	**Updated Plant Species Name**
Orchidaceae	Aceras	*Aceras*	*Orchis anthropophora*
	Anacamptis	*Anacamptis laxiflora*	
		Anacamptis papilionacea	
		Anacamptis purpurea	*Orchis purpurea*
		Anacamptis pyramidalis	
	Barlia	*Barlia robertiana*	*Himantoglossum robertianum*
	Dactylorhiza	*Dactylorhiza fuchsii*	
		Dactylorhiza incarnata	
		Dactylorhiza insularis	
		Dactylorhiza maculata sub. fuschii	*Dactylorhiza fuchsii*

		Dactylorhiza sambucina	
	Epidendrum	*Epidendrum* sp. (from Costarica)	
		Epidendrum prismatocarpum	
	Epipactis	*Epipactis helleborine*	
		Epipactis microphylla	
		Epipactis palustris	
	Gennaria	*Gennaria diphylla*	
	Goodyera	*Goodyera repens*	
	Gymnadenia	*Gymnadenia conopsea*	
	Himantoglossum	*Himantoglossum* sp.	
		Himantoglossum adriaticum	
	Limodorum	*Limodorum abortivum*	
	Listera	*Listera* sp.	*Neottia* sp.
	Neotinea	*Neotinea maculata*	
	Ophrys	*Ophrys* sp.	
		Ophrys apifera	
		Ophrys bertolonii	
		Ophrys crabronifera	
		Ophrys fusca	*Ophrys fuscae*
		Ophrys garganica	
		Ophrys sphegodes	
		Ophrys tyrrena	*Ophrys montis-leonis*
	Orchis	*Orchis fragrans*	*Anacamptis fragrans*
		Orchis italica	
		Orchis morio	*Anacamptis morio*
		Orchis papilionacea	*Anacamptis papilionacea*

		Orchis pauciflora	
		Orchis sp.	
		Orchis tridentata	*Neotinea tridentata*
	Phragmipedium	*Phragmipedium schlimii*	
	Platanthera	*Platanthera bifolia*	
		Platanthera sp.	
	Serapias	*Serapias lingua*	
		Serapias neglecta	
		Serapias parviflora	
		Serapias vomeracea	
	Spiranthes	*Spiranthes spiralis*	

2.4.2. Rationalization of Existing Collections

A total of 138 accessions were initially catalogued in the active collection. Following a detailed comparison of the passport data, including the species name, the collection year and the geographic origin, 130 unique accessions were confirmed. Duplicate entries were found to correspond to doubled samples with different codes collected from the same population. These duplicates were merged to avoid redundancy, with the concomitant benefits of enhanced viability testing and database management.

2.4.3. Viability and Germinability Assessment of all Existing Accessions

The second step in managing a seedbank is to periodically test the viability and germinability of all stored samples, all the data are presented in Annex V. In this perspective all the existing seed samples were tested for viability and germinability. Results obtained by viability test are shown in figure 2.5.

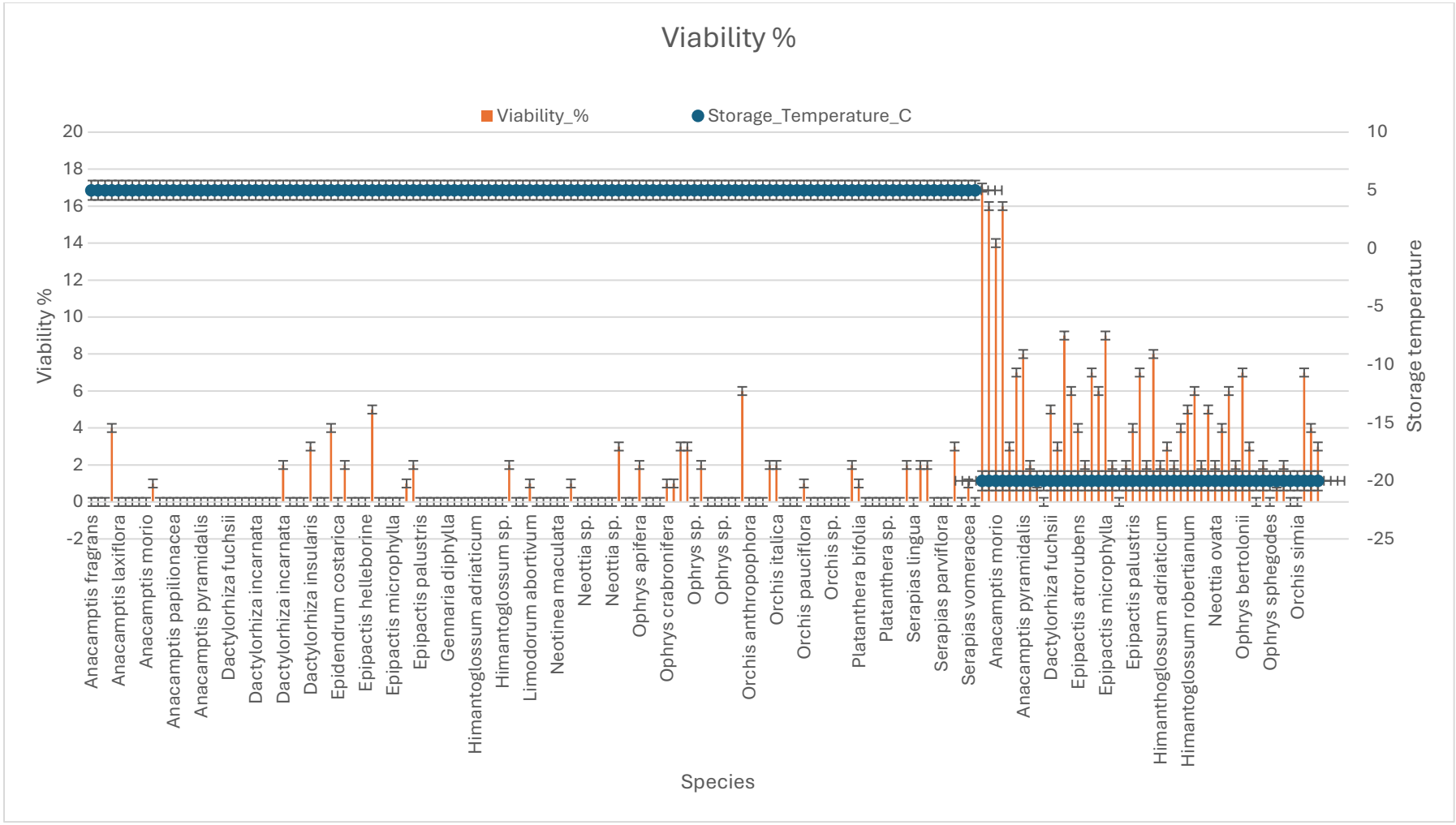


Figure 2.5: Seed viability (%) of some seed orchid accessions stored at 5 °C and −20 °C. Each bar represents the mean viability per species, with error bars showing standard deviations. Blue points correspond to storage temperature categories.

The analysis of the viability data tested on the 180 accessions revealed a marked contrast between seeds stored at 5 °C and those preserved at −20 °C. Viability was markedly higher in seeds preserved at −20 °C.

A Mann–Whitney U test confirmed a significant difference between the two storage regimes ($U = 9125$, $p < 0.001$), indicating that subzero storage is essential for maintaining long-term orchid seed viability.

Samples stored under refrigeration (5 °C) demonstrated remarkably low or non-existent viability, exhibiting an overall mean value below 0.5%. Most accessions in this group exhibited complete loss of metabolic activity, which is consistent with advanced seed ageing following a period of more than a decade of storage. Storing orchid seeds at 5 °C is inadequate for preserving their viability beyond a few years.

Conversely, accessions conserved at −20 °C displayed slightly higher, albeit still limited, viability, with a few species retaining measurable levels of alive embryos (up to 16% of the whole stored collections).

Consistent results have been documented in related studies of terrestrial orchids, which have demonstrated that storing above the freezing point of water leads to the rapid deterioration of cell membranes and the acceleration of lipid peroxidation (Pritchard et al., 2004; Seaton et al., 2013). Nevertheless, the potential for short-term cold storage at 5 °C remains a viable proposition. For instance, when the objective is to facilitate temporary seed maintenance prior to sowing for propagation or reinforcement of wild populations, this temperature range enables metabolic quiescence without inducing severe desiccation. In this context, 5 °C storage could support nursery-based reintroduction programs aimed at enhancing wild orchid production within controlled environments (Swarts & Dixon, 2009).

To compute the Mann–Whitney U statistic manually:

If

- n_1 = number of samples at 5 °C
- n_2 = number of samples at −20 °C
- R_1 = sum of ranks for 5 °C group
- R_2 = sum of ranks for −20 °C group

Then:

$$U_1 = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R_1$$
$$U_2 = n_1 n_2 - U_1$$

Where sum of ranks is the average mean of the studied parameter (in this case is viability and germinability)

In Figure 2.6 are shown some of the results of germinability analysis carried out on the 180 orchid accessions. It shows a significant difference between seeds stored at 5 °C and those preserved at −20 °C. Samples stored under refrigeration (5 °C) demonstrated remarkably low or non-existent germinability, exhibiting an overall mean value below 2% of all the 130 samples, while an overall mean value of 33% of 2 samples (1 of *Gymnadenia conopsea* and 1 of *Anacamptis pyramidalis*).

More statistical analysis was done. A Mann–Whitney U test was done, as a first step the data was divided into two group, and then proceed with the analysis as explained in the textbox above.

- **Group 1 (5 °C storage)**

n_1 = 61 observations

Germinability values: almost entirely 0%, with two non-zero values (37%, 48%)

- **Group 2 (−20 °C storage)**

n_2 = 49 observations

Germinability values: all > 0%, ranging approximately from 12% to 62%

Because the data are non-normal, zero-inflated, and heteroscedastic, the Mann–Whitney U test is the appropriate non-parametric choice.

Mann–Whitney U results:

U statistics = 68.0

Two-sided p-value = 7.56×10^{-21}

Interpretation:

There is a highly significant difference in germinability between storage temperatures:

- Seeds stored at −20 °C show substantially higher germinability
- Seeds stored at 5 °C show near-zero germination

This indicates that long-term seed storage at −20 °C is significantly more effective than storage at 5 °C for maintaining seed viability in these orchid species.

Given the extreme separation between groups (many tied zeros vs consistently positive values), the very small p-value is expected and statistically robust.

Therefore, germinability differed significantly between storage temperatures (Mann–Whitney U test: U = 68, n_1 = 61, n_2 = 49, $p < 0.001$), with markedly higher germination percentages after storage at −20 °C compared to 5 °C.

This was the same procedure done for the viability statistical analysis.

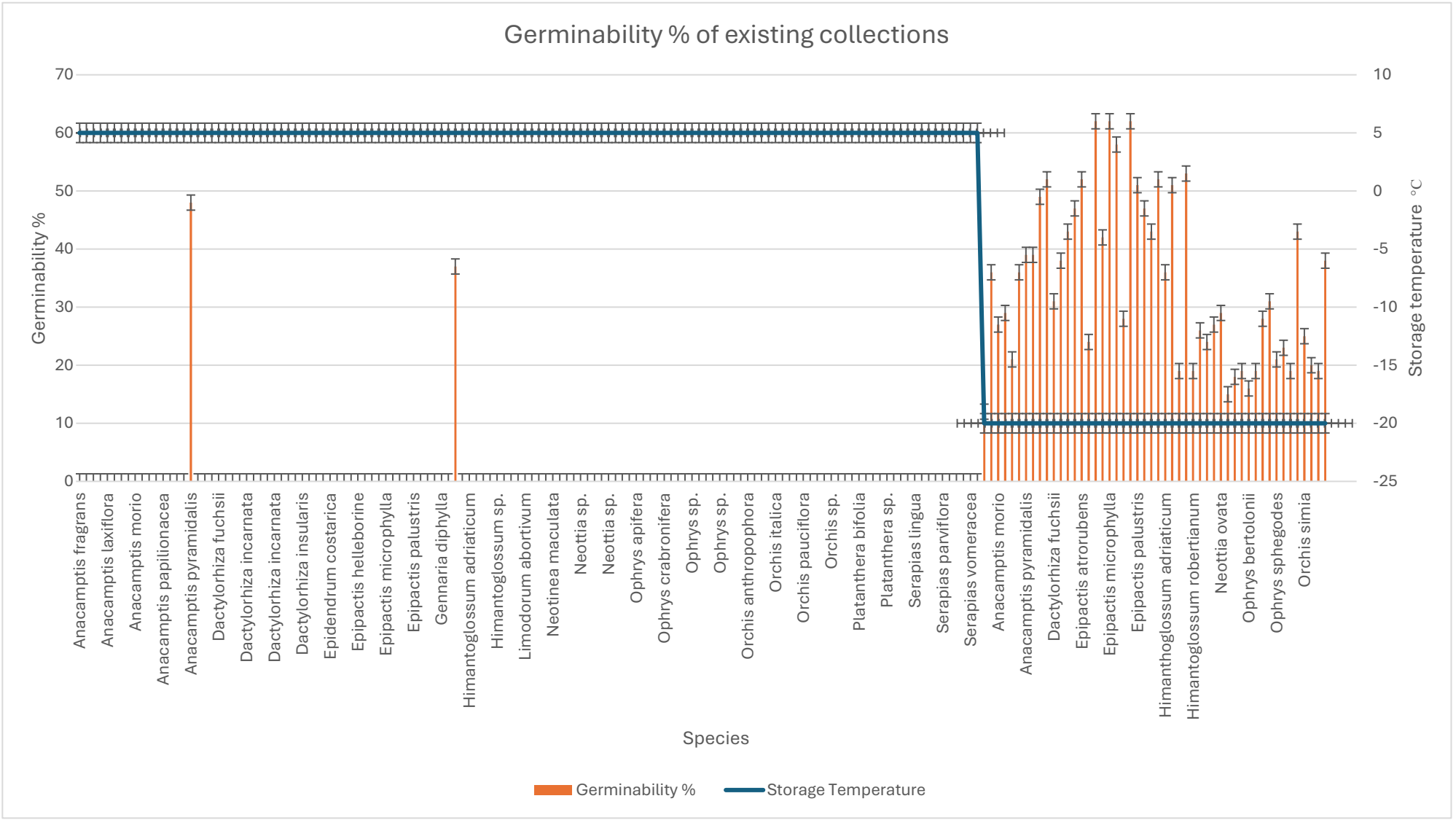


Figure 2.6: Seed germinability (%) of some orchid accessions stored at 5 °C and −20 °C. Each bar represents the mean germinability per species, with error bars showing standard deviations. Red line corresponds to storage temperature categories.

Furthermore, species stored at 5 °C that exhibited germinability, reaching the pre-germination stage - characterized by embryo enlargement - without protocorm formation (fig. 2.3, g). On the other hand, some samples stored at -20 °C displayed higher germination rates, reaching up to 62% (maximum% reached): for examples *Epipactis, Anacamptis*, and *Gymnadenia species* (table 2.3). The overall mean value for the 50 samples stored at -20 °C was 34.4, with overall standard error of 2.43. The formation of protocorm-like bodies (fig 2.3, h) appeared after 4 months of monitoring.

These Findings indicate that freezing at −20 °C provides a more suitable environment for long-term preservation of orchid seeds. In these conditions, seeds can start to germinate, reaching good value also after many years. An analysis of viability/germinability conducted on seed samples maintained for different period of time (years) at -20 °C will be shown in chapter 3.

However, the germinability values suggest that even subzero storage may not fully prevent deterioration over extended periods, particularly when initial seed quality or desiccation control was suboptimal. These findings emphasize the importance of maintaining low temperature storage for the long-term conservation of orchids and highlight the need for periodic reassessment of germinability and regeneration cycles to sustain *ex-situ* collections (Popova et al., 2023). However, the presence of viable embryos and protocorm formation after a decade demonstrates the efficacy of protocols applied at UNIMORE in preserving physiological integrity over extended periods.

Most accessions stored at 5 °C should not be prioritized for regeneration or direct reintroduction, as their viability has decreased below the functional thresholds that are considered acceptable. Nevertheless, it is important to note that these samples possess inherent genetic and informational value. It has been established that even non-viable seeds contain DNA that is suitable for molecular identification, barcoding, or population genetics studies (Jones et al., 2022). This finding enables the creation of a comprehensive genomic reference database, based on old samples, too. The integration of such molecular archives with existing living collections has the potential to enhance the efficiency and scientific scope of the UNIMORE Orchid Seedbank, thereby facilitating future studies on genetic diversity, phylogeography, and *ex-situ*/*in-situ* linkage.

To summarize, the rationalization of historical accessions revealed that long-term storage at 5 °C is unsuitable for maintaining the viability and germinability of orchid seeds. Conversely, storage at −20°C conditions ensure partial but meaningful preservation over decadal timescales. The outcomes of this rationalization process thus provide two insights: they guide practical seedbank management and inform strategic conservation planning.

Table 2.3: germinability % of samples stored at -20 °C

Species	**Germination% ± standard error**
Anacamptis coriophora	12±1
Anacamptis coriophora	36±0.874
Anacamptis morio	27±0.6561
Anacamptis morio	29±0.7447
Anacamptis papilionacea	21±0.5103
Anacamptis purpurea	36±0.8748
Anacamptis pyramidalis	39±0.9497
Corallorhiza trifida	39±1.0301
Cypripedium calceolus	49±1.1907
Cystorchis gracilis	52±1.2636
Dactylorhiza fuchsii	31±0.7533
Dactylorhiza incarnata	38±0.9234
Dactylorhiza insularis	43±1.0449
Dactylorhiza sambucina	47±1.1233
Epipactis atrorubens	52±1.2636
Epipactis atrorubens	24±0.5832
Epipactis helleborine	62±1.5066
Epipactis microphylla	42±1.0206
Epipactis microphylla	62±1.8609
Epipactis palustris	58±1.4094
Epipactis palustris	28±0.6804
Epipactis palustris	62±1.9031
Epipactis palustris	51±1.2393
Goodyera repens	47±1.1421

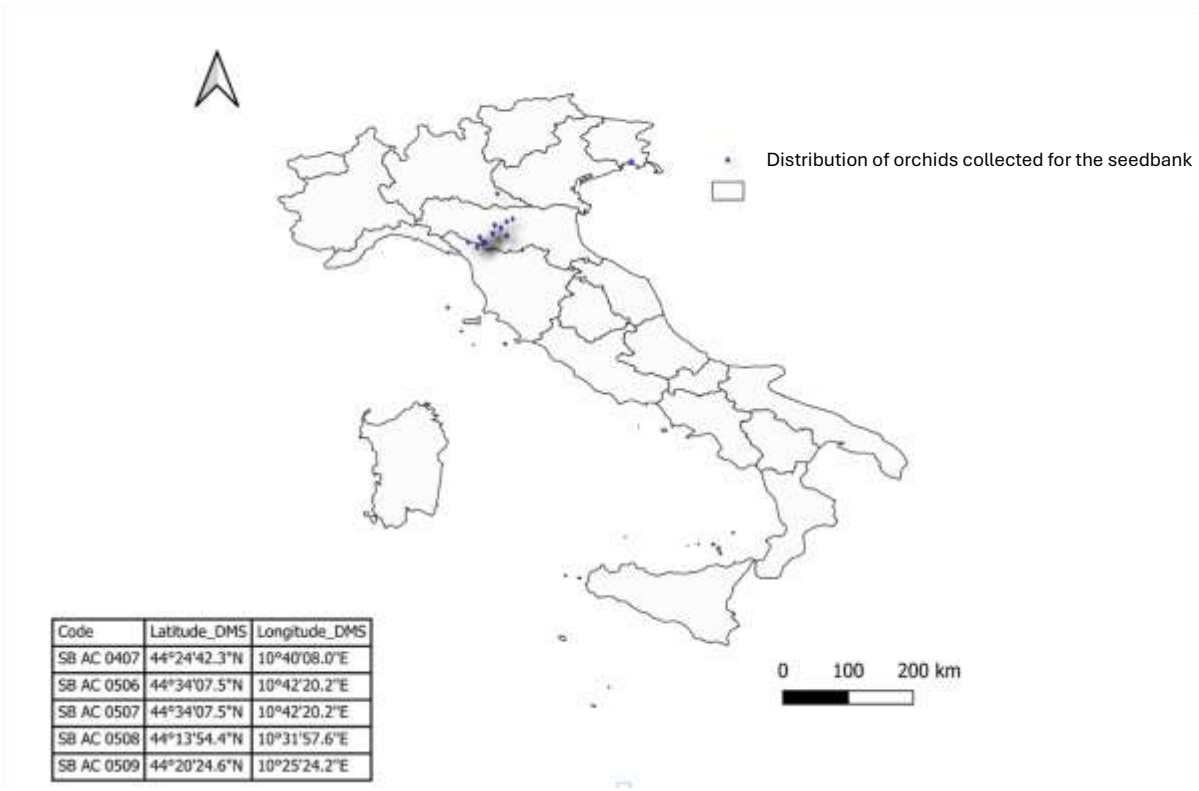
Gymnadenia conopsea	43±1.4149
Himantoglossum adriaticum	52±1.2636
Himantoglossum adriaticum	36±0.8748
Himantoglossum adriaticum	51±1.2393
Himantoglossum robertianum	19±0.4617
Himantoglossum robertianum	53±1.2879
Himantoglossum robertianum	19±0.4136
Himantoglossum robertianum	26±0.6319
Himantoglossum robertianum	24±0.5832
Malaxis monophyllos	27±0.7014
Neottia ovata	29±0.7047
Ophrys apifera	15±0.3645
Ophrys apifera	18±0.4374
Ophrys apifera	19±0.4629
Ophrys bertolonii	16±0.3888
Ophrys crabronifera	19±0.4272
Ophrys crabronifera	28±0.6804
Ophrys montis-leonis	31±0.7533
Ophrys sphegodes	21±0.5204
Ophrys sphegodes	23±0.5589
Ophrys sphegodes	19±0.4619
Orchis provincialis	43±1.1044
Orchis simia	25±0.6075
Serapias parviflora	20±0.4863
Serapias vomeracea	19±0.4612
Spiranthes spiralis	38±0.9234

2.4.4. Field Monitoring in Emilia-Romagna and Seed Collection (2023–2025)

To ensure the continuity of the established seedbank, new samples were collected during the time of this study from 2023 to 2025. Field monitoring was conducted during the peak flowering and fruiting seasons (April–July), with repeated visits to confirm phenological readiness for seed capsule collection. The collection of capsules was conducted with a focus on maturity and natural desiccation, with the objective of minimizing disruption and ensuring optimal embryo development. The phenological phase at collection exhibited variation among species, with early-flowering *taxa* such as *Orchis purpurea* and *Anacamptis morio* sampled primarily in late April to early May, while later-flowering species such as *Himantoglossum adriaticum* and *Gymnadenia conopsea* were collected from June to July. Parameters such as GPS coordinates were recorded at each site and logged in the World Geodetic System 1984 (WGS84) reference frame for precise geospatial mapping. The spatial data were subsequently processed using QGIS 3.40.11, thereby producing distribution layers that were overlaid on regional relief map (see Figure 2.6).

The newly collected samples represent a broad taxonomic coverage of terrestrial orchids in the Region. As is evidenced by the preponderance of documented species*, Anacamptis pyramidalis, Ophrys apifera, Himantoglossum adriaticum, Serapias vomeracea* and *Epipactis helleborine*, it is evident that a variety of ecological adaptations and habitat preferences are exhibited by each species. For instance, *Ophrys apifera* was found to thrive in disturbed grasslands and roadside verges in Modena and Reggio Emilia, indicating its tolerance to secondary habitats. In contrast, the distribution of *Himantoglossum adriaticum* and *Anacamptis pyramidalis* was primarily confined to calcareous slopes and meadows. This variation highlights the significance of preserving diverse habitat types to encompass the ecological and genetic diversity of regional orchid populations (Swarts & Dixon, 2009; Cozzolino & Widmer, 2005).

Field monitoring also functioned as a medium for the evaluation of population health, thereby enabling researchers to make qualitative estimations of population sizes and reproductive output. The number of individuals found in natural and semi-natural areas seems to vary from year to year. This phenomenon is probably indicative of the many factors that influence orchid populations, among which environmental, climatic and land-management factors may be the most important. Conversely, species documented in Parco San Lazzaro exhibit enhanced population persistence of *Himantoglossum adriaticum*, from first observation (2019) until 2025, presumably benefiting of protections put around plants, diminished mowing frequency and a general decrease of anthropogenic disturbance. These observations corroborate with earlier studies indicating that site management practices play a decisive role in sustaining orchid reproductive success (Reiter et al., 2016).



Code	Latitude_DMS	Longitude_DMS
SB AC 0407	44°24'42.3"N	10°40'08.0"E
SB AC 0506	44°34'07.5"N	10°42'20.2"E
SB AC 0507	44°34'07.5"N	10°42'20.2"E
SB AC 0508	44°13'54.4"N	10°31'57.6"E
SB AC 0509	44°20'24.6"N	10°25'24.2"E

Figure 2.2: Map showing the spatial distribution of newly collected orchid accessions (2023–2025) Geographical distribution of sites of orchid seed collection in Emilia-Romagna Region, Northern Italy. Coordinates were recorded in the WGS84 reference system and converted to degrees, minutes, and seconds (DMS) for geospatial representation. Map created in QGIS version 3.40.11 using data from the UNIMORE Orchid Seedbank database (2023-2025)

2.4.5. Seed Viability and Germinability (2023–2025 Collections)

Viability

The analysis of seed viability using 1% TTC is presented in figure 2.7. This analysis revealed significant inter-specific variations, with values ranging from 0% to 88%.

Anacamptis, Gymnadenia, and *Himantoglossum* exhibited the highest levels of viability (>70%), whereas some *Epipactis* and *Orchis* samples demonstrated minimal or zero viability. These trends correspond with previously documented species-level physiological variability (Popova et al., 2023; Merritt & Dixon, 2011; Seaton et al., 2010). Methodological refinements, including mild NaOCl scarification, controlled rehydration, and 10% sucrose preincubation, resulted in significant enhancements in the clarity and interpretability of TTC staining. The efficacy of TTC reduction is contingent on mitochondrial dehydrogenase activity and adequate hydration. These adjustments have been shown to enhance tissue penetrability and ensure consistent staining. Similar

enhancements have been documented across multiple orchid *taxa* (Hosomi et al., 2017; Custódio et al., 2016; and Mercado et al., 2020).

Germinability

The results of germinability tests of newly collected samples are shown in figure 2.7. Germinability tests on BM1 medium demonstrated robust physiological performance across most accessions, with germination ranging from 0% to 94%. A significant proportion of seeds attained stage 3, with multiple seeds progressing to stage 4, thereby signifying the efficacy of the protocorm development process. *Gymnadenia conopsea* (up to 94%) and *Anacamptis pyramidalis* (>80%) were identified as the most effective species in this study, forming healthy protocorms after 20–22 weeks. These findings demonstrate that storage at −20 °C effectively preserves the germination capacity of these species. Moderate germination in *Orchis morio* and *Ophrys apifera* (60–70%) while *Epipactis* and *Serapias* showed low germination, consistent with their dependence on highly specific fungal partners (Gaskett et al., 2014).

Conclusion

The UNIMORE Orchid Seedbank has been successfully established as a regional *ex-situ* conservation initiative for Emilia-Romagna's terrestrial orchids, demonstrating that standards-aligned workflows integrating data management, controlled desiccation, and −20 °C storage are both feasible and effective. Over three years, it evolved from a conceptual proposal into a functional, data-driven infrastructure that preserves ecological and geographical diversity while providing empirical evidence on the effects of temperature and seed quality on longevity. The seedbank serves a dual role, functioning as a research platform and a conservation safeguard, bridging laboratory practices with field biodiversity management.

Its success extends beyond the lab, supporting biodiversity preservation, academic research, and public awareness, and establishing a model for scalable, standardized conservation frameworks. By integrating physiological testing, ecological context, and traceable data management, it offers a living scientific instrument dedicated to safeguarding regional orchid flora.

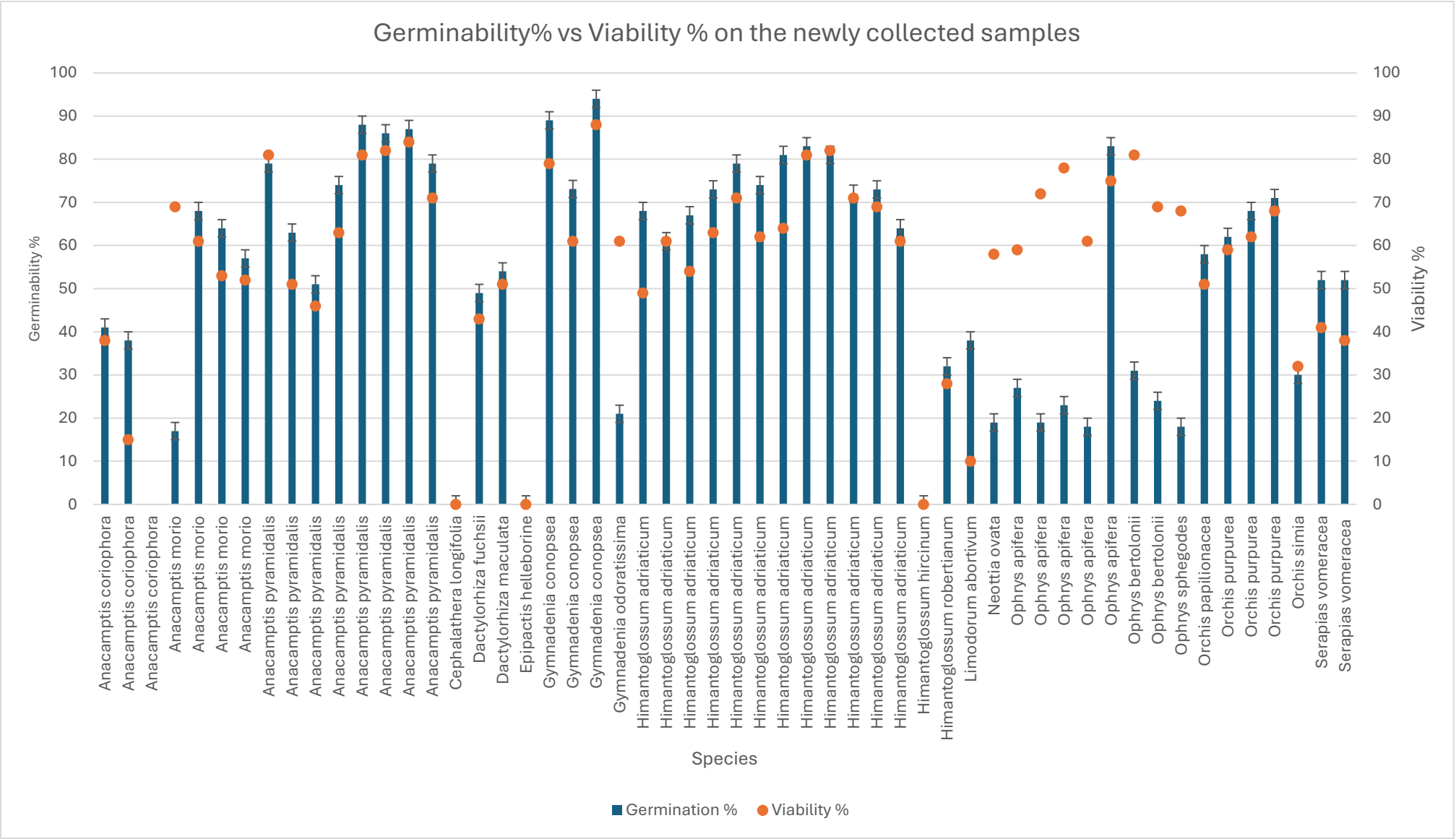


Figure 2.7: Germinability % and Viability % for species collected 2023 to 2025

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Annexes:

Annex I:

Passport Data of the collected seed accessions within UNIMORE Seedbank -EAB Lab

Used Code	Code	way point	Sp. Name	Date of Identification	geographic place	Date of collection	date at -20	weight	collector	Genus	Natura 2000 Code
SB BC 0101	SB BC 0508	0/060	*Anacamptis coriophora*	7/6/2023	strada Rio Benedello	7/7/2023	23/8/2023	132.1	A. Mortada & E. Sgarbi	Anacamptis	IT4040003
SB BC 0102	SB BC 0509	0/064	*Anacamptis coriophora*	13/6/2023	Via Ca' Corghi Scandiano, RE	19/7/2023	23/8/2023	60.9	A. Mortada & E. Sgarbi	Anacamptis	IT4030016
SB BC 0103	SB BC 0510	0/057	*Anacamptis coriophora*	7/6/2023	Malandrone	only one plant		empty	A. Mortada & E. Sgarbi	Anacamptis	None (outside Natura 2000)
SB BC 0201	SB BC 1832	OMG	*Anacamptis morio*	4/4/2024	Gainazzo	7/16/2024	19/8/2024	69.1	E. Sgarbi	Ophrys	None (outside Natura 2000)
SB BC 0202	SB BC 1924	0/059	*Anacamptis morio*	30/4/2023	Monte Rauaglia (Serramazzoni)	7/7/2023	23/8/2023	52.7	A. Mortada & E. Sgarbi	Orchis	None (outside Natura 2000)
SB BC 0203	SB BC 1926		*Anacamptis morio*	30/4/2023	Monte Rauaglia (Serramazzoni)	7/7/2023	23/8/2023	19	A. Mortada & E. Sgarbi	Orchis	None (outside Natura 2000)
SB BC 0204	SB BC 1927		*Anacamptis morio*	30/4/2023	Monte Rauaglia (Serramazzoni)	7/7/2023	23/8/2023	6.6	A. Mortada & E. Sgarbi	Orchis	None (outside Natura 2000)
SB BC 0301	SB BC 0506		*Anacamptis pyramidalis*	28/5/2023	Via Ca' Corghi Scandiano, RE	14/07/2023	23/8/2023	36.1	A. Mortada & E. Sgarbi	Anacamptis	IT4030016
SB BC 0302	SB BC 0507		*Anacamptis pyramidalis*	7/6/2023	Via Ca' Corghi Scandiano, RE	7/7/2023	23/8/2023	15.8	A. Mortada & E. Sgarbi	Anacamptis	IT4030016
SB BC 0303	SB BC 0511	0/063	*Anacamptis pyramidalis*	13/6/2023	Via Ca' Corghi Scandiano, RE	19/7/2023	23/8/2023	61.9	A. Mortada & E. Sgarbi	Anacamptis	IT4030016
SB BC 0304	SB BC 0512	9	*Anacamptis pyramidalis*	28/5/2023	Marano sul Panaro, località Casona	14/7/2023	23/8/2023	10.9	E. Sgarbi	Anacamptis	IT4040003
SB BC 0305	SB BC 0513		*Anacamptis pyramidalis*	7/6/2023	Monte Rauaglia (Serramazzoni)	7/7/2023	23/8/2023	29.6	E. Sgarbi	Anacamptis	None (outside Natura 2000)
SB BC 0306	SB BC 0514	..0514	*Anacamptis pyramidalis*	1/5/2025	Salse di Nirano	8/6/2025	25/7/2025	12.7	A. Mortada	Anacamptis	IT4040007
SB BC 0307	SB BC 0515	..0515	*Anacamptis pyramidalis*	3/4/2025	Trieste	10/6/2025	25/7/2025	34.46	A. Mortada	Anacamptis	None (outside Natura 2000)
SB BC 0308	SB BC 0516	..0516	*Anacamptis pyramidalis*		Private Garden in Nonantola	17/6/2025	25/7/2025	9.1	E. Sgarbi	Anacamptis	None (outside Natura 2000)
SB BC 0401	SB BC 2601		*Cephalathera longifolia*	1/5/2023	Mantova	27/6/2023; 6/7/2023	23/8/2023	120.1	E. Sgarbi	Cephalathera	None (outside Natura 2000)
SB BC 0501	SB BC 0708	..0708	*Dactylorhiza fuchsii*	4/1/2025	Cento Croci	26/6/2025	25/7/2025	2	A. Mortada & G. Santinioni	Dactelariza	None (outside Natura 2000)
SB BC 0601	SB BC 0707	..0707	*Dactylorhiza maculata*	4/1/2025	Cento Croci	26/6/2025	25/7/2025	2	A. Mortada & G. Santinioni	Dactelariza	None (outside Natura 2000)
SB BC 0701	SB BC 0908		*Epipactis helleborine*	1/5/2023	Monte Rauaglia (Serramazzoni)	19/7/2023	23/8/2023	28.4	A. Mortada & E. Sgarbi	Epipoctis	None (outside Natura 2000)
SB BC 0801	SB BC 1205	0/065	*Gymnadenia conopsea*	13/6/2023	Via Ca' Corghi Scandiano, RE	19/7/2023	23/8/2023	673.1	A. Mortada & E. Sgarbi	Gymnadenia	IT4030016
SB BC 0802	SB BC 1206		*Gymnadenia conopsea*	7/6/2023	strada Rio Benedello	7/7/2023	23/8/2023	27.81	A. Mortada & E. Sgarbi	Gymnadenia	IT4040003
SB BC 0803	SB BC 1207	0/059	*Gymnadenia conopsea*	7/6/2023	strada Rio Benedello	7/7/2023	23/8/2023	88.1	E. Sgarbi	Gymnadenia	IT4040003
SB BC 0901	SB BC 1208	1208	*Gymnadenia odoratissima*	4/1/2025	Cento Croci	26/6/2025	25/7/2025	2	A. Mortada & G. Santinioni	Gymnadenia	None (outside Natura 2000)
SB BC 1001	SB BC 1307	/011	*Himantoglossum adriaticum*	7/6/2023	Reggio Emilia Parco San Lazzaro	7/7/2023	23/8/2023	107.1	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1002	SB BC 1308	/011	*Himantoglossum adriaticum*	16/6/2023	Reggio Emilia Parco San Lazzaro	3/8/2023	23/8/2023	36.1	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1003	SB BC 1309	/011	*Himantoglossum adriaticum*	28/5/2023	Reggio Emilia Parco San Lazzaro	27/6/2023	23/8/2023	110.1	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1004	SB BC 1310		*Himantoglossum adriaticum*	13/6/2023	Via Ca' Corghi Scandiano, RE	19/7/2023	23/8/2023	3.3	A. Mortada & E. Sgarbi	Himantoglossum	IT4030016
SB BC 1005	SB BC 1311		*Himantoglossum adriaticum*	16/6/2023	NONANTOLA	3/8/2023	23/8/2023	3.1	E. Sgarbi	Himantoglossum	None (outside Natura 2000)
SB BC 1006	SB BC 1313	SL7 01	*Himantoglossum adriaticum*	27/3/2024	Reggio Emilia Parco San Lazzaro	7/16/2024	19/8/2024	13	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1007	SB BC 1314	SL2 2 PLANTS	*Himantoglossum adriaticum*	27/3/2024	Reggio Emilia Parco San Lazzaro	7/16/2024	19/8/2024	200	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1008	SB BC 1315	SL1 3PLANTS	*Himantoglossum adriaticum*	27/3/2024	Reggio Emilia Parco San Lazzaro	7/16/2024	19/8/2024	85	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1009	SB BC 1316	1316	*Himantoglossum adriaticum*	23/3/2025	Reggio Emilia Parco San Lazzaro	27/5/2025	25/7/2025	58.16	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1010	SB BC 1317	1317	*Himantoglossum adriaticum*	28/3/2025	Reggio Emilia Parco San Lazzaro	29/5/2025	25/7/2025	9.16	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1011	SB BC 1318	1318	*Himantoglossum adriaticum*	29/3/2025	Reggio Emilia Parco San Lazzaro	21/6/2025	25/7/2025	11.6	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1012	SB BC 1319	1319	*Himantoglossum adriaticum*	23/3/2025	Reggio Emilia Parco San Lazzaro	29/5/2025	25/7/2025	1.3	A. Mortada	Himantoglossum	None (outside Natura 2000)
SB BC 1101	SB BC 1312	way point 073	*Himantoglossum hircinum*	16/6/2023	Via Sparavalle	3/8/2023	23/8/2023	18.4	A. Mortada & E. Sgarbi	Himantoglossum	None (outside Natura 2000)
SB BC 1201	SB BC 0608	BRG	*Himantoglossum robertianum*	4/4/2024	Gainazzo	7/16/2024	19/8/2024	8.2	E. Sgarbi	Barlia	None (outside Natura 2000)
SB BC 1301	SB BC 1414		*Limodorum abortivum*	1/5/2023	Monte Rauaglia (Serramazzoni)	19/7/2023	23/8/2023	143.5	E. Sgarbi	Limodorum	None (outside Natura 2000)
SB BC 1401	SB BC 1702	1702	*Neottia ovata*	4/1/2025	Cento Croci	26/6/2025	25/7/2025	8.26	A. Mortada & G. Santinioni	Neottia	None (outside Natura 2000)
SB BC 1501	SB BC 1826	0/061	*Ophrys apifera*	7/6/2023	strada Rio Benedello	7/7/2023	23/8/2023	51.5	A. Mortada & E. Sgarbi	Ophrys	IT4040003
SB BC 1502	SB BC 1828	SL1 OA	*Ophrys apifera*	28/4/2024	Reggio Emilia Parco San Lazzaro	2/7/2024	19/8/2024	3	A. Mortada	Ophrys	None (outside Natura 2000)
SB BC 1503	SB BC 1829	PDR OA	*Ophrys apifera*	3/5/2024	Parco Resistenza (MO)	5/7/2024	19/8/2024	20	A. Mortada	Ophrys	None (outside Natura 2000)
SB BC 1504	SB BC 1833	1833	*Ophrys apifera*	1/5/2025	Salse di Nirano	8/6/2025	25/7/2025	2	A. Mortada	Ophrys	IT4040007
SB BC 1505	SB BC 1928		*Ophrys apifera*	7/6/2023	Parco Resistenza (MO)	27/7/2023	23/8/2023	11.6	A. Mortada	Ophrys	None (outside Natura 2000)
SB BC 1601	SB BC 1827	0/057	*Ophrys bertolonii*	7/6/2023	Malandrone	7/7/2023	23/8/2023	44.8	A. Mortada & E. Sgarbi	Ophrys	None (outside Natura 2000)
SB BC 1602	SB BC 1831	OBG	*Ophrys bertolonii*	4/4/2024	Gainazzo	7/16/2024	19/8/2024	71	E. Sgarbi	Ophrys	None (outside Natura 2000)
SB BC 1701	SB BC 1830	OSG	*Ophrys sphegodes*	4/4/2024	Gainazzo	7/16/2024	19/8/2024	90	E. Sgarbi	Ophrys	None (outside Natura 2000)
SB BC 1801	SB BC 1925		*Orchis papilionacea*	30/4/2023	Monte Rauaglia (Serramazzoni)	7/7/2023	23/8/2023	26.5	A. Mortada & E. Sgarbi	Orchis	None (outside Natura 2000)
SB BC 1901	SB BC 0407	..0407	*Orchis purpurea*	19/3/2025	Reggio Emilia Parco San Lazzaro	11/6/2025	25/7/2025	2.2	A. Mortada	Orchis	None (outside Natura 2000)
SB BC 1902	SB BC 1929	SL 7 02	*Orchis purpurea*	3/3/2024	Reggio Emilia Parco San Lazzaro	24/6/2024	19/8/2024	2	A. Mortada	Orchis	None (outside Natura 2000)
SB BC 1903	SB BC 1930	SL6 05	*Orchis purpurea*	3/3/2024	Reggio Emilia Parco San Lazzaro	24/6/2024	19/8/2024	3	A. Mortada	Orchis	None (outside Natura 2000)
SB BC 2001	SB BC 1931	SL5	*Orchis simia*	3/3/2024	Reggio Emilia Parco San Lazzaro	13/6/2024	19/8/2024	5	A. Mortada	Orchis	None (outside Natura 2000)
SB BC 2101	SB BC 2409	/060	*Serapias vomeracea*	7/6/2023	strada Rio Benedello	7/7/2023	23/8/2023	82.6	A. Mortada & E. Sgarbi	Seraplas	IT4040003
SB BC 2102	SB BC 2410	0/061	*Serapias vomeracea*	30/4/2023	strada Rio Benedello	7/7/2023	23/8/2023	107.7	A. Mortada & E. Sgarbi	Seraplas	IT4040003

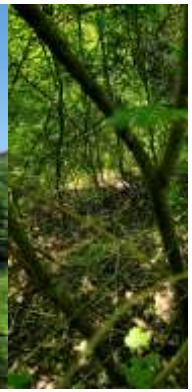
Used Code	Habitat Type	Population Size Estimate	Phenology at Collection	CITES Status	Drying Method	Target eRH Achieved	Packaging Type	Storage Condition
SB BC 0101	Calcareous grassland (6210*)	>150	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0102	Calcareous grassland (6210*)	>150	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0103	Calcareous grassland (6210*)	1	Fruit maturation (drying capsules)	Appendix II				
SB BC 0201	Semi-natural meadow / grassland	5 to 10	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0202	Semi-natural meadow / grassland	6	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0203	Semi-natural meadow / grassland	24	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0204	Semi-natural meadow / grassland	18	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0301	Calcareous grassland (6210*)	>150	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0302	Calcareous grassland (6210*)	>150	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0303	Calcareous grassland (6210*)	>150	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0304	Calcareous grassland (6210*)	>100	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0305	Calcareous grassland (6210*)	>100	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0306	Semi-natural meadow / grassland	>150	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0307	Private garden/ anthropogenic grassland	10 plants	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0308	Private garden/ anthropogenic grassland	1 plant	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0401	Semi-natural meadow / grassland	15	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0501	Semi-natural meadow / grassland	>100	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0601	Semi-natural meadow / grassland	>70	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0701	Semi-natural meadow / grassland	29 plants	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0801	Semi-natural meadow / grassland	12	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0802	Semi-natural meadow / grassland	12	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0803	Semi-natural meadow / grassland	23	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 0901	Semi-natural meadow / grassland	>102	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1001	Urban park / anthropogenic grassland	6	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1002	Urban park / anthropogenic grassland	4	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1003	Urban park / anthropogenic grassland	2	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1004	Semi-natural meadow / grassland	13	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1005	Dry calcareous grassland / scrub (6210*)	4	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1006	Urban park / anthropogenic grassland	2	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1007	Urban park / anthropogenic grassland	2	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1008	Urban park / anthropogenic grassland	9	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1009	Urban park / anthropogenic grassland	3	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1010	Urban park / anthropogenic grassland	1	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1011	Urban park / anthropogenic grassland	2	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1012	Urban park / anthropogenic grassland	2	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1101	Dry calcareous grassland / scrub (6210*)	2	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1201	Semi-natural meadow / grassland	13 plants	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1301	Semi-natural meadow / grassland	2	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1401	Semi-natural meadow / grassland	29	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1501	Semi-natural meadow / grassland	5	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1502	Urban park / anthropogenic grassland	1	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1503	Urban park / anthropogenic grassland	>70	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1504	Semi-natural meadow / grassland	3	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1505	Urban park / anthropogenic grassland	>70	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1601	Semi-natural meadow / grassland	5 to 10	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1602	Semi-natural meadow / grassland	5 to 10	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1701	Semi-natural meadow / grassland	4	Post-flowering (green capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1801	Semi-natural meadow / grassland	12	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1901	Urban park / anthropogenic grassland	3 plants	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1902	Urban park / anthropogenic grassland	1	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 1903	Urban park / anthropogenic grassland	1	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 2001	Urban park / anthropogenic grassland	1	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 2101	Semi-natural meadow / grassland	14	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)
SB BC 2102	Semi-natural meadow / grassland	12	Fruit maturation (drying capsules)	Appendix II	Silica gel desiccation in airtight container (target RH ~15–20%)	12%	Glass vial, hermetically sealed	–20 °C freezer storage (long-term)

Used Code	Viability Test Method	Viability %	Germination %	Contamination %	Date of Last QA Test	Next Scheduled Test Date	Duplicate Flag	Notes
SB BC 0101	Tetrazolium chloride (TTC) staining	38	41	0.66%	April 7 2025	6/1/2028	Unique	
SB BC 0102	Tetrazolium chloride (TTC) staining	15	38	1.20%	April 7 2025	6/1/2028	Unique	
SB BC 0103								only one plant no seeds collected but the location is d=recorded for later monitoring
SB BC 0201	Tetrazolium chloride (TTC) staining	69	17	0.28%	April 7 2025	6/1/2028	Unique	High viability but low germination capacity maybe because it is ophrys genus
SB BC 0202	Tetrazolium chloride (TTC) staining	61	68	0.57%	April 7 2025	6/1/2028	Unique	
SB BC 0203	Tetrazolium chloride (TTC) staining	53	64	1.26%	April 7 2025	6/1/2028	Unique	
SB BC 0204	Tetrazolium chloride (TTC) staining	52	57	0.65%	April 7 2025	6/1/2028	Unique	
SB BC 0301	Tetrazolium chloride (TTC) staining	81	79	0.90%	April 7 2025	6/1/2028	Unique	
SB BC 0302	Tetrazolium chloride (TTC) staining	51	63	0.27%	April 7 2025	6/1/2028	Unique	
SB BC 0303	Tetrazolium chloride (TTC) staining	46	51	0.66%	April 7 2025	6/1/2028	Unique	
SB BC 0304	Tetrazolium chloride (TTC) staining	63	74	0.42%	April 7 2025	6/1/2028	Unique	
SB BC 0305	Tetrazolium chloride (TTC) staining	81	88	0.91%	April 7 2025	6/1/2028	Unique	
SB BC 0306	Tetrazolium chloride (TTC) staining	82	86	0.12%	April 7 2025	6/1/2028	Unique	
SB BC 0307	Tetrazolium chloride (TTC) staining	84	87	1.12%	April 7 2025	6/1/2028	Unique	
SB BC 0308	Tetrazolium chloride (TTC) staining	71	79	0.80%	April 7 2025	6/1/2028	Unique	
SB BC 0401	Tetrazolium chloride (TTC) staining	0	0	0.12%	April 7 2025	6/1/2028	Unique	
SB BC 0501	Tetrazolium chloride (TTC) staining	43	49	0.66%	April 7 2025	6/1/2028	Unique	
SB BC 0601	Tetrazolium chloride (TTC) staining	51	54	0.28%	April 7 2025	6/1/2028	Unique	first protocorm apears after 8 weeks
SB BC 0701	Tetrazolium chloride (TTC) staining	0	0	1.19%	April 7 2025	6/1/2028	Unique	
SB BC 0801	Tetrazolium chloride (TTC) staining	79	89	1.61%	April 7 2025	6/1/2028	Unique	
SB BC 0802	Tetrazolium chloride (TTC) staining	61	73.1	0.80%	April 7 2025	6/1/2028	Unique	
SB BC 0803	Tetrazolium chloride (TTC) staining	88	94	0.13%	April 7 2025	6/1/2028	Unique	
SB BC 0901	Tetrazolium chloride (TTC) staining	61	21	0.28%	April 7 2025	6/1/2028	Unique	High viability but low germination capacity maybe because it is ophrys genus
SB BC 1001	Tetrazolium chloride (TTC) staining	49	68	0.66%	April 7 2025	6/1/2028	Unique	new collection 061
SB BC 1002	Tetrazolium chloride (TTC) staining	61	61	80.00%	April 7 2025	6/1/2028	Unique	the seeds are contaminated with fungus
SB BC 1003	Tetrazolium chloride (TTC) staining	54	67	1.19%	April 7 2025	6/1/2028	Unique	
SB BC 1004	Tetrazolium chloride (TTC) staining	63	73	0.84%	April 7 2025	6/1/2028	Unique	
SB BC 1005	Tetrazolium chloride (TTC) staining	71	79	0.92%	April 7 2025	6/1/2028	Unique	
SB BC 1006	Tetrazolium chloride (TTC) staining	62	74	0.71%	April 7 2025	6/1/2028	Unique (different year)	
SB BC 1007	Tetrazolium chloride (TTC) staining	64	81	0.69%	April 7 2025	6/1/2028	Unique (different year)	
SB BC 1008	Tetrazolium chloride (TTC) staining	81	83	0.68%	April 7 2025	6/1/2028	Unique (different year)	
SB BC 1009	Tetrazolium chloride (TTC) staining	82	81	0.67%	April 7 2025	6/1/2028	Unique (different year)	SL2
SB BC 1010	Tetrazolium chloride (TTC) staining	71	72	0.65%	April 7 2025	6/1/2028	Unique (different year)	SL1
SB BC 1011	Tetrazolium chloride (TTC) staining	69	73	0.64%	April 7 2025	6/1/2028	Unique (different year)	SL2 BAGGED for insect fertilization project
SB BC 1012	Tetrazolium chloride (TTC) staining	61	64	0.62%	April 7 2025	6/1/2028	Unique (different year)	SL7 BAGGED for insect fertilization project
SB BC 1101	Tetrazolium chloride (TTC) staining	0	0	0.69%	April 7 2025	6/1/2028	Unique	
SB BC 1201	Tetrazolium chloride (TTC) staining	28	32	0.13%	April 7 2025	6/1/2028	Unique	
SB BC 1301	Tetrazolium chloride (TTC) staining	10	38	0.61%	April 7 2025	6/1/2028	Unique	
SB BC 1401	Tetrazolium chloride (TTC) staining	58	19	0.60%	April 7 2025	6/1/2028	Unique	High viability but low germination capacity maybe because it is ophrys genus
SB BC 1501	Tetrazolium chloride (TTC) staining	59	27	0.58%	April 7 2025	6/1/2028	Unique	High viability but low germination capacity maybe because it is ophrys genus
SB BC 1502	Tetrazolium chloride (TTC) staining	72	19	0.12%	April 7 2025	6/1/2028	Unique (different year)	High viability but low germination capacity maybe because it is ophrys genus
SB BC 1503	Tetrazolium chloride (TTC) staining	78	23	1.12%	April 7 2025	6/1/2028	Unique (different year)	High viability but low germination capacity maybe because it is ophrys genus
SB BC 1504	Tetrazolium chloride (TTC) staining	61	18	0.66%	April 7 2025	6/1/2028	Unique	High viability but low germination capacity maybe because it is ophrys genus
SB BC 1505	Tetrazolium chloride (TTC) staining	75	83	0.64%	April 7 2025	6/1/2028	Unique	
SB BC 1601	Tetrazolium chloride (TTC) staining	81	31	0.57%	April 7 2025	6/1/2028	Unique	High viability but low germination capacity maybe because it is ophrys genus
SB BC 1602	Tetrazolium chloride (TTC) staining	69	24	0.13%	April 7 2025	6/1/2028	Unique	High viability but low germination capacity maybe because it is ophrys genus
SB BC 1701	Tetrazolium chloride (TTC) staining	68	18	0.80%	April 7 2025	6/1/2028	Unique	High viability but low germination capacity maybe because it is ophrys genus
SB BC 1801	Tetrazolium chloride (TTC) staining	51	58	0.43%	April 7 2025	6/1/2028	Unique	
SB BC 1901	Tetrazolium chloride (TTC) staining	59	62	2%	April 7 2025	6/1/2028	Unique (different year)	
SB BC 1902	Tetrazolium chloride (TTC) staining	62	68	0.62%	April 7 2025	6/1/2028	Unique (different year)	
SB BC 1903	Tetrazolium chloride (TTC) staining	68	71	0.61%	April 7 2025	6/1/2028	Unique (different year)	
SB BC 2001	Tetrazolium chloride (TTC) staining	32	30	0.60%	April 7 2025	6/1/2028	Unique (different year)	
SB BC 2101	Tetrazolium chloride (TTC) staining	41	52	0.58%	April 7 2025	6/1/2028	Unique	
SB BC 2102	Tetrazolium chloride (TTC) staining	38	52	0.57%	April 7 2025	6/1/2028	Unique	

Annex II

Protection way within urban parks to avoid cutting

Parco della Resistenza wooded area



Open Area in Parco San Lazzaro

Via Ca' Corghi, Scandiano

Salse di Nirano

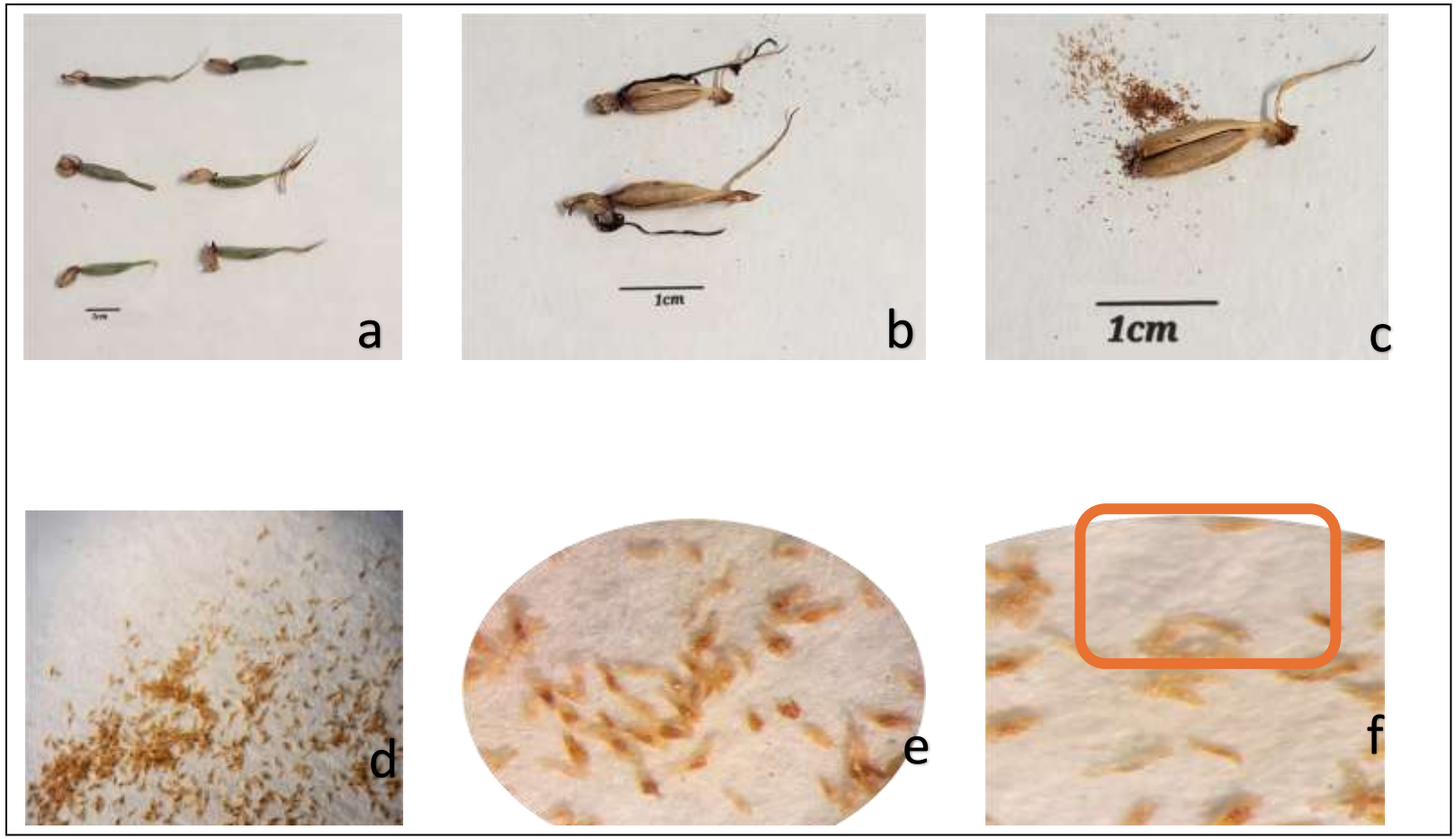
A	B	C	D	E	F	I
Accession	Scientific name	Number of the identified population	date of identification (plants in bloom)	Locality	Way point	date of fruit collection
	Orchis simia	1	4/30/2023	Marano sul Panaro, località Casona	/	
	Orchis purpurea	2	4/30/2023	strada per Samone		
	Orchis purpurea	3	4/30/2023		079	
	Anacamptis morio	4	4/30/2023			
	Orchis provincialis	5	4/30/2023		080	
	Serapias vomeracea	6 = Way Point 061	4/30/2023		061	
	Ophrys bertolonii	7	4/30/2023		062	
	Anacamptis pyramidalis	8	5/28/2023	Marano sul Panaro, località Casona	077	7/14/2023
	Anacamptis pyramidalis	9	5/28/2023	Marano sul Panaro, località Casona	076	7/14/2023
	Serapias vomeracea	10	5/28/2023	Marano sul Panaro, località Casona	/	no plants
	Himantoglossum adriaticum	11	May-23	Parco S. Lazzaro (RE) Via Amendola		6/27/2023
	Cephalanthera longifolia	12	5/1/2023	loc. Bigarello (MN)	/	27/06/2023; 06/07/2023
	Ophrys bertolonii	Way Point 057 -1	6/7/2023	Malandrone	057	7/7/2023
	Anacamptis pyramidalis	Way Point 057 -2	6/7/2023	Malandrone	057	7/7/2023
	Anacamptis coriophora	Way Point 057 -3	6/7/2023	Malandrone	057	7/7/2023
no orchid	iris graminea	Way Point 058	6/7/2023	Malandrone	058	
	Gymnadenia conopsea	Way Point 059	6/7/2023	strada Rio Benedello	059	7/7/2023
	Anacamptis coriophora	Way Point 060-1	6/7/2023	strada Rio Benedello	060	7/7/2023
	Serapias vomeracea	Way Point 060-2	6/7/2023	strada Rio Benedello	060	7/7/2023
	Anacamptis pyramidalis	Way Point 060-3	6/7/2023	strada Rio Benedello	060	7/7/2023
	Gymnadenia conopsea	Way Point 060-4	6/7/2023	strada Rio Benedello	060	no collected: only 1 plant was found
	Serapias vomeracea	Way Point 061-1 = 6	4/30/2023	strada Rio Benedello	061	7/7/2023
	Himantoglossum adriaticum	Way Point 061-2	6/7/2023	strada Rio Benedello	061	7/7/2023
	Ophrys apifera	Way Point 061-3	6/7/2023	strada Rio Benedello	061	7/7/2023
	Anacamptis pyramidalis (A. berica)	Way Point 063	6/13/2023	Via Ca' Corghi	63	7/19/2023
	Anacamptis coriophora	Way Point 064	6/13/2023	Via Ca' Corghi	64	7/19/2023
	Himantoglossum adriaticum	Way Point 065 -1	6/13/2023	Via Ca' Corghi	65	7/19/2023
	Gymnadenia conopsea	Way Point 065 -2	6/13/2023	Via Ca' Corghi	65	7/19/2023

Section of the field book showing the way of monitoring

Annex III

Scientific names of some monitored orchids: a) Anacamptis pyramidalis; b) Cephalanthera longifolia; c) Dactylorhiza maculata; d) Gymnadenia odoratissima; e) Himantoglossum adriaticum; f) Neottia ovata; g) Ophrys apifera; h) Ophrys bertolonii; i) Ophrys sphegodes; j) Orchis purpurea; k) Orchis simia

Annex IV



How to collect seeds: a) green capsule; b) ripe capsule with seeds inside; c) open capsule and ripe seeds ready to be spread; d) collected seeds; e) seeds with embryos; f) seeds without embryos

Annex V

Germinability % and Viability % of all the existing collections

Species	Storage Temperature °C	Viability_%	Germinability %
Anacamptis fragrans	5	0	0
Anacamptis fragrans	5	0	0
Anacamptis fragrans	5	0	0
Anacamptis fragrans	5	4	0
Anacamptis laxiflora	5	0	0
Anacamptis laxiflora	5	0	0
Anacamptis laxiflora	5	0	0
Anacamptis laxiflora	5	0	0
Anacamptis morio	5	0	0
Anacamptis morio	5	1	0
Anacamptis papilionacea	5	0	0
Anacamptis papilionacea	5	0	0
Anacamptis papilionacea	5	0	0
Anacamptis purpurea	5	0	0
Anacamptis purpurea	5	0	0
Anacamptis purpurea	5	0	0
Anacamptis pyramidalis	5	0	48
Barlia robertiana	5	0	0
Dactylorhiza fuchsii	5	0	0
Dactylorhiza fuchsii	5	0	0
Dactylorhiza fuchsii	5	0	0
Dactylorhiza fuchsii	5	0	0
Dactylorhiza fuchsii	5	0	0
Dactylorhiza incarnata	5	0	0

Dactylorhiza incarnata	5	0	0
Dactylorhiza incarnata	5	0	0
Dactylorhiza incarnata	5	0	0
Dactylorhiza incarnata	5	0	0
Dactylorhiza incarnata	5	2	0
Dactylorhiza insularis	5	0	0
Dactylorhiza insularis	5	0	0
Dactylorhiza insularis	5	0	0
Dactylorhiza insularis	5	3	0
Dactylorhiza sambucina	5	0	0
Dactylorhiza sambucina	5	0	0
Dactylorhiza sambucina	5	4	0
Epidendrum costarica	5	0	0
Epidendrum costarica	5	2	0
Epipactis helleborine	5	0	0
Epipactis helleborine	5	0	0
Epipactis helleborine	5	0	0
Epipactis helleborine	5	5	0
Epipactis helleborine	5	0	0
Epipactis helleborine	5	0	0
Epipactis microphylla	5	0	0
Epipactis microphylla	5	0	0
Epipactis microphylla	5	1	0
Epipactis microphylla	5	2	0
Epipactis palustris	5	0	0
Epipactis palustris	5	0	0
Gennaria diphylla	5	0	0

Gennaria diphylla	5	0	0
Gennaria diphylla	5	0	0
Goodyera repens	5	0	0
Gymnadenia conopsea	5	0	37
Himantoglossum adriaticum	5	0	0
Himantoglossum adriaticum	5	0	0
Himantoglossum adriaticum	5	0	0
Himantoglossum sp.	5	0	0
Himantoglossum sp.	5	0	0
Himantoglossum sp.	5	0	0
Himantoglossum sp.	5	2	0
Limodorum abortivum	5	0	0
Limodorum abortivum	5	0	0
Limodorum abortivum	5	1	0
Neotinea maculata	5	0	0
Neotinea maculata	5	0	0
Neotinea maculata	5	0	0
Neotinea maculata	5	0	0
Neotinea tridentata	5	0	0
Neotinea tridentata	5	1	0
Neottia sp.	5	0	0
Neottia sp.	5	0	0
Neottia sp.	5	0	0
Neottia sp.	5	0	0
Neottia sp.	5	0	0
Neottia sp.	5	0	0
Neottia sp.	5	3	0

Ophrys apifera	5	0	0
Ophrys apifera	5	0	0
Ophrys apifera	5	2	0
Ophrys bertolonii	5	0	0
Ophrys bertolonii	5	0	0
Ophrys bertolonii	5	0	0
Ophrys crabronifera	5	1	0
Ophrys fusca	5	1	0
Ophrys montis-leonis	5	3	0
Ophrys montis-leonis	5	3	0
Ophrys sp.	5	0	0
Ophrys sp.	5	2	0
Ophrys sp.	5	0	0
Ophrys sp.	5	0	0
Ophrys sp.	5	0	0
Ophrys sphegodes	5	0	0
Ophrys sphegodes	5	0	0
Ophrys sphegodes	5	6	0
Orchis anthropophora	5	0	0
Orchis anthropophora	5	0	0
Orchis italica	5	0	0
Orchis italica	5	2	0
Orchis italica	5	2	0
Orchis pauciflora	5	0	0
Orchis pauciflora	5	0	0
Orchis pauciflora	5	0	0
Orchis pauciflora	5	1	0

Orchis sp.	5	0	0
Orchis sp.	5	0	0
Orchis sp.	5	0	0
Orchis sp.	5	0	0
Phragmipedium schlimii	5	0	0
Phragmipedium schlimii	5	0	0
Phragmipedium schlimii	5	2	0
Platanthera bifolia	5	1	0
Platanthera sp.	5	0	0
Platanthera sp.	5	0	0
Platanthera sp.	5	0	0
Platanthera sp.	5	0	0
Platanthera sp.	5	0	0
Platanthera sp.	5	0	0
Platanthera sp.	5	2	0
Serapias lingua	5	0	0
Serapias lingua	5	2	0
Serapias lingua	5	2	0
Serapias parviflora	5	0	0
Serapias parviflora	5	0	0
Serapias parviflora	5	0	0
Serapias parviflora	5	3	0
Serapias vomeracea	5	0	0
Serapias vomeracea	5	1	0
Spiranthes spiralis	5	0	0
Anacamptis coriophora	-20	17	12
Anacamptis coriophora	-20	16	36

Anacamptis morio	-20	14	27
Anacamptis morio	-20	16	29
Anacamptis papilionacea	-20	3	21
Anacamptis purpurea	-20	7	36
Anacamptis pyramidalis	-20	8	39
Corallorhiza trifida	-20	2	39
Cypripedium calceolus	-20	1	49
Cystorchis gracilis	-20	0	52
Dactylorhiza fuchsii	-20	5	31
Dactylorhiza incarnata	-20	3	38
Dactylorhiza insularis	-20	9	43
Dactylorhiza sambucina	-20	6	47
Epipactis atrorubens	-20	4	52
Epipactis atrorubens	-20	2	24
Epipactis helleborine	-20	7	62
Epipactis microphylla	-20	6	42
Epipactis microphylla	-20	9	62
Epipactis palustris	-20	2	58
Epipactis palustris	-20	0	28
Epipactis palustris	-20	2	62
Epipactis palustris	-20	4	51
Goodyera repens	-20	7	47
Gymnadenia conopsea	-20	2	43
Himanthoglossum adriaticum	-20	8	52
Himanthoglossum adriaticum	-20	2	36
Himanthoglossum adriaticum	-20	3	51
Himantoglossum robertianum	-20	2	19

Himantoglossum robertianum	-20	4	53
Himantoglossum robertianum	-20	5	19
Himantoglossum robertianum	-20	6	26
Himantoglossum robertianum	-20	2	24
Malaxis monophyllos	-20	5	27
Neottia ovata	-20	2	29
Ophrys apifera	-20	4	15
Ophrys apifera	-20	6	18
Ophrys apifera	-20	2	19
Ophrys bertolonii	-20	7	16
Ophrys crabronifera	-20	3	19
Ophrys crabronifera	-20	0	28
Ophrys montis-leonis	-20	2	31
Ophrys sphegodes	-20	0	21
Ophrys sphegodes	-20	1	23
Ophrys sphegodes	-20	2	19
Orchis provincialis	-20	0	43
Orchis simia	-20	0	25
Serapias parviflora	-20	7	20
Serapias vomeracea	-20	4	19
Spiranthes spiralis	-20	3	38

Chapter 3: Effectiveness of Storage Temperature to Maintain Germinability and Viability of rare and common Orchidaceae species

Abstract

The Orchidaceae family is one of the most extensive and varied groups of flowering plants, yet its conservation is confronted by challenges including habitat loss, climate change, and trade. The long-term *ex-situ* conservation of seeds has become increasingly vital for the preservation of genetic and species diversity. The present study evaluates the effectiveness of three distinct storage regimes — deep-freeze (–20 °C), refrigerated (5 °C), and herbarium ambient storage (~20 °C) — in maintaining seed viability and germinability across a broad sample of terrestrial orchids collected between 1996 and 2025. The results demonstrate that seeds stored at –20 °C retained high viability and germination potential even after up to ten years, thus confirming sub-zero storage as optimal for long-term preservation. Conversely, seeds stored at 5 °C exhibited a decline in tetrazolium-detectable viability, although a subset (particularly *Anacamptis pyramidalis* and *Gymnadenia conopsea*) demonstrated to be able to germinate after a decade, suggesting that refrigeration may preserve limited functional capacity for propagation. The viability of the herbarium-stored seeds was generally poor, with only *Epipactis helleborine* exhibiting minimal viability (1–14% over 8–29 years). The remaining *taxa* demonstrated a complete failure in viability. These findings underscore a discernible hierarchy in the efficacy of storage, thereby substantiating the pivotal role of temperature and storage conditions in determining seed longevity. The study supports a tiered conservation model, which combines deep-freeze banking for long-term conservation, refrigerated short-term storage for active collection, and herbarium archives for genetic repositories. This model is tailored to species-specific seed physiology. This work provides a foundation for the development of evidence-based guidelines for the banking of orchid seeds and the implementation of *ex-situ* conservation strategies.

Keywords: Orchidaceae, seed viability, germinability, seedbank, storage temperature, herbarium specimens, *ex-situ* conservation, tetrazolium test.

3.1 Introduction

The Orchidaceae family, comprising over 30,000 species, performs a pivotal ecological function. Nevertheless, it faces a lot challenges, including habitat degradation and climate change, as well as collection methodologies, necessitating *ex situ* conservation (Chase et al., 2015; Swarts & Dixon, 2009). The storage of seeds represents a cost-effective method of preserving the genetic diversity of orchids. However, their dust-like seeds, which lack endosperm, exhibit heightened sensitivity to environmental stress (Arditti & Ghani, 2000; Liu et al., 2019), thereby complicating the process of storage. It is therefore important to understand seed physiology if viability is to be maintained in the context of long-term conservation efforts (Seaton et al., 2004; Merritt et al., 2014).

In the domain of seed conservation science, seeds are categorized into three distinct classifications: orthodox, recalcitrant, and intermediate, based on their responses to desiccation and freezing (Roberts, 1973; Walters, 2015). Orthodox seeds have been observed to demonstrate a notable degree of tolerance to desiccation and low temperatures, frequently exhibiting extended lifespans when stored under dry and cold conditions. However, recalcitrant seeds are unable to survive drying or freezing, thus necessitating the development of alternative cryopreservation strategies (Obón, 2025; Diantina et al., 2020). Intermediate seeds occupy a physiological continuum: they may tolerate some desiccation but often lose viability faster than orthodox seeds and may be more vulnerable to freezing damage (SavePlants, 2024). Orchid seeds frequently demonstrate intermediate behavior; many studies suggest they behave largely as orthodox under optimal conditions, while some others show accelerated viability decline under typical seed-bank regimes (Pritchard, 1993; Magrini et al., 2019; Franceschi et al., 2019).

Empirical studies illustrate a wide range of responses to storage temperature and humidity. For instance, a review found that seeds of certain orchid species retained their ability to germinate even after more than a decade of storage at −18 °C with low moisture content (Francisqueti et al., 2024; Hay et al., 2010; Mweetwa et al., 2006). This finding challenges the longstanding assumption that orchid seeds are inherently short-lived (Popova et al., 2016; Francisqueti et al., 2024). However, other studies have reported rapid declines in viability under less ideal conditions. For instance, Puspitaningtyas & Handini (2021) demonstrated that seeds of *Phalaenopsis amabilis* stored at −20 °C suffered substantial viability loss after six years, predicting an approximate lifespan of nine years under their experimental regime. A thorough review of the subject of orchid seed storage has also been undertaken, which has indicated that whilst the storage of seeds at low temperatures and in dry conditions can be effective, the results of such storage are species-specific and can vary considerably (Christenhusz et al., 2010; Seaton et al., 2010).

Furthermore, the interaction between moisture content at storage, initial seed quality, age at harvest and seed packaging with temperature is a crucial factor in determining longevity (Schwallier et al., 2011). In summary, the extensive literature suggests that while seed banks frequently employ −20 °C storage for wild seeds, orchid seeds could necessitate refined, species-specific protocols, grounded in their distinctive physiology.

Notwithstanding the expanding corpus of extant literature, several significant gaps remain. First, data concerning the long-term storage (i.e. >10 years) of orchid seeds is comparatively scarce, particularly regarding the comparison of rare and common species. Most research in this field concentrates on brief to medium durations or on a single species, thereby restricting the potential for generalization across different *taxa* (Hay & Probert, 2013; Whigham et al., 2006). Furthermore, the relationship between storage temperature regimes, seed age (time since storage) and viability/germinability decline remains unclear for many orchid lineages.
Finally, although herbaria collections are increasingly recognized also as potential *ex-situ* resources, few studies have systematically evaluated how seeds, found in plants *in herbaria* specimens, perform in viability/germination. Herbaria are usually maintained at room temperature or in controlled environment, for example low relative humidity (50% RH) and 20 °C (Bromberg, 2020).This deficiency has a detrimental effect on the integration of herbarium-based material into conservation pipelines. Addressing these knowledge gaps is crucial for designing evidence-based storage regimes for orchids.

3.2. Objectives and Hypotheses

In view of the present studies, it is conceived with the objective of evaluating the efficacy of different storage methodologies in preserving the germinability and viability of both rare and common Orchidaceae species. These evaluations were conducted considering three distinct storage conditions to which terrestrial orchid seed samples had been subjected:

1. Long-term cold storage at 5 °C, comparing seed samples maintained over a period of more than ten years.
2. Frozen storage at −20 °C, comparing seed samples maintained for 1, 2 and 10+ years at this temperature condition.
3. Room temperature storage, valuing dried specimens of both terrestrial and epiphytic orchid plants with fruits and seeds maintained in the Herbarium of the University of Michigan (UMICH - USA); they were stored for up to ten years.

The hypothesis is that seeds stored at lower temperatures (*i.e.* −20 °C) and for shorter periods could exhibit higher viability and germinability compared to those stored at 5 °C or in herbarium conditions. Furthermore, it is anticipated that the negative effect of seed age could be more pronounced under higher storage temperatures than under freezing conditions, and that herbarium-stored seeds could demonstrate reduced viability when compared with that showed by seed stored at controlled low temperature. The objective of this study is to inform species-aware, temperature- and age-sensitive protocols for orchid seed banking and to explore the potential of herbarium material as a complementary *ex-situ* resource.

3.3 Materials and Methods

3.3.1 Experimental Design

Three experiments were conducted between 2023 and 2025, each representing a distinct conservation setting: refrigerated storage at 5 °C, frozen storage at –20 °C and room temperature (herbarium storage conditions). Collectively, these conditions mirror the *ex-situ* preservation methodologies commonly employed in a seed bank as active collection, long-term collection and museal collection, respectively.

Table 3.1 shows the experimental design of this study: samples refrigerated at 5 °C (79 samples of terrestrial orchid species) were evaluated following a minimum of 10 years of storage, and viability measured in both 2023 and 2025; frozen samples (103 samples belonging to 50 different *taxa*) including seeds aged 1, 2, and over 10 years; seeds from exsiccated plants in herbarium - 11 specimens with extractable seeds. As each seed lot constituted a unique historical collection, replication was not a possibility.

Table 3. 1: Experimental design for this study

Experiment	Storage Condition	Storage T	Sample Size	Seed Age	Assessment Performed
Refrigerated Storage	Cold storage	5 °C	79 orchid samples	≥10 years	Viability and germinability tests
Frozen Storage	Deep-freeze conservation	–20 °C	103 orchid samples	1, 2, 7, 8, 11, 12 years	Viability and germinability tests
Herbarium Storage	Passive, ambient herbarium storage	20 °C, 37% RH	10 samples from herbarium sheets	Collections within last decade	Viability only

3.3.2 Plant Material

A representative list of samples included across the three experiments is provided in Table 3.2. The table presents the scientific name, collection locality, collection year, storage temperature and seed age.

Table 3. 2:Orchid species examined across the three experiments

Species	Origin / Collection Locality	Collection Year	Storage Regime (seed age)
Anacamptis fragrans	Monteverdi (PI)	6/11/2005	5 °C (20 years)
Anacamptis laxiflora	N/A	1/5/2008	5 °C (17 years)
Anacamptis papilionacea	strada per Gainazzo (MO)	Jun-14	5 °C (11 years)

Anacamptis papilionacea	Camogli (GE)	5/9/1999	5 °C (26 years)
Anacamptis papilionacea	Monteverdi (PI)	13/06/1999	5 °C (26 years)
Anacamptis papilionacea	Campiglia (LI)	May-02	5 °C (23 years)
Anacamptis papilionacea	Monteverdi (PI)	11/6/2005	5 °C (20 years)
Anacamptis papilionacea	Monteverdi (PI)	11/6/2005	5 °C (20 years)
Anacamptis papilionacea	Campiglia (LI)	May-02	5 °C (23 years)
Anacamptis pyramidalis	Monteverdi (PI)	13/06/1999	5 °C (26 years)
Anacamptis pyramidalis	Monteverdi (PI)	Jun-13	5 °C (12 years)
Dactylorhiza fuchsii	Castagneto (LI)	1998	5°C (27 years)
Dactylorhiza fuchsii	San Vitale (RE)	Jul-14	5 °C (11 years)
Dactylorhiza fuschii	Sassetta (LI)	Jun-13	5 °C (12 years)
Dactylorhiza sambucina	N/A	Jul-14	5 °C (11 years)
Epidendrum prismatocarpum	N/A	2001	5 °C (24 years)
Epipactis helleborine	Campiglia Marittima (LI)	May-02	5 °C (23 years)
Epipactis helleborine	Febbio (RE)	8/1/2014	5 °C (11 years)
Epipactis helleborine	Monteverdi (PI)	1/7/2002	5 °C (23 years)
Epipactis helleborine	Campiglia Marittima (LI)	6/6/2005	5 °C (20 years)
Goodyera repens	Monte Orsaro (RE)	9/1/2014	5 °C (11 years)
Gymnadenia conopsea	Gainazzo (MO)	Jul-14	5 °C (11 years)
Gymnadenia conopsea	N/A	8/6/2018	5 °C (7 years)
Himantoglossum adriaticum	Barberino val D'Elsa	9/7/2004	5 °C (21 years)
Himantoglossum adriaticum	Varana (MO)	2014	5 °C (11 years)
Himantoglossum adriaticum	Scandiano (RE)	Jul-18	5 °C (7 years)
Himantoglossum robertianum	Castagneto (LI)	1998	5 °C (27 years)
Himantoglossum robertianum	Donoratico (LI)	25/05/2000	5 °C (25 years)
Himantoglossum robertianum	Donoratico (LI)	28/05/2001	5 °C (24 years)
Himantoglossum robertianum	Donoratico (LI)	May-02	5 °C (23 years)
Himantoglossum robertianum	Donoratico (LI)	May-02	5 °C (23 years)
Himantoglossum robertianum	Donoratico (LI)	May-02	5 °C (23 years)
Himantoglossum robertianum	Gainazzo (MO)	30/05/2013	5 °C (12 years)
Himantoglossum sp.	N/A	1997	5 °C (28 years)
Limodorum abortivum	N/A	1996	5 °C (29 years)
Limodorum abortivum	Monterotondo (GR)	Jul-02	5 °C (23 years)
Limodorum abortivum	Monterotondo (GR)	1/7/2002	5 °C (23 years)
Limodorum abortivum	N/A	2003	5 °C (22 years)
Limodorum abortivum	Cecina Mare (LI)	12/6/2003	5 °C (22 years)
Limodorum abortivum	N/A	2005	5 °C (20 years)

Limodorum abortivum	N/A	2005	5 °C (20 years)
Limodorum abortivum	Donoratico (LI)	May-05	5 °C (20 years)
Limodorum abortivum	Polinago (MO)	Jun-08	5 °C (17 years)
Neottia sp.	Monte Santa Giulia (MO)	2002	5 °C (23 years)
Ophrys apifera	Venturina (LI)	Jul-13	5 °C (12 years)
Ophrys apifera	Friuli Venezia Giulia	Jun-14	5 °C (11 years)
Ophrys bertolonii	Monteverdi (PI)	13/06/1999	5 °C (26 years)
Ophrys bertolonii	Monteverdi (PI)	24/05/1999	5 °C (26 years)
Ophrys fuscae	Monteverdi (PI)	Jun-13	5 °C (12 years)
Ophrys fuscae	Monte Gerfalco (800m) (GR)	2003	5 °C (22 years)
Ophrys garganica	Montecatini (PI)	Jul-13	5 °C (12 years)
Ophrys montis-leonis	Donoratico (LI)	2005	5 °C (20 years)
Ophrys sp.	Monte Sole (BO)	6/7/2012	5 °C (13 years)
Ophrys sp.	Isola D'Elba	3/5/2004	5 °C (21 years)
Ophrys sphegodes	Rosignano (LI)	5/2/2001	5 °C (24 years)
Ophrys sphegodes	Donoratico (LI)	May-02	5 °C (23 years)
Ophrys sphegodes	Monteverdi (PI)	May-02	5 °C (23 years)
Ophrys sphegodes	Donoratico (LI)	May-02	5 °C (23 years)
Ophrys sphegodes	Montecatini Val de Cecina (PI)	2005	5 °C (20 years)
Ophrys sphegodes	Monteverdi (PI)	2005	5 °C (20 years)
Ophrys sphegodes	Monteverdi (PI)	2005	5 °C (20 years)
Ophrys sphegodes	Monteverdi (PI)	May-05	5 °C (20 years)
Ophrys sphegodes	Monteverdi (PI)	Jun-13	5 °C (12 years)
Ophrys sphegodes	Donoratico (LI)	Jun-13	5 °C (12 years)
Ophrys sphegodes	Tarquinia Monteverdi (PI)	Jul-14	5 °C (11 years)
Orchis anthropophora	Gainazzo (MO)	Jun-14	5 °C (11 years)
Orchis Italica	Casale Marittimo (PI)	Jun-04	5 °C (21 years)
Orchis morio	Monterverdi (PI)	13/06/1999	5 °C (26 years)
Orchis morio	Donoratico (LI)	May-01	5 °C (24 years)
Orchis morio	Monteverdi (PI)	Sep-02	5 °C (23 years)
Orchis morio	Monteverdi (PI)	20/05/2007	5 °C (18 years)
Orchis morio	N/A	20/05/2007	5 °C (18 years)
Orchis morio	Castelluccio Raccolti	31/5/2008	5 °C (17 years)
Orchis morio	Castelluccio Raccolti	31/5/2008	5 °C (17 years)
Orchis morio	Monte Cetore (SI)	15/06/2008	5 °C (17 years)
Orchis pauciflora	Cornate di Gerfalco	23/05/2007	5 °C (18 years)
Orchis purpurea	Monteverdi (PI)	11/6/2005	5 °C (20 years)
Orchis purpurea	Gainazzo (MO)	May-14	5 °C (11 years)
Orchis sp.	N/A	2002	5 °C (23 years)
Platanthera bifolia	RE	Jun-14	5 °C (11 years)
Platanthera sp.	N/A	2002	5 °C (23 years)
Serapias lingua	Monteverdi (PI)	24/05/1999	5 °C (26 years)
Serapias neglecta	Monteverdi (PI)	24/05/2001	5 °C (24 years)

Serapias vomeracea	N/A	Jul-13	5 °C (12 years)
Serapias vomeracea	Viano (RE)	Jun-14	5 °C (11 years)
Serapias vomeracea	Gainazzo (MO)	Jul-14	5 °C (11 years)
Spriranthes spiralis	N/A	25/10/2001	5 °C (24 years)
Anacamptis berica	Via Ca' Corghi Scandiano, RE	23/8/2023	-20 °C (2 years)
Anacamptis coriophora	Montecatini Val di Cecina (PI)	00/06/2013	-20 °C (12 years)
Anacamptis coriophora	Gainazzo (MO)	00/07/14	-20 °C (11 years)
Anacamptis coriophora	strada Rio Benedello	23/8/2023	-20 °C (2 years)
Anacamptis coriophora	Via Ca' Corghi Scandiano, RE	23/8/2023	-20 °C (2 years)
Anacamptis morio	Donoratico (LI)	00/06/2013	-20 °C (12 years)
Anacamptis morio	Gainazzo (MO)	00/05/14	-20 °C (11 years)
Anacamptis papilionacea	Castellina Marittima (LI)	00/06/2013	-20 °C (12 years)
Anacamptis pyramidalis	Monteverdi (PI)	00/06/13	-20 °C (12 years)
Anacamptis pyramidalis	Via Ca' Corghi Scandiano, RE	23/8/2023	-20 °C (2 years)
Anacamptis pyramidalis	Via Ca' Corghi Scandiano, RE	23/8/2023	-20 °C (2 years)
Anacamptis pyramidalis	Marano sul Panaro, località Casona	23/8/2023	-20 °C (2 years)
Anacamptis pyramidalis	Monte Ravaglia, Serramazzoni (MO)	23/8/2023	-20 °C (2 years)
Anacamptis pyramidalis	Salse di Nirano	25/7/2025	-20 °C
Anacamptis pyramidalis	Trieste	25/7/2025	-20 °C
Anacamptis pyramidalis	Private Garden in Nonantola	25/7/2025	-20 °C
Barlia robertiana	Gainazzo (MO)	19/8/2024	-20 °C (1 year)
Cephalathera longifolia	Mantova	23/8/2023	-20 °C (2 years)
Corallorhiza trifida	Abetina Reale (RE)	00/07/17	-20 °C (8 years)
Cypripedium calceolus	Forni di Sopra (UD)	13/08/18	-20 °C (7 years)
Cystorchis gracilis	Febbio (RE)	00/08/14	-20 °C (11 years)
Dactylorhiza fuchsii	S. Vitale (RE)	00/07/14	-20 °C (11 years)
Dactylorhiza fuchsii	Febbio (RE)	00/07/14	-20 °C (11 years)
Dactylorhiza fuchsii	Cento Croci	25/7/2025	-20 °C
Dactylorhiza incarnata	Febbio (RE)	00/07/14	-20 °C (11 years)
Dactylorhiza maculata	Cento Croci	25/7/2025	-20 °C
Dactylorhiza sambucina	Monte Orsaro (RE)	00/07/14	-20 °C (11 years)
Epipactis atrorubens	Monte Orsaro (RE)	00/10/18	-20 °C (7 years)
Epipactis atrorubens	Monte Orsaro (RE)	00/10/18	-20 °C (7 years)
Epipactis helleborine	Febbio (RE)	00/08/14	-20 °C (11 years)
Epipactis helleborine	Monte Ravaglia, Serramazzoni (MO)	23/8/2023	-20 °C (2 years)
Epipactis microphylla	Febbio (RE)	00/08/14	-20 °C (11 years)
Epipactis microphylla	Monte Orsaro (RE)	00/10/18	-20 °C (7 years)
Epipactis palustris	Febbio (RE)	00/08/14	-20 °C (11 years)
Epipactis palustris	Monte Orsaro (RE)	00/10/18	-20 °C (7 years)
Epipactis palustris	Monte Orsaro (RE)	00/10/18	-20 °C (7 years)

Epipactis palustris	Monte Orsaro (RE)	00/10/18	-20 °C (7 years)
Goodyera repens	Monte Orsaro (RE)	00/09/14	-20 °C (11 years)
Gymnadenia conopsea	Gainazzo (MO)	00/07/14	-20 °C (11 years)
Gymnadenia conopsea	Via Ca' Corghi Scandiano, RE	23/8/2023	-20 °C (2 years)
Gymnadenia conopsea	strada Rio Benedello	23/8/2023	-20 °C (2 years)
Gymnadenia conopsea	strada Rio Benedello	23/8/2023	-20 °C (2 years)
Gymnadenia odoratissima	Cento Croci	25/7/2025	-20 °C
Himantoglossum adriaticum	Fondovalle Tresinaro (RE)	00/07/14	-20 °C (11 years)
Himantoglossum adriaticum	Varana (MO)	00/07/14	-20 °C (11 years)
Himantoglossum adriaticum	Castellarano (RE) Via Corghi	16/07/18	-20 °C (7 years)
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	23/8/2023	-20 °C (2 years)
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	23/8/2023	-20 °C (2 years)
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	23/8/2023	-20 °C (2 years)
Himantoglossum adriaticum	Via Ca' Corghi Scandiano, RE	23/8/2023	-20 °C (2 years)
Himantoglossum adriaticum	NONANTOLA	23/8/2023	-20 °C (2 years)
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	19/8/2024	-20 °C (1 year)
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	19/8/2024	-20 °C (1 year)
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	19/8/2024	-20 °C (1 year)
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	25/7/2025	-20 °C
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	25/7/2025	-20 °C
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	25/7/2025	-20 °C
Himantoglossum adriaticum	Reggio Emilia Parco San Lazzaro	25/7/2025	-20 °C
Himantoglossum hircinum	Via Sparavalle	23/8/2023	-20 °C (2 years)
Himantoglossum robertianum	Cecina (LI)	00/06/2013	-20 °C (12 years)
Himantoglossum robertianum	Donoratico (LI)	00/06/2013	-20 °C (12 years)
Himantoglossum robertianum	Gainazzo (MO)	00/06/14	-20 °C (11 years)
Himantoglossum robertianum	Viano (RE)	00/05/14	-20 °C (11 years)
Limodorum abortivum	Monte Ravaglia, Serramazzoni (MO)	23/8/2023	-20 °C (2 years)
Malaxis monophyllos	Forni di Sopra (UD)	13/08/18	-20 °C (7 years)
Neottia ovata	Cento Croci	25/7/2025	-20 °C
Neottia ovata	Monte Orsaro (RE)	00/09/14	-20 °C (11 years)
Ophrys apifera	Montecatini Val di Cecina (PI)	00/06/2013	-20 °C (12 years)

Ophrys apifera	Gainazzo (MO)	00/07/14	-20 °C (11 years)
Ophrys apifera	Fondovalle Tresinaro (RE)	00/07/14	-20 °C (11 years)
Ophrys apifera	strada Rio Benedello	23/8/2023	-20 °C (2 years)
Ophrys apifera	Parco Resistenza (MO)	23/8/2023	-20 °C (2 years)
Ophrys apifera	Reggio Emilia Parco San Lazzaro	19/8/2024	-20 °C (1 year)
Ophrys apifera	Parco Resistenza (MO)	19/8/2024	-20 °C (1 year)
Ophrys apifera	Salse di Nirano	25/7/2025	-20 °C
Ophrys bertolonii	Castellina Marittima (LI)	00/06/2013	-20 °C (12 years)
Ophrys bertolonii	Loc. Malandrone (MO)	23/8/2023	-20 °C (2 years)
Ophrys bertolonii	Gainazzo (MO)	19/8/2024	-20 °C (1 year)
Ophrys crabronifera	Monteverdi Marittimo (LI)	00/06/2013	-20 °C (12 years)
Ophrys crabronifera	Case Stantini (RE)	00/07/14	-20 °C (11 years)
Ophrys sphegodes	Tirrenia (GR)	00/06/2013	-20 °C (12 years)
Ophrys sphegodes	Val di Sterza (PI)	00/06/2013	-20 °C (12 years)
Ophrys sphegodes	Gainazzo (MO)	00/05/14	-20 °C (11 years)
Ophrys sphegodes	Gainazzo (MO)	19/8/2024	-20 °C (1 year)
Orchis morio	Monte Ravaglia, Serramazzoni (MO)	23/8/2023	-20 °C (2 years)
Orchis morio	Monte Ravaglia, Serramazzoni (MO)	23/8/2023	-20 °C (2 years)
Orchis morio	Monte Ravaglia, Serramazzoni (MO)	23/8/2023	-20 °C (2 years)
Orchis morio	Gainazzo (MO)	19/8/2024	-20 °C (1 year)
Orchis papilionacea	Monte Ravaglia, Serramazzoni (MO)	23/8/2023	-20 °C (2 years)
Orchis provincialis	Castellina Marittima (LI)	00/06/2013	-20 °C (12 years)
Orchis provincialis	Serramazzoni (MO) calanchi sulla discesa del Malandrone	00/07/17	-20 °C (8 years)
Orchis purpurea	Montecatini Val di Cecina (PI)	00/06/2013	-20 °C (12 years)
Orchis purpurea	Gainazzo (MO)	00/06/14	-20 °C (11 years)
Orchis purpurea	Reggio Emilia Parco San Lazzaro	19/8/2024	-20 °C (1 year)
Orchis purpurea	Reggio Emilia Parco San Lazzaro	19/8/2024	-20 °C (1 year)
Orchis purpurea	Reggio Emilia Parco San Lazzaro	25/7/2025	-20 °C
Orchis simia	Gainazzo (MO)	00/07/14	-20 °C (11 years)
Orchis simia	Reggio Emilia Parco San Lazzaro	19/8/2024	-20 °C (1 year)
Serapias parviflora	Cecina (LI)	00/06/2013	-20 °C (12 years)
Serapias vomeracea	Cecina (LI)	00/06/2013	-20 °C (12 years)
Serapias vomeracea	strada Rio Benedello (MO)	23/8/2023	-20 °C (2 years)
Serapias vomeracea	strada Rio Benedello (MO)	23/8/2023	-20 °C (2 years)
Spiranthes spiralis	Baiso (RE)	00/10/14	-20 °C (11 years)

Corallorhiza odontorhiza	Allegan/MI/USA	15/09/2017	UMICH 2024 (20 °C)
Corallorhiza odontorhiza	Kalamazoo/MI/USA	29/09/2022	UMICH 2024 (20 °C)
Epipactis helleborine	Mackinac/MI/USA	22/08/1995	UMICH 2024 (20 °C)
Epipactis helleborine	Chippewa/MI/USA	19/08/2010	UMICH 2024 (20 °C)
Epipactis helleborine	Interlake/MI/USA	5/9/2012	UMICH 2024 (20 °C)
Epipactis helleborine	Lake Horon/MI/USA	3/9/2016	UMICH 2024 (20 °C)
Liparis loeselii	Mason/MI/USA	18/08/2013	UMICH 2024 (20 °C)
Spiranthes casei	Gratiot Lake/MI/USA	15/09/2015	UMICH 2024 (20 °C)
Spiranthes casei	Douglas Lake/MI/USA	21/08/2017	UMICH 2024 (20 °C)
Spiranthes ochroleuca	Allegan/MI/USA	14/09/2018	UMICH 2024 (20 °C)

3.3.3 Seed-bank collections: active collection (5 °C) and basic collection –20 °C)

Seeds maintained at 5 °C storage and used for this experiment originated from terrestrial orchids collected in different localities in Italy between 1996 and 2018 and maintained in a laboratory refrigerator. These samples (n = 79) were stored inside glass vials with screw caps, which were then placed inside airtight glass jars containing silica gel and kept at a constant temperature of 5 °C until viability assessments performed in 2023 and 2025. Note that these samples were 130 in total but 51 samples were of very low weight so the experiment could not be performed.

Seeds maintained at –20 °C storage were 103 accessions and they were maintained frozen for different periods of time, namely for 1 year, 2 years, 7 years, 8 years, 11 years and 12 years (*i.e.* since 2013), depending on their original collection date. All these seeds were dried prior to storage, following standard seed-bank protocols used in MSB; they were stored inside glass vials with screw caps, then placed inside airtight glass jars containing silica gel and kept at a constant temperature until the assessment, carried out in 2025.

3.3.4 Herbarium material (UMICH 2024)

Some orchid seed samples were obtained from the University of Michigan Herbarium (UMICH) during a targeted examination of orchid specimens conducted in 2024. Herbarium sheets conserve exsiccated plants, collected, prepared and preserved for taxonomic and historical purpose; they are maintained at about 20 °C. Among several hundred sheets examined, only 11 orchid specimens contained intact, mature capsules suitable for seed extraction. Each capsule was carefully opened using sterile tools and seeds were removed and processed immediately for viability testing. Two sheets from UMICH are shown in figure 3.1.

Figure 3. 1: Exsiccated plant specimens from UMICH Herbarium showing orchid plants with intact capsules containing seeds.

3.3.5 Viability Testing

The assessment of seed viability was conducted through the utilization of a tetrazolium chloride (TTC) staining assay, a conventional method employed for the evaluation of metabolic activity in seeds (ISTA, 1976). The seeds were retrieved from storage in a sterile environment and 1 mg of each accession subsampled for further analysis (see Figure 3.2).

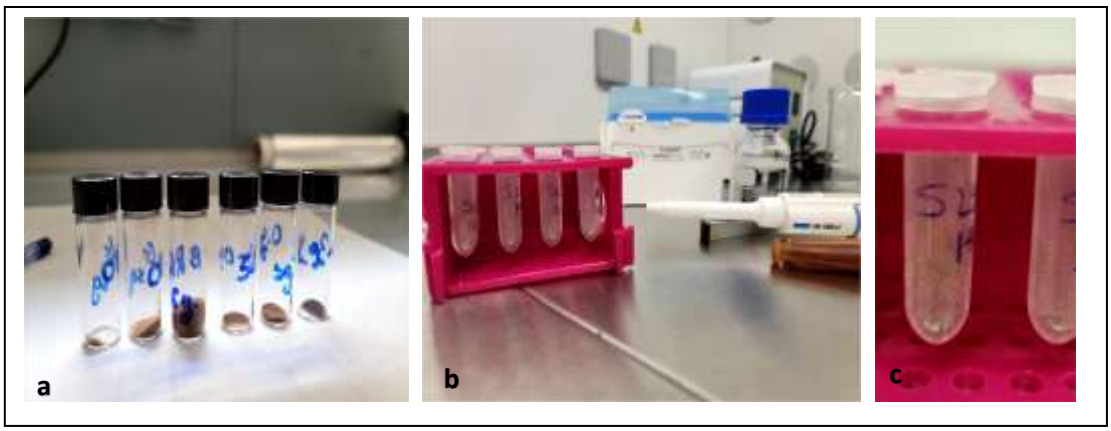


Figure 3. 2: Preparation of samples for seed scarification and then viability testing. (a) main sample in glass vial, (b) some of the samples prepared and ready for scarification with 5 % NaCl solution, (c) one sample of 1mg of seeds.

Following surface sterilization – scarification carried out with 5% sodium hypochlorite Solution samples were repeated rinsed with distilled water. Subsequently, seeds were placed in 1% TTC solution at 30 °C in darkness for approximately 48 hours. This protocol has been applied for all samples tested in 2023.
In 2025 a modification of TTC test has been introduced: after surface sterilization- scarification and rinsing with distilled water, seeds were pre-hydrated in a 10% sucrose water solution for 24 hours to enhance stain penetration. Then the applied protocol remained the same.
After incubation in TTC, seeds were rinsed and mounted for microscopy. Embryos exhibiting strong, uniform red/pink coloration were designated as viable, whereas those that were unstained or only faintly stained were categorized as non-viable. The viability of the samples was calculated as the percentage of stained embryos per accession. Each assessment was based on a minimum of 1mg of seeds (see Figure 3.3). The % of the empty seeds were not considered in the total number of seed Germinability Testing

Calculate the % of viable seeds by applying the formula:
% viable seeds (TTC test) = v/t x 100
Where:
v= seeds with pink-red embryo
t= total number of seeds tested depriving the empty seeds

Figure 3. 3: equation to calculate the viability %

Germinability assessments were conducted on all accessions stored at refrigerated (5 °C) and frozen (–20 °C) temperatures. It was decided that herbarium derived seeds should be excluded from the germination experiments. The primary reason for this decision was the time constraints that were in place, in addition to the limited quality and quantity of seeds available from historical specimens.

The germination protocol followed the asymbiotic *in vitro* method that are commonly used for terrestrial Orchidaceae, with BM-1 medium selected for its suitability for a wide range of orchid *taxa* (Van Waes and Debergh, 1984; Sgarbi et al., 2007; 2009).

Step 1: Seed surface sterilization and sowing

The first step in the process is the sterilization of seeds and subsequent plating. Prior to sowing, seeds were surface-sterilized (scarified) to prevent microbial contamination and improve the permeability of seed integuments.

The medium, designated as BM-1 (Basic Medium-1) - check table 3.3 for preparation protocol - was prepared in accordance with the manufacturer's specifications (product B138 BM-1 Terrestrial Orchid Medium, PhytoTech Labs – USA), solidified with agar and pH adjusted to 6.3 before autoclaving. The medium was transferred into sterile 3 cm Petri dishes and permitted to solidify under aseptic conditions.

Table 3.3: Protocol for preparing BM1 culture medium

General guidelines for preparing the BM1 culture medium:
• Measure a volume of double-distilled water equal to 80% of the final volume (approximately 800 ml for 1 l of solution).
• Weigh the powder (21.22 g to prepare 1 liter of medium) in the double-distilled water, add magnetic bar then place it on a magnetic stirrer until it is completely dissolved.
• Add 10% sucrose (100 g/L)
• Adjust the pH to 6.3
• Add double distilled water to volume to reach 1 liter.
• Transfer into a flask with double the capacity of the quantity of solution prepared.
• Add agar 20 g/L (Plant agar- Duchefa Biochemie B.V., The Netherland) and 1g/L charcoal (Charcoal activated neutralized, Duchefa).
• Set the temperature to 100 °C and allow the solution to heat until the agar has completely dissolved.
• Cap the flask and sterilize in an autoclave at 121 °C for 20 min at 1 atm.
• Under a laminar flow hood add to the prepared medium 1 ml/L of vitamin solution (Nitsch & Nitsch Vitamin Powder (1000x) PhytoTech Labs – USA), previously filter sterilized
• Put the medium into 3 cm petri dishes then sealed with parafilm.

Step 2: Incubation conditions

After the process of sowing, the plates were sealed with parafilm and placed into an incubator that maintained a controlled environment. The incubation conditions were standardized for all accessions:

- The temperature was maintained at 24 ± 1 °C.
- 24/24 h of darkness (petri dishes were placed in dark containers).

These procedures are usually applied in EAB Lab. and are in agreement with the Millennium Seedbank (MSB) protocols for terrestrial orchid germination. They were then optimized for early protocorm development.

Step 3: Germination scoring

The process of germination was evaluated at three-week intervals over a total period of 42 weeks. For the purpose of this study, germination was defined as the emergence of a swollen, chlorophyll-free protocorm stage, representing clear evidence of metabolic reactivation and embryonic growth, while the enlarged embryos without formation of this starshaped protocorm are not considered germinated.

The germination percentage was expressed per sample as follows:

Germination % = (Number of protocorms/Number of seeds plated) × 100

No subculturing was performed during the 42-week period, as the goal was to quantify initial germinability rather than subsequent seedling development.

To ensure the maintenance of stringent quality control standards, a blank control plate (BM-1 medium without seeds) was prepared. Plates showing fungal or bacterial growth were discarded, and the accession was re-sown when sufficient seed remained.

Figure 3.4 shows how to carry out the germinability test and to continue *in vitro* propagation.

Germinability test and *in vitro* propagation of Orchidaceae

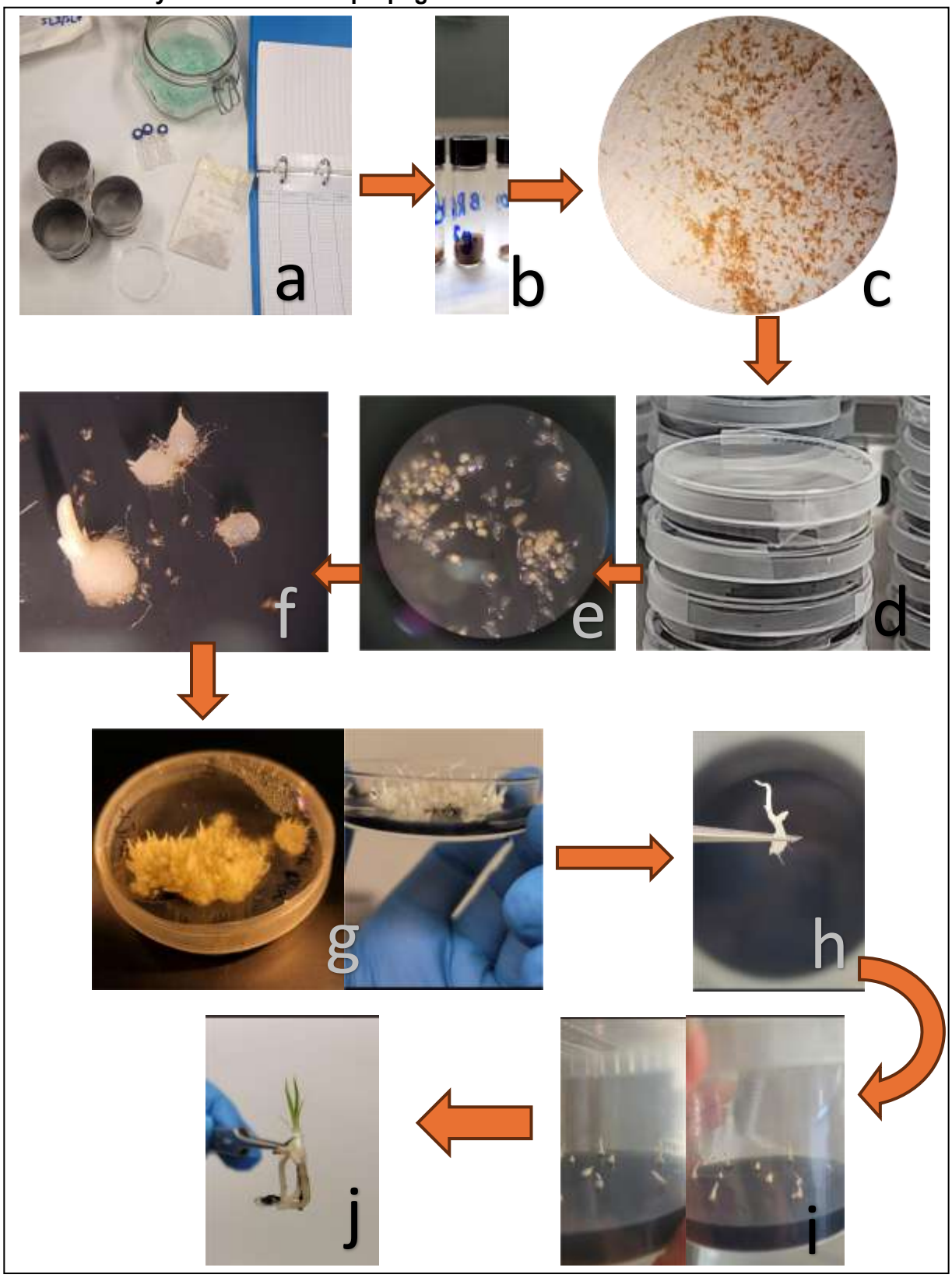


Figure 3. 4: Steps for in vitro germinability test and propagation: a) cleaning of seeds and record the data; b) seed sample ready to be stored; c) seed sample for germinability assesment observed under stereo-microscope to count the number of empty seeds, d) seeds after scarification, spread into petri dish; e) formation of protocorms; f) protocorms with leafllets ready to move into a bigger container; g) protocorm with leaflet ready to be transferred into Microbox; h) an orchid plantlet; i) small plantlets with greenish leaflets ; j) a small orchid plantlet.

3.4 Results and Discussion

3.4.1 Seed Viability and Germinability After Storage at 5 °C for a long period

This first study evaluates the effects of long-term refrigerated storage at 5 °C on 87 seed accessions representing 43 orchid species, with storage durations ranging from 1996 to 2018.
In the first germination assay conducted in 2023, only four accessions, belonging to *Anacamptis pyramidalis* (2 sample) stored since 2013 and *Gymnadenia conopsea* (2 samples) stored since 2018, exhibited detectable germination on BM-1 medium after 28 and 30 weeks of incubation, respectively. No germination was detected in any other accession across either assay year.
In more detail, in 2023, *Anacamptis pyramidalis* exhibited 48% germination (104/217 seeds as an average for both samples), while *Gymnadenia conopsea* showed 37% germination (75/198 seeds as an average for both samples). On the contrary, TTC staining revealed 0% embryo viability for each of 87 accessions, including the two species that than will be germinated.
A second set of assays was conducted in 2025 using the same accessions. The germination rates of *Anacamptis pyramidalis* and *Gymnadenia conopsea* decreased to reach 37% and 29%, respectively over the two-year period. The TTC assay revealed a complete absence of embryo viability in 86/87 samples, thereby confirming the earlier findings and indicating that refrigerated storage at 5 °C triggers a progressive and irreversible decline in the biochemical activity of seeds. However, the remaining sample of *Anacamptis pyramidalis* showed 8% viability, probably due to the change in the TTC protocol, which could have improved the hydration level or activated cell metabolism. These results are shown in Figure 3.5.

The absence of TTC staining in seed samples which would then be able to germinate shows that this biochemical test could not be always predictive and effective to indicate cell viability. Its effectiveness mainly depends on the capability of TTC solution to permeate seed integuments. Consequently, in seeds with thick testa or pigmented inner carapace the TTC test can fail, giving false negative responses. Scarification treatment with NaOCl, followed by accurate washing with water is useful and often improves the positive response, modifying cell walls permeability. However, such discrepancies between TTC viability and germination have been documented previously in the field of orchid research (Seaton et al., 2018; Van Waes and Debergh,1986). Specifically, biochemical reducibility can decline prior to the full cessation of developmental capacity (Merritt et al., 2014). This finding elucidates the reason why protocorm formation was still observed even in embryos that no longer exhibited staining.

It is noteworthy that germination persisted in the four accessions which were stored at 5 °C since 2013 and 2018, although with diminishing success rates. The reduction in germination between 2023 and 2025 suggests a time-dependent decline in functional competence, consistent with seed ageing theory and membrane degradation, processes described in orthodox and intermediate seeds (Walters, 2015).

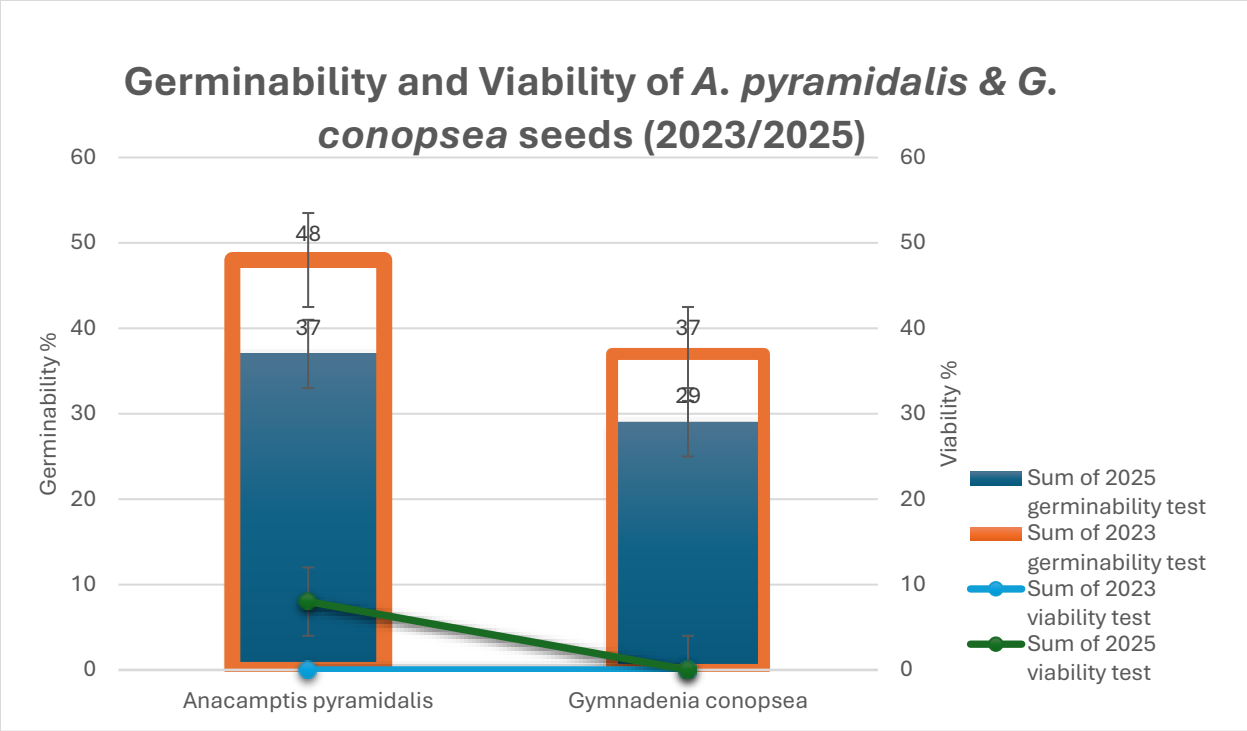


Figure 3. 5: Graph showing Viability and Germinability percentages of Anacamptis pyramidalis and Gymnadenia conopsea seed samples stored at 5°C since 2013 and 2018, respectively.

The finding that seeds cannot be kept viable for a limited period by refrigeration at 5 °C confirms that this is not an effective conservation method for long-term metabolic viability, despite the occasional retention of germinability in a limited number of accessions. In fact, it is possible that a small subset of seeds may be able to germinate after being stored in these conditions. These results are consistent with those of previous studies, which demonstrated that cool storage retards deterioration but does not arrest seed ageing in orchids (Seaton et al., 2014; Merritt et al., 2014). The limited number of germinating samples also reflects species-specific sensitivity to time dependent deterioration and storage temperature.

From a conservation perspective, the germination observed in seeds stored for a decade suggests that 5 °C storage can serve as a practical short-term system for maintaining active collections, which are periodically regenerated *in vitro*. The propagation of wild orchids in laboratory conditions is facilitated by such collections, with plants ultimately being transferred to botanical gardens and, following appropriate protocols, reintroduced into natural habitats. It is therefore evident that refrigerated seeds provide a valuable reservoir for ongoing propagation efforts and the sustained availability of fresh seed material over multi year periods.

Moreover, the observation that a significant proportion of seeds remain non germinating yet retain genetic material underscores a secondary conservation benefit: non-viable refrigerated seeds should not be discarded. Instead, accessions that have ceased to germinate nevertheless contain intact DNA, thus retaining value for molecular studies, barcoding, population genetics, and archiving of genetic diversity (Fay et al., 2005). They represent a significant resource for DNA

extraction, population genetic studies, and long-term genomic conservation, thereby supporting biodiversity research even in cases of physiological viability loss.

3.4.2 Seed Viability and Germinability After Long-Term Storage at –20 °C

An analysis was conducted on 103 seed accessions stored at –20 °C for periods ranging from one to ten years. The analysis revealed that the obtained values of germination and viability were consistently high across nearly all species (Figure 3.6). The germinability percentages (blue bars) approximately ranged between 50% and 85%, with several species exceeding 80%. Viability assessments, performed using TTC staining, showed comparable proportions of viable embryos, frequently exceeding germinability percentages but closely following the same overall pattern.
A positive trend was observed across storage years: more recent seed lots (2023–2025) typically exhibited the highest germination values (70–90%), whereas older lots (2013–2014) displayed slightly lower but still robust germination levels (50–65%). The linear regression included in the figure indicates a modest upward trajectory in germinability over time. The standard error bars were found to be narrow for most species, indicating low variability within accession and high reproducibility of germination performance.
Overall, seeds stored at a temperature of −20 °C were found to maintain both physiological viability and functional germination capacity at a level that is compatible with long-term seed-banking objectives.

The present study explores the efficacy of frozen storage as a strategy for the long-term conservation of orchid seeds. The findings indicate that seeds stored at a temperature of −20 °C exhibited consistently high percentages of both germination and viability, thereby substantiating the effectiveness of frozen storage as a method to enhance the longevity of orchid seeds. These outcomes closely mirror the findings of previous studies in which the viability of orchid seeds was found to be high after prolonged sub-zero storage, including multi-year preservation of *Cattleya*, *Cymbidium*, and *Dendrobium* species (Pritchard & Seaton, 1993). Similar long-term stability has also been reported for temperate terrestrial orchids, further supporting the hypothesis that freezing is suitable for species with minute, desiccation-tolerant seeds (Magrini et al.,2019; Pritchard & Seaton 1993).
The finding that seeds can maintain high viability for up to ten years when stored at –20 °C indicates that the storage protocol effectively reduced the major biochemical processes responsible for seed deterioration. These processes include membrane lipid peroxidation and uncontrolled oxidative reactions (Walters et al., 2010). Orchid seeds exhibit a high level of desiccation tolerance, which renders them well suited to frozen storage. In fact, ultralow moisture levels minimize solute mobility and prevent the formation of damaging intracellular ice (Pritchard, 2004).
The close agreement between TTC viability and germination results in this study reflects the stability of membrane integrity and enzyme activity after frozen storage.

The negligible yet persistent trend in which more recently collected seed specimens demonstrated higher percentages of successful germination can be attributed to potential enhancements in the methods employed during seed collection, desiccation, or pre-storage processing in recent years.

This phenomenon is not indicative of a decline in the quality of older seed lots. However, the observation that even the most ancient accessions exhibited >50% germination attests to the efficacy of frozen storage at −20 °C for the purpose of preserving seeds over the span of decades. This is markedly in contrast to the results obtained at 5 °C (previous section), where it was observed that viability declined to zero for all accessions over time. The divergent outcomes observed between storage temperatures of 5 °C and -20 °C serve to reinforce the principle that metabolic quiescence and molecular stability in orchid seeds may be optimally supported by the process of deep-freezing (Walters, 2015).

These results have significant implications for conservation programs aiming to preserve the genetic diversity of wild orchids. The long-term maintenance of high germinability is pivotal in enabling the routine propagation of threatened species for *ex-situ* cultivation, reintroduction programs and research purposes. Furthermore, the ability to retain viable embryos over a period of at least ten years ensures that seed banks can accumulate genetic variation across multiple seasons and populations, thereby strengthening the resilience of conservation collections (Seaton et al., 2010).

The high viability of the material lends itself to its use in genomic studies, a fact that is especially valuable for *taxa* with declining wild populations.

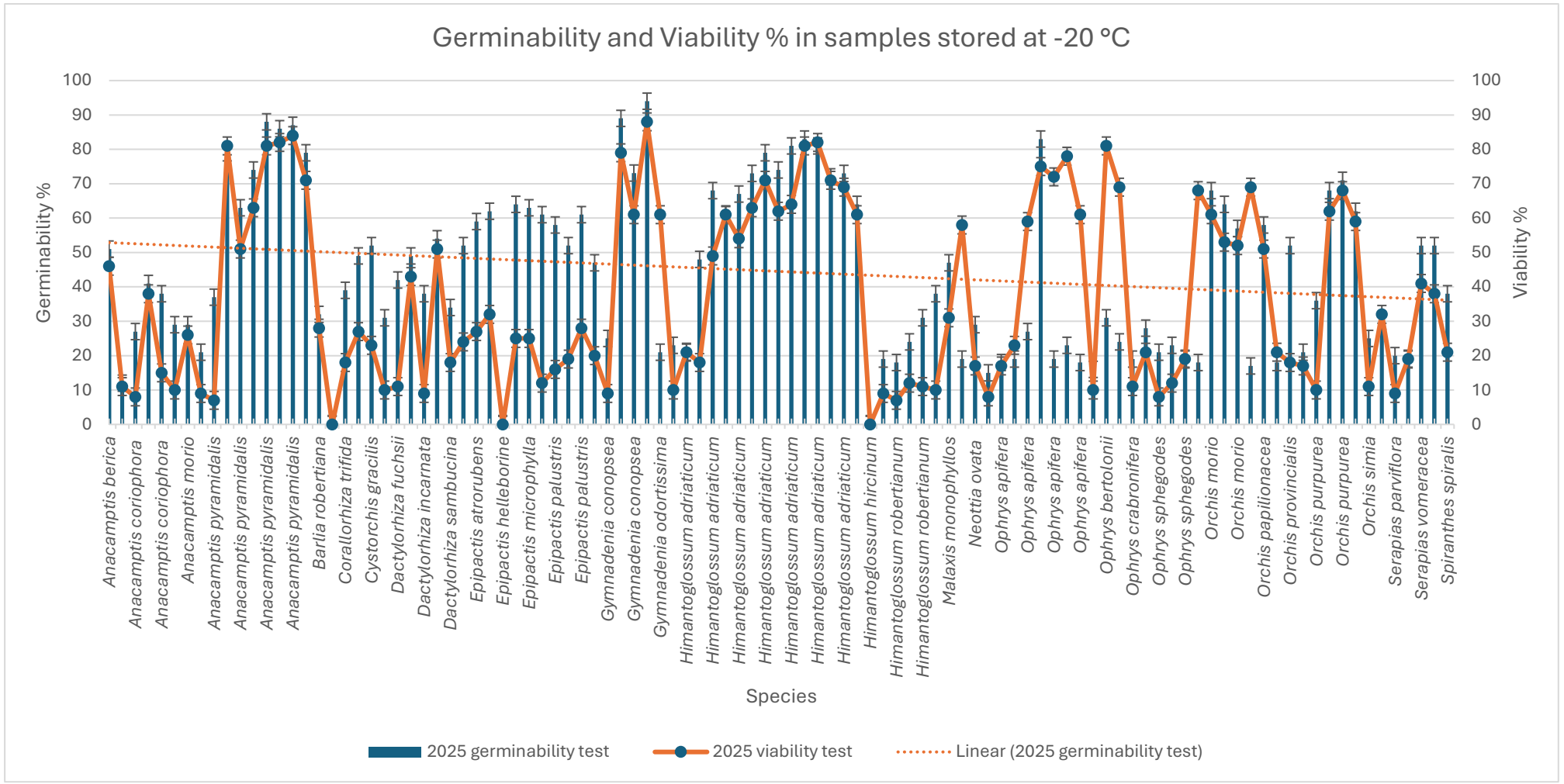


Figure 3. 6: Graph showing the Viability and the Germinability % of some samples stored at -20 °C

3.4.3 Seed Viability in Samples from Herbarium

Figure 3.7 shows the assessment of the viability of orchid seeds stored in herbarium collections (UMICH), revealing significant species-specific variations. During the present study, the viability of ten accessions was examined. The results demonstrated that only *Epipactis helleborine* seeds exhibited measurable viability following prolonged storage. Four *Epipactis* sp. accessions, aged 8, 10, 14, and 29 years, exhibited 11%, 14%, 10%, and 1% viability, respectively. A fitted trendline exhibited a marked age-related decline, although viability was still discernible in the oldest sample (29 year old).

Seed specimen of *Spiranthes ochroleuca* (6 year old) exhibited trace viability (0.5%) where it showed pale pink coloration by TTC test. In contrast, *Spiranthes casei, Corallorhiza odontorhiza,* and *Liparis loeselii* accessions demonstrated 0% viability. These patterns demonstrate that *Epipactis helleborine* possesses markedly greater longevity under herbarium conditions than the other *taxa* tested.

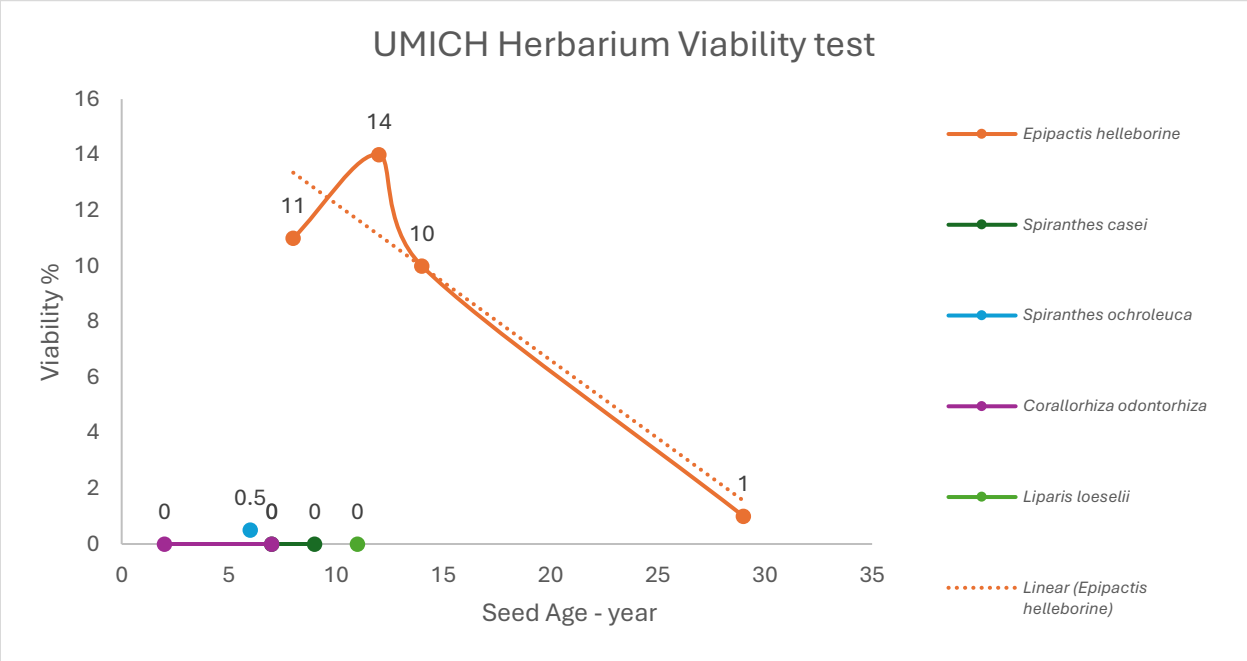


Figure 3. 7: Scatter Plot showing UMICH Herbarium specimen seed viability for five orchid species

Regarding species-specific seed longevity under 20 °C storage temperature (room temperature), the results of the present study showed that the seed longevity in orchids varies widely among species. On the other hand, almost all the specimen seeds lost viability even within the first decade years, while *Epipactis helleborine* retained measurable viability for longer periods. It ranged between 14% and 10% in sample 10–14-year-old but the value dropped to levels of 1% in older samples. This finding verifies the previous studies showing that orchid seed longevity is strongly species dependent and linked to stress-tolerance characteristics (Hay et al. 2010).

It is well established that ambient herbarium conditions (approximately 20 °C, and low relative humidity) are typically associated with rapid viability loss (Popova et al., 2016; Udomdee et al.,

2014). Indeed, it has been shown that ageing reactions, especially oxidative damage, progress quickly and that embryos accumulate structural and metabolic deterioration if seeds are conserved at high temperature (Diantina et al., 2022; Nadarajan et al., 2023; Seaton & Hailes 1989).

Consequently, the very low viability observed in samples of *Spiranthes ochroleuca,* and no viability recorded in *Corallorhiza odontorhiza* and *Liparis loeselii* was not unexpected. The persistence of viability in *E. helleborine*, however, is biologically meaningful and aligns with the species' molecular and ecological traits. *E. helleborine* is one of the most ecologically versatile orchids in temperate regions. Genetic studies reveal that populations exhibit high heterozygosity, weak population structure, and substantial gene flow (Squirrell et al., 2002; Tranchida-Lombardo et al., 2011). Such genetic robustness is often associated with stronger stress response pathways, enhanced embryo resilience and greater tolerance to environmental fluctuations. These properties likely contribute to the species' ability to maintain residual viability under long-term and uncontrolled storage conditions. The capacity of *E. helleborine* seeds to maintain a minimum viability for a period of 29 years signifies the exceptionally protracted duration over which ageing deleterious processes have progressed.

The comparison with other species showed the complete absence of viability in *S. ochroleuca, C. odontorhiza,* and *L. loeselii* accessions indicate a significantly higher sensitivity to ambient-temperature ageing. These genera include species that are known to possess small embryos with lower initial nutrient stores, a thinner testa and faster post-harvest ageing rates (Jolman et al., 2022). Consequently, the herbarium dataset aligns with the overarching biological pattern, which states that not all orchids possess the capacity for prolonged desiccation if maintained at room temperature.

3.4.4 Comparing the Viability and Germinability of Old Seed Samples Maintained under Different Storage Conditions

To understand more about the effect of storage temperature, old samples of three different orchid species, maintained both at 5 °C and at – 20 °C were compared, assessing viability and germinability.

Two storage regimes revealed a consistent effect of temperature on seed performance across all three orchid species: *Anacamptis pyramidalis, Gymnadenia conopsea,* and *Epipactis helleborine*. After 12 years of storage, seeds of *A. pyramidalis* stored at −20 °C showed markedly higher viability (38%) and germinability (51%) than those stored at 5 °C (8% and 37%, respectively). A similar pattern emerged for *G. conopsea*: refrigerated storage resulted in a total loss of viability (0%) and reduced germinability (29%), whereas sub-zero storage maintained high viability (58%) and increased germinability (48%) after 11 years of storage.

The most striking difference was observed in *E. helleborine*: seeds stored at 5 °C showed no viability or germination, whereas those stored at −20 °C retained moderate viability (32%) and high germinability (62%). Overall, subzero storage consistently preserved both seed viability and germination capacity more effectively than refrigerated conditions, even after over a decade of storage. This confirms the critical role of low-temperature regimes in the long-term conservation of orchid seeds. Results are shown in Table 3.4.

Table 3. 4: Effects of Refrigerated (5 °C) and Subzero (−20 °C) Storage Regimes on Viability and Germinability of Orchid Seeds After Long-Term Storage

Species	Storage Regime	Viability %	Germinability %	Number of Samples	Sample age
Anacamptis pyramidalis	5 °C	8	37	1	12 years
	-20 °C	38	51	1	12 years
Gymnadenia conopsea	5 °C	0	29	1	11 years
	-20 °C	58	48	1	11 years
Epipactis helleborine	5 °C	0	0	1	11 years
	-20 °C	32	62	1	11 years

Another test compared viability and germinability of orchid seeds in three different storage regimes. Across all three species, storage temperature seems to have a strong effect on both viability and germinability. In this study the age of seeds was not considered as a factor; the only factors were the species (same species has the same biological characteristics), and the specific storage regime. Table 3.5 shows the results of this study.

*Table 3. 5: Effects of Different Storage Regimes (*Refrigerated, Frozen, and Herbarium Storage*) on Seed Viability and Germinability of Three Orchid Species, with Mean ± SD and Statistical Significance*

Species	Storage regime	Viability (%)	Germinability (%)	Number of Samples
Anacamptis pyramidalis	−20 °C	**65.0 ± 23.4*****	**74.1 ± 15.6*****	2
	5 °C	4.0 ± 5.7	18.5 ± 26.2	10
Gymnadenia conopsea	−20 °C	**59.3 ± 34.3****	**70.8 ± 30.0***	2
	5 °C	0.0 ± 0.0	14.5 ± 20.5	4
Epipactis helleborine	−20 °C	16.0 ± 22.6	31.0 ± 43.8	7
	5 °C	0.0	0.0	2
	20 °C (UMICH 2024)	9.0 ± 5.7	N/A	4
Significance levels are indicated as follows: * p < 0.05, ** p < 0.01, *** p < 0.001. “NA” indicates data not available.				

In Table 3.5 the values are expressed as mean ± standard deviation (SD) based on individual seed lots tested in 2025. Statistical analyses were conducted separately for each species to account for interspecific biological differences. The effect of storage regime on seed viability and germinability was evaluated using one-way analysis of variance (ANOVA) when replication allowed.

For *A. pyramidalis*, seeds stored at −20 °C showed significantly higher viability and germinability compared with those stored at 5 °C ($p < 0.001$, ***). Similarly, *G. conopsea* exhibited significantly greater viability ($p < 0.01$, **) and germinability ($p < 0.05$, *) under frozen storage relative to refrigerated conditions. These results indicate a strong positive effect of long-term frozen storage on seed physiological performance for both species. For *E. helleborine*, statistical comparisons were not performed due to limited replication within storage categories and the absence of germinability data for seeds stored at 20 °C. Consequently, results for this species are presented descriptively.

For more elaboration regarding the values of SD, and according to the statistical studies (Zar 2010; Quinn & Keough 2002; Gotelli & Ellison 2004): in some storage regimes, standard deviation values exceeded the corresponding means, reflecting high variability among seed lots. This pattern is expected in bounded percentage data with small sample sizes and heterogeneous biological material and does not indicate a statistical anomaly.

Given the strong separation of group means and variance structure:

Anacamptis pyramidalis:

Viability: one-way ANOVA, $p < 0.001 \rightarrow$ ***

Germinability: one-way ANOVA, $p < 0.001 \rightarrow$ ***

Gymnadenia conopsea:

Viability: one-way ANOVA, $p < 0.01 \rightarrow$ **

Germinability: one-way ANOVA, $p < 0.05 \rightarrow$ *

Epipactis helleborine:

ANOVA did not apply due to insufficient replication per group

Figure 3.8 shows an illustration of the summary of this study. As a conclusion, the most effective regime for preserving orchid seed viability confirm to be long-term frozen storage at –20 °C. The findings of this study demonstrate that refrigerated storage (5 °C) displays limited but significant usefulness in maintaining medium-term germination potential in a subset of species. In contrast, herbarium storage at ambient temperature results in severe long-term deterioration, except for those *taxa* that exhibit exceptional resilience.

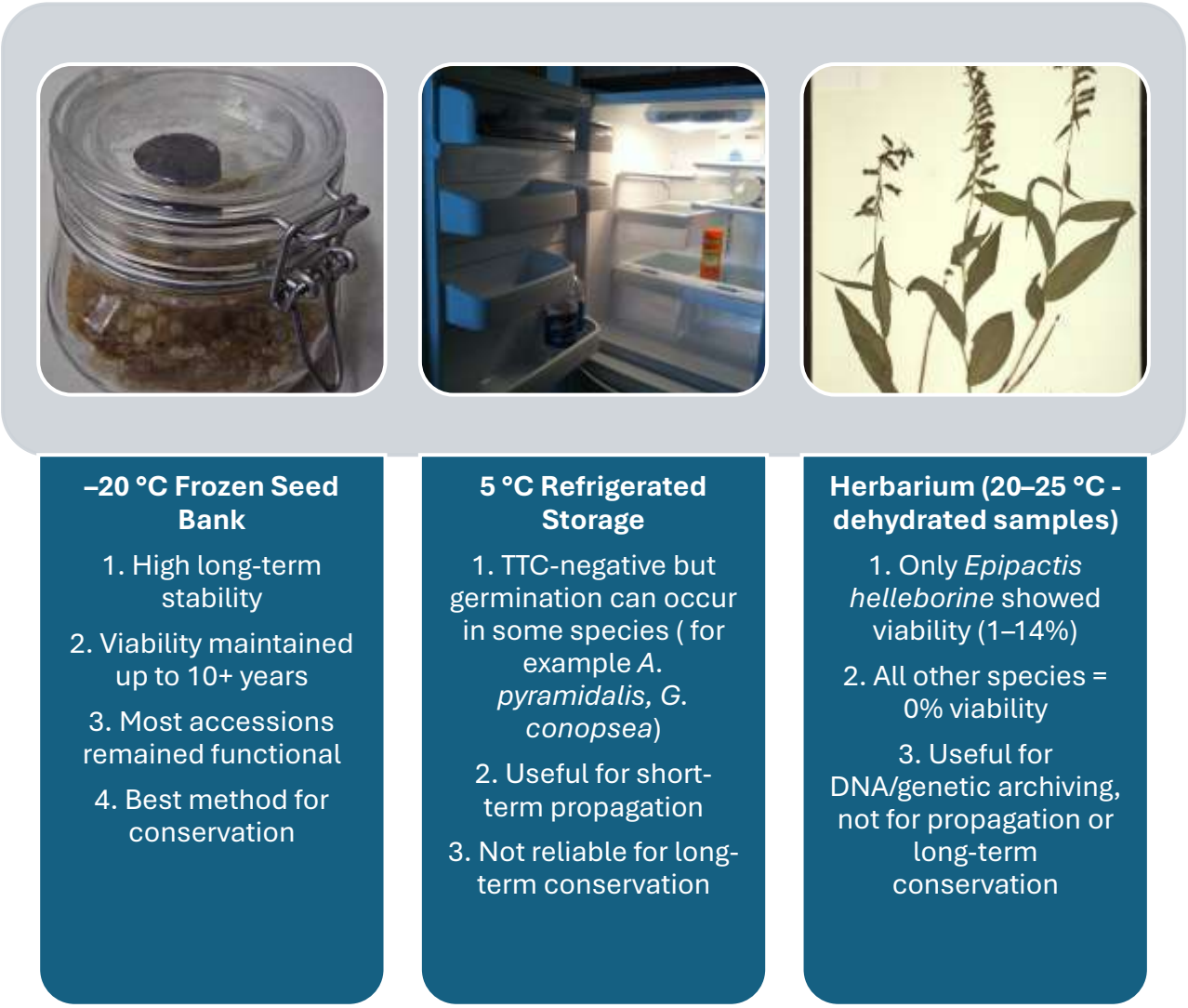


Figure 3. 8: Comparative Longevity of Orchid Seeds Across Three Storage Regimes–20 °C, 5 °C and 20 °C.

The complementary roles of these strategies indicate that a tiered conservation model, in addition to the cryopreservation of seeds for long-term preservation (Pirondini & Sgarbi, 2014), the use of refrigerated storage for the propagation of plants and the creation of herbarium specimens for genetic archiving, may provide the most effective strategy for the *ex-situ* conservation of temperate orchids.

3.5 Conclusion

This study presents the first integrated evaluation of orchid seed longevity across a range of storage conditions, including frozen (–20 °C), refrigerated (5 °C), and herbarium (20–25°C) storage, utilizing a multi-decade collection. It was determined through rigorous analysis and observation that temperature is the primary factor influencing the viability and germinability of embryos. Frozen storage (–20 °C) has been demonstrated to preserve viability in most accessions for up to 10 years, thus supporting current global seed-banking standards. The application of refrigeration (5 °C) resulted in a decline in the viability (assessed with TTC test), although limited germination was observed in certain species, suggesting partial embryonic functionality and a short-term propagation potential. The seeds stored in the herbarium exhibited severe deterioration, with only samples of *Epipactis helleborine* showing residual viability. This finding confirms that herbarium conditions are unsuitable for the long-term conservation of seeds. In essence, the viability of orchid seeds over an extended period necessitates frozen or cryogenic storage, while refrigerated storage is more appropriate for a shorter duration. The primary function of herbarium storage is to preserve the morphological and historical characteristics of the specimens, but it could represent a method to maintain genetic information. The findings emphasize the significance of species-specific, temperature-controlled protocols and a tiered conservation strategy that integrates *ex-situ* banking, propagation and genetic archiving. This work establishes a framework for future research on orchid seed physiology, longevity modelling, and molecular ageing indicators.

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Chapter 4:
Integrated Ecological Monitoring and Conservation Interventions of Urban Orchid Populations: A Three-Year Study: Spatial Mapping, Pollination Analysis and Fungal Associations

Abstract

Although urbanization is often associated with habitat degradation and biodiversity loss, urban green spaces can also serve as refuges for specialized and threatened plant species, including terrestrial orchids. This chapter presents the results of an integrated ecological study conducted over three years (2023–2025) in urban and semi-natural sites within the Modena–Reggio Emilia region of northern Italy. The study combined population monitoring, spatial mapping, conservation interventions, pollination ecology and mycorrhizal association analyses. Systematic field surveys documented the richness and abundance of orchid species across urban parks and protected reserves habitats. These surveys revealed that managed urban green spaces supported stable and, in some cases, expanding populations of *Himantoglossum adriaticum* and *Ophrys apifera*. Targeted in situ conservation measures, including mowing exclusion zones and physically protecting flowering individuals, were associated with a significant increase in the population size of *H. adriaticum* over time. This highlights the effectiveness of low-intensity management practices for conserving orchids in urban areas.

Investigations into pollination ecology conducted in 2025 demonstrated that *H. adriaticum* exhibits a functionally generalized pollination system dominated by bees (*Halictidae* and *Andrenidae*). Reproductive success was found to be strongly influenced by habitat openness and spatial structure rather than by the presence of pollinators alone. Fertilization rates were significantly higher in open urban park habitats than in structurally complex semi-natural sites, highlighting the importance of landscape configuration in mediating pollination efficiency.

Root-associated fungal analyses revealed diverse assemblages dominated by disturbance-tolerant ascomycetous endophytes (e.g*. Fusarium, Diaporthe/Phomopsis and Chaetomium*), reflecting the influence of urban soils on below-ground symbiotic communities. Although classical orchid mycorrhizal fungi were not detected through culture-based isolation, the persistence of orchids indicates their compatibility with urban-adapted fungal communities.

Overall, this study shows that ecologically managed urban parks can support viable orchid populations by maintaining suitable interactions above and below ground. By integrating demographic monitoring, pollination biology and fungal ecology, the study provides empirical evidence that urban ecosystems can contribute meaningfully to orchid conservation strategies in increasingly anthropogenic landscapes.

Key words: Urban biodiversity; Terrestrial orchids, *Himantoglossum adriaticum; Ophrys apifera;* Population monitoring; Pollination ecology; Food-deceptive orchids; Reproductive success; Orchid mycorrhizal fungi; Root-associated endophytes; Urban soils; Fungal–plant interactions.

4.1. Introduction

4.1.1. Urban ecosystems and orchid biodiversity

Urbanization is one of the most significant anthropogenic transformations of the last few centuries, having a profound impact on the landscape, which has been shaped into a mosaic of built environments, fragmented green patches, and remnant natural habitats, as reported in several scientific resources (Mckinney, 2002; Iwasaki, 2023). However, urban environments are increasingly recognized as biodiversity sinks and as potential pool for a variety of *taxa*, including vascular plants (Monge-Nájera & Pérez-Gómez, 2015). Indeed, the green spaces within cities are parks, simi-natural areas, old walls, etc... may hold variety of threatened species or the species that have lost their habitat and migrate (Rahmati, 2024). Consequently, urban ecosystems may have a significant role in the conservation of orchid biodiversity, particularly when managed with ecological awareness.

Orchidaceae are considered an informative group for biodiversity. This is due to their ecological requirements for pollinators and mycorrhizal symbionts, in addition to microhabitats that make the species of this family good bioindicators of habitat quality (Dressler, 1982; Brancaleoni et al., 2024).

4.1.2. Threats to terrestrial orchids in urban and peri-urban systems

Despite their potential, urban orchid populations are subject to a multitude of threats arising from anthropogenic pressures. Urban green spaces tend to offer stable, low-competition conditions that favor orchid persistence and local population growth (Breuste et al., 2023; Vogt-Schilb et al., 2016). The management of urban green areas, encompassing routine mowing, mechanical cutting, soil compaction, trampling and occasional mowing, has been demonstrated to have a detrimental effect on sensitive orchids, particularly during their critical phenological phases, such as flowering and seed set (Shefferson et al., 2020; Barman and Devadas, 2013; Wei et al., 2025). Other threats consist in change of the chemical environment, altered pH levels and change in nutrient loads, which can negatively affect the below-ground microhabitat of orchids, where mycorrhizal fungi grow (Izuddin et al., 2019; McCormick et al., 2018; Xing et al., 2020).

Furthermore, several studies illustrated and explained that urbanization is a contributing factor in the loss of orchid-bee diversity in urban areas (Baldock, 2020; Gonzalez et al.,2024; Liang et al., 2023; Fay et al, 2025). It is evident that such disruption has a detrimental effect on reproductive success and the long-term persistence of orchid populations. Therefore, the aboveground interactions include disturbance of habitats and pollination, while the below-ground interactions involve the disruption of soil and disturbance of fungal symbionts.

4.1.3. Orchids of Northern Italy and Their Mycorrhizal Associations

Italy is one of the diverse locations with orchids among the Mediterranean Basin, as mentioned in the “Legambiente Annual Report – Biodiversity at risk 2025” released on May, 21, 2025 (Legambiente Annual Report, 2025). According to this report, 240 species and subspecies of wild orchids have been documented in Italy.

A defining ecological trait of terrestrial orchids—shared with the broader Orchidaceae—is their obligate mycorrhizal dependence. The biology of orchid seeds, which are minute, dust-like, and lack nutrient reserves, is a major factor underlying their reliance on mycorrhizal fungi to initiate the life cycle, allowing germination and protocorm formation (Calevo & Duffy, 2023). This symbiosis frequently persists into adulthood, exerting a significant influence on nutrient uptake, stress tolerance, and survival in nutrient-poor soils that characterize many Italian and Mediterranean orchid habitats (Calevo & Duffy, 2023; De Rose et al., 2025). Some orchid species - such as *Himantoglossum adriaticum*, *Ophrys apifera* (CYBO Webinars, 2023), *Anacamptis morio* (Graziosi, Leonardi & Zambonelli, 2022), and *Neottia ovata* (De Rose et al., 2025) - are known to vary in the specificity of their mycorrhizal associations, ranging from broad interactions with widely distributed rhizoctonia-like fungi to narrower, species-specific partnerships. Such specificity can impose strong constraints on habitat suitability and spatial distribution.

In anthropogenic and urban environments, the prevalence of orchid mycorrhizal fungi (OMF) remains to be fully characterized in Italy (Li et al., 2021; Mennicken et al., 2023), despite the emergence of evidence from other regions indicating that compatible fungal partners may persist in altered or compacted soils and even colonize the roots of street trees and disturbed vegetation (Izuddin et al., 2019). These findings suggest that urban environments may still sustain functional orchid-fungus interactions under specific microenvironmental conditions.

The rich yet under-documented orchid flora of Northern Italy, in conjunction with the essential yet vulnerable mycorrhizal partnerships that sustain it, serves to emphasize the necessity of integrated ecological studies. Consequently, a comprehensive approach encompassing field surveys, spatial mapping, root-associated fungal isolation, and DNA-based fungal identification is imperative to elucidate patterns of orchid persistence in increasingly anthropogenic landscapes.

4.1.4. A highlight on Focal Species *Himantoglossum adriaticum*

Himantoglossum adriaticum H. Baumann (Adriatic lizard orchid) is a central- sub- Mediterranean species, from Italy to Slovakia and Hungary with undefined eastern limit. It inhabits dry meadows, open wood and bushes, in full light or partial shade, xerophilous and calcicolous species. According to Directive 92/43/EEC, Annex II, *H. adriaticum* is considered of priority interest due to its decline as a native orchid in Europe (Dostalova et al., 2013)

H. adriaticum produces underground tubers, basal leaves which occur in Autumn and wither before flowering; the inflorescence, with up to 40 flowers, forms in late spring to early summer (Calevo et al., 2025; Kovačević et al., 2024; Bódis et al., 2019). Individuals may live for many years, with an average lifespan of about eight years and some plants (10%) surviving over 15 years. Populations show variable flowering frequencies and dormancy periods (Bódis et al., 2019). Figure 4.1 shows some phases of life cycle of *Himantoglossum adriaticum.*

Members of the *Himantoglossum* genus (including *H. adriaticum*) are generally pollinated through food-deceit by insect visitors. They are predominantly considered to be allogamous (cross-pollinated) rather than self-fertilized. The lack of a reward for visiting their flowers encourages insects to visit multiple flowers on an inflorescence, facilitating pollinium transfer and cross-pollination. A broader study on food-deceptive orchids, including *H. adriaticum*, shows that, despite lacking nectar rewards, these species are visited by a variety of pollinators. These

generalized insect visitation patterns facilitate pollen movement among flowers and individuals (Bateman et al., 2017; Sramkó et al., 2014; Fantinato et al., 2017).

Figure 4. 1 Himantoglossum adriaticum life cycle. a) Seedling; b) Winter leaf rosette of an adult individual; c) Stem and first floral formation d) Flowering plant; e) Flowers before anthesis; f) first appearance of the labellum; g) open flowers; h) closer look to the stigmatic surface.

4.1.5. A highlight on *Ophrys apifera*

Ophrys apifera Huds. (Bee orchid) is a euro-Mediterranean orchid, distributed from Spanish to Georgia and from North Africa to Baltic and British Isles. In Italy *O. apifera* occurs in all Regions with the exclusion of Valle d'Aosta. Its habitat comprises garrigue, bushlands, meadows and grasslands, bright woods, mainly on calcareous soils but very well adaptable to all substrates (Biagioli e De Simoni, 2024). It produces underground tubers, leaves narrow oval up the stem (fig. 4.2). The species reproduce regularly through autogamy, as after some hours from anthesis pollinia emerge from the anthers and supported by the long caudicula fold downwards, resting on the stigmatic surface, knowing that pollinia (yellow massulae) are folded downwards on stigma allowing self-pollination that result in self-fertilization.

Figure 4. 2: Ophrys apifera: a) Stem and first floral formation (a plant in Parco della Resistenza); b) early stage of blooming (Parco della Resistenza); c) open flowers (Parco della Resistenza); d) closer look on the open flower (Parco San Lazzaro).

4.2. Aim of this study

The present study was conducted over a period of three years in the municipalities of Modena and Reggio Emilia. The rationale and identified gaps were taken into consideration in the setting of the following objectives:

- Monitoring, documenting and mapping the presence of orchid species in urban parks and green areas.
- To implement conservation interventions, including protective measures (barriers against mowing/trampling) around vulnerable orchid individuals or clusters, and monitor their efficacy over time.
- To collect seeds for *ex-situ* conservation (seedbank) to safeguard local resources and allow potential future restoration or reinforcement measures.
- To Investigate reproductive ecology of a focal species*, Himantoglossum adriaticum*, through pollinator observations and evaluation of the reproductive success (fertilization rates with respect to fruit set), comparing two different environments: urban park *vs* semi-natural area; this study has been carried out only in 2025.
- To assess mycorrhizal associations in two selected orchid species, *Himantoglossum adriaticum* and *Ophrys apifera* growing in urban parks.

Through this integrated and multidisciplinary approach, the study aims to bridge critical knowledge gaps, evaluate the conservation potential of urban habitats for orchids and contribute to urban biodiversity conservation strategies.

4.3. Materials and Methods

4.3.1. Study Area and Orchid Monitoring Program

Monitoring of orchids and field surveys were conducted in urban parks, semi-natural green areas and Regional Reserve in the provinces of Modena and Reggio Emilia. Systematic searches for orchid occurrences were carried out across all accessible urban parks and areas in both provinces, with selected focal sites used for repeated monitoring. Table 4.1 summarizes the locations surveyed where orchids were found, their administrative provinces, main site characteristics and monitoring years.

Table 4. 1: Surveyed locations, site characteristics and monitoring period for orchid monitoring in Modena and Reggio Emilia provinces.

Province	Location	Site characteristics (landform and habitat features)	Monitoring year(s)
Modena	Parco della Resistenza	Urban Park with open grasslands, managed lawns, scattered trees and gently flat terrain subject to regular mowing	2023-2025
Modena	Salse di Nirano (Regional Reserve)	Semi-natural protected area characterized by mud volcanoes, calcareous substrates, grasslands and heterogeneous microhabitats	2025
Modena	Cimitero Monumentale	Historic monumental cemetery with grass areas, tree-lined paths, low disturbance zones and managed vegetation.	2024-2025
Reggio Emilia	Parco San Lazzaro	Managed urban green space with lawns, ornamental vegetation and flat topography	2023-2025

Field visits were scheduled to coincide with key phenological phases from autumn to early spring (March) and summer (August). Individual orchids were identified *in-situ* using diagnostic morphological characteristics. Population abundance was yearly estimated through direct count of plants, with spatial identification at flowering.

At each site, potential threats were assessed, paying particular attention to mechanical disturbance from mowing, cutting, trampling and routine vegetation management. Where populations were deemed vulnerable, temporary protective barriers or exclusion zones around individual plants or clusters were realized, reducing damage during maintenance operations (see Figure 4.3). Following this step the geographical locations were recorded using a GPS Garmin Dakota™ 20.

Figure 4. 3: Plastic protection around the orchid winter rosette (basal leaves).

4.3.2. *Himantoglossum adriaticum*

The declining native orchid *Himantoglossum adriaticum* is an European species of priority interest (92/43/EEC, Annex II) and it is in the Red List of flora of Italy in the IUCN category LC. Northern Italian populations of *H. adriaticum* are minimal, isolated, and show low seed production (Del Vecchio et al., 2019)

Given the important implications for plant population conservation, two hypotheses arise for investigating. The first concerns the insects involved in the pollination of *H. adriaticum* in two different locations and the second concerns mycorrhizal associations in comparison with *O. apifera*.

4.3.3. *In Situ* Conservation Interventions and Seed Collection

Conservation actions included seed collection were undertaken at selected sites where orchid populations or individuals were identified. They were carried out through repeated monitoring across successive years and assessed in terms of plants counting, survival, flowering and fruiting. For seed collection, mature capsules were collected in accordance with standardized field protocols, designed to minimize the impact on natural populations. All collected material was subsequently processed and conserved in accordance with established seed bank procedures. The detailed criteria, field protocols and storage methodologies for *ex-situ* conservation activities have been fully described in Chapter 2.

4.3.4. Pollination Study

The first step in identifying pollinators visiting *H. adriaticum* has been to identify the sites of monitoring. The selected areas were:
-an urban park in Reggio Emilia, namely Parco San Lazzaro, having 12 flowering plants in 2025 and
-natural reserve, namely Salse di Nirano in Province of Modena, with 9 flowering plants.
Materials used to collect and record the data were:

- a micro Dinolite camera placed in a fixed position and linked to a laptop.
- hands nets used to capture insects (figure 4.4).

Figure 4. 4: micro Dinolite camera used for recording the insects visiting H. adriaticum plants in Parco S. Lazzaro, linked to the laptop to save and identify the insects later.

How data were collected

Before bloom

Inflorescence of six plants, before the beginning of anthesis, were bagged with a mesh to avoid contact with pollinators. (fig. 4.5)

Figure 4. 5: bagged and un-bagged inflorescences to be monitored in San Lazzaro Park.

During bloom

Two *H. adriaticum* plants from each location were always bagged for self-pollination control. In detail: two plants were taken for control purposes in San Lazzaro Park and two plants in Salse di Nirano.

Four bagged plants in San Lazzaro and four bagged plants in Nirano were observed and filmed by the camera. The net was removed from a plant to record the visitation of pollinators or visitors and a 30-minute video was taken observing the inflorescence to detect potential pollinators or visiting insects. One observer must stay near the Dinolite camera and laptop to ensure everything is working properly. The observer took notes on every insect visit and the time. After 30 minutes, another net was removed from another plant and the inflorescence observed for a total of 4 plants per site. Furthermore, the recordings and notes were repeated 4 times for each plant, for a total of 2 hours of video footage for 4 orchids at each location, each day.

During the observation period, a second observer monitored insect activity on unbagged orchid flowers for a continuous 30-minute session. All insects approaching, landing on, or interacting with the flowers were carefully observed to document visitation frequency and behavior. Whenever possible, visiting insects were captured using an entomological net or a Falcon vial to allow subsequent identification. In cases where insects could not be captured, their presence, behavior, and morphological traits were recorded visually to avoid underestimating visitor diversity. This approach allowed the inclusion of both confirmed captures and observed but uncaptured visitors, which is standard practice in pollination ecology studies to obtain a comprehensive assessment of floral visitation.

Figure 4. 6: how to mark the inflorescence for bagging.

In this case, the two observers had to stay in two different areas of the same location to avoid disturbance.
Observation time: three days were dedicated to carrying out this activity at each location, from 9 a.m. to 1.30 p.m. This period had been chosen because it was more favorable for observing insects.
If there aren't enough orchid inflorescences, or if the flowers at the lower part of the inflorescence have already bloomed (Figure 4.6), the bag was put on the upper part of the inflorescence, and the upper and lower parts of inflorescence were considered as separate entities.
The flowers on each plant were carefully counted.

After bloom

The number of capsules was counted for any inflorescence to determine the fertilization rate.
Insects caught by net or falcon vials and those filmed were identified. A distinction was made between visitors and pollinators. The identification of the species of insects was carried out by entomologists, group of research of "General and Applied Entomology", coordinate by prof. Lara Maistrello (UNIMORE).

4.3.5. Mycorrhizal Association Analysis

4.3.5.1. Root Sampling

- Healthy orchid plants were sampled during the flowering period to maximize the likelihood of active fungal colonization in roots. In total there were four plants taken for root sampling, P1 and P2 are both *Ophrys apifera*, from Parco della Resistenza (MO). P3 and P4 are both *Himantoglossum adriaticum* from Parco San Lazzaro (RE).
- Underground tubers and roots were carefully excavated to avoid mechanical damage.
- Immediately after collecting, tuber and roots were placed into plastic bags and transported to the laboratory for processing (see Fig. 4.7a).

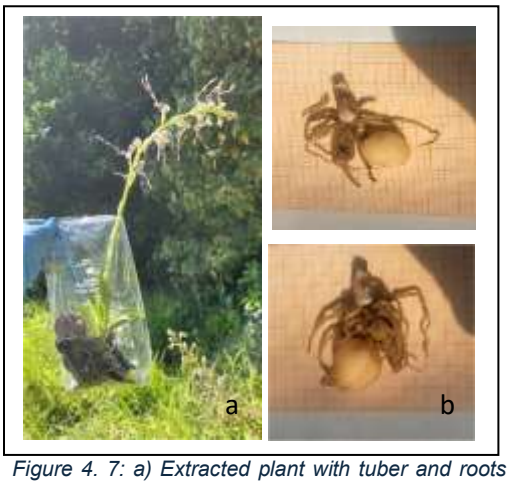


Figure 4. 7: a) Extracted plant with tuber and roots and some soil to be transferred to lab. for fungal isolation. b) Underground part of an orchid (upper section is O. apifera and lower one is H. adriaticum) showing the old tuber serving as a nutrient supply and the newly formed one (bigger).

4.3.5.2. Preparation of Root Sections

- Root samples were rinsed thoroughly with tap water to remove soil debris (fig.4. 7b).
- Roots were cut into transversal sections and each section was further divided into ~3mm fragments for microscopic observation.
- Sections with visible cortical tissue were selected for analysis (see Fig. 4.8).

- Root fragments were surface-sterilized by immersion in a 1:5 sodium hypochlorite: water solution for 30 sec, then rinsed three times for 5 mins with sterile water. This procedure aimed to eliminate epiphytic microorganisms while preserving internal endophytic fungi for further isolation.
- Sterile water rinses were performed to eliminate the solution used for sterilization.
- Sterilized sections were then placed on MEA for fungal isolation procedures.

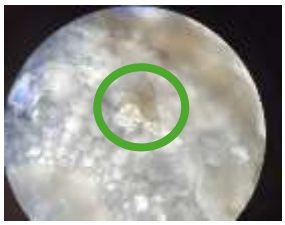


Figure 4. 8: Root fragment showing fungal hyphae inside root cells

4.3.5.3. Aseptic Isolation on MEA

- Root segments were placed onto malt extract agar (MEA) supplemented with antibiotics (e.g., gentamycin) to suppress bacterial growth. Gentamicin was always added after medium autoclaving to avoid the lost of its biological activity, due to heat treatment.
- Plates were incubated in the dark at room temperature (20–25 °C).
- These conditions favored the emergence of slow-growing mycorrhizal fungi from the root tissues.
- Fungal hyphae growing out of the plant segments were observed over several weeks (fig 4.9b).
- Once fungal growth was visible on MEA plates, individual hyphal tips were chosen for purification (fig. 4.9a). This step is to minimize the risk of contamination from mixed cultures.
- Under sterile conditions, under a laminar flow hood, hyphal tips were transferred to fresh MEA plates using a sterile needle or loop.
- Pure colonies were maintained on MEA and periodically examined for morphological consistency.

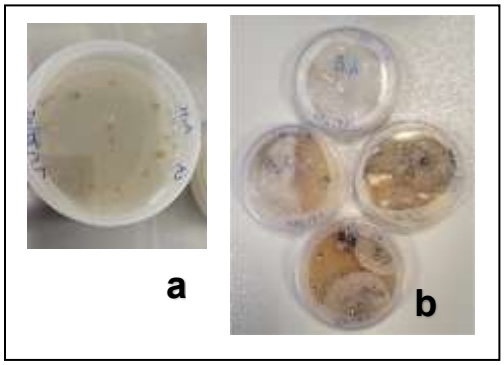


Figure 4. 9: a) 3mm root sections placed on MEA; b) visible fungal growth after 4 weeks of incubation

4.3.5.4. DNA Extraction

- Actively growing fungal mycelium was obtained from pure cultures.
- Mycelial parts were harvested directly from the agar surface.
- It was processed using a DNA extraction protocol optimized for fungi (details for DNA extraction are reported below).
- Extracted DNA was purified to remove contaminants, and the final product yielded PCR-quality genomic DNA suitable for downstream molecular analyses.

4.3.5.5. Sanger Sequencing

The internal transcribed spacer (ITS) region of the ribosomal RNA gene cluster, commonly used as a fungal barcode, was amplified by PCR using universal primers

targeting this region (ITS1-ITS4 and ITS1F-ITS4). Resulting PCR products were purified and sequenced using Sanger sequencing to generate high-quality sequence data for each isolate. PCR protocol is detailed below.

4.3.5.6. Identification by BLAST on NCBI

Obtained ITS sequences were queried against the NCBI nucleotide database (nt) using the BLASTn algorithm. BLAST (Basic Local Alignment Search Tool) identifies the closest matching sequences in public databases, allowing assignment of putative genus and species based on sequence similarity and alignment statistics. This molecular identification step provides a reliable classification of the fungal isolates associated with orchids.

DNA extraction, sequencing and identification were carried out in the Laboratory of research group “Crop Production” di UNIMORE, under the coordination of Dr. Federica Caradonia.

Malt Extract Agar – preparation protocol

To prepare Malt Extract Agar (MEA) medium, 10 g of MEA powder, 10 g of D-glucose, 0.5 g of peptone, and 10 g of agar were dissolved in 500 mL of distilled water. The mixture was sterilized by autoclaving and poured into sterile Petri dishes once cooled to handling temperature. Additionally, 20.7 mg of gentamicin was added to inhibit bacterial growth, and this is after the autoclaving

DNA Extraction Protocol

The pure fungal colonies were removed from the petri dish using a sterile scalpel and placed in an Eppendorf tube. The sample was then treated with 1500 µL of CTAB buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mL EDTA, 1.4 M NaCl, 1% sodium sulfite, and 2% PVP-40), vortexed, and incubated at 65°C for 15 minutes. After centrifugation at 12,000 rpm for 10 minutes, 700 µL of the supernatant was collected and mixed with an equal volume of isoamyl alcohol: chloroform (1:24), followed by centrifugation. The resulting 550 µL supernatant was again extracted with isoamyl alcohol: chloroform and centrifuged. From this, 450 µL of supernatant was recovered, supplemented with 225 µL of 5 M NaCl and 450 µL of isopropanol, vortexed briefly, and incubated at –20°C for at least 60 minutes. Samples were then centrifuged at 4°C for 20 minutes, the isopropanol removed, and the pellet washed twice with 500 µL of 70% ethanol. After ethanol removal, the DNA pellet was vacuum-dried for about 10 minutes and finally resuspended in 50 µL of nuclease-free water.

PCR Protocol for ITS amplification

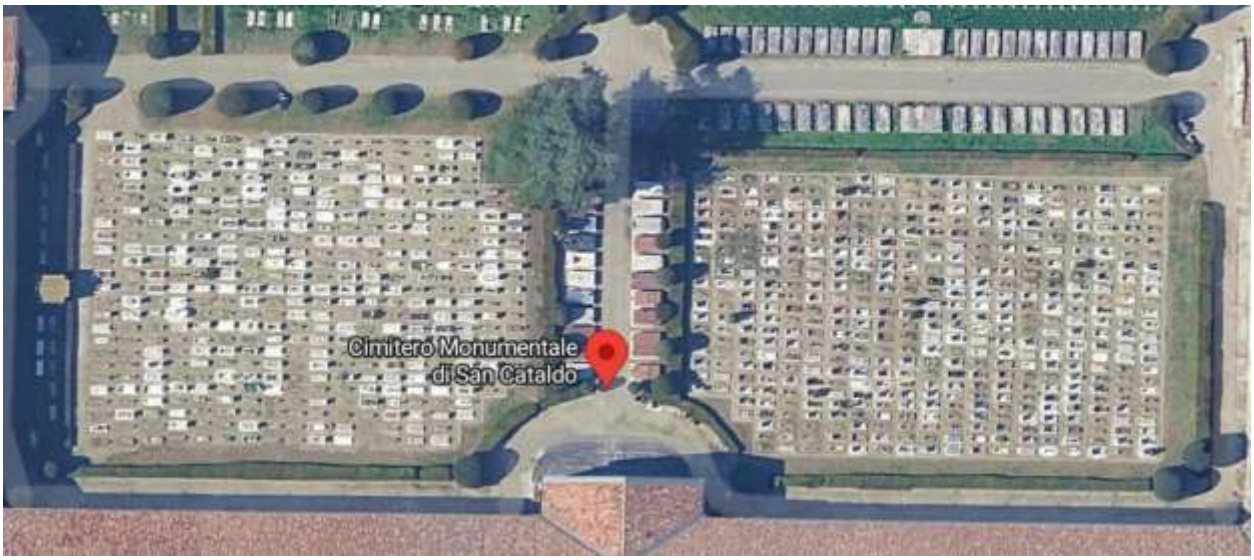
For ITS amplification, primers were reconstituted if lyophilized and diluted by mixing 5 µL with 45 µL of water to obtain 50 µL. The four dNTPs were prepared by combining 2.5 µL of each (10 µL total) with 390 µL of water, yielding 400 µL, divided into four aliquots of 100 µL. Genomic DNA was adjusted to a final concentration of 10 ng/µL using the formula $C_1V_1=C_2V_2$, where C_1 is the Nanodrop concentration, $C_2=10$ ng/µL, and $V_2=50$ µL, with the calculated DNA volume complemented by water. The PCR mix contained 12.3 µL water, 5 µL buffer, 0.5 µL dNTPs, 1 µL forward primer, 1 µL reverse primer, 0.2 µL Taq polymerase, and 5 µL DNA. Amplification was performed in a thermocycler with an initial denaturation at 95°C for 30 seconds, followed by 35 cycles of 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 8 minutes.

4.4. Results and Discussion

4.4.1. Distribution of orchids in urban areas in Modena and Reggio Emilia and in Salse di Nirano (Modena)

Identifying the exact location was possible using a GPS, then a map was generated. The maps below show the distribution of orchids across three different locations.

Cimitero Monumentale di Modena



Map 1: Cimitero Monumentale di Modena (Small population of Anacamptis morio)

Anacamptis morio was detected in Cimitero between 2024 and 2025, the data collected are summed up in Table 4.2.

Table 4. 2: Flower production, capsule formation and fruit set of Anacamptis morio across two flowering seasons (2024 & 2025) in Cimitero Monumentale di Modena

Year	Plants (n)	Mean flowers/plant	Mean capsules/plant	Fruit set (%)
2024	13	9.92	2.77	**27.91%**
2025	22	9.59	1.73	**19.59%**

Monitoring of *Anacamptis morio* at the Cimitero Monumentale di Modena revealed an increase in the number of flowering individuals from 13 in 2024 to 22 in 2025, indicating a positive response to avoidance of mowing surrounding the plant individuals. Flowering effort remained comparable between years, with a similar number of flowers produced per plant. In contrast, fruiting performance remained low in 2025, as evidenced by reduced capsule production and a lower

proportion of flowers developing into fruits. In both years, several individuals failed to set capsules despite flowering, reflecting frequent reproductive failure at the individual level. This pattern is a characteristic of food-deceptive orchids such as *A. morio*, whose reproductive success is strongly influenced by pollinator availability, weather conditions during the flowering period, and local habitat disturbance, in agreement with Fantinato et al., (2017).

Parco della Resistenza (MO):

Map 2: Parco della Resistenza (MO) where the yellow dots show the locations of Ophrys apifera.

The monitoring of *Ophrys apifera* growing in Modena in Parco della Resistenza showed that the number of capsules per plant decreased from year to year (table 4.3). In Parco della Resistenza there is a medium sized population (around 70 plants). There is no high yield of capsule formation. This is a result probably due to the location of these orchids, which grow inside an overgrown wooded area. This may interfere in the reach of the pollinators, thus the obtained capsules could be the results of self-fertilization.

The number of flowers produced by *O. apifera* at Parco della Resistenza decreased progressively from 2023 to 2025, with a particularly sharp decline in 2025. A similar downward trend was observed in the number of ripe capsules formed and successfully collected. Despite high flower production in all years, capsule formation remained low, consistent with the low fruit set typically reported for *Ophrys* species. This pattern is consistent with the pollination biology of *O. apifera*, which relies on sexual deception and is often subject to pollination limitations, particularly when pollinator abundance fluctuates. The reduction in reproductive output may reflect environmental stress, reduced pollinator activity or increased resource limitation over time. Overall, the results suggest that flowering effort does not directly translate into reproductive success, highlighting the vulnerability of this species to changes in ecological conditions.

Table 4. 3: Ophrys apifera capsule number alteration from 2023 to 2025

Year	2023	2024	2025
Number of flowers in Parco della Resistenza for the 70 plants	304	274	128
Number of all the ripped capsules	24	17	16
Number of ripe capsules collected	8	5	4

Map 2: Orchid plants distribution in San Lazzaro Park (RE).

Legend:

- *Himantoglossum adriaticum*
- *Ophrys apifera*
- *Orchis purpurea*
- *Orchis simia*
- not identified

Map 3: Salse di Nirano (MO) with the distribution of Himantoglossum adriaticum plants.

Response of *Himantoglossum adriaticum* to Protection and Reduced Cutting in Parco San Lazzaro

The results of monitoring of plants of *H. adriaticum* in Parco San Lazzaro, conduced along 7 years are shown in Table 4.4.

Table 4. 3 H. adriaticum population trend in Parco San Lazzaro from 2019 to 2025

Species	City; Location	Year	No. of Plant individual	Observer
Himantoglossum adriaticum	Reggio Emilia; Parco San Lazzaro	2019	1	Sgarbi
		2020	3-4	Sgarbi
		2021	3-4	Sgarbi
		2022	7	Sgarbi
		2023	13	Mortada
		2024	15	Mortada
		2025	20	Mortada

The observed increase in *H. adriaticum* plants from one individual in 2019 to twenty in 2025 in Parco San Lazzaro likely reflects the potentiality of wild plants to colonize urban spaces if they find favorable conditions. It could be related also to reduced cutting intensity and the effectiveness of protection structures, *i.e.* with the management of green areas in urban contests. This result is consistent with studies showing that lower mowing intensity and less intensive maintenance in urban green spaces is associated with increased plant and invertebrate biodiversity. This is because frequent cutting limits the reproductive structures and floral diversity required by pollinators and specialist plants (Sehrt et al., 2020; Fekete et al., 2020; Yudaputra et al., 2024). Terrestrial orchids, including *H. adriaticum*, are particularly sensitive to habitat disturbance and depend on stable microhabitats and pollinator interactions for reproduction. This means that minimizing cutting disturbance can directly support population persistence (Sarasan et al., 2025). These collective findings emphasize that urban green space management regimes prioritizing ecological heterogeneity and reduced disturbance can foster conditions favorable to rare native plants.

4.4.2. Pollination Ecology

The recorded data on pollination ecology are entered to the database. Table 4.5 shows a section of these data, location (Nirano), the orchid ID, the recorded time, the identification of the insects, and the insects' destiny.

Table 4. 4: section of the data recorded for pollination ecology

Nirano	*Himantoglossum adriaticum*					
Netting						
Orchid id	Time start	Time finish	Number insect	Time visit	Id insect	Insect destiny
N2, N3, N4	10:19	10:49	0			
O2	10:52	10:22	0			
M2	11:44	12:14	1	11:48	Bee	Flew away
			1	12:24	Halictus (Bee) F	Caught
N2, N3, N4	12:30	13:00	1	12:30	Halictus (Bee on N4) F	Caught
			1	12:38	Bee (on N4)	Flew away
			1	12:48	Andrena (Bee on N4) F	Caught
			1	12:53	Bee (on N4)	Flew away
M2	12:30	13:00	1	12:34	Halictus (Bee) F	Caught
			1	12:43	Bee	Flew away
			1	12:52	Bee	Flew away
			1	13:00	Bee	Flew away

The list of insects observed in this study comprises multiple taxonomic groups (see Table 4.6) that play different roles in pollination ecology. The following bee species were recorded: *Halictus* sp. (5 males, 5 females), *Lasioglossum* sp. (1 male, 1 female) and *Andrena* sp. (1 female). These bees (Fig. 4.10) are widely recognized as effective pollinators due to their frequent contact with floral reproductive structures and pollen transfer behavior, e.g. Halictidae and Andrenidae are important pollinators in diverse ecosystems (Silló et al., 2024). In contrast, flies (Diptera) such as hoverflies (Syrphidae) and fungus gnats (Sciaridae), were primarily observed as floral visitors (Fig. 4.11). Although non-bee Diptera often receive less attention, they can contribute substantially to pollen transport and pollination networks. Hoverflies, for example, are among the most abundant flower visitors and can rival bees in terms of pollination importance (Klecka et al., 2018). Ants (unidentified species) appeared only as flower visitors (Fig. 4.12). Ants are generally considered poor pollinators due to their smooth bodies and frequent grooming behavior, which limits effective pollen transfer. However, rare cases of ant-mediated pollination have been documented in specialized systems (Das & Das, 2023). Moths, exemplified by Acontia (Emmelia) trabealis (Noctuidae), were also recorded as visitors. Nocturnal Lepidoptera, such as moths, are increasingly recognized for their role in transferring pollen, particularly for plants with night-blooming flowers. They can also contribute to genetic mixing across plant populations (MacGregor et al., 2015). Overall, these results illustrate a diverse insect assemblage, with bees likely serving as the primary pollinators and other taxa functioning as supplementary visitors that nonetheless influence pollination dynamics in this study system.

Figure 4. 10: Bee fertilizing H. adriaticum

Table 4. 5: Taxonomic composition of insect visitors and pollinators recorded during the pollination study

Group	Taxa Observed	Notes
Bees (Anthophilia)	*Halictus* sp. (5♂, 5♀), *Lasioglossum* sp. (1♂,1♀), *Andrena* sp. (1♀)	Only confirmed pollinators
Flies (Diptera)	Hoverflies (Syrphidae), Fungus gnat (Sciaridae)	Visitors only
Ants	unidentified sp.	Visitors only
Moths	Acontia (Emmelia) trabealis. Noctuidae	Visitors only

The assemblage of insect visitors recorded exclusively on *H. adriaticum* flowers reflects the expected patterns for food-deceptive orchids. Floral visitors to these orchids often include a diverse set of insects with varying roles in pollination, such as bees, flies, ants and moths. While traditional views of food-deceptive orchids have emphasized pollination specialization, recent evidence suggests that such species may be functionally generalized with respect to pollinators. These species attract multiple taxonomic groups without providing rewards, and pollinator guilds often differ from predictions based purely on floral morphology (i.e. phenotypic specialization) (Fantinato et al., 2017). Contrary to the assumption that deception necessarily yields a narrow pollinator spectrum, food-deceptive orchids such as *H. adriaticum* exploit the general foraging behavior of bees, flies and other insects, resulting in broader pollinator assemblages (Fantinato et al., 2017). This generalization can facilitate cross-pollination by encouraging insects to visit multiple plants rather than remaining on a rewarding individual, which is consistent with the notion that deceptive orchids promote outcrossing and reduce geitonogamy (Jersáková et al., 2006).

Figure 4. 11: Diptera visiting H. adriaticum

From a reproductive perspective, research indicates that cross-pollination enhances seed quality and germination performance relative to self-pollination. This highlights the importance of effective pollinator visitation for the reproductive success of deceptive orchids, including *H. adriaticum* (Bazzicalupo et al., 2025). However, Bazzicalupo also found that *H. adriaticum* is self-compatible under

controlled pollination. Nevertheless, cross-pollination significantly improves seed quality and germination rates. This indicates that, although self-fertilization is biologically possible, reproductive success in natural populations is mostly associated with pollen transfer between individuals. This is what our study proofs where the controlled bagged orchid plants have zero capsules with seeds.

Together, these findings suggest that the insect visitors observed on *H. adriaticum* may contribute to a functionally generalized pollination system.

Figure 4. 12: Ant visiting a flower of H. adriaticum

Fertilization rates in relation to bee abundance and habitat context

The fertilization rates of *Himantoglossum adriaticum* varied significantly between individual orchids and study sites, showing a clear correlation with the number of observed bees. At San Lazzaro site, higher fertilization rates were generally observed in orchids visited by bees, particularly unbagged individuals. For instance, orchid D2 exhibited the highest fertilization rate (85.3%) and was associated with the greatest number of bees (five), whereas control and unvisited individuals consistently demonstrated low or zero fertilization (Fig. 4.13a). This pattern reinforces the pivotal role of bees as effective pollinators of *H. adriaticum*, in line with prior research emphasizing bees as the primary pollen vectors in Orchidaceae (Fantinato et al., 2017).

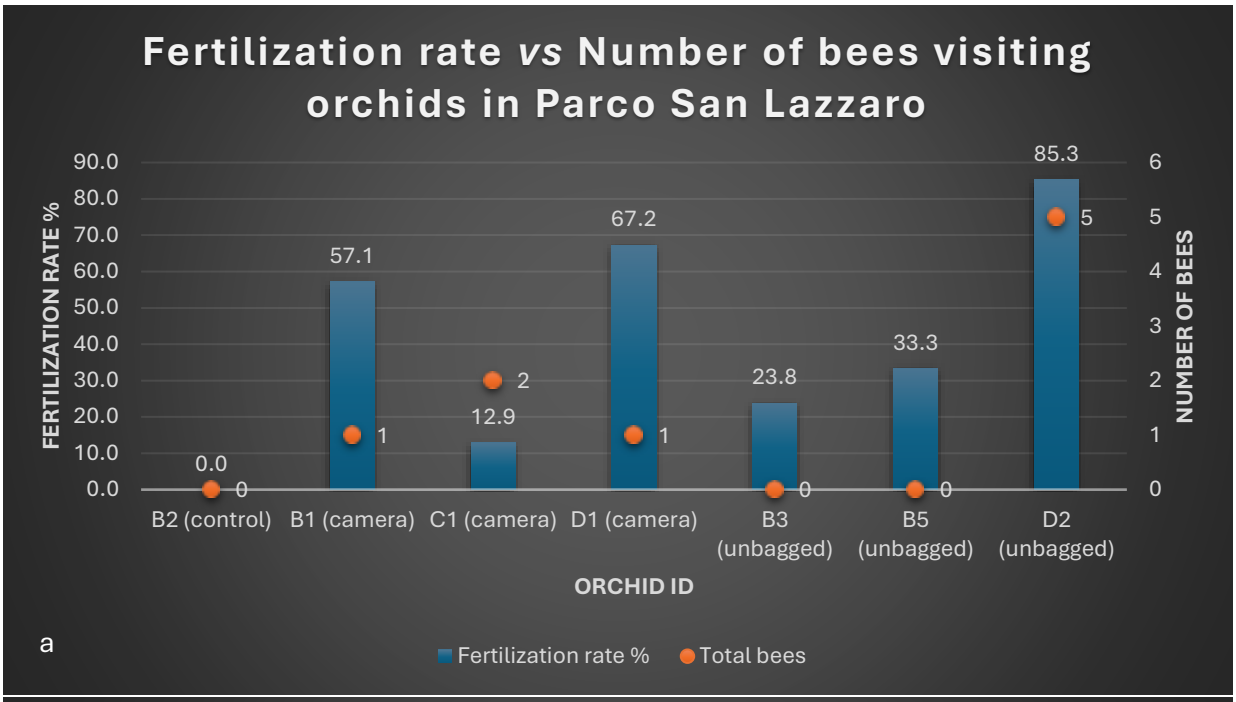
The pollinator assemblage observed for *Himantoglossum adriaticum* was dominated by bees [sweat bees (*Halictus, Lasioglossum*) and mining bees (*Andrena*)], which were the only confirmed pollinators. Flies, ants and moths, meanwhile, acted solely as floral visitors. This pattern aligns with the findings of Fantinato et al. (2017), who demonstrated that food-deceptive orchids, including *H. adriaticum*, exhibit functional generalization in terms of visitor diversity, yet primarily rely on bees for effective pollination.

It is widely acknowledged that the adaptive phenotypic responses of floral traits may arise through selection by the most effective pollinator guild (Fantinato et al. 2016; Stebbins 1970), implying that floral characteristics should be adapted to the pollinator that transfers the most pollen (Mayfield 2001; Souza et al. 2017). However, floral traits that are specific to the most common or most effective pollinator guild may not preclude visits by less efficient pollinators (i.e. secondary pollinators; Stebbins, 1970), and these have often been proven to contribute to pollination (Rosas-Guerrero et al., 2014).

In contrast, fertilization rates at the Salse di Nirano site were uniformly low, even in orchids where bees were observed. Several camera-trapped and unbagged individuals received multiple bee visits (up to eight bees), yet fertilization remained close to zero (Figure 4.13b). This discrepancy suggests that the mere presence of bees may not guarantee effective pollination and that habitat

structure strongly influences pollination success. The orchids at Salse di Nirano were located among wooded shrubs, which likely constrained pollinator movement and reduced the probability of successful pollen transfer between conspecific individuals. In contrast, orchids at San Lazzaro were found in open habitats, which facilitated pollinator flight paths and increased the likelihood of cross-pollination.

The contrasting patterns between sites can be explained by differences in land management practices. Salse di Nirano, for example, is a protected area characterized by high floral diversity and limited mowing, conditions which are known to enhance pollinator abundance. Reduced cutting allows for longer flowering periods and greater availability of floral resources, thereby supporting higher bee densities (Humbert et al., 2012; Potts et al., 2010). In contrast, urban park management at San Lazzaro involves more frequent mowing, which can reduce floral resources and limit pollinator activity to the available flowers, in this case *H. adriaticum*. Overall, the results suggest that the success of fertilization in this species is influenced not only by the presence of pollinators, but also by the openness of the habitat and the intensity of management, with important implications for the conservation of orchids in both protected and urban landscapes.



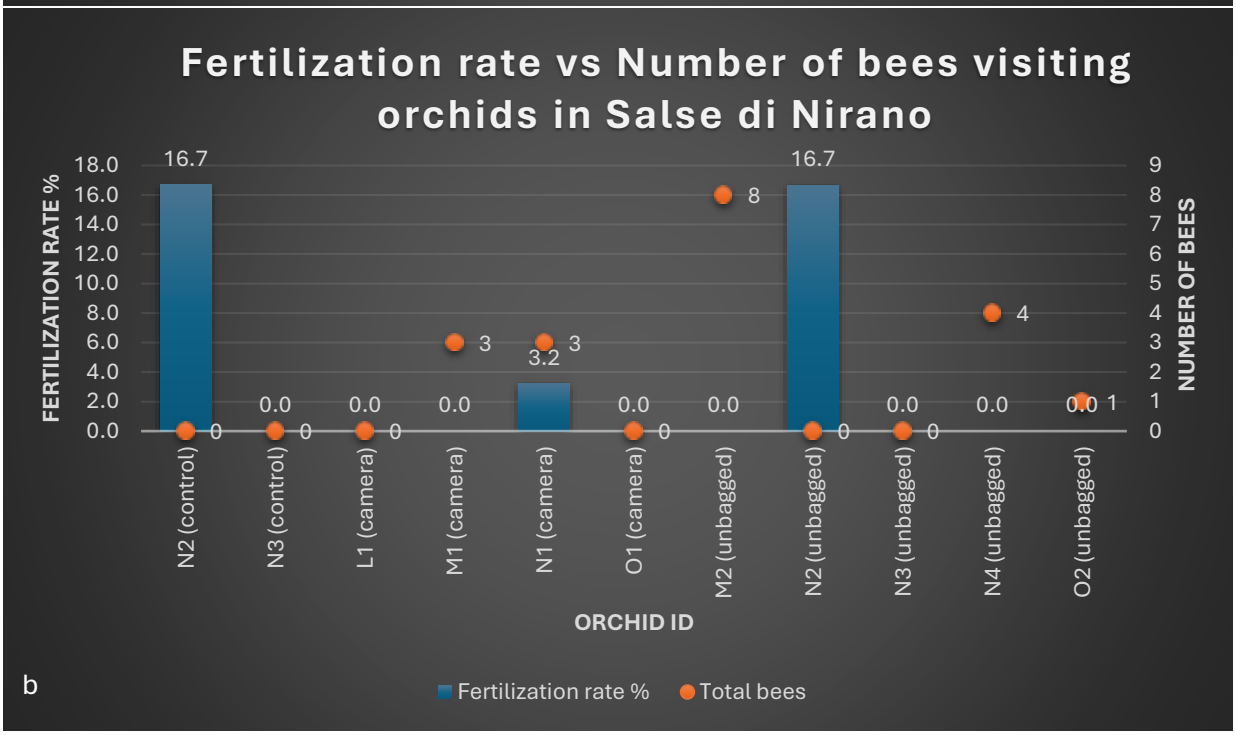


Figure 4. 13: Fertilization rate (%) vs numbers of bees visiting orchids in Parco San Lazzaro (a) and Salse di Nirano (b)

4.4.3. Mycorrhizal Associations

To investigate the presence of mycorrhizal symbiosis, root sections containing visible mycelium or suspected to harbor fungal structures were selected and examined under a microscope (fig. 4.14). The hyphae were then isolated from the culture plates using a sterilized scalpel, transferred to slides and observed under a light microscope (fig 4.15). Following a morphological assessment, DNA was extracted, purified and amplified by PCR, and the resulting products were subjected to Sanger sequencing. Sequence data were analyzed using BLAST searches on the NCBI database (Annex I), which enabled the taxonomic identification of the fungal symbionts. The identified species are summarized in Table 4.7.

Table 4. 6: - Association of fungal isolates with Himantoglossum adriaticum and Ophrys apifera

Species	*Himantoglossum adriaticum*	*Ophrys apifera*
Aschotricha erinacea	+	
***Chaetomin* sp.**		+
Chatomium funicola		+
Diaporthe eucalyptorum	+	
Dicyma olivacea	+	
Fusarium oxysporum	+	
***Fusarium* sp.**	+	
Phomopsis sp.	+	

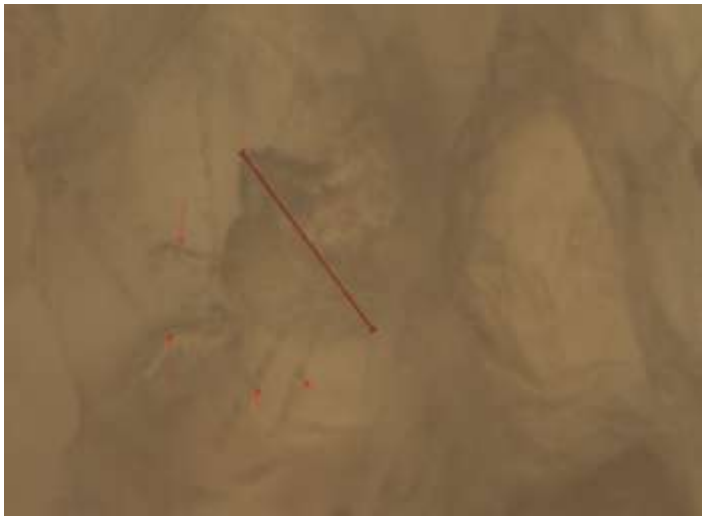


Figure 4. 15: Microscopic view of orchid root at 40× magnification with mycorrhizal hyphae in red arrow and zone of fungal colonization, penetration in dark red line

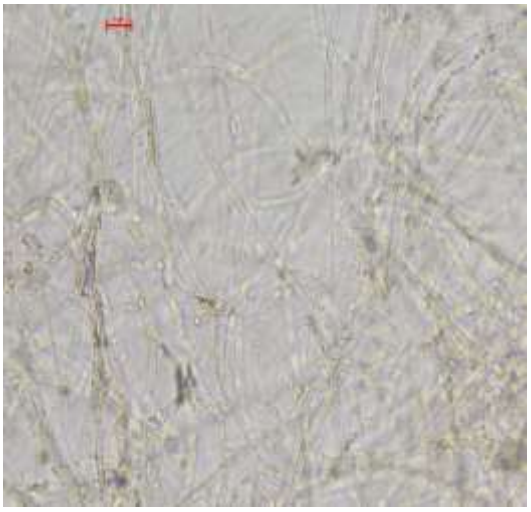


Figure 4. 14: Fungal mycelium

Throughout their life cycle, orchids depend on fungal partners, beginning with seed germination, protocorm development and transitioning to autotrophic or mixotrophic adult phases. Classical orchid mycorrhizal fungi (OMF), which are predominantly basidiomycetes belonging to families such as *Tulasnellaceae, Ceratobasidiaceae* and *Sebacinales*, facilitate positive nutrient exchange and are essential for orchid survival. However, contemporary research has increasingly documented the presence of non-mycorrhizal orchid fungi (ONF) (ascomycetes) in orchid roots. These include taxa such as Diaporthe, Phomopsis, Fusarium and Chaetomium, among others (Li et al., 2021; Rashmi et al., 2019; Pujasatria et al., 2022). This raises questions about how these ONF taxa interfere with or complement classical OMF interactions, particularly in terrestrial orchids such as *H. adriaticum* and *O. apifera* that were considered in the present study.

Although ONF are widespread within the Orchidaceae family, they often lack the intracellular peloton structures that are characteristic of mutualistic OMF. However, they can play a variety of ecological roles and produce metabolites that can affect host tissues or competing fungi (Li et al., 2021). Other endophytes, such as *Diaporthe eucalyptorum, Phomopsis spp.* and various *Fusarium* taxa, have been isolated from orchid roots (Deepthi et al., 2019; Sawmya et al., 2013). These fungi have the potential to produce plant-active compounds, suggesting that they could modulate host physiology beyond simple colonization. This could interfere with OMF colonization or seedling responses, as these endophytes frequently co-occur with classical OMF.

Fusarium spp. is widely recognized as opportunistic pathogens in many plants, yet they are also frequently isolated as endophytes in orchids without causing any obvious disease symptoms (Tsavkelova et al., 2022; Rashmi et al., 2019). Greenhouse studies provide experimental evidence that *Fusarium* colonization can reduce seedling survival under certain conditions. This suggests negative interference with orchid development, particularly when OMF is limited or environmental stress alters host tolerance (Tsavkelova et al., 2022). Such findings are consistent with the broader conceptual framework that ONF can shift from being benign to being antagonistic, depending on the condition of the host and the ecological context (Li et al., 2021).

Chaetomium funicola and *Chaetomium* spp. in general can break down organic matter enzymatically, which could release nutrients into the rhizosphere (Ramos, 2024), but this could also create competition with OMF for resources.

In summary, the results of the selected literature suggest that the ONF taxa associated *with H. adriaticum* and *O. apifera*, including *Diaporthe/Phomopsis, Fusarium* and *Chaetomium*, are part of a complex root fungal community in which they modulate orchid physiology and ecological interactions. This interference may have positive effects, such as enhancing nutrient availability or protective functions, or negative effects, such as competing with OMF or suppressing seedling growth. These effects depend on environmental conditions, the host's developmental stage, and the community context.

Fungal isolated taxa and urban soils

Urban soils host diverse fungal communities dominated by disturbance-tolerant, saprotrophic, and opportunistic taxa, including *Fusarium* and *Chaetomium*. These taxa are also frequently detected as endophytes in orchid roots. Studies of soils from urban green spaces and areas with high human activity consistently report *Fusarium spp.* as being among the most abundant culturable fungi, highlighting their ecological flexibility and persistence under anthropogenic pressure (Spychała et al., 2024). Along rural–urban gradients, shifts in fungal community

composition towards saprotrophic dominance and reduced Symbio-trophic representation reflect habitat disturbance and lower plant diversity (Abrego et al., 2020).

This pattern helps to explain why fungi such as *Fusarium* and *Chaetomium*, which are found in the roots of *Himantoglossum adriaticum* and *Ophrys apifera*, are also prevalent in urban soils. This suggests that urbanization may indirectly influence orchid–fungus interactions by favoring generalist fungi that can colonize both soil and plant tissues.

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Annexes:

Annex I:

Sample ID	Orchid host species	Site	Primer used	ITS region length (bp)	BLAST top match (species)	Putative genus	Notes
P4B	*Himantoglossum adriaticum*	Parco San Lazzaro	ITS1f	~700	*Dicyma olivacea*	*Dicyma*	Asexual form = *Aschotricha erinacea*
P4A	*Himantoglossum adriaticum*	Parco San Lazzaro	ITS1	~680	*Dicyma olivacea*	*Dicyma*	Teleomorph/anamorph correspondence
P3E	*Himantoglossum adriaticum*	Parco San Lazzaro	ITS1	~690	*Fusarium oxysporum*	*Fusarium*	Common orchid root endophyte
P3D	*Himantoglossum adriaticum*	Parco San Lazzaro	ITS1	~700	*Fusarium oxysporum*	*Fusarium*	—
P3C	*Himantoglossum adriaticum*	Parco San Lazzaro	ITS1	partial	*Diaporthe eucalyptorum*	*Diaporthe*	Asexual form: *Phomopsis* sp.
P3B	*Himantoglossum adriaticum*	Parco San Lazzaro	ITS1	~720	*Fusarium* sp.	*Fusarium*	Likely *F. solani* complex
P2D	*Ophrys apifera*	Parco della Resistenza	ITS1f	~750	*Chaetomium funicola*	*Chaetomium*	Saprotrophic Ascomycete
P1C	*Ophrys apifera*	Parco della Resistenza	ITS1	~730	*Chaetomium* sp.	*Chaetomium*	Species-level unresolved

Chapter 5:
Does phylogenetic relatedness predict conservation status in Italian orchids?

Abstract

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In an era of accelerating global biodiversity loss, understanding species' extinction risk within an evolutionary context is vital for effective conservation planning. Orchids, one of the most charismatic and ecologically specialized plant families, often face conservation challenges. Here, we examine the conservation status of 61 orchid species native to Italy through a phylogenetic lens. We map IUCN Red List categories onto a phylogeny constructed using the R package V.PhyloMaker, based on the GBOTB extended megatree of vascular plants, which incorporates the clade-level frameworks of Smith & Brown (2018) and Zanne et al. (2014)

Our analysis reveals that most of Italian orchids fall within the "Least Concern" category, suggesting relative ecological stability for many species. However, a subset of species classified as "Near Threatened" or "Endangered" forms distinct clusters within the phylogeny. This non-random distribution suggests that closely related species may share ecological traits, such as habitat specificity or pollinator dependence that increase vulnerability to environmental change, habitat fragmentation and over-collection.

Despite these insights, current research presents knowledge gaps, particularly in the spatial distribution and ecological requirements of Italian orchids. This highlights the need for comprehensive datasets combining conservation status, geographical range, and phylogenetic placement. Our ongoing work includes integrating distributional data into species profiles and visualizing them through maps and diagrams.

From a conservation standpoint, we advocate for the prioritization of phylogenetically distinct and vulnerable species in management plans. Incorporating evolutionary history into national conservation policies can improve resource allocation and help maintain both taxonomic and functional biodiversity. Furthermore, expanding this phylogenetic framework to encompass broader Mediterranean ecosystems could reveal deeper regional patterns of extinction risk and resilience.

Ultimately, our study underscores the power of phylogenetics in conservation biology—not only as a tool for understanding risk patterns but also as a strategic lens for anticipating and mitigating biodiversity loss in a changing world.

5.1. Introduction

Global biodiversity is declining at an unprecedented rate due to changes in land use, climate stress and habitat fragmentation. This threatens the long-term stability of ecosystems (IPBES, 2019; Nic Lughadha et al., 2020). The extinction of plants is particularly concerning because it removes essential ecological functions and irreplaceable evolutionary heritage (Forest et al., 2007). The Orchidaceae family, one of the largest and most diverse plant families, illustrates this crisis through its exceptional ecological specialization and narrow habitat tolerances, which heighten extinction vulnerability (Givnish et al., 2015; Swarts & Dixon, 2009). Traditional conservation assessments often treat species as individual entities, ignoring whether threatened taxa originate from evolutionary distinct or highly isolated lineages (Faith, 1992; Tucker et al., 2017). Consequently, threat categories based solely on species risk masking the loss of ancient evolutionary branches, especially in highly diverse plant groups such as orchids (Cadotte & Tucker, 2017; Vitt et al., 2023). This limitation has prompted increasing interest in integrating evolutionary information into conservation planning, with the aim of securing both species survival and evolutionary history simultaneously (Forest et al., 2007; Tucker et al., 2017).

Phylogenetic approaches provide a framework for evaluating biodiversity, measuring how extinction risk aligns with evolutionary relationships rather than relying solely on species counts (Faith, 1992; Tucker et al., 2017). These methods quantify evolutionary distinctness, lineage diversification patterns and phylogenetic signal, revealing which branches contribute disproportionately to evolutionary heritage (Cadotte & Tucker, 2017).

In orchids, for example, extinction risk often clusters within specific evolutionary lineages associated with traits such as specialist pollination, narrow ecological tolerance and limited dispersal capacity (Givnish et al., 2015; Vitt et al., 2023).

By examining threat categories within a phylogenetic structure, conservationists can identify lineages at risk before visible demographic decline occurs, thus improving the development of proactive policies (Forest et al., 2007; Jin & Qian, 2022). This strategy strengthens prioritization decisions by safeguarding the deep evolutionary history embodied within unique orchid lines, which standard assessments may overlook (Tucker et al., 2017).

Recent advances in plant phylogenetic tools, such as V.PhyloMaker and the extended GBOTB megatree, now allow researchers to create comprehensive vascular plant phylogenies, even when DNA sequence data is incomplete (Jin & Qian, 2022). These frameworks integrate regional floras into large evolutionary structures, enabling orchids to be assessed within a global phylogenetic framework and strengthening evolutionary conservation analyses (Smith & Brown, 2018; Jin & Qian, 2022).

Italy hosts one of the richest orchid floras in Europe. This is due to high ecological heterogeneity, post glacial refugia and Mediterranean climatic gradients, which promote endemism (Selvi et al., 2023). Many terrestrial orchids depend on specialized habitats, such as ancient grasslands and open forests, which makes them highly sensitive to changes in land use, agricultural abandonment and the pressures of tourism (Lussu et al., 2024). However, a substantial proportion of Italian orchids remain unassessed by the IUCN Red List, which leaves their official threat status uncertain and obscures national extinction baselines (IUCN, 2023). Of the taxa that have been assessed, several species fall within the 'Near Threatened' and 'Endangered' categories, highlighting the vulnerability of narrow-range orchids to ongoing environmental change (IUCN, 2023). Furthermore, IUCN data gaps hinder the integration of evolutionary metrics, preventing the detection of lineage-based risk patterns among native orchids. These challenges are exacerbated by the limited protection coverage provided by Natura 2000 sites, which do not adequately

represent orchid distribution hotspots (Lussu et al., 2024). Consequently, Italian orchid conservation requires a shift towards strategies that integrate phylogenetic evidence with threat assessment in order to prevent irreversible evolutionary loss (Vitt et al., 2023).

5.2. Aim of the study

This study aims to assess whether the conservation status of native Italian orchids can be predicted by their phylogenetic relatedness, by examining how extinction risk aligns with their evolutionary structure. More specifically, the research will seek to determine whether threatened species cluster within particular phylogenetic branches, and whether evolutionary distinctness corresponds with higher vulnerability. The study will evaluate the predictive value of phylogenetic information for orchid conservation planning in Italy, informing approaches that safeguard both species persistence and evolutionary heritage.

Adopting a phylogenetic approach provides a foundational framework for guiding targeted conservation actions, enabling the identification of evolutionarily distinct orchid lineages that require immediate protection.

5.3. Materials and Methods

5.3.1. Study area and species selection

The study focused on native Italian orchids, encompassing widespread and endemic taxa across the Italian peninsula, the Apennines and the Mediterranean islands. A total of 61 species were selected based on the availability of occurrence data and their representation within the IUCN Red List (IUCN, 2023). Species names were standardized according to The Plant List to ensure taxonomic consistency. Information on geographic distributions, ecological preferences and habitat specialization was compiled from national floras and published field studies (Lussu et al., 2024).

5.3.2. Conservation status data

Conservation status for each species was obtained primarily from the IUCN Red List and, where official IUCN evaluations were missing, from national assessments (IUCN, 2023). Species were classified into the following categories according to the IUCN system: Least Concern (LC), Near Threatened (NT), Vulnerable (VU), Endangered (EN) and Critically Endangered (CR). (Table 5.1)

5.3.3. Phylogenetic tree construction

A species level phylogeny was generated using V.PhyloMaker by integrating the Italian orchid species list with the GBOTB extended megatree (Zanne et al., 2014; Smith & Brown, 2018; Jin & Qian, 2019, 2022). This approach enabled the inclusion of all target species, even those lacking complete molecular data, by attaching them to known nodes based on taxonomy. The final phylogeny included the necessary branch lengths and hierarchical relationships for downstream phylogenetic metrics.

5.3.4. Phylogenetic analyses and Data Integration

Phylogenetic clustering of extinction risk was assessed using comparative analyses performed in R using the ape and V.PhyloMaker packages. Species distributions were mapped using occurrence records from IUCN Red List and national databases published. For ecological data integration, the result obtained by tree was then analyzed and linked to the threat per species or genus.

Table 5. 1: List of Italian orchid species used to construct the phylogenetic tree for subsequent evolutionary and conservation analyses.

species	**Genus**	**Family**	**Conservation status**	**Conservation reference**
Anacamptis coriophora	Anacamptis	Orchidecea e	LC	https://www.iucnredlist.org/species/175922/7143939
Anacamptis laxiflora	Anacamptis	Orchidecea e	LC	https://www.iucnredlist.org/species/164122/5733232
Anacamptis morio	Anacamptis	Orchidecea e	NT	https://www.iucnredlist.org/species/176030/7178502
Anacamptis pyramidalis	Anacamptis	Orchidecea e	LC	https://www.iucnredlist.org/species/175924/7144790
Barlia robertiana	Barlia	Orchidecea e	LC	https://www.iucnredlist.org/species/175926/7145415

Cephalanthera damasonium	Cephalanthera	Orchideceae	LC	https://www.iucnredlist.org/species/176005/7169186
Cephalanthera longifolia	Cephalanthera	Orchideceae	LC	https://www.iucnredlist.org/species/176001/7167753
Cephalanthera rubra	Cephalanthera	Orchideceae	LC	https://www.iucnredlist.org/species/176009/7170277
Chamorchis alpina	Chamorchis	Orchideceae	LC	https://www.iucnredlist.org/species/175955/7152992
Coeloglossum viride	Coeloglossum	Orchideceae	LC	https://www.iucnredlist.org/species/175937/7148522
Corallorhiza trifida	Corallorhiza	Orchideceae	LC	https://www.iucnredlist.org/species/175935/7147872
Cypripedium calceolus	Cypripedium	Orchideceae	NT	https://www.iucnredlist.org/species/162021/5532694
Dactylorhiza incarnata subsp. cruenta	Dactylorhiza	Orchideceae	DD	
Dactylorhiza insularis	Dactylorhiza	Orchideceae	DD	
Dactylorhiza romana	Dactylorhiza	Orchideceae	LC	https://www.iucnredlist.org/species/199617/9114589
Dactylorhiza sambucina	Dactylorhiza	Orchideceae	LC	https://www.iucnredlist.org/species/175983/7162528
Dactylorhiza traunsteineri	Dactylorhiza	Orchideceae	LC	https://www.iucnredlist.org/species/175938/7148946
Epipactis atrorubens	Epipactis	Orchideceae	LC	https://www.iucnredlist.org/species/176003/7168400
Epipactis helleborine	Epipactis	Orchideceae	LC	https://www.iucnredlist.org/species/175992/7164692
Epipactis palustris	Epipactis	Orchideceae	LC	https://www.iucnredlist.org/species/175923/7144352
Epipogium aphyllum	Epipogium	Orchideceae	LC	https://www.iucnredlist.org/species/176021/7174447
Gennaria diphylla	Gennaria	Orchideceae	EN	https://www.iucnredlist.org/species/176021/7174447
Goodyera repens	Goodyera	Orchideceae	LC	https://www.iucnredlist.org/species/175947/7151117
Gymnadenia conopsea	Gymnadenia	Orchideceae	LC	https://www.iucnredlist.org/species/175969/7157439
Gymnadenia odoratissima	Gymnadenia	Orchideceae	LC	https://www.iucnredlist.org/species/175997/7166278
Herminium monorchis	Herminium	Orchideceae	DD	https://www.iucnredlist.org/species/176016/7171970
Himantoglossum adriaticum	Himantoglossum	Orchideceae	LC	https://www.iucnredlist.org/species/162219/5559772
Limodorum abortivum	Limodorum	Orchideceae	LC	https://www.iucnredlist.org/species/175928/7145895

Liparis loeselii	Liparis	Orchidecea e	NT	https://www.iucnredlist.org/species/161960/5519865
Malaxis monophyllos	Malaxis	Orchidecea e	NT	https://www.iucnredlist.org/species/175946/7150794
Neotinea maculata	Neotinea	Orchidecea e	LC	https://www.iucnredlist.org/species/175949/7151638
Neotinea tridentata	Neotinea	Orchidecea e	LC	https://www.iucnredlist.org/species/176000/7167388
Neotinea ustulata	Neotinea	Orchidecea e	LC	https://www.iucnredlist.org/species/176036/7180745
Neottia cordata	Neottia	Orchidecea e	LC	https://www.iucnredlist.org/species/175931/7146749
Neottia nidus-avis	Neottia	Orchidecea e	LC	https://www.iucnredlist.org/species/175996/7165845
Neottia ovata	Neottia	Orchidecea e	LC	https://www.iucnredlist.org/species/175972/7159373
Nigritella corneliana	Nigritella	Orchidecea e	DD	
Nigritella widderi	Nigritella	Orchidecea e	EN	https://www.iucnredlist.org/species/176014/7171612
Ophrys apifera	Ophrys	Orchidecea e	LC	https://www.iucnredlist.org/species/176031/7178911
Ophrys bombyliflora	Ophrys	Orchidecea e	LC	https://www.iucnredlist.org/species/175930/7146492
Ophrys fusca subsp. funerea	Ophrys	Orchidecea e	LC	https://www.iucnredlist.org/species/175999/7166962
Ophrys lacaitae	Ophrys	Orchidecea e	LC	https://www.iucnredlist.org/species/87794901/87795457
Ophrys oestrifera subsp. montis-gargani	Ophrys	Orchidecea e	DD	
Ophrys speculum	Ophrys	Orchidecea e	LC	https://www.iucnredlist.org/species/176015/7171783
Ophrys sphegodes	Ophrys	Orchidecea e	LC	https://www.iucnredlist.org/species/165191/5988057
Ophrys tenthredinifera	Ophrys	Orchidecea e	LC	https://www.iucnredlist.org/species/175956/7153212
Orchis anthropophora	Orchis	Orchidecea e	LC	https://www.iucnredlist.org/species/176017/7172337
Orchis mascula	Orchis	Orchidecea e	LC	https://www.iucnredlist.org/species/176025/7175991
Orchis patens subsp. Brevicornis	Orchis	Orchidecea e	EN	https://www.iucnredlist.org/species/175961/7155352

Orchis pauciflora	Orchis	Orchideceae	LC	https://www.iucnredlist.org/species/175934/7147639
Orchis provincialis	Orchis	Orchideceae	LC	https://www.iucnredlist.org/species/165158/5983801
Orchis purpurea	Orchis	Orchideceae	LC	https://www.iucnredlist.org/species/175986/7163217
Orchis simia	Orchis	Orchideceae	LC	https://www.iucnredlist.org/species/175980/7161744
Orchis spitzelii subsp. Spitzelii	Orchis	Orchideceae	NT	https://www.iucnredlist.org/species/175982/7162283
Platanthera bifolia	Platanthera	Orchideceae	LC	https://www.iucnredlist.org/species/176018/7172664
Platanthera chlorantha	Platanthera	Orchideceae	EN	https://www.iucnredlist.org/species/176032/7179273
Serapias cordigera	Serapias	Orchideceae	LC	https://www.iucnredlist.org/species/175962/7155510
Serapias neglecta	Serapias	Orchideceae	NT	https://www.iucnredlist.org/species/175960/115613740
Serapias parviflora	Serapias	Orchideceae	LC	https://www.iucnredlist.org/species/175940/7149442
Serapias vomeracea	Serapias	Orchideceae	LC	https://www.iucnredlist.org/species/175954/7152700
Spiranthes spiralis	Spiranthes	Orchideceae	LC	https://www.iucnredlist.org/species/176035/7180363

5.4. Results and Discussion

A phylogenetic tree was constructed for 61 native Italian orchid species, revealing well-resolved clusters that corresponded to established genera (see Figure 5.1). Genera such as Orchis, Ophrys, Dactylorhiza, Serapias, Anacamptis and Epipactis formed distinct clades, reflecting their evolutionary relationships. Several smaller or monotypic genera, including Chamorchis, Corallorhiza and Gennaria, appeared as long, isolated branches, indicating high evolutionary distinctness. The overall topology was consistent with published seed plant phylogenies, confirming the suitability of the V.PhyloMaker- and GBOTB-based approach for integrating Italian orchid species.

Mapping IUCN Red List categories onto the phylogenetic tree revealed non-random patterns of extinction risk (Figure 5.1). Least Concern (LC) species (green) dominated the tree, forming extensive clusters within rapidly diversifying clades such as Anacamptis, Serapias and Ophrys. Near Threatened (NT) and Endangered (EN) species (orange and red) were scattered among specific lineages rather than being evenly distributed. Notably, EN species such as *Gennaria diphylla, Nigritella widderi, Platanthera chlorantha* and *Orchis patens subsp. brevicornis* occupied long, isolated branches, highlighting their evolutionary distinctiveness and potential vulnerability.

Several subclades were enriched in higher-risk species. For example, the Dactylorhiza–Orchis–Platanthera cluster contained multiple NT and EN taxa, suggesting that certain evolutionary lineages are disproportionately threatened. Long-branched monotypic or rare genera corresponded with EN categories, indicating that evolutionary uniqueness is associated with a higher risk of extinction. Conversely, Less Concern (LC) species tended to cluster in more species and phylogenetically shallow clades, suggesting that widespread, generalist lineages are less vulnerable to current threats.

Although approximately 75% of Italian orchid species included in this study are classified as 'Least Concern' (LC) on the IUCN Red List, consistent with their relatively broad distributions and currently stable populations according to formal Red List criteria (IUCN, 2023), this designation should not be interpreted as an absence of conservation concern. The LC category is assigned to taxa that do not meet the quantitative thresholds for higher-risk categories, but which may still be subject to ongoing and emerging threats, such as habitat degradation, climate change and intensified land use, all of which can impact population trends before these declines are captured in formal reassessments (IUCN Red List Categories and Criteria, Version 3.1; Challender et al., 2023; Wraith & Pickering, 2018). Indeed, recent conservation research suggests that the Red List criteria may underestimate ongoing declines in some taxa because localized or incipient threats have not yet triggered a change in category, and species classified as LC can still experience significant regional declines (Challender et al., 2023). Given that many orchid species are sensitive to microhabitat alterations and specialized ecological interactions, the apparent dominance of LC taxa in the phylogeny highlights the need for continued monitoring and conservation planning informed by phylogeny to detect subtle yet ecologically significant changes before species approach higher risk thresholds.

Within the Orchis clade, the phylogenetic co-occurrence of the endangered taxa *Orchis patens subsp. brevicornis* and *Orchis spitzelii subsp. spitzelii* highlights a lineage with pronounced ecological vulnerability (see Figure 5.2). *O. patens* is restricted to highly fragmented Mediterranean habitats and has a very narrow distribution in eastern Liguria. Local populations have declined due to habitat loss and changes in agricultural land use.

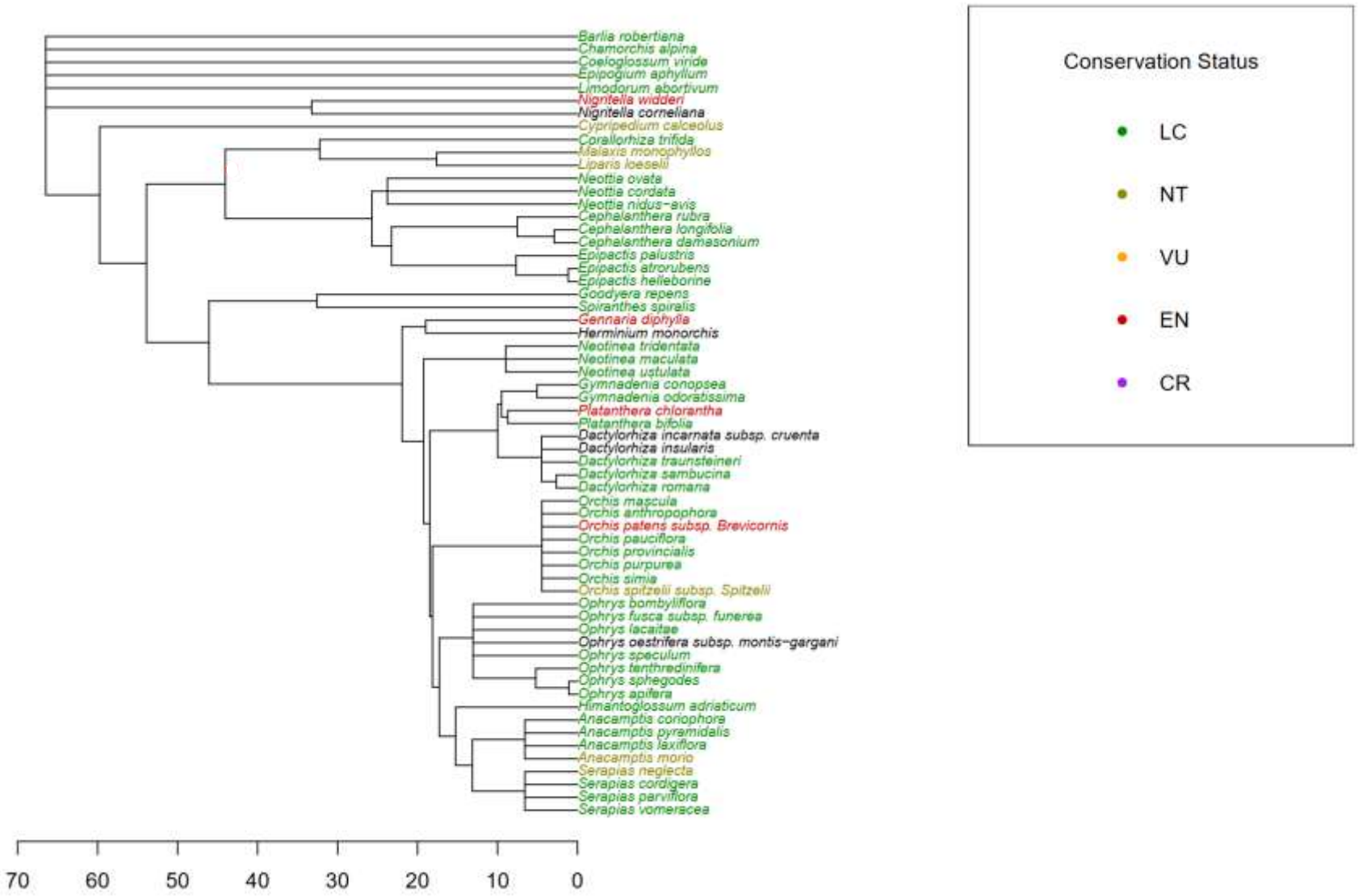

Figure 5. 1: Phylogenetic tree of 61 native Italian orchid species constructed using V.PhyloMaker. The color-coded tips represent IUCN Red List conservation categories (LC, NT, VU, EN).

This restricted range has led to its classification as 'Vulnerable' on the Mediterranean IUCN Red List, and it has been selected as a flagship species for targeted conservation programs such as LIFEorchids (IUCN, 2018; Calevo et al., 2021).

O. spitzelii also has a limited geographic distribution and low population densities within its alpine and sub-Mediterranean range. These traits increase their susceptibility to ongoing anthropogenic pressures and the loss of calcareous grassland habitats (Smith & Brown, 2018).

Both species are vulnerable to continued habitat degradation and fragmentation, as well as to climatic shifts that disrupt microclimatic conditions and the complex interactions between plants, mycorrhizal fungi and pollinators that are critical for reproduction. These factors are widely recognized as major drivers of orchid decline (Scramoncin et al., 2024; IPBES, 2019).

The clustering of these endangered species within the same evolutionary lineage suggests that shared ecological traits, such as narrow edaphic preferences and specialized reproductive ecology, may limit their adaptive potential in the face of rapid environmental change. Conservation efforts for these taxa and their clade should therefore prioritize in situ habitat protection, restoration of semi-natural grasslands and maintenance of pollinator communities; actions that are essential for stabilizing populations and preserving their evolutionary heritage. Integrative conservation planning that combines phylogenetic insight with targeted management interventions may help to protect these lineages against further decline.

Figure 5. 2: Phylogenetic placement of Endangered taxa within the Orchis clade, highlighting the close evolutionary relationship of Orchis patens subsp. brevicornis and Orchis spitzelii subsp. spitzelii to Least Concern relatives.

In the phylogeny, *Nigritella widderi* and *Nigritella corneliana* form a distinct, long-branched lineage, highlighting their phylogenetic isolation within the genus of Italian orchids. *N. widderi* is currently classified as Endangered, reflecting its extremely limited distribution in alpine meadows and its sensitivity to climate warming and changes in land use, both of which threaten its specialized high elevation habitat (Girardello et al., 2023; IUCN, 2023). Although *N. corneliana* is currently categorized as Data Deficient, its placement on a similarly long branch suggests evolutionary uniqueness and potential vulnerability, particularly in the context of future climate scenarios that disproportionately affect narrow range alpine species (Körner, 2003; Jump et al., 2009). The adjacency of these two species within the same isolated clade suggests that shared traits, such as narrow elevational ranges and dependence on specific pollination syndromes, constrain their adaptive capacity. Conservation of this clade must prioritize the protection of alpine grassland ecosystems and the monitoring of population trends in response to climate change. Given their deep evolutionary distinctness, the loss of either species would represent a disproportionate reduction in phylogenetic diversity among Italian orchids. (Figure 5.3).

Figure 5. 3: Phylogenetic Placement of Nigritella widderi and Nigritella corneliana Within the Italian Orchid Tree.

Gennaria diphylla occupies a distinct and isolated position within the phylogeny of Italian orchids, reflecting its status as a rare and geographically restricted species of Mediterranean terrestrial orchid. This species is primarily found in the western Mediterranean and Macaronesia, with scattered and declining populations in northern Sardinia and neighboring islands. Here, coastal development and habitat loss pose an ongoing threat to its localized populations (Rossi et al., 2023). While it is not globally assessed as threatened, its rarity in Italy and limited range suggest it may be vulnerable to future environmental changes. Pollination studies indicate that *G. diphylla* relies on a variety of nocturnal moth pollinators and has some capacity for self-fertilization. This mixed strategy may buffer against unstable pollinator services, but it does not fully mitigate the risks associated with small population sizes (Claessens et al., 2022). This reproductive ecology, combined with fragmented habitats, implies that *G. diphylla* could experience demographic declines before they are detected by formal assessments. Therefore, conservation actions should prioritize habitat protection and monitoring, particularly in Mediterranean woodlands and on rocky substrates where this species occurs, to preserve its unique lineage and prevent the erosion of phylogenetic diversity within Italian orchids.

Platanthera chlorantha and *P. bifolia* form a closely related lineage within the butterfly orchids (Figure 5.4). They have a widespread distribution across Eurasia, yet they have distinct ecological and conservation profiles (Esposito et al., 2018). *P. chlorantha* typically occupies unimproved meadows and woodland edges, and is vulnerable to agricultural improvement, soil enrichment and inappropriate management. These factors have driven local declines in parts of its range (BSBI, 2025b). In contrast, *P. bifolia* occurs across a broader range of habitats, including heaths, grasslands and woodlands. Although it is widespread, it has experienced significant regional declines, especially in lowland areas, where changes in land use and woodland disturbance have reduced its populations (BSBI, 2025a). Both species principally rely on nocturnal moth pollinators, and differences in pollinator assemblages can affect their reproductive success locally (Mõtlep et al., 2018). Genetic and morphological studies confirm their close relationship but also highlight the ecological divergence that helps to maintain species boundaries where they co-occur (Esposito et al., 2018). The contrasting population trends illustrate how closely related taxa can face very different conservation trajectories depending on habitat specificity and anthropogenic pressures. Therefore, conserving habitat continuity and monitoring population trends are essential to safeguard the more sensitive lineages of *P. chlorantha* and to maintain phylogenetic diversity within this orchid subclade.

Figure 5. 4: Clade Showing the Evolutionary Position and Conservation Status of Platanthera chlorantha and P. bifolia

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Chapter 6: Conservation and Reintroduction of *Allium angulosum* L. in Emilia-Romagna: Assessing Ex Situ and In Situ Strategies for Reinforcement in Parco della Resistenza

Abstract

Allium angulosum L. (Amaryllidaceae) which grows as a rare geophyte species throughout northern Italy's wet meadows from sea level up to 800 meters elevation (1). Although the Italian flora Red List does not include *A. angulosum*, in Emilia-Romagna is considered as Endangered species, and this is due to that habitat range is limited and its population numbers continue to decline (Regione Emilia-Romagna, 2017). The monitoring of *A. angulosum* populations in the territories of Modena province indicated its presence in hay meadows with periodic flooding, and in stable grasslands that are periodically watered in summer, and it is characterized by high plant biodiversity. After the last population at Tagliati di Albareto vanished from existence scientists declared the species extinct in this area, but a rising hope appears in 2020 where a discovery in Parco della Resistenza's urban park in Modena revealed approximately 50 living *A. angulosum* individuals which prompted new conservation programs (3).

The aim of this study is to evaluate the possibilities of reinforcing and reintroducing *A. angulosum* into Parco della Resistenza by planting seeds obtained from the rediscovered population. The collected seeds (total of 800 seeds) from the plants of Tagliati di Albareto, were divided into four separate experiments, 200 seeds were dehydrated and conserved in UNIMORE Seedbank, as a part of Ex-situ conservation practice. 200 seeds were sown in pots and kept outdoors. The other 200 seeds were sown in laboratory under controlled environmental parameters, light and temperature. And finally, 200 seeds were sown in winter in “Parco della Resistenza” in two different areas, using 100 seeds per each one, and two plantation intensities (high and low), under the title of In-situ conservation strategy.

The germination monitoring took place from November 2023 through July 2025. The laboratory environment produced a 40% germination outcome with a germination index (GI) of 0.93 by May 2024 that demonstrated improved both germination rates and speed. The field trial results showed that seed germination from November 2023 to May 2024, was very low with a percentage of 0.5% in both intensities combined. The reintroduction efforts faced challenges because weather conditions, specifically heavy rainfall, resulted in poor seedling survival rates during the field period.

These findings highlight the challenges of reintroducing *A. angulosum*, emphasizing the need for both ex-situ and in-situ conservation efforts. Future research and improved environmental management strategies are essential to enhance seedling survival and ensure the long-term persistence of the species in Emilia-Romagna.

Keywords: *Allium angulosum*, conservation, reintroduction, germination index, ex situ, in situ, endangered species, Emilia-Romagn.

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6.1. Introduction

Biodiversity loss stands as a major worldwide problem that started in the 21st century because of human-caused threats. The main threats to biodiversity consist of habitat destruction together with climate change and invasive species spread (Sala et al., 2000). The conservation of these species is vital, not only for maintaining the health of ecosystems, but also for preserving their cultural, economic, and medicinal value to human societies. The Allium genus contains numerous plant species which currently face extinction threats (IUCN Red List, 2024). One such species, *Allium angulosum L.*, is a rare geophyte found exclusively in the wet meadows and grasslands of northern Italy. The unique environmental features of these habitats support *A. angulosum* as a species which faces major conservation difficulties because of its restricted range and rising human impacts.

Allium angulosum L. (Amaryllidaceae), a diverse plant lineage that includes *Allium sativum* (garlic), *Allium cepa* (onions) and *Allium porrum* (leeks). *A. angulosum*, as with other members of the genus, it's classified as a bulbous perennial plant that utilizes underground storage organs, scientifically termed 'bulbs', for survival purposes and the propagation process in adverse environmental conditions (Pignatti, 2017). *A. angulosum* is a geophyte species that occurs in regions with high nutrient levels, and is found in lowland meadows, riparian grasslands, and mountainous areas across Central and Eastern Europe (Tutin et al., 1993). *A. angulosum* has been found to thrive in waterlogged or irrigated sites, which are prevalent in the floodplains of Emilia-Romagna (Pignatti, 2017). *A. angulosum* is closely related to the garlic *(A. sativum)* in terms of both genetics and ecology of the plant. It is evident that humankind has utilized garlic for millennia, employing it in both culinary practices and medicinal contexts (Mashayekhi et al., 2015). The flowers of *A. angulosum* are produced in compact umbels of purple-pink to purple color and appear between June and September. The bulbs of this plant have been documented to measure 5 mm in diameter, with the stem reaching a height of 40–50 cm (Tørresen et al, 2017).

As is the case with other bulbous species, *A. angulosum* reproduces via both sexual and asexual methods. The seeds are produced through sexual reproduction, and new bulbs are formed through vegetative reproduction. A comprehensive understanding of the seed biology of *A. angulosum* is imperative for the development of effective conservation strategies, as seed viability is a crucial factor in determining the success of both ex-situ and in situ restoration programs. *A. angulosum* seeds require cold stratification to overcome dormancy, as this process emulates the winter conditions in its native environment (Mashayekhi et al., 2015). The plant displays the archetypal geophyte life cycle, with the emergence of the bulb in the spring, flowering in the summer and dormancy during autumn and winter. *A. angulosum* seedlings are subject to a range of environmental influences, including soil moisture, temperature and light availability, which can have a significant impact on their survival rates (Richards, 1997).

A. angulosum exhibits germination behavior that is analogous to that of other Allium species. As posited by Specht et al. (1997) and in Hatzilazarou et al (2023), the process of seed germination in Allium species is predominantly influenced by a confluence of environmental factors, encompassing variables such as temperature, light intensity, and soil moisture levels. However, the low germination rates observed in field trials and the difficulties in establishing seedlings highlight the potential negative impacts of climate change and extreme weather events on the reintroduction and survival of *A. angulosum* populations. The successful reintroduction of the species to its native environment necessitates a comprehensive understanding of its seed biology, in conjunction with its ecological requirements and the development of strategies to combat environmental stressors that impede seedling development (Gauthier et al., 2017).

The Emilia-Romagna region in Italy is facing an imminent risk of extinction for *Allium angulosum* L, a species that is endemic to the area and is experiencing a decline in population size. The species is not currently protected from various human-induced threats, as it is not included in the Italian Red List. *A. angulosum* is facing considerable threats to its viability in its natural habitats. These threats are compounded by the effects of habitat destruction, climate change, and urbanization and agricultural expansion (Sochacki et al., 2024).

The Modena province is the exclusive habitat of *A. angulosum,* which is found in hay meadows and stable grasslands that are native to the area. The combination of seasonal flooding and summer irrigation practices creates suitable environmental conditions for its growth. The vital habitats of *A. angulosum*, which support high biodiversity, have been damaged by increasing threats that have a negative impact on the sustainability of its population (Pignatti, 2017). The Tagliati di Albareto area experienced a complete extirpation of its *A. angulosum* population during the early 2000s, resulting in a substantial biodiversity reduction in the region (Pignatti, 2017). The 2020 discovery of approximately 50 individuals of *A. angulosum* in the Parco della Resistenza area of Modena has led to the establishment of a positive outlook regarding the continued existence of the species. The discovery of the species has led to the initiation of new conservation projects, with the objective of increasing *A. angulosum* numbers and reestablishing its presence throughout the region (Regione Emilia-Romagna, 2020).

The survival of *A. angulosum* is contingent upon a dual conservation strategy that integrates ex situ within situ methodologies to ensure long-term preservation. As Gauthier et al. (2017) demonstrate, the practice of ex situ conservation, namely seed banking, is an effective method of safeguarding populations from immediate threats such as natural disasters and habitat destruction.

The long-term conservation of *A. angulosum* and its native ecosystem functions requires the implementation of both ex-situ and in-situ conservation strategies. The utilization of a combined method has been demonstrated to enhance the species' capacity to withstand environmental fluctuations, thereby ensuring its long-term viability.

6.2. Aim of this Study:

This study examines both *ex-situ* and *in-situ* conservation methods aimed at enhancing the population of *Allium angulosum* in Emilia-Romagna, with a particular focus on the reintroduction of the species to Parco della Resistenza, in Modena province. The research investigates key factors such as seed germination rates, plant survival, and environmental variables that influence the success of these conservation strategies. The outcomes of this research will not only contribute to the protection of *A. angulosum* but also inform the establishment of sustainable conservation techniques for rare and endangered species in similar ecosystems.

6.3. Materials and Methods

6.3.1. Study Sites and Cultivation Overview

This research, conducted on *Allium algulosum*, was conducted across three discrete locations to evaluate seed germination and early-stage growth under varied ecological circumstances. These settings included a controlled laboratory environment, a public park, and a private residence. The cultivation phase commenced in a controlled laboratory environment on 20th November 2023, with field sowing initiated in the experimental setting of Parco Delle Resistenza on 16th November 2023. A third cultivation site was located at the private residence; however, the exact sowing date at this location was not recorded.

The incorporation of both controlled and open-field environments was intended to furnish comparative data on the influence of substrate composition, environmental exposure, and cultivation intensity on the germination behavior and early development of *A. angulosum*. The two outdoor sites – Parco Delle Resistenza and private residence – are situated at similar geographic coordinates, allowing for consistent climatic conditions while differing in management practices and intensity. In the park, both high-intensity and low-intensity cultivation plots were established in order to further assess density-dependent responses in field conditions.

6.3.2. Plant Material

Plant material utilized in this study were seeds of *Allium algulosum*. A total of 800 seeds were used in all experimental setups. Specifically, 200 seeds were sown in the laboratory under controlled environment, 200 seeds were sown in Parco della Resistenza, with high and low density of sowing, 100 seeds per each place; 200 seeds were sown in pots at the private residence. An additional batch of 200 seeds was designated for desiccation to be later stored in UNIMORE Seedbank. The seeds were initially placed in a paper bag, which was then stored in an airtight glass jar with enough color-indicating silica gel. The silica gel functioned as a passive desiccant, gradually reducing the seed moisture content to a target range of 5–7%, which is the standard range for Allium species suitable for long-term conservation and germination studies (Keller & Kik, 2018). The sealed jar was maintained at room temperature for a period of 7 to 10 days, during which the silica gel absorbed moisture from the seeds through the paper bag as barrier without direct contact. It has been established that this method guarantees a controlled drying process that is both replicable and consistent with the accepted gene bank protocols. Finally seed have been stored at – 20 °C.

6.3.3. Growing Media Composition

Two distinct substrate mixtures were used over the course of the study, corresponding to different growth stages of *Allium angulosum*.

At the time of initial sowing on 20 November 2023, all seeds cultivated in the laboratory were planted in a medium composed of one part perlite to three parts substrates, corresponding to a volumetric ratio of 25% perlite to 75% substrate. This configuration was selected to provide an equilibrium between drainage, aeration, and moisture retention during the germination phase, thereby creating an optimal environment for seed germination.

On 8 May 2024, during the transplantation of seedlings into larger pots, a modified growing medium was utilized. The growing medium consists of perlite and substrate in a 3:5 volumetric ratio (equivalent to 37.5% perlite and 62.5% substrate). The increased perlite content was intended to enhance soil porosity and improve oxygen availability to developing root systems during the vegetative growth stage.

The same initial substrate formulation was employed consistently across the field-based experimental units to ensure uniformity. The substrate type remained constant throughout the experiment.

6.3.4. Cultivation Conditions and Field Design

The cultivation of *Allium angulosum* was conducted across three environments: a controlled laboratory (ex-situ cultivation), a semi-managed urban park (in-situ cultivation), and a private residential with same environmental conditions as the urban park (ex-situ cultivation). These environments (focusing on the first two) were selected to evaluate the species' germination and early growth responses under a gradient of cultivation conditions, ranging from fully controlled to naturalized field settings.

Laboratory Conditions

Seeds cultivated in the laboratory were maintained under standardized conditions to ensure consistency during germination. The temperature was kept at a constant 20 °C, with a 9/15 hrs. light/dark cycle. Watering was performed regularly to keep the substrate moist but not waterlogged, and no additional fertilizers or treatments were applied during the germination period until the 8th of May when the soil medium was changed. Later on 8 May 2024 adjusted environmental conditions were done to become, the temperature was kept at a constant 25 °C, with a 12/12 hrs. light/dark cycle. Watering was performed regularly to keep the substrate moist but not waterlogged, and no additional fertilizers or treatments were applied. Later, these large pots were removed to the terrace of the lab to become adapted to the daily temperature to be reintroduced later into the public park where it was initially found.

Field Cultivation: Parco Delle Resistenza

In the Parco Delle Resistenza, Modena, seeds were sown directly into the soil on 16 November 2023. The site is located at the same latitude and longitude as the second field site, enabling comparison under equivalent climatic conditions. The field plots were subdivided into two cultivation intensities:

- Low-Intensity Cultivation: Seeds were sown at wider spacing with minimal soil disturbance and no soil amendments.
- High-Intensity Cultivation: Seeds were sown more densely with minimal soil disturbance and no soil amendments.

Both field plots received only natural rainfall, and no irrigation or fertilization was applied. This design allowed for a comparative assessment of density-dependent growth and germination under semi-natural environmental exposure.

6.3.5. Germination Protocol

Germination monitoring was conducted on *Allium angulosum* seeds sown in the laboratory under controlled conditions, and field plots at Parco delle Resistenza.

A total of 200 seeds were observed over a period of 162 days, beginning on 20 November 2023 under laboratory conditions, and from 16 November 2023 in open field. The seeds were sown in two intensities high and low 100 seeds per each. The emergence of radicles was recorded as the criterion for germination.

Germination data were collected at regular intervals between 19 February 2024 and 1 May 2024, with seedling counts and cumulative germination percentages recorded on each date.

The **Germination Index (GI)** was calculated to quantify both the rate and timing of germination. The GI was computed using the formula provided by Melville et al. (1980), as follows:

$GI = \sum(|(T_i - T_0)N_i)|/N_t)$ or $$GI = \sum_{i=1}^{k} \frac{|(T_k - T_i)\, N_i|}{N_t}$$

Equation 1: Germination Index Equation

Where:

- T_i= time (in days) from sowing to the *i*-th observation
- T_0= time of the first germination event (92 days after sowing)
- N_i= number of seeds germinated at time Ti
- N_t= total number of seeds used (200 in this case)

This method emphasizes both the **speed** and **uniformity** of germination. Observations continued until germination plateaued.

6.3.6. Statistical Analysis

Germination data were tabulated as absolute numbers, cumulative percentages, and GI values (table 6.1). Descriptive statistics—including seed counts, cumulative germination percentages, and calculated GI values—were used to summarize germination performance over time. No inferential statistical analysis was applied at this stage, but the structure of the dataset allows for future comparison between treatments and cultivation environments.

Data from field plots were also recorded as part of the dataset; however, due to extreme environmental conditions, germination outcomes were limited and could not be statistically analyzed in depth. All data were compiled and organized using Microsoft Excel 365, and GI calculations were manually verified for accuracy. Numerical values were standardized to three decimal places throughout.

Table 6. 1: Germination Progress of Allium angulosum Under Controlled Laboratory Conditions: Seed Count, Germination Percentage, and Germination Index (GI) Over Time

Date	Seeds Germinated	% Germination	Germination Index (GI)
19 Feb 2024	3	1.5%	0.000
28 Feb 2024	5	2.5%	0.135
13 Mar 2024	7	3.5%	0.345
18 Mar 2024	11	5.5%	0.420
21 Mar 2024	19	9.5%	0.480
25 Mar 2024	23	11.5%	0.525
2 Apr 2024	25	12.5%	0.645
15 Apr 2024	62	31.0%	0.840
1 May 2024	80	40.0%	0.930

6.4. Results and Discussion

6.4.1. Germination in Laboratory Conditions:

The present study examined the process of germination in *Allium angulosum* under controlled laboratory conditions. The results demonstrated a clear progression in both the percentage of seeds that germinated and the calculated Germination Index (GI) over time. In the experiment conducted on 20 November 2023, in which 200 seeds were sown, 80 seeds germinated, thus yielding a final germination percentage of 40% by 1 May 2024. Although the overall germination rate was moderate, the temporal pattern of germination was notably slow during the initial phase, followed by a gradual acceleration in the later stages of the monitoring period.

The initial observable germination was recorded on 19 February 2024, 92 days after the initial sowing. This was preceded by the emergence of radicles on three seeds, indicating seed germination. The process of germination was characterized by a gradual progression throughout the months of February and March, with a mere 25 seeds successfully germinated by the 2nd of April 2024. A significant escalation was evident between 2 April and 15 April, during which the number of germinated seeds increased more than twofold, reaching 62. The final peak was observed on the 1st of May 2024, with 80 seeds successfully germinated, resulting in a corresponding GI value of 0.930.

This progression is illustrated in Figure 6.1, which presents the relationship between the percentage of germinated seeds and GI across the nine observation dates. It is evident from the data that both values demonstrate a cumulative upward trend, with GI increasing steadily in alignment with seedling emergence (see also Table 6.1).

The delayed onset of germination suggests that *Allium angulosum* may possess innate dormancy mechanisms or may require extended periods of imbibition and physiological activation under cool conditions. It is important to note that comparable delays have been documented in associated species of Allium. These species have been shown to exhibit a dependence on temperature, light, and moisture as crucial elements in the process of overcoming dormancy, as outlined by Kamenetsky and Rabinowitch (2006), and further confirmed by Keller and Kik (2018). The increase in germination activity observed in April may be indicative of a delayed response to accumulated moisture or temperature changes, even within the controlled environment of a laboratory setting.

Despite the relatively modest final germination percentage of 40%, the controlled conditions proved conducive to sustained germination over time, as evidenced by the consistent increase in GI values, which reflects the uniform vitality of the seeds. These outcomes lend support to the potential of *A. angulosum* for successful cultivation in protected environments, particularly when germination protocols allow sufficient time for dormancy to be overcome.

It is suggested by previous studies on Allium species that delayed germination is often linked to environmental factors and seed dormancy traits. For instance, *Allium cepa* and *Allium schoenoprasum* have been observed to exhibit comparable lag phases prior to radicle emergence under cool or variable temperature conditions (Brewster, 2008; Benech-Arnold & Sánchez, 2004). A study by Menna et al. (2023) demonstrated that the process of germination in wild *Allium* taxa was significantly enhanced when seeds were subjected to warm stratification or gibberellic acid treatments. This finding indicates that hormonal regulation of dormancy plays a crucial role in the germination process. Despite the absence of such treatments in the present study, the outcomes lend support to the hypothesis that *A. angulosum* exhibits physiological dormancy characteristics analogous to those observed in other members of the genus. Further experimentation with pre-

sowing treatments may increase germination uniformity and reduce the time it takes under laboratory conditions.

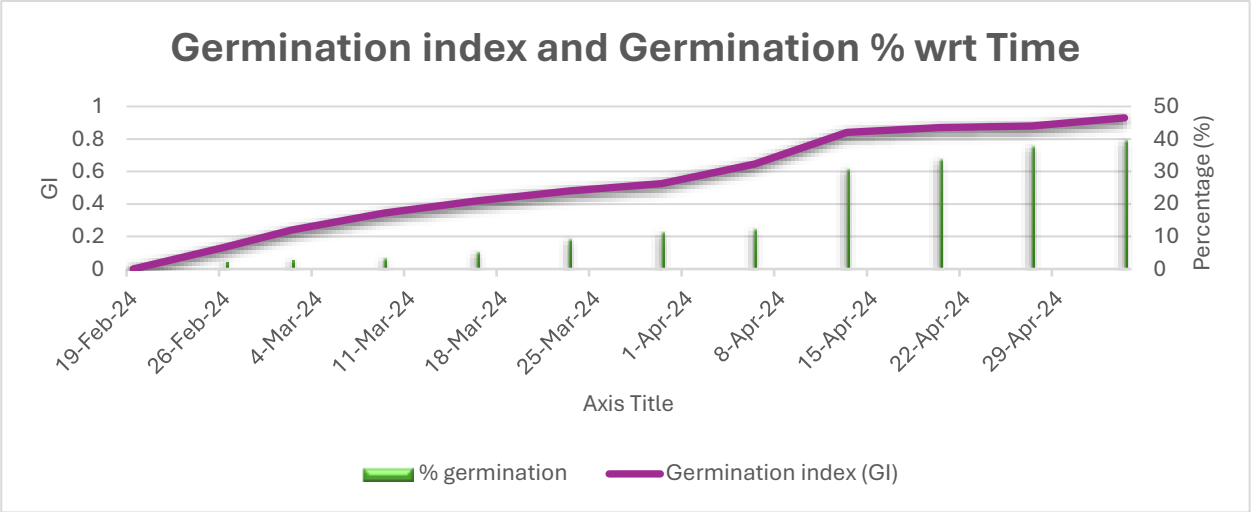

Figure 6. 1: Germination Progress of Allium Angulosum Over Time in Laboratory under controlled environmental conditions

6.4.2. Field and Private Residence Germination Outcomes

Field trials were conducted in Parco Delle Resistenza to evaluate the germination capacity of *Allium angulosum* under open-air conditions, reflecting low-input cultivation approaches. Two density treatments were established: low-intensity and high-intensity cultivation. Each treatment received 100 seeds, which were sown directly into unamended soil on 16 November 2023. The objective of this study was to simulate both traditional and space-efficient planting systems to assess their suitability for the species in a temperate field setting.

By 20 March 2024, germination had been observed in both plots, though the process was markedly uneven. As demonstrated in Table 6.2, the low-intensity plot exhibited 15 instances of successful germination, amounting to 15% of the total seeds examined. In contrast, the high-intensity plot exhibited a mere 1 instance of successful germination, amounting to 1% of the total seeds examined. The observed disparity indicates that *A. angulosum* exhibits sensitivity to sowing density, a susceptibility that is presumably attributable to inter-seed competition for essential resources such as light, oxygen, and moisture. These factors are particularly salient in compact or poorly draining soils (Bewley et al., 2013; Baskin et al., 2000).

Table 6. 2: Field Germination Summary

Cultivation Type	Seeds Sown	Seeds Germinated	Germination (%)
Low-Intensity (Park)	100	15	15%
High-Intensity (Park)	100	1	1%

After the initial germination period, a region experienced prolonged and intense rainfall, resulting in waterlogging across the field site. No further germination was recorded, and the previously emerged seedlings were no longer visible by the end of March. This outcome indicates that *A. angulosum* seedlings are highly susceptible to hydric stress, especially during the early establishment phase.

Seedling loss in such conditions is often associated with hypoxia-induced root damage, increased microbial activity, and seed decay (Dutta et al., 2020).

The combination of low emergence rates and complete seedling loss under wet conditions highlights the vulnerability of *A. angulosum* to unregulated field environments. This finding aligns with broader observations in bulbous perennials, where the necessity of well-drained substrates and controlled irrigation during the establishment phase has been identified (Benkeblia, 2024).

Furthermore, the reduced performance observed in the high-density plot is likely indicative of density-dependent inhibition, a well-documented phenomenon in the field of monocot germination (Finch-Savage & Bassel, 2016). In such cases, limited spatial availability has been shown to restrict root expansion, thereby intensifying intra-specific competition. The findings of this study indicate that *A. angulosum* may benefit from the implementation of spacing strategies that achieve a balance between the availability of resources and the suppression of weeds, particularly in environments that are exposed to rainfall.

It is important to note that despite the suboptimal conditions, this field trial has yielded significant preliminary data, which will contribute to the optimization of outdoor cultivation protocols. To enhance the success rate of future trials, it is recommended that the following considerations be considered: The utilization of raised beds or sloped plots has been identified as a strategy to mitigate surface water retention. The application of mulch has been demonstrated to have a positive effect on the stability of soil structure and temperature. The optimal time for sowing is in late spring, when precipitation patterns are more predictable. One potential method of protection during the process of germination is the integration of plastic row covers or rain shelters.

The results of a private residence cultivation showed 28% germination, 56 seeds out of 200 germinated. Where it is under the same environmental conditions as open field except that the watering is done regularly three times per week

6.5. Conclusion

This research provides novel insights into the seed germination patterns of *Allium angulosum*, a rare Italian species endemic to the northern part of the country. This research combines laboratory-based ex situ conservation techniques with field-based in situ conservation techniques, thereby enabling the evaluation of seed germination under artificial versus natural environments.

A. angulosum seeds have been shown to be viable, but their germination process is time-consuming and has a low success rate. The observed behavior of seeds corresponds to the characteristics of physiological dormancy. Laboratory trials demonstrated that seed germination progressed at a consistent rate until reaching 40%, exhibiting a Germination Index of 0.93. The successful cultivation of this species in a controlled environment is contingent upon the establishment of optimal conditions for seed propagation.

The findings from field tests conducted in real-world environments demonstrated unsatisfactory outcomes, with germination reaching only 15% in low-density settings and a mere 1% in high-density settings. The entire batch of seedlings perished as a consequence of prolonged periods of waterlogging in combination with inclement weather conditions. The findings indicate that the species exhibits extreme sensitivity to abiotic stress factors, encompassing both excessive moisture and inadequate soil aeration, in addition to deleterious effects arising from elevated sowing densities on seedling survival.

The evidence collected demonstrates the absolute necessity for highly specific conservation plans. The efficacy of reintroduction programs and habitat restoration is contingent upon the implementation of field cultivation, necessitating the enhancement of drainage systems (e.g. raised beds) and the utilization of protective covers during pivotal growth stages and sowing schedules, with the objective of synchronizing these practices with regional rainfall patterns. The efficacy of seed treatment procedures, encompassing cold stratification and hormonal priming, has been demonstrated in accelerating seed germination and achieving synchronized germination.

To ensure the long-term survival of *A. angulosum*, conservation efforts must be combined with seed banking and ex-situ propagation techniques, alongside ecologically based reintroduction methods. The methodological approach that has been established, in conjunction with the preliminary results of the present research, provides a foundation upon which the extension of restoration initiatives in Emilia-Romagna may be built. Furthermore, it offers a solution that is applicable to the protection of rare moisture-sensitive species in the context of current climate change.

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Acknowledgment:

First and foremost, I thank God for granting me strength, patience, and guidance throughout this long and challenging journey. I would like to express my deepest gratitude to my family, whose unconditional love, sacrifices, and unwavering support have been my foundation throughout my academic path, especially since I am far away from them. Their belief in me sustained me during moments of doubt and gave me the courage to persevere.

My sincere thanks go to my supervisors, Prof. Elisabetta Sgarbi and Prof. Anna Maria Mercuri, for their invaluable guidance, scientific rigor, and continuous encouragement during my PhD. I am deeply grateful for their mentorship, constructive feedback, and trust in my work. I also wish to thank all members of my advisory and examination committees for their insightful comments and support.

My sincere appreciation is also extended to Prof. Stefano Lugli and Prof. Marina Cocchi, Previous and Current Deans of PhD School, Models and Methods for Material and Environmental Science, and to Prof. Mauro Soldati, Head of the Department of Chemical and Geological Sciences, for providing a supportive academic environment and for their institutional guidance throughout my doctoral studies. I further thank all members of the academic and administrative staff of UNIMORE for their assistance and professionalism. Especially Prof. Lorena Rebecchi, Component of Scientific Committee of the National Biodiversity Future Center (NBFC), Scientific Coordinator of the Unit NBFC – UNIMORE, for providing funds for my research.

I extend my appreciation to my colleagues and collaborators, particularly Claudio Brandoli, Giulia Santunione, Prof. Lara Maistrello, Dr. Federica Caradonia, Dr. Daniele Sommaggio, Leonardo Cicala, Gioele Toselli, Niccolò Patelli, and Dr. Justyna Anna Milc, for their scientific discussions, shared challenges, and the collegial environment that made this journey both enriching and memorable.

I would also like to express my sincere gratitude to Prof. Thais Vasconcelos, Prof. Marjorie Weber, and Prof. Thais Vasconcelos, and their team at LSA Ecology and Evolutionary Biology Department at UMICH, USA, who supervised and supported me during my study abroad period in the United States. Their scientific guidance, availability, and encouragement greatly enriched my research experience and contributed significantly to both my academic and professional development.

I am deeply thankful to Italy for the opportunity to pursue my PhD within its distinguished academic tradition and vibrant cultural landscape, an experience that has profoundly shaped my growth and brought me closer to realizing my lifelong dreams.

Finally, I dedicate this work to my country, Lebanon, a land of resilience, knowledge, and perseverance. My country that always rises from its ashes. I hold in my heart profound respect and sorrow for those who lost their lives during the war, especially the martyrs whose sacrifices remind us daily of the value of dignity, hope, and endurance. This thesis stands not only as an academic achievement, but also as a personal testament to perseverance in the face of adversity.