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Manual for the collection, study, *ex situ* processing and conservation of germplasm

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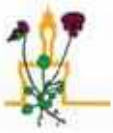

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PRESENTATION

In 2001 the Governments of the European Union pledged to reach an ambitious objective: to arrest the loss of biodiversity in Europe by 2010. This goal appears in the EU Strategy sustainable development as well as in the Sixth Action Program for the Environment (2002 - 2012) and, in the course of the meeting of the 9th March 2006 of the European Council, this commitment was again unanimously confirmed. The biological resources are fundamental for our survival and their loss jeopardizes the existence of individual species, habitats and whole ecosystems. The loss of biological diversity though variable in different locations, is estimated to be taking place at a rate of between 50 and 1,000 times the natural extinction rate.

APAT is engaged on many fronts in the struggle to defend biodiversity. This document discusses conservation aspects , particularly the conservation of germplasm outside the natural environment (*ex situ*), that is the material capable of transmitting the hereditary characters, that allows us to protect in a direct manner the biodiversity of species at the genetic level, while indirectly contributing to the variety of ecosystems. The term germplasm was often limited only to seeds but today, thanks to effective modern technologies, is frequent applied to preserve other entities capable of transmitting the hereditary material such as pollen, explants, cuttings and spore. For cultivated plants, *ex situ* conservation has been relentlessly practiced on a scientific bases for almost a century, even at the level of highly organized global networks. For instance, in these very days, the Norwegian Government has just given the go ahead for the construction of the largest World Bank for the conservation of seeds of agricultural plants, and will eventually contain the cumulative result of 10,000 years of plant domestication.

In the past, the necessity to preserve plants for alimentary and industrial uses was given priority over wild and non domesticated species, but today there exists sufficient scientific reason for very obvious reasons to conserve even these wild species and their ecosystems, since these translate in benefit for all living things included man. Recently RIBES – the Italian Network of germplasm banks, formed from around twenty public and private foundations as well as non profit organisations, was constituted for the *ex situ* preservation of indigenous wild flora, and is actively associated with analogous European networks. The task of undertaking the *ex situ* conservation of all the genetic resources that we currently possess and which we will need in the future, must be undertaken in the best possible manner. It is important to understand the entire cycle involved in preserving the material entrusted for safekeeping, from beginning to the end.

And precisely for this reason we are proposing the present manual that undertakes to describe all that we know about the collection, preservation and ex situ management of the germplasm derived from taxonomic units of the native flora of the Mediterranean lands. Being aware that the work is far from finished, APAT will work to collect more information and suggestions and to maintain a constant updating of this manual.

Giancarlo Viglione

Commissario straordinario

PRESENTATION

As an institution, APAT is responsible for technical-scientific activities for the protection of nature and the environmental and more specifically ones related to the protection of the natural resources, all of which are of national interest. These responsibilities result from the objectives outlined in the three-year activity plan of the Department for Conservation of Nature. By virtue of its different institutional tasks, several manuals on the protection of nature have been compiled over the past years; in particular, those dealing with the *ex situ* conservation of plant biodiversity.

For this reason, APAT decided to compile a manual that was useful to technical persons operating in the specific area of the germplasm conservation and management. To execute this task, the Department for the Conservation of Nature, in collaboration with the Centre for Conservation of Biodiversity (CCB) of the University of Calgiari, has compiled this manual using easy to understand language and terminology whilst maintaining the level of scientific rigor that is expected from such a manual. Throughout its preparation, the subjects have been treated with the highest possible detail as merits the high level of specialization that has been reached in the field of germplasm conservation.

The main objective was that of uniting authors with different areas of specialisation (21 authors from 15 institutions) present in various countries (Italy, Spain and France), with the purpose of creating a useful tool to on how to operate in institutional and academic fields, as well as in technical areas like germplasm banks or in a productive area such as in the flower nurseries. This volume, therefore, is the fruit of authors in various areas of expertise, field workers, and the results from the Interreg III B Genmedoc project, which led to the setting up of a Mediterranean network of germplasm banks. It needs to be pointed out that, inspite of the flora of such territories being quite well known and backed by numerous scientific publications specifically dealing with conservation, there is no working manual or software to guarantee effective results in *in situ* conservation throughout the Mediterranean, and especially in our country. This publication is being issued to address such a deficit, and to provide a starting point for future operations to succeed in a more coordinated manner, following ethical criteria and methods for effective *ex situ* conservation of biodiversity.

The ultimate aim is for Italy to contribute actively to plant conservation in compliance with the Convention on Biological Diversity to which Italy is a signatory, the Global Strategy for Plant Conservation and the European Strategy for Plant Conservation.

Marisa Amadei
Director, Nature Conservation, APAT

PRESENTATION

My comments after reading this manual must start with a personal memory. As a young investigator of one of the more active laboratories in Italy in the study of the biology of seeds, I was invited in the then Czechoslovakian Republic to hold a series of seminars in some research centres members of the Czech Academy of Sciences. My research was linked to the study of factors that control the dormancy of seeds, giving me my first opportunity to visit a germplasm bank. Immediately I took note of the great interest that colleagues placed in this area of research and the attention dedicated for the collection, cataloguing and conservation of the plant genetic diversity of their nation. Their speeches underlined the need to reduce and eliminate the possible loss of biodiversity especially those plants of great agronomic interest. The contrast between the organisation of that area of research and mine was clear. In fact, in the case of the germplasm bank the simplicity of organisation and near total absence of expensive instruments was a dominating factor. This concept of research clearly contrasted with my illusion that the most promising research required the latest models of scientific equipment. For the past thirty years, mass-media of well-developed countries have constantly highlighted the loss of existing biodiversity and outlined the need to put into effect measures of protection and conservation. I must therefore recognise with bitter irony, that even without expensive equipment, these colleagues were already in a line of research of the greatest and contemporary importance. Moreover, even without the aid of the first computers that began to appear in research laboratories in the West, I had noticed how that structure of research took care to participate in an efficient communication between all the germplasm banks of Eastern Europe.

Nowadays in a Europe with enlarged borders, we are made aware of the setting up of new germplasm banks: each answering to its own characteristic traits and strongly reflecting the needs of their territories. Obviously different structure are equipped and have at their disposal different technologies, sometimes even within the same institution, and this has created differences that contrast even in the management of a single structure. These differences in the organisation of germplasm banks brings out the urgent need to re-unite them with the scope of guaranteeing greater cooperation and integration and also ensure an increase in total efficiency and the possibility for each to move beyond the institutional needs, both local and national. At the same time, in a world increasingly dedicated to globalisation, the requirement exists to connect these national initiatives with those at a global level. Attainment of this final objective is rendered easier with computerised networks and the web, and will be the only way to guarantee the conservation of biodiversity in all its aspects. Therefore this handbook arrives at a good time and is a necessary instrument for all those involved in the management of germplasm banks. It represents a clear synthesis of the aims of these research structures and exhaustively examines the activities carried out in conservation both *in situ* and *ex situ*. The formulation is to delineate and therefore it will be able to carry out, beyond the role of practical guide, the role of public awareness on the fundamentals of the protection and conservation of biodiversity.

In conclusion I must pronounce, in the name of the Italian Botanical Society and my staff, of being in debt to all those who have contributed in some way to the realisation of this handbook. At the same time I augur that the limitations of scientific research can be overcome in order to contribute to all citizens, and in particular to public

administrators, the level of knowledge necessary to guarantee that these structures are in the best position to carry out their indispensable research activity.

Donato Chiatante
President of the Italian Botanical Society

PRESENTATION

Existence according to the laws of Nature has been seen, has been observed in an ignorant manner, by those peoples that although with little distances from the proper habitats, increased and decreased on the basis of natural disposition, without damaging the environment, in a complete connection with homeostatic knowledge of the earth. An harmonic cycle in which harmony does not refer to aesthetics but to equilibrium, to sustainability.

Man then started, this time in shorter periods, to make excessive use of resources available, to an unbalanced exploitation. Two examples suffice: transformation of the territory starting with deforestation undertaken by the Roman Empire and the present consumption per capita of Europeans (triple of what the Earth offers per each individual). One cannot apologise for the life of primitive man, that is unthinkable, but it is important that original natural habitats, capable of generating and protecting diversity, are under siege from urbanisation, industry, by allochthonous plants and animals, and climatic changes. Even the best of management is not always enough, alone, to take care of our treasures. Human history, on the whole, is continuously on the search of equilibrium.

Therefore, in front of such limitations, if not an impossibility, to manage all the resources *in situ* there is a need for germplasm banks or designated areas to protect with care the necessary material to perpetuate biodiversity. It is not by chance that one speaks of “seed banks”, “spore banks”, “tissue banks”; banks able to safeguard in the long term these precious materials and banks because institutions able to make the best possible use of the capital deposited. It is not therefore passive conservation: in germplasm banks many biological aspects are observed, for example, a seed collection of a rare species serves for nothing unless there is no knowledge on its propagation.

This Manual, which APAT has prepared in collaboration with numerous Spanish and Italian researchers, aims to be a guide for the operations of gathering, cleaning, processing, conservation, qualitative analysis and propagation of germplasm of natural plants in Mediterranean areas. The work is not complete but we believe it can become a work of reference for those who work in this field.

Paolo Gasparri
Responsible for Parks and Natural Resources at APAT

PREFACE

The objective of this manual is constituted by the germplasm of taxonomic units relative to autochthonous flora of Mediterranean territories and, in more general terms, European ones. This text is predisposed for operations on field and in laboratories following simple and clear instructions in the context of a rigorous conservation *in situ* and *ex situ*, respecting biodiversity, the territory and culture from which it is obtained. It is retained that the following methodologies can make easier the work of gatherers, bank curators, guaranteeing the collection, treatment and best management possible of germplasm, with respect to procedures and the national and international standards.This work is directed not only to those working in specific sectors (bank technicians, nurseries, those assigned to institutional work, researchers and university lecturers) as well as those persons interested in the subject of *ex situ* conservation in its wider context. Particular attention is given to students, to whom we tried to transfer, in simple terms, the research experience and results of experimentation undertaken by the Authors as well as the scientific literature available, with the objective of furnishing them with the most important elements of this field of study. It is important to remember that the protection of biodiversity through conservation of genetic resources is not always reserved to endemic species or species of phytogeographic interest, but also plants of more widespread distribution in Europe, as well as those potentially threatened by increased restrictions on their area of distribution. Suffice it to reflect on the drastic reduction in area and highly fragmented forest formations of the Italian Padana Plains, which due to agricultural expansion now occupy a territory equivalent to 1% of the original (Gorian, *in verbis).* The manual is structured into 14 chapters that describe the commonly used methodologies and covers the entire cycle from the point of field collection of the germplasm to its handling up to storage and conservation. The actions described in the manual refer to the conservation of modest quantities of germplasm; however it does not exclude the possibility of adapting the manual to larger quantities that may entail the use mechanically operated techniques. In the text several field, laboratory and nursery schedules and protocols are included, which permit the management and the monitoring of seeds lots during all the stages of preservation; furthermore, such schedules have provisions through the use of appropriate software, for the storage of all relevant data in electronic format for future reference. Finally, a technical glossary of terms considered important for a better understanding of the text, has been included. For the systematic nomenclature reference has been made to the recent work of Conti et al., (2005) a work executed on assignment by the Directorate for the Protection of the Nature within the Ministry of the Environment and Protection of the Territory. The present work cannot and does not want to be described as the definitive guide, but should be considered as a dynamic tool in a constant state of development that can serve as a reference point for common methodologies. Space is left open for useful suggestions and comments that will help to improve the quality of this manual. It circulation to a wider public and updates will be possible via the web on the internet site www.apat.it. the translation of the manual in more languages is also envisaged to make it accessible to a wider public

The Authors

1. Introduction

In simplified definition, germplasm is considered to be "that material capable of transmission of hereditary characteristics from one generation to another" (Witt, 1985). More precisely, germplasm is the physical unit of heredity, or the sum of genes as well as cytoplasmic factors governing heredity. Therefore when using the term germplasm, one must encompass various structures including spores, pollen, parts and tissues of plants, individual cells, DNA and RNA as well as seeds, which represent the most commonly used organ in the replication of higher plants. Seeds are also the most widely conserved material. The term 'germplasm' is composed of the root words 'germ' meaning beginning or origin and 'plasm', meaning material capable of generation of other living material similar or equal to the starting point (Perrino *et* Terzi, 2003). In brief, the word 'germplasm' means any form of life and can be reported, in virtue of the diverse taxonomic ranges, to a genus (e.g. germplasm of *Olea*) or to more a specific unit (e.g. germplasm of *Olea europaea* L.) or even to an infraspecific rank (e.g. *Olea europaea* L. var. *sylvestris* Brot.). The expression 'genetic resources' frequently substitutes the concept of 'germplasm' when referring contextually to several species or genera derived from different kingdoms (floral genetic resources, microbial genetic resources, etc.).

Since the beginning of civilisation and agriculture, farmers (and plant propagators) have been responsible for seed collection and preservation for successive seasons. The idea to conserve seeds of numerous species throughout the world and under conditions capable of maintaining long term viability, started in the beginning of the last century through the Russian student Nikolai Vavilov (Koo *et al*., 2004). Being a large and poor nation, Russia, asked Valivov to increase the species' yield for food and industry through genetic improvements. To achieve this objective, within thirty years he created and catalogued an immense scientific collection. This germplasm, conserved ex-situ, outlines one such effort to protect floral biodiversity. The structures that nowadays house and preserve the biodiversity contained in the different types of germplasm are called germplasm banks (*gene banks*), or alternatively *seed banks*, if the material is mostly conserved in the form of seeds. The samples of collected material that are introduced in germplasm banks for conservation purposes are technically referred to as 'accessions'. Each accession represents the acquisition by the bank of a germplasm sample related to a single collection, for a particular taxon of a specific population that has been identified with certainty.

It is important to underline that up to a few years ago the seed banks had focused their attention almost exclusively on the conservation of seeds or plant varieties of agricultural importance or their wild relatives. Ninety percent of all accessions present in seed banks is in fact represented by food species and, common plants which on a global scale are intensively cultivated and represent economically important crop species. The recent change of direction by seed banks to preserve rare or nearly extinct flora is a result of the implementation of specific obligations as dictated by specific conservation rules such as those set by the Convention on Biological Diversity (CBD). For such banks, the criteria of rarity, vulnerability and endemicity are the prime considerations in the choice of material to protect and cultivate, without omission of entities often considered to be marginal, but equally important in their contribution to biodiversity. As a result of agreements with the FAO, nowadays there are around a million floral accessions conserved thanks to the activity of 1,300 seed banks. These

represent only a small fraction of worldwide biodiversity and many important regions in our planet have yet to plan this type of action. The importance to conserve diversity has been demonstrated and is nowadays an accepted concept. It is enough to consider that everyone's life is directly or indirectly independent on the conservation of this biological diversity as this guarantees the existence and prolongation of suitable conditions for the environment and evolution of life itself (Perrino *et* Terzo, *op. cit.*).

1.1 Ethics and philosophy of conservation *ex situ* and *in situ*

For 10,000 years different forms of germplasm but more specifically the seeds, have been conserved by those taking care of the propagation of the plants and this for a multitude of reasons. Germplasm and seed banks in a modern sense are one of the best means available to prevent the loss of genetic biodiversity, and therefore to guarantee a future to a species in danger of extinction. Like the majority of structures identified for the conservation of the biodiversity, they are born with the aim of decreasing the exponential loss of species, whether natural phenomena, destructive anthropogenic activities and atmospheric pollution. Their function is not only to safeguard seeds of the species in danger of extinction, but also to conserve over a long time span the use of appropriate techniques to conserve the spore, the wood, the tissues and whichever other structure that constitutes the genetic biodiversity of the planet. In these centres, the best conservation strategies are also studied and put into effect for a future *in situ* conservation of species in danger of extinction (Bacchetta, 2006).

The first seed banks, truly intended for such a use, were created as part of university institutions or botanic gardens in the United States at the end of the nineteen fifties. In particular the first bank worth mentioning, was the national laboratory for the conservation of seeds, opened in 1958 on the campus of the State University of Colorado (Hartmann *et* Kester, 1990). Until some years ago the activity of the germplasm banks focused on retrieval and conservation, generally within the native territories, the greater number of entities being representatives of rare and threatened flora. Their role was therefore, almost exclusively concerned with *ex situ* conservation.

The creation of many varied and species rich collections has however created problems of space management and planning of activities of the banks. This has resulted in lowering of conservation standards and in an insufficient representation of the gene pool relative to the taxons collected, by not taking into account the inter- and intra-population genetic variations even though these are known to be extremely important considerations. Furthermore various families and genera are difficult to conserve and above all to regenerate (e.g. *Orchidaceae*). The conservation of exotic or ornamental species is often the results of collections completed through the use of the *Index Seminum* or collaborations with other agencies has, in some cases modified main research focus of the banks, and in so doing putting at risk the conservation of autochthonous flora.

Many years of work and study has resulted into valuable experience that has helped modify the old concept of *ex situ* conservation: adapting and extending it to the real demands of a particular region and also addressing other aims. The conservation philosophy has been changed, especially for the taxa in imminent or serious danger of extinction, towards *in situ* conservation with the aim of direct preservation of the plants as they occur in their wild habitat, aiming to limit the collection, and with actions

targeted towards information and awareness campaigns. For this reason, studies of the biology of the conservation have intensified and the consequent remedial actions have turned to *in situ* conservation (micro-reservoirs networks of flora, ecological networks for territorial consolidation, etc.).

Using this method, landowners and local administrations have been given direct involvement in field activities as well as in monitoring and following studies in publicity and educating campaigns for the public. As understood today, *ex situ* conservation has developed into the creation of representative collections of the different habitat typologies present in a specific region through the identification of existing genetic variability and by widening research to include entities of marginal interest. In this sense, *ex situ* conservation must be considered a very useful tool, indispensable for *in situ* interventions and in extremes, as the only possible means to prevent the extinction of a taxon from its wild habitat.

The criteria for rarity, vulnerability and endemicity are the prime considerations in the choice of material to protect and regenerate. In addition, germplasm banks act as research centres with regards to taxa representative of habitats and other entities that are fundamental in the regeneration of degraded and strongly compromised areas. In this context, pioneering, structural and highly adaptable species are also taken into consideration, conserved and regenerated. The intention is not only to conserve a large number of rare seeds in the germplasm banks, but also to understand from different aspects whether the stored germplasm guarantees the long term conservation of biodiversity.

The plant tissues conserved *ex situ* must be used to increase knowledge on the biology and ecology of entities, and in particular the reproductive cycle, to identify strengths and weaknesses and to initiate *ex situ* strategies for successive experimentation in the region intended for reconstruction or reinforcement of populations. It is fundamental to develop propagation techniques, either sexual or vegetative, at the end assuring an effective conservation *ex situ* and successful regeneration.

Particular caution must be exercised on the use of seeds to execute for germination tests, and seed viability or the different types of study and analysis that results in their destruction. Conserving germplasm within seed banks is not the same as storing books on the shelves of a library. It is a living and dynamic process, that is perhaps similar to management of a bio-park or a botanical garden, in that seeds are living entities that require specific technique and attention for proper and long term conservation (Koo *et al.*, *op. cit.*).

1.2 Information and participation

The demarcation of a geographical region of competence for banks constitutes a fundamental aspect for the establishment of programmes and policies of intervention[1]. Proper territorial management including a coherent biodiversity conservation and protection strategy, is necessary to demark the geographical area of origin of the accession, activating the creation of a channel for communication and bidirectional exchange of information and its valuation (local administrations, parks, universities,

[1] Banks can also assume responsibility for a specific responsibility of a certain material e.g. forest species or fungal spores or pteridophyte spores.

botanical gardens, gardens and botanical research stations, research laboratories, associations, interested individuals etc.). In this context it is easier to render possible collaboration and exchange of germplasm, financial resources as well as specialised knowledge. In pursuing this objective, each bank must be structured in such a way as to guarantee that the collection of material concurs with the legislation in force at regional and national levels, as well as the principal regulations and international conventions that regulate the sector (v. 2.1 and 2.2). The aim of each bank, apart from releasing special permissions and/or authorisation, is to train, educate and continuously update collaborators to ensure the correct culture preparation and technique employed by collaborators, whilst respecting the scientific and conservation aims of the collection (excluding cases where specific conventions intervene).

The role of the bank must also go beyond making its initiatives public and include initiatives such as the creation of a proficient network of collaborators at various levels, allowing for an initial and healthy exchange of knowledge and updates on work. The involvement of collectors necessitates periodical exchange of information on the management plans of entities with the intent to involve them even more in achieving the project's aims and motivating them to extent of executing the collaboration. All those involved are furnished with an index of botanical entities with a local interest, a specific index for the collection of germplasm (v. 13.1) and for the characterisation of populations (v. 13.3).

2. Regulations and Conventions on Conservation of Biodiversity

2.1 International regulations and conventions.

The commitment of national bodies to safeguard plant biodiversity is regulated by treaties and international conventions, European Community regulations and laws of a national and regional character; hereunder are described in a chronological sequence, the principal regulations approved at various levels to date.

2.1.1 Washington Convention - CITES

The CITES convention (1973) "on the international trade of endangered species threatened by extinction", was adopted with Reg. (CE) n. 338/1997 by the European Union and ratified in Italy by law on 19th December, 1975, n. 874; the modalities of the application of legislation are found in Reg. (CE) n. 1808 of 30th August, 2001 (CE, 2001). Dossier I of the convention reports on the species in danger of extinction that are or can be damaged by commercial activity; Dossier II indicates species that although not in danger of extinction, could be so in case commercial activity is not subject to rigid regulations; Dossier III details the species identified by interested parties where commercial activity must be subject to regulation. Dossier IV outlines conclusions of a regulatory permit if this is granted. For the import, export, re-export and introduction of species in the aforementioned dossiers it is necessary to obtain, for every single expedition, under the terms of article 3, 4 and 5 of the Convention, a certificate conforming to the terms of article 6, according to the scheme stipulated in Dossier IV. This permit, issued by the Administrative Authority of the State concerned, has a validity of six months from its release. In Italy, the terms of the same articles cannot be applied/ are not applicable for exchanges between scientists or scientific institutions registered with the Ministry for the Environment, Nature Conservation Service, and adhering to the terms of the Ministerial Decree of 23rd March, 1994, in which germplasm and labelled exsiccate are exchanged herbarium as reported in Dossier IV of Regulation (CE) 1808/2001.

With Regulation (CE) 349/2003 of 25th February, 2003, the European Commission has suspended the introduction into the EU territory, specimens of wild flora and fauna that are reported in the above dossiers.
The latter, updated in 23rd June, 2005, are available from www.cites.org/.

Figure 1 ***Gentiana lutea L. subsp. lutea*, a species protected under the Washington Convention. (Photo: R. Guarino)**

2.1.2 The Bern Convention

The Bern Convention, which specifically caters "for the protection of wildlife and the wild environment of Europe", was signed by member states in 1979 and ratified by Italy in 1981 (Law 503/81), reports in Dossier I the index of wild floral species that are under rigorous protection, for whom the gathering, collection, cutting or the intentional uprooting is banned (CEE, 1982). Article 9 of the Convention however states that all the interested parties may benefit from the derogations in article 5, which can only be applied "*in the interest of safeguarding the protection of flora and fauna; to prevent serious damage to crops, livestock, forests, fisheries, water and other forms of property; in the interests of public health and safety, air safety or other overriding public interests; for the purposes of research and education, of reinforcement, of reintroduction and for the necessary breeding; to permit, under strictly supervised conditions, on a selective basis and to a limited extent, the taking, keeping or other judicious exploitation of certain wild animals and plants in small numbers.*"

2.1.3 First Inter-ministerial Conference on the Protection of European Forests

In the interest of forest genetic resources, the first inter-ministerial conference for the protection of European forests at Strasbourg in 1990, dealt with conservation of such resources and initiated collective decision making at a Pan-European level with respect to European forests. Numerous common strategies were explored; taking into account the trans-frontier character of genetic resource, the responsibility must be shared between stakeholders to ensure major benefits in conservation of intra-species variability that can be obtained with this philosophy. Hence, the proposal for technical and scientific co-operation through the implementation of a series of acts and resolutions. In particular resolution 2, that regards the conservation of forest genetic resources, is based on the following principles:

- Immediate intervention commensurate with the available resource;
- Preferential use of simple methodologies assuring their application over a long period;
- Conservation of genotypic variability at all levels;
- The application and integration of *in situ* methods in forest management, complemented when necessary, with *ex situ* conservation;
- Conserve both rare species as well as rare forest ecosystems;
- Implement at a national level, specific measures for the conservation of genetic resource on the basis of principles stated above, in particular with regards to sylvicultural techniques and the handling/management of reproductive forest material.

2.1.4 Convention on Biological Diversity (CBD)

The convention on biological diversity signed by a hundred and fifty nations in the course of the conference of the United Nations on the environment and development, held in Rio de Janeiro between the 3rd and 14th June, 1992, represents the first initiative on a world scale to conserve biodiversity and defines the guidelines to elaborate common strategies towards the safeguard of animal and plant species as well as habitats introducing the concept of conservation *in situ* and *ex situ* (Williams *et al*., 2003). This convention was ratified by Italy with Legislation n. 124 of 14th February, 1994.

2.1.5 Directive 92/43/CEE on the conservation of natural and semi-natural habitats, wild flora and fauna

This represents the principal instrument for the protection of species of community interest. The Directive (EC, 1992) was acknowledged in Italy with DPR 357/97[2] and listed in Appendix B[3] (All. 2 Directive) (fig. 2) that includes "animal and plant species of community interest for whom conservation needs the designation of Special Zones of Conservation", in Appendix D (All. 4 Directive) that include "animal and plant species of community interest which require rigorous protection", and in Appendix E (All. 5 Directive) that includes "animal and plant species of community species whose removal from nature and whose exploitation can be subject to regulations". For the species in Appendices B and D of DPR 357/97, any form of gathering is banned; however the Ministry for the Environment (art. 11 DPR 357/97) can authorise its removal, in as a derogation of this article, "for didactic and research purposes, repopulation and reintroduction of such species and multiplication intervention necessary including *in vitro* multiplication."

Figure 2 *Brassica insularis* Moris, species included in Directive 92/43/CEE. (photo: G. Bachetta)

2.1.6 Directive 1999/105/CE relating to the marketing of forest reproductive material

Legislative decree 386, approved at the end of 2003, acknowledges the contents of Directive 1999/105/CE relating to the marketing of forest derived reproductive material. Directive 105 introduced, in the interest of forestry problems, is based on the concept of "sustainable development" and "biodiversity protection" and foresees that "the member states establish an index of regions of provenance that specify precisely the origin of basic material" and that "the demarcation of regions of provenance must be indicated to the member states through the publication of appropriate maps".

The Legislative Directive classified reproductive forest material in four categories:

- Source-identified: Reproductive material derived from basic material which may be either a seed source or stand located within a single region of provenance;
- Selected: Reproductive material derived from basic material which shall be a forest stand located within a single region of provenance, which has been phenotypically selected at the population level and meets the requisites namely origin, isolation, effective size of population, age and development, uniformity, adaptedness, health and resistance, volume production, wood quality;

[2] Modified by DPR 120/03

[3] Appendix modified by DM 20th January, 1999 that acknowledges DIR. 97/62/CE

- Qualified: Reproductive material derived from basic material which can be seed orchards, parents of families, clones or clonal mixtures, the components of which have been phenotypically selected at the individual level, and which meets the requirements;
- Tested: Reproductive material whose reproductive superiority must have been demonstrated by comparative testing.

The production, conservation, marketing and distribution of whichever type of material is subject to regulations by decree and subject to the issue of the appropriate license (article 4) released by the official body. Such dispositions do not apply to universities and institutes, public entities of research and experimentation, as well as national centres for the conservation of forest reproductive material and biodiversity used exclusively for research and experimentation. Within a year of the Decree's (article 10) being put into practice, the regions and autonomous provinces of Trento and Bolzano must establish a register of basic material from species listed in Dossier I in the proper territory. The demarcation of regions of provenance must be indicated appropriately by documentation and publication of detailed maps. The maps are sent to the Ministry and, through it, to the European Commission and other member states.

2.2 Associated National Regulations and Measures

To date, at a national level, there is no law that safeguards flora, with respect to the two legislative acts (DPR 616/1977 and L. 984/77), and the State has delegated to its relative functions of protection of sites of natural beauty, the administrative functions concerning intervention for nature protection, as well as the obligation to safeguard wild flora. The only regulations at a national level for the protection of flora are the Royal Decree n. 3267 of 1923 for reorganisation and reform of legislation for the protection of forest and mountainous terrain known as the "Forestal Law", the law for safeguard of olives (Law 144/51 and DPR 10/06/1955) and Law 06/01/1931 for the "Regulation of cultivation, collection and commerce of medicinal plants".

2.2.1 DM 22nd December, 1992 "Official methods for analysis of seeds"

The official methods for seed analysis (Ministry of Agriculture and Forests, 1993) constitute the manner by which someone in Italy can determine seed quality of herbaceous, arboreus, shrubby, floricultural and medicinal species, whether cultivated or not. They are updated with a certain frequency and acknowledge international regulations in this area. They are of particular importance in the commerce of seeds where the price is linked to quality. The dossier of the decree illustrates in detail the methods for sampling, analysis for purity, tests for germinability (with specificity of conditions that must be conducted for every species), the determination of seed viability with biochemical tests (calorimetric), the calculation of seed humidity, determination of weight of a 1,000 seeds, analysis of coated seeds and various other tests.

2.2.2 Proposal for a National Plan on Biodiversity

A proposal for a national plan on Biodiversity was compiled by the consulting committee on Biodiversity and Bioethics of the Ministry for the Environment with D.M. 97/568 of the 15th May, 1997. This instrument was born as a follow up act to the Conference of Rio de Janeiro in 1992 and the relative Convention on Biological

Biodiversity signed by Italy in 1994. It contains numerous objectives, including the following which are of importance to this manual:

- Objective 3 "Education and Awareness" Act 3.1.2 and 3.3.1 "Centres for Biodiversity", Act 3.2.1 "Professional education"; Act 3.3.3 "Information campaign";
- Objective 4 "*in situ* Conservation", ob. 4.3 "Restoration and rehabilitation of degraded ecosystems, protection and recovery of threatened species";
- Objective 7 "*ex situ* Conservation", ob. 7.1 "Realisation of an integrated network of centres of germplasm conservation", Act 7.1.1 "Census of collections of germplasm", Act 7.1.2 "Institution of a databank accessible via a website", Act 7.2.2 "Renewal and enlargement of collections", Act 7.2.3 "Institution of new centres for conservation", Act 7.2.4 "Institution of nurseries for the production of autochthonous species";
- Objective 9 "International co-operation and eco-diplomacy"

From the spirit of this document emerges the need to create a national structure that has the knowledge to operate with adequate instruments, to develop and share this with agencies concerned with biodiversity.

2.3 Regional regulations and authorisations

Nearly all Regions and Autonomous Provinces have in place specific provisions and most of these have compiled a list of protected species. Some provinces have enacted regulations for the protection of wild flora. One region has legislation for the safeguard of flora, but does not have a list of species (Marche). Some regions (Puglia, Sardegna, Sicily) have no legislation in place.

Laws for the protection of flora for Autonomous Regions and Provinces:

Italian Regional/Autonomous Province	**Year of enactment**
Abruzzo	1979
Basilicata	2005
Bolzano	1972
Calabria	2001
Campania	1994
Emilia Romagna	1977
Friuli Venezia Giulia	1981
Lazio	1974
Liguria	1984
Lombardia	1979
Marche	1987
Italian Regional/Autonomous Province	**Year of enactment**
Molise	1999
Piemonte	1982
Puglia	Not enacted
Sardegna	Not enacted
Sicilia	Not enacted
Toscana	2000
Trento	1973
Umbria	1987
Valle d'Aosta	1977
Veneto	1974

2.4 Safeguard instruments strategies adopted by the International Community

2.4.1 Red and Blue Lists of the "International Union of the Conservation of Nature **(IUCN)"**

Although they do not represent a form of safeguard, the red and blue lists rank species according to the categories set by the IUCN and are a fundamental instrument in the protection of flora. Inserting a species in the lists requires, in fact, the collection and processing of a large amount of data, which becomes useful in taking up adequate protection measures. According to the data available a *taxon* can be inserted in one of the following categories (Pignatti *et al*., *op. cit*.; IUCN, 1994 and 2001):

- Extinct (EX)
- Extinct in the Wild (EW)
- Critically Endangered (CR)
- Endangered (EN)
- Vulnerable (VU)
- Near Threatened (NT)
- Least Concern (LC)
- Data Deficient (DD)
- Not Evaluated (NE)

The criteria for the insertion of *taxa* in diverse categories are codified at a national and regional level, according to the international standards of the IUCN (2003a; 2003b). With respect to Italy, the reference works relative to the IUCN lists are those by Conti *et al*., (1992 and 1997). Recently another red list was drawn up, updated in 2005, with regards the species threatened by extinction compiled by Scoppola and Spampinato (2005) and a checklist of fifty highly endangered species in the Mediterranean denominated "TOP 50 Mediterranean Island Plants" (Montmollin *et* Strahm, 2005) (Fig. 3).

Figure 3- *Lamyropsis microscephala* (Moris) Dittrich *et* Greuter, a critically endengared (CR) species listed by the IUCN in the 'Top 50 Mediterrenean Island Plants". (photo: E. Mattana)

2.4.2 Global Strategy for Plant Conservation (GSPC)

This is a strategic plan at a global scale enacted in 2002 (Decision VI/9), promoted by the secretariat of the Convention on Biodiversity (CBD) of the UN and the United Nations Environment Programs (UNEP), in association with Botanic Garden Conservation International (BCGI). Amongst various objectives it recommends *ex situ* conservation of 60% of threatened species, prioritising the country of origin for such entities, and the initiation of reproduction and reintroduction projects for these species by 2010 (objective 8). The strategy foresees that no wild plant must be put in danger as a result of commercial activity or of unsustainable exploitation and that a minimum of 30% of products of plant origin must come from resources managed in a sustainable manner. The importance of plant diversity and the necessity for its conservation must be incorporated in publicity programmes and campaigns. With the aim of achieving such objectives, the GSPC makes initiatives for the creation of and reinforcement of networks for the conservation of plants at a regional, national and international level.

2.4.3 European Strategy for Plant Conservation (ESPC)

This was adapted by the European Council in April, 2002 following a proposal by Planta Europa, as a European contribution to the implementation of GSPC. It recommends that the European Union should undertake *ex situ* conservation of 80% of species at risk of extinction, by 2010. This strategy also underlines the adoption of strategies for effective conservation of 10% of ecological regions in the world and protection of at least 50% of the more important areas for plant diversity. With regards to exotic species it is hoped that plans will be drawn up for the *in situ* management for at least one hundred of the principal invasive species that threaten plants communities and their relative habitats and ecosystems. For the selected plant entities and fungi of commercial value, it is necessary to elaborate a national monitoring programme and, if necessary, regulate collection and trading, with the aim of achieving sustainability. ESPC underlines the importance of plant diversity and the necessity for its conservation must be incorporated in programmes of communication and awareness for public opinion. The ESPC also make initiatives for the creation and reinforcement of networks for conservation of plants at a regional, national and international level, via the identification of economic resources such as national and international financing, via ample use of EU Life programme funds, etc.

2.4.4 Global 200

The conservation and management of the territories, landscapes or ecosystems on the basis of a known process such as Eco-Regional Conservation (ERC), is rapidly confirming itself as an effective strategy, which is necessary for the attainment of consistent and functional results in maintaining Life on Earth. The campaign dedicated to the promotion of the process was launched by the WWF in 1996 as "Global 200". The initiative places as its principal target of conservation, the largest possible number of species, habitat, and ecological processes characteristic of a specific eco-region. In 2003, two hundred and thirty-eight eco-regions were identified as being priorities of the various land, sea and freshwater habitats indicated in the shortlist for Global 200. Maintenance and correct management of these 238 regions at a global level can guarantee the protection of the maximum area possible. The objective is therefore to safeguard the larger areas that retain the best environmental and conservation conditions. In other words, each eco-region in Global 200 list, identifies the more significant eco-region of each habitat type in each bio-geographic region (Bulgarini *et al*., 2003).

2.5 Access to genetic resource

In recent years the theme of access to genetic resource has been given importance, in particular for those with an existing or potential economic interest, and the spread of benefits derived from their utilisation. The importance of facing up this argument was manifested in numerous congresses and conferences. In the case of species of agricultural importance the "International treaty on plant genetic resources for food and agriculture", a binding agreement born within the FAO in 2001 (FAO, 2001), establishes a multilateral system to facilitate access to genetic resources of a series of agricultural species and a mechanism for the distribution of eventual benefits. The

‘Conference on Biological Diversity” in 1992, fixes as an objective, other than their conservation and sustainable utilisation, the just and equal participation in the benefits derived from the use of these genetic resources.

This convention recognizes the sovereignty of States (art. 15) over their genetic resources. At the same time, it recommends the creation of conditions that facilitate the access to resources for employment of adequate environmental character, in line with conditions of mutual agreement between interested parties, with the objective of assuring participation in benefits. It also encourages interested parties to promote and realise scientific research based on genetic resources in collaboration with other parties. In the interest of CBD, the “The Bonn Guidelines on Access to Genetic Resources and Benefit Sharing”, were created and adopted in 2002. The directive places the identification of strategies for access to the benefits pending the identification of steps to follow in such cases, of the fundamental requirement of the terms of the agreement and of the participation and responsibilities of all parties. The document deals with, amongst other things, the incentives, the means for supervision, verification and solutions for controversies in litigation. Finally the directives propose a series of points to be taken into consideration with regards to agreements on the transport or exchange of material, such as a list of possible economic benefits that may ensue. Even if the Bonn Directives are a voluntary instrument, one considers their implementation by centres responsible for management of genetic resources, as being motivated by the establishment of transparent and equal relationships, reinforcing the credibility of institution and facilitating the achievement of the CBD objectives.

3. Networks for germplasm banks

At this point in time, there are around 1,300 germplasm banks in the entire world, mostly distributed in industrialized countries, above all in Anglo-Saxon countries. In Europe there around 150, of which around eighty are in the Nordic countries and seventy are in the Mediterranean region, the latter being essentially in Italy, France, Greece and Spain (Bacchetta, *op.cit.*). Today, in the field of *ex situ* conservation of biodiversity, each institution has built on its own experience, devised and improved protocols and different methodologies with respect to manpower and financial capabilities at their disposal. The pursuit of a major area of research with respect to conservation and given the need for a stronger collaboration and exchange of germplasm, data and knowledge, makes co-ordination at a network level a necessity. In Europe, both at national and community levels, numerous networks have been set up with the aim of coordinating the activities of germplasm banks.

3.1 National networks

3.1.1 Network for Italian Germplasm Banks for the conservation Ex Situ of wild Italian flora (RIBES[4])

In Italy, the number of germplasm banks does not exceed 20 in number , the most important being the Botanical Gardens of the Universities of Cagliari (BG-SAR), Catania, Pavia (LSB), Palermo, Pisa and Rome; that of 'Provincia Autonoma di Trento' (TSB) and the Institute of Germplasm at Bari, managed by the National Research Centre (Centro Nazionale per le Ricerche or CNR). The first germplasm bank was that at Lucca, born nearly thirty years ago with the collaboration of the regional development agency and another centre responsible for innovation in the agricultural. In this bank, species of agricultural interest are conserved, in particular cultivars of vegetable and forage plant varieties. At the CNR of Bari, storage facilities primarily concerns material of agricultural interest, with germplasm of wild flora being of secondary interest. Conversely storage facilities with universities and botanical gardens concerned with long-term seed storage at low temperatures are almost exclusively for autochthonous species representative of the Italian flora. The Pisa bank is specialised in the flora of Tuscany and the Tuscan archipelago, while that of Palermo conserves germplasm of wild and cultivated species in the Mediterranean area, the Cagliari bank concentrates its effort on conservation of western and insular (found on islands) Mediterranean species (Bacchetta, *op. cit.*).

In order to address the absence of an institutional network for the national coordination of the *ex situ* conservation of wild flora in the Italian territory, numerous groups in the sector agreed on setting up a national network of germplasm banks. The first step, was to approve the text of the memorandum of understanding to initiate "Network for Italian Germplasm Banks for the *ex situ* conservation of wild Italian flora" or RIBES in short. This network has occupied itself mainly with projects concerning species in danger of extinction or which are useful in reintroduction at a national level. The memorandum of understanding, signed at Pavia on the 9th February, 2005, laid the foundation for the

[4] RIBES is the Italian acronym that stands for Rete Italiana Banche del germoplasma per la conservazione Ex Situ della flora spontanea italiana

formal definition of the network as a legally recognised body. Today it represents a non-profit scientific association, that operates primarily for the *ex situ* conservation of the autochthonous Italian floral biodiversity. The RIBeS statute was signed by the eighteen founding members (fig. 4) on the 3rd December, 2005 at Trento. To achieve its primary aim RIBeS has formulated a detailed action primary plan with the general objective of improving the quality and safety of germplasm reserves of wild plant species in Italy. This plan has been implemented via the organisation of workshops dedicated to specific lines of action namely acquisition of germplasm, its treatment within banks, management of data and activities linked with education and popularisation.

These workshops were formally launched in the first ordinary assembly organised in Pisa in March 2006. During the meeting several needs were established, namely: to define the priorities for action at a national level; to identifying possible work methodologies; to indicate the minimum requisites for member institutions; and to suggest the best solutions applicable with the human and material resources available (Bedini *et al*., 2005).

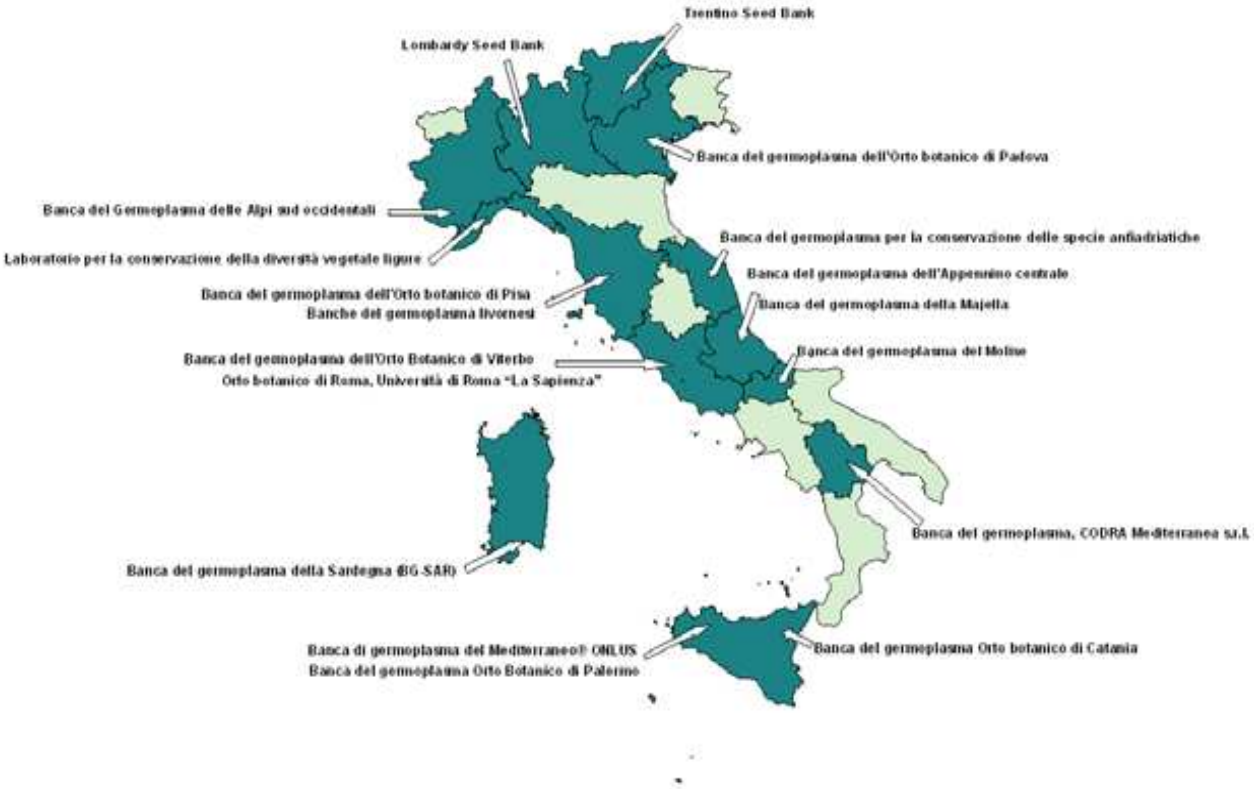


Figure 4 RIBES: regional nodes of the network (dark colouring indicating regions where germplasm banks are present.

3.1.2 Red Espanola de Bancos de Germoplasmas de Plantas Silvestres (REDBAG)

In November 1992, the members of the Ibero-Macronesian Association of Botanical Gardens (AIMJB), that concern themselves primarily with the management of germplasm, and in collaboration with the Department of Plant Biology of the Polytechnic University of Madrid, set up the "Red Espanola de Bancose de Germplasm

de Plantas Silvestres" (REDBAG). REDBAG is open to all those institutions involved in management of germplasm banks of wild species or other genetic resources of plants; the partners in this network are divided in three categories:

- Stable partners: institutions with germplasm banks which for medium and long term conservation of seeds representative of populations of Spanish wild flora by means of appropriate technologies and structures. In particular they form part of the following institutions: Departamento de Biologia Vegetative UPM-BGVA de Andalucia and Cordoba Botanice Garden, University of Valencia Botanic Garden, Real Jardin Botanico de Madrid, Jardin Botanico de La Concepcion, Marimurta Botanic Garden and Soller Botanice Garden (Hernandez Bermejo and Herrera Molina, 2005).
- Partners in phase of consolidation: institutions whose germplasm banks are under development under the guidance of an established partner.
- Potential partners: institutions that are establishing germplasm banks or have projects that are aiming to set one up. Institutions with a germplasm banks but not belonging to the AIMJB are considered as invited partners.

At the moment this network is not officially established and is not recognized at a national level, but it actively works for the conservation *in situ* of plant biodiversity and some partners have participated in international projects such as "Genmedoc" (v. 3.2.1) and the "Ensconet" network (v. 3.3.2).

3.1.3 Istituenda rete della Banche del germoplasma foreste spagnole

The Spanish Ministry for Environment , in synergy with autonomous administration, ha detailed a "Spanish strategy for the conservation and sustainable use of genetic resources of forests". The document plans, amongst other things, the creation of a "Banca del germoplasma foreste in rete", to which various institutions dedicated to conservation *ex situ* can adhere to. The aim of conservation *ex situ* of genetic resources relative to forest species, creating base collections of different material of reproduction and using diverse strategies and in particular: seeds or pollen, live collections from the wild and others needing major technological support, such as *in vitro* maintenance or the cryoconservation. At the same time this germplasm bank in the network will provide material for eventual activities *in situ*, as well as the availability of genetic material necessary for genetic analysis and programmes to improve it, as stated in the protocol for access of genetic resources that would be identified. The bank will be organised like a virtual node, functioning as a manager of accession registers, co-ordinator and divulger of numerous initiatives in programme. Adhesion to this structure will require accepting a protocol that contains a series of requisites and obligations to respect. It is expected that there will be the creation of a virtual laboratory for the evaluation of forest material for propagation both in the immediate and in the long term future; characterisation of which requires amongst other things, the evaluation of the quality of the exterior of seed cover or the quality of the seedbeds, the molecular characterisation, etc.

3.1.4 Federation Conservatoires Botaniques Nationaux Francais (FCBN)

The first French *Conservatoires Botaniques National* was instituted in 1990; by the 1st January 2004 their number had reached eight units and their area of competence covers 78 departments relative to national territories (fig. 5). There are several projects under discussion for creation of new *Conservatoires* for the regions of Aquitania, Poitou-

Charentes, Alsace-Franche-Comte-Lorraine and in the Antilles. The *Ministere de la ecologie et du developpement durable* has acted in this manner to complete the network of *Conservatoires* with the aim of covering the entire national territory. Since 2000, the *Conservatoires* have been reunited in a federation that coordinates and harmonises work practices, brings to life its national programmes of study and conservation of flora and habitat and gives its technical support to the creation of new *Conservatoires*.

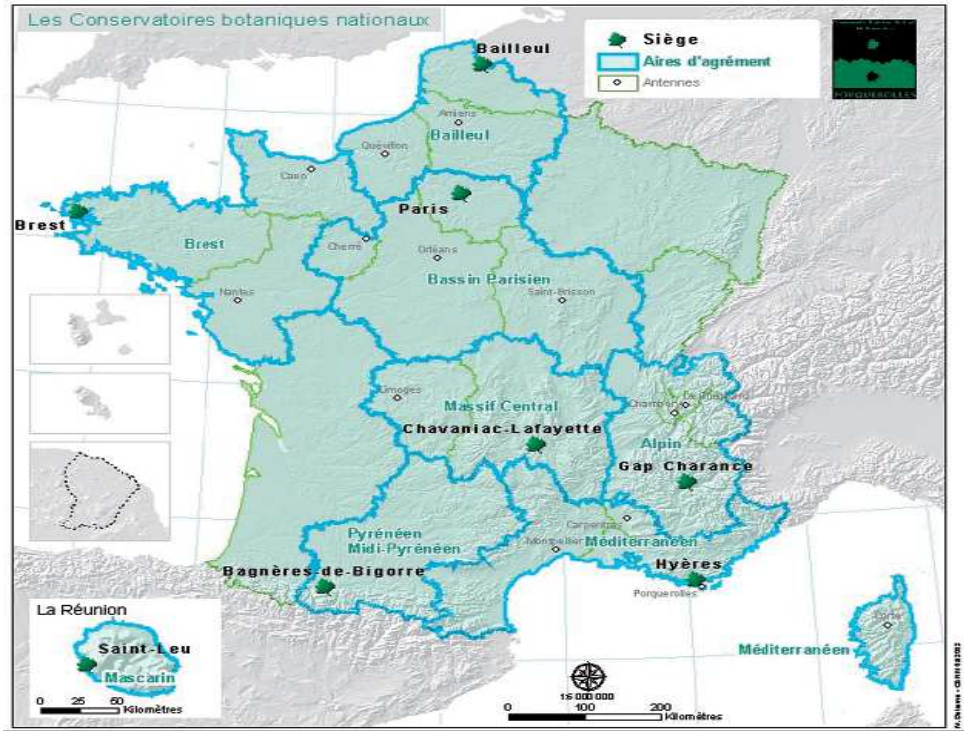


Figure 5- The network of *Conservatoires* with relative areas of competence.

The articles D416-1 and observers of environment laws define precisely the role and method of operation of the *Conservatoires Botaniques Nationaux*, recognized as institutions of a scientific character pursuing the following objectives:

1. Understanding of the state and the evolution of wild flora and natural habitat and wild seeds. They conduct surveys, manage bank dealing with wild flora present in the relevant region of competence with the objective of classifying according to a hierarchical order the natural patrimony (at regional, national and international levels), following their application in field practice. This information is indispensable for the implantation of regional and national policies dealing with protection of nature.
2. Identification and conservation of entities of rarity and under threat by wild flora and by natural or semi natural habitats. They detail and propose lists of species to protect (especially at a regional level), following their implantation in practice. They intervene in the *in situ* protection of species proposing adequate measures, jurisdictional or contractual, to protect plants threatened in their natural environment. With respect to conservation *ex situ*, they implement conservation techniques in the nurseries and conservation of seeds by freezing (seed banks) with the aim of avoiding the disappearance of the most threatened species and prepare a stock of seeds for numerous purposes (research, valorisation, reintroduction in the natural environment, etc.).

3. Technical and scientific support to the State, and its public entities, local entities and their aggregation for themes relative to the conservation of spontaneous flora and natural or semi natural habitats.
4. Information and education with the aim of knowledge of and for protection of plant diversity. The *Conservatoires* publish documents and conduct programmes of awareness and popularisation on protection of natural species, both through the public at large and also to a specialised public (local administrators and professional categories, etc.).

The certification of quality "*Conservatoire Botanique National*" is released by the "Ministere de l' ecologie et du development durable". The candidatures are examined by the commission of the *Conservatoires*. The qualification, granted by a ministerial decree for duration of five years on a renewable basis, is awarded on the approval of a programme that admitted institution is expected to respect. The certification gives right to the appellation "*Conservatoire Botanique National*" which is a trade name. The authorisation is released for a specific territory constituted altogether of departments that present common biological and geographical characteristics. This qualification can be revoked if the activity or functioning of the institution does not respect the fixed objectives. This control is done via an annual report on the activity achieved and a programme document, which is presented during the reunion of the scientific counsel. The *Conservatoires* have a detailed and thorough knowledge of the distribution of wild plants, of their biology and their ecological demands. The specificity of their actions and the responsibilities that are derived from their certification are to assure, in any possible means, the transfer of this knowledge to all those involved in the management of the natural environment: public, private, departmental or regional administrative services, forest management bodies, etc. The aim of their intervention is to evidence the importance of the presence of threatened species in the management and planning operations that involved the natural environment.

3.2 European Networks

3.2.1 GENMEDOC

Genmedoc, "Creation d'un reseau de centres de conservation du material genetique de la flore des regions mediterraneennes de l'espace MEDOCC", re-enters in the common action policies for the environment of the European Union for the safeguard lf biodiversity and conservation of species and habitat. These objectives are pursued through an exchange of technical information, adoption of strategies and common work protocols for the conservation of genetic resources of Mediterranean taxa and principally prioritising those present in habitats stated in the directive 92/43/CEE.

The project Interreg IIIB "Genmedoc" places the prioritised objective of a network of germplasm conservation centres in the western Mediterranean. With the network "Genmedoc" numerous European partners consenting to the coverage of most of the MEDOCC space, therein included the larger islands in the Mediterranean (Balearics, Corsica, Sardinia, Sicily and Crete) and a Tunisian partner for the southern coastline. The ten centres involved (fig. 6) are: Banc de Llavors Forestals (CIEF) for the region of Valencia; Centre for Conservation of Biodiversity (CCB) of the Department of Botanical Science of the University of Cagliari; Conservaitore Botanique National Mediterraneen de Porquerolles (Isole Hyeres); Department of Botany of the University

of Catania; Jardi Botanic of the University of Valencia; Foundation Jardi Botanic de Soller (Balearic isles); Mediterranean Agronomic Institute of Chania (Crete); Institut Botanic e Jardi Botanic de Barcelona; Institut des Regions Arides (IRA) di Madenine (Tunisia); Direccion General del Medio Natural della Regione di Murcia.

The principle objectives of the project are:

- the detailing of common models for management of taxa combining conservation *ex situ* (collection and conservation of germplasm) with that *in situ* (protection, recovery, implementation of natural populations);
- the exchange of knowledge on conservation of germplasm (collection, treatment, conservation and multiplication);
- the duplication of collections between partners, in a way to guarantee their effective conservation;
- the study of structural taxa of habitat and of an endemic, rare or threatened nature.

The final objective is to significantly contribute to the development of the European network NATURA 2000 (in addition to the 300 plant species and 40 Mediterranean habitats), born with the scope of conservation of biodiversity in Europe, in synergy with the dictates of the CBD. The basis for selection of species has been agreed on by partners in particular their structural role in coenosis, their singularities, known to be rare and/or endemic, the level of protection and the level of the threat. For species of particular interest, there have been detailed numerous protocols of efficient germination, with the aim of guaranteeing the possibility for multiplication of germplasm for use in reinforcing populations or reintroduction in the natural environment. All the information relative to the project, on biodiversity and in particular conservation *ex situ*, are available from the internet on its official site (www.genmedoc.org).

The project has given rise to numerous interregional collaborations between partners, favouring the collaboration and inter-exchange of information that might contribute within the near future to a real effort for conservation of flora at risk of extinction.

Figure 6 Partners in Interreg IIIB Genmedoc

3.2.2 ENSCONET

ENSCONET "European native seed COnservation NETwork", is a network that reunites various European seed banks with the scope of putting place a common operative policy, coordinating efforts to avoid wastage and optimising management of available resources. Ensconet has also proposed assistance to the European Union's conservation policies in its obligations to the CBD and GSDPC via the protection of seeds and avoiding the extinction of European natural flora.

The project was initiated thanks to financial help from the European Union as part of its Sixth Framework Programme for Research and Technological Development (FP6), via infrastructural activity explicitly integrated with coordinated actions (N. 506109/2003) and comprehends 19 European institutes belonging to 12 member states, representative of five European bio geographic regions. The network is coordinated from the Royal Botanical Gardens of Kew and is organised in four workshops with the following targets:

- analysis of existing collections and identify species and areas of limited representation, in such cases initiate new collection campaigns;
- improvement of the quality of seed conservation practices, standardising structures practices between institutions;
- propose the basis for integration of numerous databases of diverse institutions;
- dissemination of new knowledge and placing practices acquired at the disposition of others.

4. Collection of Germplasm

The identification of the target species to sample is a function of ethical criteria, the scientific aims of previous collections and of the technical necessities determining the management of a region. For this reason there is a need to clarify that some of the following indications are an appeal to the principles of selection of stations and to work methodologies relative to widespread entities and/or with gastronomical interests. This context of activity is difficult to trace in the area of conservation of rare, vulnerable or strongly endangered species. It is however important, at the end of presenting the complete picture, to refer to these practical applications inviting the collector to make use of experience gained field practice, to common sense and to indications that might be forwarded from bank curators or those that have experience on *in situ* conservation.

4.1 Criteria for selection of collecting sites

The methodologies for seed collection applied by the collector are known to be of crucial importance in the manner they are executed as an interface between genetic variability in the field and its representation in the sample taken into the bank (Namkoong, 1988). To proceed with the collection of a population destined for conservation, based on criteria established by the Royal Botanical Gardens, Kew, one must satisfy a series of conditions relative to the population selected for seed collection, namely that the population:

- must be "genetically diverse" occurring in different soil types, climates, altitudes, having different pollinators, and physical barriers to cross-pollination;
- must be indigenous, wild and self-sown and not planted or originating from cultivation;
- allows for sampling from a random representative cross-section, consisting of a minimum of fifty individuals, with the exceptions of threatened populations, where the numbers can be reduced;
- permits the collection of between 10,000 to 20,000 seeds; in the case of critically endangered species, severely depleted populations or individuals producing a small number of seeds, a lower number may be collected;
- must have ripe seeds, preferably still attached to the parent plant itself and ready for dispersal;
- does not have a high percentage of seed loss due to physical damage, consumption by predators or dead/ aborted embryos.

The criteria for the choice of stations is based on stable parameters such as the inter-population geographical distances, altitude, climate and habitats, as well as parameters of a genetic nature aimed to identify genetic variation existing within populations. Before initiating activities on site it is therefore necessary to evaluate the availability of information on the environmental characteristics present in the area and the distribution of species. The basis of this knowledge is to divide the region into a limited number of areas, which are distinguishable from the ecological point of view, stability of characteristics and use of the territory. The choice of a station, given to genetic sampling, is applied where the *taxa* show distinct polymorphism, always taking into account of the presence/absence of genetic barriers between populations and related species. If the species is noted in various stations, one can initiate with sampling of not less than 50% of populations; if on the other hand the entity is of a very rare nature and

is identified in a limited number of stations, it is advised to proceed with the sampling of all the population, with respects for the limits needed to ensure survival of the parent population (v. 4.2). In whatever situation one finds himself, the first thing to do is to organise a preliminary excursion to the identified station to confirm the identification of the entities to gather, to determine the possibilities of a collection and the probable period for maturation of the seeds.

4.2 Methods of sampling

4.2.1 Genetic sampling

The strategies for sampling a population depend on the extent of genetic variability in the population and of the biodiversity existing within different populations. As a general rule populations with a high percentage of diversity are more genetically heterogeneous and therefore merit more sampling. To sample the genetic resources of a specific population it is fundamental, as a starting point, to evaluate the wealth of available alleles or the number of distant alleles of a single locus. The wealth of available alleles of a sample is definable as a measure of its quality (Brown *et* Marshall, 1995). For the numerous potential uses of a seed collection one must therefore maximise the number of alleles present in the sample, by collection of the largest possible proportion of representatives in a population. The objective must be that of including a copy of 95% of the alleles present in a target population in the sample. This must be assured of by collection of seeds or other plant material of thirty genotypes randomly chosen in the case of entities with cross-pollination or of fifty-nine individuals chosen randomly in the case of entities with auto-pollination (Brown *et* Marshall, *op. cit.*). This is possible if information about the method of pollination used by the *taxon* under study is available.

Given the fact that the reproductive biology of most entities has not been studied and that collection of alleles entails conservation of a substantial number of samples;,it is advisable to proceed with sampling for at least 50% of individuals within a single population, when possible. This number can be increased in duplication of some samples, if one suspects that not all the seeds are alive or not in optimum conditions and to avoid possible loss of material during transportation or in quarantine (Brown *et* Marshall, *op. cit.*). As a general rule, each accession to be taken in by the bank must constitute a minimum of a number of individuals representing 50% of alleles. For this reason, a batch of seeds collected from a single population must have the potential to re-establish the population in the station of origin or in other compatible sites where the natural ecological range of the *taxon* and the genetic characteristics are the same.

Collection of seeds from trees and shrubs: considerations on the maintenance of genetic variability

The procedure for acquisition of propagation material of forest tree and shrub species depends, to a large extent, on the final utilisation purpose or the material. In the case of woody tree species, where the production aspects are overriding, the phenotype of individuals assumes the highest consideration. Therefore one can exercise selective criteria notable on individuals destined for the production of seeds and other propogative material (e.g. cuttings), eventually creating specialised systems like a seed stand. If on the contrary, the final scope of the intervention is naturalistic sylviculture,

or restoration of the environment, it would be fundamental to select characterised material of a high genetic variability. This in fact, is strictly correlated to adaptability and thus capable of considerably improving the probability of success of the intervention, as well as contributing to the creation of stable populations and increased naturalistic value. Loss of genetic variability can be verified at all levels of the reproduction purpose which from the original population leads to material available for reforestation. In any case the critical moments appear to be connected with seed collection, their processing in the seed bank and the growing techniques applied within nurseries (Ducci, 2003).

Naturally, the genetic variability must be considered as a function of the ecological conditions of the environment in which the intervention is to be carried out: the introduction of germplasm from localities, especially if characterised by diverse pedoclimatic conditions, could indeed be counter-productive, even if this result in an improvement of the genetic pool. One cannot undervalue the possibility of pollution at a genetic level, modifying the gene pool of the natural population which would have resulted from a long process of evolution and therefore suitably adapted to local conditions. In the case of afforestation activities as part of *in situ* conservation; it is unavoidable to make use of autochthonous germplasm of local origin. The ecological aspects that define the adaptation of a population are: climatic characteristics, especially those concerning temperature fluctuations. Topographical aspects such as altitude, latitude and aspect are also of major importance, whilst the substrate type has less importance (Mentelone *et al*., 2005a)). In today's reality the situation is worrying since certification on the origin of forest and shrub seed is only done for the more important species, while in other cases material of unknown origin is used, often being of an imported origin. In the area of forest species, the problem can be resolved by resorting to the place of origin, as explained in the European Union Directive number 105/99 and repeated in Legislative Decree number 386/03. The provenance must be a "territory or group of territories exposed to sufficiently uniform ecological conditions, and on which the topsoil and soil seed bank are sufficiently homogenous from a phenotypic and genotypic perspective (where it is possible to evaluate the latter), keeping note also of altitude limits where appropriate."

However there is still the problem of provision of material. Nowadays there are few criteria with adequate codification; collection is often based exclusively on considerations relative to production: favouring few individuals (the more productive, or the more accessible). The material obtained is consequently of a high genetic uniformity. On the contrary, it is necessary to collect material from the highest number of diverse individuals, allowing for conservation of highest possible levels of biodiversity of the original population: roughly considered to be suitable for the collection of areas not inferior to ten hectares and the that areas are proportionally increased for those species that are habitually isolated or occur sporodically, such as walnuts, cherry trees, chequer trees, lime, etc., (Duce *et al*., 2001).
Scientific studies on this aspect are limited in number. The studies show that the number of unrelated genotypes to utilise for the production of seeds must not be inferior to thirty, and in cases where there are exigencies of *in situ* conservation of biodiversity, this number must be increased. Therefore sampling must consider trees with a sufficient distance between them, as a general rule sampling cannot be effected on individuals at an inferior distance to 100-200m (FAO, 1995). It is infact evident how the process of dissemination tends to give birth to new individuals in proximity of the tree of origin: in

many species, a natural mechanism of vegetative propagation (e.g. root suckering or root cuttings) determines the presence of a single genotype (clone) in individuals of apparent diversity (Gradel *et al*., 1995; Wilson *et* Samuel, 2003). In addition to the above studies, a recent study between the University of Torino and the National Centre for the Study of Conservation of Biodiversity (Corpo Forestale dello Stato), who analysed the genetic characteristics of an arboretum of a Scots pine starting from seeds of nineteen mother plants, revealed a decrease in biodiversity of biochemical and molecular markers both when passing from forest seeds to arboretum specimens derived from seeds as well as in successive process from teh arboretum to vegetative plant material obtained from those specimens (Monteleone *et al*., 2005b). Similar conclusions have been obtained in research, that analysed an oak stand in Lombardy to evaluate the potential of using this as a seed stand. Of the two provences used, one has manifested consistent loss of genetic variability with respect to the forest of origin whereas the second had conserved genetic variability at sufficiently elevated levels; reinforcing the hypothesis that the difference is due to a variable number of seed-bearing plants used for the production of the stand (Castagna *et al*., 2005). The importance of ensuring that reproductive material destined for afforestation and reforestation is collected according to standard practices (aimed at maintaining variability and the geographical component of genetic diversity), is confirmed in the study conducted by Burgerella *et al*, (2006) on *Quercus ilex* L. in Andulucia. By utilisating molecular markers, they demonstrated a marked reduction in the genetic variability of planted stands when compared to the natural population of origin.

4.2.2 Selection of individuals for sampling

Randomised sampling is considered to be the most appropriate however it can be confirmed, especially in case of autochthonous entities, that natural populations develop sub-populations or sometimes its own meta-populations. Once such a situation is identified, they must be randomly collected, and treated as separate accessions.
A randomised collection further implies, that every single plant present in the population has the same probability of being included in the sample as any others (Marshall *et* Brown, 1983). In practice, the seed collectors either gather in a randomised manner or follow line transects. The point of departure and direction of these transects in the area under study must be carefully chosen so as to avoid sampling closely related individuals (Brown *et* Marshall, *op. cit.*). The distance and consequently the individuals sampled are related to the biology of the species, therefore one cannot make use of a single criterion or unique method but must employ a species-specific protocol.

4.2.3 Number and type of vegetative material per plant

An important factor is to identify the method and quantity of material to be collected from a single individual. Collection of seeds or spores as distinct from vegetative material in other genera is distinguished by the strict dependence on the correct timing of the collection. It can be quite challenging in cases of individuals or species still understudied and this may involved several field-studies before one proceeds to collect fruits and seeds or spores at the correct stage of maturation. The collection of other vegetative material is less heavily dependent on time of collection e.g. bulbs, rhizomes or aerial parts, is not rigidly time constrained, and one can work preferably during the months of dormancy. The quantity of germplasm to be collected is always a function of

the level of threat of the species or its vulnerability and rarity. In the case of collection of seeds or spores the sampling must be adapted to the availability of germplasm produced in the current season. The pressures exerted by the collection must be adjusted from time to time and matched to the increase or decrease of the population. For this reason, the collection must adhere to an indicative protocol and consider amongst other options even the possibility of discontinuing the sampling exercise or on the other extreme to collect all the available germplasm. Even the collection of vegetative material for other uses e.g. for bio molecular analysis must be carried out within the protocol constraints acceptable for *in situ* conservation and *ex situ* research activity. Normally in the collection of seeds destined for the production of seedlings or for *ex situ* conservation are not held in separate lots by individuals to avoid the possibility of favouring the presence of certain genotypes (e.g. more productive, easily accessible, etc.), one must collect an analogous amount of seeds from every individual.

4.2.4 Important considerations to be observed during collections

- As a general rule, one is not to collect more than 20% of seeds available on the day of the fieldwork. This assures that the population is not negatively affected by an excessive collection. The only exception to this rule is in particular situations, such as the security and imminent destruction of the population.
- For rare and/or endangered *taxa*, in the case of availability of *ex situ* material, assuming that the first (F1) generation is free of the phenomenon of hybridisation, it is possible to employ the material available for the execution of germination trials necessary for the identification of effective protocols (germination and propagation) and instead, conserve all the germplasm collected *in situ*, reproducing it once the protocols are defined.
- An entity found only in restricted geographical regions, merits to be sampled more intensively, with an increase in the number of individuals per location and an increase in the number of propagules for each individual. The same principles apply for very rare *taxa* where it is impossible to sample an elevated number of individuals; in this case one proceeds with a collection in more stations, obtaining maximum quantity of seeds from a single individual.

- The entities that grow in a wide range of ecological conditions diverge easily in terms of genetic content. In these situations it is useful to increase the population number and sub-population being sampled, keeping them as distinct accessions from others.
- Populations of perennial plants comprise individuals of different age groups and the population can have an age-dependent structure. In this case the individuals must be sampled randomly without taking into account the size or age to maximise sampling of existing genetic diversity.
- If the entity can be regenerated by vegetative means, it may be useful to collect those tissues other than seeds, which can be used for its regeneration. This alternative type of regeneration is favoured when the *taxon* is in serious danger of extinction.
- Simultaneous collections can be effected for a species where numbers are phenologically synchronous (flower at the same time) though this must still follow precise planning of the fieldworks. On the other hand, when the flowering season is spread out over a long period, not all the individuals at the time of sampling would bear seeds at the same stage of maturation. Genetic variability can also be

influenced by diverse site-specific factors and it is therefore important to collect samples from the highest number of plants as found in diverse environmental and ecological conditions.

- For species manifesting high levels of polymorphism it is necessary to increase the collection size. Populations of self-pollinating species can still have sub-populations and this may justify a randomised collection.
- -In the case of anemophilous pollination, one has to take into account that a plant with numerous flowers can be pollinated with pollen from diverse provenances. Conversely in the case of animal pollination, the source of pollen can be one plant for diverse flowers (Brown *et* Marshall *op. cit.*).

Particular attention goes to isolated populations and those on the edge of their distribution, because these can present various rare alleles. In the contact zone between subspecies one can note contrasting genetic and morphological variations. This is obviously the result of hybridisation and of segregation. Collections from the different morphotypes are to be kept as separate as possible. Collectors must give particular attention to sites and areas where transition of *taxa* may occur. It is fundamental that collectors are alert enough to note and record any change in frequency and geographical ranges of the entity and suggest reasons why this process is manifested (Von Bothmer *et* Sebers, 1995).

Summary:

- Whenever possible, not less than ten populations should be sampled for a homogenous ecogeographical area.
- If doable, sample about 50% of the individuals in every population.
- Sample randomly, but keep collection from meta-populations separate if the habitat is distinctly heterogenous.
- Sample seeds or vegetative material in sufficient quantities to ascertain satisfactory representation.
- Randomise the collection over the area under study and indicate on the collection field maps/schedule the methodology followed (central collection, diagonal line, marginal collection, etc.).
- Take note of various physical parameters of the site, (altitude, aspect, soil, slope, shading) to ensure that the sample represents the highest level of diversity possible.
- Take note on the appropriate collection schedule of the phonological phases during the first site visit and correlate these with the time of collection. This information will help to compile a phenological calendar, allow for monitoring variation in the vegetative and reproductive cycle over the years and to save time planning and executing future seed collection expeditions.
- Take note on the collection schedules of all anomalies or doubts that emerged during sampling.

4.3 Collection of germplasm on site

During the gathering operation it is important to take note of the stage of maturation of fruits and seeds even in relation to their position on the plant. The position in the inflorescence can result in a staggered maturation of seeds. For example, in *Pastinaca sativa* L., the primary umbels mature around 10-14 days before the secondary

umbellules, which mature 10-14 dys before the tertiary group. For the entire population, seeds are naturally dispersed between August and October (Hendrix, 1984). Even observation of the pollen process can give some indication as to how to proceed with the collection procedure: pollination from different (pollen) donors can result in diverse fruit maturation time, and early fertilisation can result in more rapid seed development as opposed to late fertilisation (Lee, 1988). To reduce the risk of loss of mature seeds, the collection must be staggered during the period of seed dispersion, recording every single collection as a separate accession. The longevity of a seed sample depends heavily on their quality at the time of collection especially with respect to so-called "orthodox" seeds (v. 6.9.1). The idea is to obtain the same number of seeds (or fruits) from each plant that is sampled, at the same stage of maturation, immediately before dispersal. The methodology of seed collection can influence laboratory tests on germination and affect their capability to overcome any eventual dormancy. It has been demonstrated that for some species such variations can depend on the position that the seeds have inside the fruit (e.g. basal seeds are more dormant than those at the apex) and their distribution on plants (Toole *et al*., 1964). Even the weight and dimension of seeds can influence the quality of the batch and its response to tests on the seeds' viability. Some (*Poacea*); develop two different seed types, thicker seeds giving more vigorous plants and with an increased germinative capacity with respect to smaller ones (Lehiri *et* Kharabanda, 1961).

4.3.1 Identification of ideal timeframe for Collection

In many cases the seeds cannot be collected as a single entity and must gathered together with the fruit (fig. 7-9) that contains them. In this way one avoids interrupting the physiological maturation of seeds as well as allowing for the seeds to gain tolerance to dehydration. It is a known fact that that seeds produced from fleshy, dehiscent and indehiscent fruits are intolerant to dehydration in the initial stages of development, and that collection is best effected in the successive phase, when the seed becomes hygroscopic and therefore tolerant to dehydration. Identifying this moment is not easy. When experience is not useful, several indicators on the fruit (fleshy or not) can give useful pointers as to the procedure:

- Change of colouration can be a good indicator, but this is not reliable. In the tomato (*Lycopersicon esculentum* Mill.), for example, berries of a different colour (green to red) can contain seeds at the same stage of maturation (Ellis *et* Roberts, 1981).
- The size of the fruit in a drupe is correlated to the complete development of its seeds (Smith, 1995).
- The hardening of the pericarp in certain fruits is manifested only after the development of the embryo is complete.

Fleshy fruits must be collected at the point of optimal maturation. An early collection, can infact yield material with low germination capacity. A late collection, can result in losses due to natural dispersal, to predation by animals and a number of meteorological events such as hailstorms and heavy rain.

Figure 7 Fruits of *Astragalus verrucosus* Moris in maturation phase. (photo: E. Mattana)

Figure 8 Seeds of *Heliodiceros muscivorus* (L. f.) Engl. (photo: L. Podda)

Figure 9 Cones of *Juniperus oxycedrus* L. subsp. *macrocarpa* (Sibth. *et* Sm.) Neilr. For their correct conservation the seeds must be extracted from the cones on their intake in the bank. (photo: G. Bacchetta)

4.3.2 Seed sectioning test

After identifying the population to be sampled, the collector must examine with care the first sample of seeds using the "seed sectioning test" technique (fig. 10) and for very small seeds using a magnifying glass. This preliminary analysis is a rapid method to estimate the quality of the material being collected, the frequency of empty or damaged seeds and the expediency of the collection. Seed sectioning test is a rapid method that can be carried out at the same time of the collection. The test consists of making use of a sharp razor or scalpel to cut the seed in half: seeds of high quality show turgid and healthy tissues with a colour typical of the species (white or ivory), and without pathogenic or insect damage. In the case of a batch of seeds of poor quality, one needs to estimate the number of healthy seeds (Suszka *et al*., 1994). The difficulties in undertaking this preliminary analysis can arise in collectors with limited

Figure 10 – Seed sectioning test in a seed of Pancratiun maritimum (Photo: E. Mattano)

experience, or when working with very small seeds (Piotti *et al*., 2001). In this situation, if the magnifying glass is not of help, one proceeds with the collection making use of transparent bags, delegating the study of the batch quality to the seed bank, where the tests is executed making use of three replicates of thirty seeds each (Crosti *et al*., 2006).

4.3.3 Collection protocol

If there are sufficient seeds that appear in good condition, one can follow the following protocol:

- Mature and dry seeds should be stored in cotton/paper bags.
- Seeds still contained in fruits should be stored as such, in paper bags.
- Fleshy fruits must be immediately stored in plastic bags with adequate aeration, as they are likely to decompose rapidly; poor storage may reduce the seed's viability. The cleaning of seeds must be left to the seed bank staff.
- A herbarium specimen must be obtained and prepared using normal procedures for precise identification.
- Collection of seeds from rare/endangered species' must be repeated for two consecutive years, or several samples taken during the same year, without collecting any further herbarium material.
- The quantity of viable seeds that must be made in a collection must be large enough to allow for:
  - the long-term storage of a representative sample as a precaution against the population's possible disappearance and as a resource for genetic and biological studies of the species.
  - germination and viability testing.
  - regular periodic monitoring of viability during the storage period.
  - the potential use of parts of the collection to enhance other collections in other seed-banks/research centres.

Seed collection must not be carried out if this could result in the further depletion of endangered species' populations. In such situations, alternative methods of propagation must be sought, eg, *in vitro* reproduction; germination and reproduction of germplasm already conserved in other banks.

4.3.4 Typical procedures for the collection of seeds

The relative procedures to be followed for the compilation of the germplasm information data sheet/card as well as details needed are specified below (v. 13,1:

- correct identification of the taxon;
- if the identification is of a doubtful status, one is to make use of floras and field guides to arrive at an accurate determination (in such a case one is to indicate on the collection card the name of the field guide or flora that has been used); in case it is still not possible to accurately determine the taxon, one is to highlight on the card " urgent need for determination";
- accurate delimitation of the collection station;
- in cases where the station size are known and measurable, to verify these limits, together with any new extensions in the original range and the presence of eventual microstations, updating all the data on the collection card; apart from information of a geographic nature, it is important to include the reference to ownership of the property and any levels of protection that may exist for the site;

- to collect the material;
- to indicate in the card the number of individuals on which the collection has been carried out as well as the type of material collected.
- Complete the information date sheet by adding any other information that may be useful for the seed bank.
- Register also the sample taken for the herbarium.

4.4 Collection of data and information in field: compilation of the cards

The characterisation of populations is the most basic way for the diagnosis of their status, not only as regards their future variability (García, 2002) but also as regards any *in situ* conservation programme and management action. As far as *ex situ* conservation action is concerned, the results derived from studies of population structure and dynamics as well as a good knowledge of the distribution of a taxon (comprising the pattern of genetic variability and reproductive biology), can contribute to a more effective and representative sampling of the genetic pool of the taxon under consideration .

Population field studies specifically foresee the acquisition of information and data that allows one to become acquainted with the auto ecology of a taxon. In order to guarantee comparability and homogeneity of the data collected by the various banks, field cards whose compilation requisites concur have been formulated by agreement. In this way the relative data collected for the taxa of interest from a particular station can be consistently analysed (v. 13). Every time it becomes necessary to execute a specific survey, the relative data sheet of the taxon that one agrees to conserve must be compiled. .

4.4.1 Equipment required for collection of germplasm and the scientific data:

The equipment and materials used for the field activities (fig.11) is in the greater part similar to that normally used for outdoor research activities or for outdoor sports in the pursuit of nature. A checklist of useful equipment needed for the collection of material, data is given below but also includes other useful things that may occasionally become useful:

- Field note-book
- Tape-recorder
- Field files (PC or notebook)
- Cotton bags / paper envelopes of various sizes
- Polythene bags of various sizes
- Plastic containers (for keeping trapped parasites, pollinators, etc)
- Transparent and transparent philately envelopes
- Adhesive tape
- Rubber bands
- Labels and cards to place inside each envelope or bag
- Denatured alcohol 95% and glycerine
- Formaldehyde (30%) for preserving live tissue
- Floras and field guides
- Altimeter/barometer

- Thermometer
- Hygrometer
- Clinometer
- pH meter (hand held)
- GPS or dGPS
- Gardener's gloves / latex gloves
- Gas masks
- Magnifying glass 3x or 6x
- Razor blades
- Scalpel
- Portable display boxes
- Small knives
- Pruning shears
- Drill
- Pick-axe
- Spade
- Ice-pick
- Climbing equipment (helmets, ropes, shackles, etc)
- Pencils, pens, erasers
- Photographic camera (reflex or compact)
- Binoculars
- Newspaper pages / absorbent paper / field press
- Plastic pots
- Silica gel

4.5 Procedure to follow in special cases

4.5.1 Failed Collection of Germplasm

For failed expeditions, the schedule for the collection must still be compiled, explaining the reason for the failed collection and indicating how to make up for the missing germplasm (by defining a new period for collection, choosing another location for the collection, collecting a different type of germplasm, etc.). In the case of affecting other types of surveys, compile the record sheet (phenological record, demographics, phytol-sociological survey, pedological survey, etc.) and attach them to the collection record to consign to the bank.

Figure 11. The moment of collection of nutlets of A*nchusa formosa* Selvi, Bigazzi *et* Bacch. (photo: C. Pontecorvo)

In case it is impossible to repeat a collection (unfavourable weather conditions, precarious plant health, absence of germplasm, etc.), one proceeds with a single sample of one of the specimens (v. 4.5.6) or, in the case of infection by a pathogenic agent, of a complete sample of the plant. This material is conserved in nylon bags with a name tag.

4.5.2 Populations of extremely reduced size

When a population is very small (less than one hundred individuals), but there is sufficient material and the collection does not affect in any way the species' future, one keeps samples from each plant separate, utilising a sealed packet in each case. In the collection record, one must repeat the batch number and the indications for treating separately material contained in each packet. This contributes to the conservation of genetic diversity of the population.

Such a situation reappears when working with endemic *taxa* or those threatened by extinction, whose distribution is limited to small localised stations, such as, for example *Centranthus amazonum* Fridlender *et* A. Reynal and *Ribes Sardonum* Martelli exclusively present in Oliena (NU), in Sos Prados.

4.5.3 Biotic damage to population

The observation skills in a collector are an important element in identifying factors of disturbance or threat to the site and plant species in question. One must continuously understand the general conditions of the species and individuals from whom the germplasm is taken. In the case of infestation by insects, fungi or other pathogenic agents, it is necessary to sample the host and pathogen, taking care to conserve the sample in a well sealed packet, keeping it separate from any material that could be contaminated. Such material can also be conserved in glass test-tubes with 30% formaldehyde or denatured alcohol and glycerine at a 1:1 ratio.

4.5.4 Unfavourable weather conditions

When weather conditions are not favourable (e.g. rain, hail, snow) one must not proceed with the collection; in case of rain in the preceding days, one can proceed with sampling giving particular attention to the conditions of the seed and in particular if these have already been dispersed, especially if they are very wet or damaged. If the material is suitable for collection, one can proceed with the collection, at the same time taking care to dry the seeds in the open, but not in direct sunlight, and henceforth processing the germplasm in the bank as quickly as possible.

4.5.5 Necessity to collect Herbarium samples and/or living plants

In the eventuality that no herbarium sample of the population is present in the collection, it is necessary to provide for its collection. The herbarium specimen must have the same reference number of the corresponding seed collection and ideally must be a complete sample (fig. 12) including flowers, fruits, vegetative parts and roots (for herbaceous plants). To ensure a good herbarium specimen it is suggested, that during long excursions, the plant must be dried completely in the shortest time possible, using

absorbent paper (newspaper) or sheets of foam or using a portable press. When this is not possible, the samples must be kept in a dry place, with frequent changes of the absorbent paper. If drying is impossible, it is nevertheless recommended to press the material and conserve it in plastic bags, taking care to sprinkle with denatured alcohol before sealing any packets. This procedure is adopted during expeditions and collection trips in hot and humid places, especially in tropical areas where fermentation processes lead to deterioration and risk to compromise its quality. No herbarium specimens must be collected for extremely rare or threatened entities, especially if already well studied taxonomically. An alternative technique for the study, conservation and reproduction of the germplasm of a *taxon* is the collection of living plants to conserve in pots or nurseries. Such a collection is undertaken only by specialised personnel when its removal does not prejudice or reduce the reproductive potential of the *taxon* within the already threatened population. Such a technique is of great utility when the reproductive biology and the phenology of the species is unknown, or when the species lends itself well to vegetative reproduction.

Figure 12- Scans of a herbarium sample of *Serapias parviflora* Parl. (Tornabene collection) available over the internet web pages of Department of Botany, University of Catania, Italy.

4.5.6 Collection of Soil Samples

Sometimes it is important to gather concurrent to the collection of the germplasm one or more soil samples. The samples can then be used for the characterisation of the soil parameters through physical-chemical analysis. The sol data will give a better understanding of the ecology of the particular taxonomic unit. To obtain a complete characterisation of a soil sample, the following parameters are usually determined: soil texture, pH in water, pH in aqueous potassium chloride (KCl), carbonate content, humus content, organic carbon, total nitrogen, absorbable phosphorus, the pF (4.2 and 2.5), total acidity, exchangeable base, base saturation and cation exchange capacity. Once the data is obtained, one can proceed to classify of the soil according to recognised standards of Soil Taxonomy (Soil Survey Staff, 1998). Of great importance for the studies of an ecological nature is the soil analysis to determine the presence of a soil seed bank, intended to identify the abundance and persistence of viable seeds in the soil. (v.10.7).

4.6 Collection of pollen

4.6.1 Introduction

Pollen is a haploid entity, that develops inside cavities known as the loculi forming part of the pollen sacs of anthers, surrounded by a layer of nutritive tissue, the tapetal layer, which has the function to feed and regulate development of pollen grains (Pavini, 1997).

When the grains are physiologically mature the anther opens to expose pollen to agents of dispersal. This process of dehiscence is preceded by a partial loss of water from the anther, as well as the outermost pollen grains which lose some water during and after aperture (Pacini, 2000). At the moment of aperture of the anther, pollen can be:

- ejected from the anther, as in some anemophilous plants (e.g. *Morus, Parietaria*) and in certain entomophilies species of the genus *Genista*;
- slowly dispersed subsequent to opening, because there are no mechanisms which withhold it, as in most anemophilous plants (e.g. *Poaceae*);
- retained within the anther by pollenkitt, a viscous gelatinous substance derived from degeneration of tapetal layer of the anther (Pacini *et* Hesse, 2005), as long as the adhesive strength of the pollen to the anther is not displaced by wind, or the pollen is accidentally transferred on to animals visiting the flowers.

In all three situations, at the moment of the anther's aperture, the pollen is exposed to changes in temperature and relative humidity. If the pollen grains have no mechanisms to survive these stressful conditions, they can lose or gain water, are subject to invasion by fungi and bacteria, especially in conditions of high humidity, and therefore prone to an accelerated death. The intervening time and distance between the point of aperture of the anther and the arrival of pollen on its landing site, that is the stigma in *Angiosperms* or in the micropylar drop (also called the pollination drop) in *Gymnosperms*, can vary substantially. This can be from a few seconds and a few centimetres in annuals such as the *Poaceae* to several days and several kilometres, as in most anemophilous trees such as conifers; of several days but short distances, as happens in entomophilous plants that are present in small numbers per unit area (e.g. *Orchidaceae*)[5].

4.6.2 Categories of pollen granules

At the moment of dehiscence, one can distinguish pollen grains on the basis of several characteristics namely, size, shape and structure, stage of development, water content, and clumping characteristics. The grain size can range from 30 to 200 microns, with most species having grain sizes in the 60-80 micron range. The pollen grain shape is often oval, sometimes spherical, and only rarely are there other forms. Such is the case in marine monocotyledons, as for example the pollen of *Posedonia oceanica* (L) Delile, which is needle shaped, several millimetres long and several tens of microns wide.

The external and internal features of pollen grains are important for its recognition. Having two covering layers of different composition, the exine and intine, this determines the geometry of the granule and is important characteristics for its recognition. Also, the presence or absence of starch inside the pollen grains is often a systematic characteristic (Franchi *et al.*, 1996). In fact during its development there is always accumulation of starch, but in the mature pollen this starch is partially or totally hydrolysed (Pacini *et al.*, 1996). Its absence does not mean there are no carbohydrate reserves, but that these are located within cytoplasmic vesicles instead of inside amyloplasts (Franchi *et al.*, 1996). In mature granules, there exist soluble reserves of carbohydrate, especially glucose, fructose and sucrose (Speranza *et al.*, 1997). Varying according to the systematic grouping it belongs to, pollen grains can either complete their development before dispersal or do so as the pollen tube grows. Pollen grains can either be trinucleate or binucleate, the former having completed their development.

[5] The Orchidacea are considered as a special case since pollination involves the transfer of pollinia (specialised anthers with a sticky pad at the base of the filaments) rather than individual pollen grains. Here the pollen is retained until the pollinia are transferred to another flower.

They are composed of two male gamete nuclei and a tube nucleus. On the other hand, in binucleates, one nucleus must still differentiate into two male gametes via mitotic division. Recently it has been shown that similar to what happens in seeds, there are two categories of pollen grains: partially dehydrated, similar to orthodox seeds and partially hydrated similar to recalcitrant seeds (Franch *et al*., 2002; Napi *et al*., 2001; Pacini *et* Hesse, 2004; Pacini *et al*., 2006). As in seeds, discrimination between the two categories of pollen grains is based on the 30% water content limit present in pollen. Pollen grains that are naturally partially dehydrated, with water content less than 30%, can resist temperature stress and changes in relative humidity and are easily conserved. Grains with a water content greater than 30%, do not survive dehydration, but undergo a quicker germinative process, even after a few minutes, because the rehydration phase is very short. When the whole anther opens, the grain can affect dispersion in different ways, this depending on the type of mechanism that binds the granules together to form small clumps. The term "Dispersion unit of pollen" refers to the mechanism by which pollen is dispersed. In fact, pollen grains can be dispersed individually, as in anemophilous plants, or grouped together. The four pollen granules derived from meiotic division can separate, or remain together to give a tetrad, with common membranous walls joining them together. Thirteen different units of dispersion have been identified by Pacini and Franchi (1998). Pollen grains in monad or tetrad form can be dispersed as more complex dispersion units held together by viscous adhesive substances such as pollenkitt and adhesive filaments. This brings out the fact that pollen granules can arrive on the stigma as single units, or in large masses (pollenia) comprising even hundreds of thousands, as in *Orchidaceae*. From a genetic perspective, one realises that the larger the number of pollen granules making up a dispersion unit that arrives on a stigma, the larger the number of seeds that have the same "father". Vice-versa, if pollen is dispersed individually it is easier for pollen granules from different plants to arrive on the stigma, i.e. different "fathers"; in this case the presence of strong male competition (gametophytic selection). All these characteristics are important, directly or indirectly, for the modalities of pollen collection and for its eventual conservation.

4.6.3 Why pollen is collected

Pollen is collected for research and development purposes (Hoekstre, 1995; Dafni *et al*., 2004); and depending on the reasons for its collection, the method and quantity of pollen collected can vary according to one of the reasons listed hereunder:

- To identify the form and type of the dispersion unit;
- To verify its state of hydration, i.e. establish whether it is partially hydrated or dehydrated and therefore evaluate possibility and methods for its conservation;
- To check for viability, and therefore fertilisation activity;
- To check the number of nuclei present ie whether it is formed by two or three cells, therefore its stage of development;
- To store and use for research into plant biology;
- To test and use the pollen for therapy in the field of pollen allergy;
- To store and later pollinate plants of economic interest, as in the Kiwi where the flowers that are abundantly pollinated form fruits that are larger than normal and fetch a better market price;
- To conserve germplasm of a determined species.

4.6.4 Control for Viability

Whatever the reasons for pollen collection, by analogy to conservation procedures used for seeds, it is useful to identify the pollen's viability and whether it has the capacity for fertilisation. This characteristic is important not only at the point of the anther's dehiscence, but also at the moment of collection and successive phases. If the pollen is partially hydrated and therefore particularly vulnerable, it is useful to understand the time period for retention of viability at low humidity. There are five different approaches to evaluate pollen's viability, giving direct or indirect results. Indirect results are those that have data that indicates whether the pollen is viable or not. Direct results rely on measurements of its fertilisation activity. These different approaches are outlined below as follows:

- Analysis of pollen to verify the presence and quantity of enzymes involved in the respiratory process, where their presence indicates whether pollen is alive or not;
- Staining of cytoplasm or other generic molecules that indicate its presence or metabolic activity, however this does not confirm that pollen is alive;
- Pollen is germinated *in vitro*, on solid or liquid media. If pollen grains are capable of giving off a pollen tube, this indicates they are alive;
- Pollen is used for pollination and one observes fruit setting and the number of seeds that complete maturation.

The last method is the most reliable however it requires weeks, sometimes months. The other methods, on the other hand, give results in a few hours, in some cases even minutes.

The indirect method that is most frequently used is that of florochromatic reaction (Heslop-Harrison *et al*., 1984). This method requires the use of a non-polar stain "fluorescein diacetate" which is capable of penetrating inside pollen grains. Inside their cytoplasm the presence of esterase enzymes permit the release of fluorescein, a polar fluorescent compound that permeates to the inside of the granules only if the plasma membrane is intact. This method demonstrates the presence of active enzymes and the integrity of plasma membrane. Viable pollen grains, that have accumulated fluorescein within them, appear intensely fluorescent when observed under a UV microscope, while dead granules are weakly fluorescent or completely non-fluorescent. For reliable data it is necessary to base observation on a minimum of a hundred granules and three replicates to proceed to allow for reliable statistical analysis. With this method it is possible to evaluate the sample's viability in less than an hour, given access to a fluorescent microscope. If only a simple light microscope is available, it is possible to utilise other indirect methods to demonstrate enzymatic activity such as dehydrogenases and peroxidases, which tests are also simple and rapid, but can give false positives (Dafni *et al*., 2004). Another indirect method that makes use of a light microscope is that of Palfi and Mihalik (1985) using a specific stain for proline, a vital amino acid in viable cells. Not all pollen grains maintain viability over time, this depends on their water and carbohydrate content (Pacini *et al*., 2006). Partially dehydrated granules are conserved for longer periods because they are able to vary internal turgor pressure irrespective of external conditions. The mechanism involved in this type of homeostasis is the polymerisation and depolymerisation of sucrose, glucose, fructose and amides. An increase in temperature encourages the depolymerisation of sucrose and amides, such that this increased turgor pressure and water in cytoplasmic colloid does not evaporate. When the temperature is near zero there is also an increase in turgor pressure, which helps to reduce the formation of ice crystals. The same increase in turgor pressure is observed when the relative humidity decreases a lot and evaporation is prevented.

Conversely, when the relative humidity is very high, even above 90%, turgor pressure decreases due to polymerisation of glucose, and fructose to sucrose, and even the formation of a glucose amide (Vesprini *et al*., 2002; Pacini *et* Hesse, 2005). Partially hydrated pollen grains are normally equipped with homeostatic mechanisms through which most of them lose water, decrease in volume and die within a few hours of the anther's aperture, especially if kept in a dry environment. This has been shown via different methodologies with *Cucurbita pepo* L., whose presentation of pollen to agents of dispersal lasts only six hours (Nepi *et* Pacini, 1993). *Poaceae* have partially hydrated granules and it is difficult to conserve them for long periods. Naturally their viability is prolonged if conserved in high relative humidity (Pacini *et al*., 1997). Barnabas *et* Rajki (1981) have identified a method for conservation of maize pollen granules by slowly decreasing their water content. This method is however not in common use.

4.6.5 Methods of collection

Given that pollen cannot leave the anther until it opens, or until it remains, the modalities for its collection are different. One has to keep track of the length of time, from the point that the anthers dehisce, one can still collect pollen. Thus it is imperative to identify time zero (T_0), after which several characteristics of the grains can change depending on external conditions, especially for pollen grains that are partially hydrated (Pacini, 2000). Pollen, which is left to naturally disperse from the anther, is collected on sheets of wax paper. If dealing with plants that eject pollen from the anthers, or if this leaves at a point other than the anther's aperture, the plant or portions of the plant with flowers or male inflorescences are gathered, as close to the time of dehiscence of the anther, and are placed in a vase at the centre of the sheet of wax paper, aiming to orient them to the vase's outside. The operation is best done in the evening. The morning after, if the paper is not in a place accessible to sunlight, it is better to illuminate the flower vase with a lamp to facilitate a rise in temperature and the anther's aperture. It is recommended to collect pollen day by day and repeat the procedure every evening. The collection is done in an environment with no air currents or wind-dispersed contaminants. The same method can be used in anthers that retain pollen, for example due to the presence of a pollen adhesive (pollenkitt), however good results are still obtained even if cut flowers as in the case of the *Astaraceae*, are placed face down on wax paper. Even in this case, it is recommended not to leave flowers from which pollen is collected for more than a day, as this may accelerate release of immature pollen. In some cases, one can collect the anthers by cutting the filaments, especially if they are long, leaving them for some time in a dry atmosphere to facilitate their opening, and pass these through a sieve with a mesh just wider than a pollen grain's diameter. In these cases the yield is very low especially if the grains are surrounded by pollenkitt, while if the mesh is too wide the epidermal cover and other anther residues pass through. A method giving good results is to use a small suction pump to collect grains from the unopened anthers. In these cases pollen grains are collected in a test-tube that is subsequently used for storage purposes. Whatever the manner of pollen collection, it is important to control purity: this may include foreign particles from the same plant or plants of different origin, or extraneous pollen. It is important that grains are used to make sera for use in the study of allergies (Cour *et* Loublier, 1980). If the pollen is to be used for research purposes, it is necessary to assay its viability.

5. TRANSFER OF GERMPLASM

5.1 Temporary Preservation of Germplasm

5.1.1 Preservation of the consignments of seeds collected in the field.

Before being dispatched to a seed-bank, seed samples collected in the field must be kept in a cool, dry and well-shaded location. This may be done by following the suggestions listed below:

- Germplasm must never be left in the car or any other place with high temperature. Exposure to high temperatures and direct sunlight may damage and/or severely compromise the seed quality.
- Good ventilation around the germplasm must be maintained; seed should only be kept in paper envelopes or cotton bags[6], which allow adequate ventilation.
- Attention must be given sealing envelopes and bags in order to prevent loss and contamination of the collected germplasm.
- Envelopes and bags must be closed with pins and clips; adhesives may only be used on the external parts. Very small seeds may get stuck in the adhesive, becoming unusable.
- Under no circumstance may germplasm be frozen before arriving at the seed bank.
- In case of fleshy fruits collected at the right ripening stage, it is best to remove the fleshy pulp from the seed as soon as possible, thus avoiding fermentation processes that may reduce the germination potential of the seeds[7]. This is best done at the seed-bank. If it is not possible to consign the seeds immediately to the bank or to remove the fleshy pulp, the fruits must be kept in a refrigerator at 0-5^{o}C above zero.

5.1.2 Extraction of seeds from fruits

Seed extraction from fruits is best done at the seed-bank by an experienced person. In case of fully mature seeds enclosed in desiccated fruits/capsules, the seeds may be manually extracted rapidly and completely. In case of fleshy fruits, seeds become tolerant to desiccation at a relatively late stage of development. The seed's developmental progress is not always obvious from the fruits' ripening state and the seeds may retain high levels of internal humidity. If it is not possible to immediately dispatch the seed collection to the seed-bank, one must spread out the seeds in a single or thin layer on absorbent paper to allow maximum aeration, thus allowing them to desiccate and acclimatize to the surrounding environmental conditions. Some environmental conditions, particularly relative humidity and temperature, must be kept constant and regularly monitored.

[6] Use of plastic bags is not recommended since this is impermeable to gases and water vapour. Moisture if present in sufficient amounts would encourage moulding.

[7] In some instances, the presence of moisture can cause the seeds to start germination, again spoiling them completely or rendering them unusable for banking.

5.2 Consigning seeds to the seed-bank

5.2.1 Accessions accepted by the bank

Seed collections of indigenous species supplied by seed-collectors are often small and of inconsistent quality. Notwithstanding this, they must be accepted and enhanced by more extensive collecting from the same provenance in successive years. However, seed collection must not be accepted when collection data such as species identification, provenance, etc. are missing or uncertain.

5.2.2 Accession Documentation

For the purpose of ensuring precise information on an accession and subsequent analysis of that information, a specific germplasm collection field data card has been formulated (v. 13,1). This field data card should be completed with all the requested details and used each time that a sample is taken from this accession for whatever use. It should refer to a collection made on a single and specified entity, in a single and specific station (or microstation) and on a single and specified collection date. The seed collection card from a given population will subsequently be assigned an identification code or accession number. When carrying out collection of the seed material, it is fundamental to take care to indicate on labels the fundamental data to be able to facilitate the recognition of the sample and to limit any cause of doubt of identification. The same procedure should be adopted when one comes to take a sample from field seed banks.

If the material comes from other activities conducted *in situ* other than specifically from seed collection expeditions (floristic collections, vegetation surveys, demographic and phenological studies) besides the germplasm collection card, it is necessary to attach also a copy of the other data cards compiled in field. A list summarising all the details of material collected and the collector's contact details must be attached to the seed-container at the bank, so that the collector may be consulted should queries regarding the collection arise or in case a phytosanitary certificate (v.5.2.3) is required. When filling out data sheets, writing out labels and summary lists, the following simple guidelines must be followed:

- One must not abbreviate common and scientific names, as this may lead to erroneous identification.
- Use of clear hand writing in block letters is recommended.
- Use of indelible pencils and pens.
- Avoid corrections that make writing illegible or difficult to comprehend.

5.2.3 Phytosanitary condition of collection material

The movement of plant germplasm has the potential to transfer various pests, pathologies and pathological agents. For this reason, most countries have detailed legislation that regulate the importation and in some cases, internal movement of plants.

The spread of pathogenic agents can seriously compromise the state of viability of the collected seeds and the material, if reproduced, can spread infection to other species and territories. On the basis of such risks, one needs to document the presence of pathologies and sanitary state of health of the population where the seed sample was

collected, as well as eventual treatments to which the germplasm has been exposed (e.g. fumigation, fungicidal and insecticidal pre-treatment). It may be necessary to consult a phytopathologist or an entomologist (Frison *et* Jackson, 1995). In general, the risk for spread of disease is higher when plant roots are also transported as most pathological agents are harboured within the soil, and can therefore be carried with the sample. For viable vegetative entities the transfer of germplasm in vitro reduces most of these problems. The pathologies present in the transported germplasm, if present, must still be noted, as well as any diseases found in the region where sampling occurred. It is also important to indicate if the plants are healthy in an area notoriously or historically subject to infestations.

Before dispatching any material for scientific and conservation aims, one must ascertain that there is no need for phytosanitary certificate; at the present moment European Community regulations consent free circulation of germplasm inside European Union countries, whilst a certificate of provenance and phytosanitary document is required for third countries. In particular, the Ministerial decree of 11/05/2005, acknowledges the directive of the Commission of 15th October, 2004 that determines models for official phytosanitary certificates or phytosanitary certificates for re-exportation that accompany plants, plant products or other type of biotic material from third countries as explained in Directive 2000/29/CE.

5.2.4 Accession Dispatch Methods

The following are the details of the general regulations on the dispatch of germplasm adopted by the Royal Botanical Gardens of Kew, and taken in this manual as a point of reference. Seed containers must be tagged both on the inside and on the outside and must be carefully sealed. The following packaging rules are recommended:

- Cotton or cloth bags;
- Transparent nylon or PVC textile bags;
- Boxes of water resistant cartoon, inside which one deposits the bags containing the seed material.

It is not recommended to pack up the seeds in non-transparent, plastic or PVC containers or envelopes. For the dispatch of accessions one must follow this procedure in the order listed below:

- Pack the seeds at the last possible moment before dispatch;
- Enclose the relevant details, the accession and the number of bags in the box, taking care to keep a copy of this information;
- Use bubble wrapping, expanded polyester and other material to fill in empty spaces and limit germplasm movement within the box;
- Enclose the card showing the details of the collection expedition and keep a copy;
- Seal the boxes and tag the destination and sender's addresses;
- Measure and weigh the boxes;

Relevant photos, herbarium samples and other useful material can be sent preferably as a separate package on an alternative date.

5.2.5 Management of accessions originating from other entities

The aforementioned modalities for conservation and expedition of accessions, is not only valid for material arriving directly from an *in situ* collection but it is also

applicable for seeds originating from other bodies operating *ex situ*, e.g. another germplasm bank, botanic garden or nursery. It is of great importance to manage correctly the accessions originating from other centres collecting all the information about the management and treatment that the seeds were exposed to before dispatch. As part of this normal routine the bank curator must be furnished with the chronological details of the material received, a photocopy of the original collection programme and information to allow for contact with collectors and curators.

If sending several batches containing different accessions, it is important to prepare a list of material contained in the package. The personnel at the bank must conserve and manage the batch according to the most suitable modality applicable for the individual accessions. Each accession received into the bank must have attached to it the collection schedule, any pest treatment information, modalities for conservation and any additional notes including a note on whether protocols on germination or nursery production are available. In case where the information is not available or cannot be supplied, on e is to furnish a telephone, email or postal address reference of the persons responsible for curating the accessions previous to its consignment to the bank.

5.2.6 Management of material by the bank

Once the verifications on material delivered and on accompanying documentation is complete, the bank becomes responsible for its correct management, identifying timeframes for processing and the more suitable modality for cleaning, storage, viability testing and germination of the germplasm, excepting cases where specific conventions dictate a different procedure. Furthermore, once the material is consigned, the seed bank curators must evaluate and decide whether to work on the entire batch or part of it, taking in consideration the priorities identified by the bank, as well as the importance of the material and its quantity.

6. PRE-CONSERVATION TREATMENT OF GERMPLASM

This section of the manual gives a standard pre-conservation procedure, and is the result of the integration of procedures described in protocols used by the principal European germplasm banks; in particular it is based on the experience of the seed banks in Sardegna (BG-SAR, Centro Conservazione Biodiversita- CCB), Porquerolles (Conservation Botanique Mediterrenean), Cordoba (Banco de Semilas – Jardin Botanico de Cordoba), Kew (Royal Botanic Gardens, Millenium Seed Bank Project) and Valencia (CIEF Centre d' Investigacio; Experiences Forestales – Generalitat Valenciana). This does not exclude alternative procedures employed by other centres, especially for large quantities of seed.

6.1 Admission of seed batches into germplasm banks

Having completed the necessary phytosanitary controls, the material is introduced into the germplasm bank. One proceeds to register the relevant data on the database, verifying need to adopt any precautions for its manipulation, indicating these in the seed cleaning and storage data sheet as shown in the appropriate schedule (v. 13.9). Verification of the integrity and suitability of the germplasm is carried out on the premises, followed with the seed sectioning test if this was not executed in the field.
The registration of accessions is a fundamental routine for the correct management of germplasm as well as the initiation and execution of the correct procedures for batch admission. The bank ensures that there is complete correspondence between the list of accessions furnished by the collectors and the enclosed envelopes, noting discrepancies and any missing information. In the registration process it is fundamental to indicate:

- Name of the *taxon*;
- Number of accessions in the batch;
- Date of admission into the bank;
- The level of seed purity and the type of treatment used;
- Provenance of the batch, denoted with the name of collection station, and the code/name of the collector or the body that furnished the material;
- Any specific objectives for the collection or the name of a specific project for which the collection was undertaken.

The accession number can be an alpha-numeric code. For example, the Germplasm Bank of South-Western Alps, Chiusa di Pesio (CN), has adopted a code with the form: "NA/06/89" where NA stands for Numero Accessione or accession number in the bank, 06 indicates the collection year (2006), 89 is the sequence number generated for each new accession. The following year, the date (07) changes but the final number continues to increase. In this manner, one has a tentative indication of the material's provenance, its age and the identification number against other batches. During the Genmedoc (v.3.2.1) project, a new code was identified which has the following format:

GM 1234 SA 01 00/00A

GM - two letters identifying the project for which the batch was collected;
1234- four numbers corresponding to the collection's taxonomic;
SA – two letters that identify the partner institutions participating in the project (Sardinia)
01- two numbers that identify population number the collection was effected from;
00/00- first two numbers indicating the year/ last two numbers indicating the progressive no of the collection to identify the collection details;

A- letter to indicate the sample in storage after all the treatment phases.

The code GM 0245/SA010502-B (fig.13) identifies sample B of the collection 2 of 2005 carried out on population 01 "Monte Lattias- Uta (CA)" by the partner SARDENIA in the taxon *Anchusa formosa* Selvi, Bigazzi *et* Bacch, as part of the

Genmedoc project.

Figure 13- Example of an automatic tag generated by the Genmedoc database.

This accession number accompanies the corresponding germplasm at all stages, permitting the reconstruction of its chronology even after germination tests, dehydration, cold storage, dispatching to other institutes and the eventual germination in the nursery or in research laboratories. All the data sheets, tags, references, data analysis and database pages must bear the same code. It stands to reason that the accession code must be in a format that facilitates electronic cataloguing and data manipulation. Apart form these systems, there are others including bar-coding, the latter frequently utilised in a commercial setting, and is actually in use at the Onlus Germplasm Bank, Palermo.

6.2 Quarantine

Before the material is introduced on the bank's premises, it is useful to allow for a quarantine period of variable duration-time, during which germplasm is stored in an environment which is isolated from the main bank structures. Such procedures permit the evaluation of the phytosanitary state of the collected material and in particular identify the possible presence of fungal infections as well as phytophagous insects or other damaging parasites. It is not rare to find, even in perfectly cleaned and treated accessions, damaged material, or harmful organisms capable of damaging the seed (fig. 14).

Figure 14- Parasitised seeds of *Austragalus nitiflorus* Jiminez Mun. *et* Pau. (photo: E. Mattana)

6.3 Preliminary tests evaluating new accession batches

Whenever the batch size permits, one proceeds to conduct a series of tests (germination, viability, calculation of internal moisture, initial seed weight, etc.) on the fresh material (fruits, seeds, etc.) to acquire useful data that will help in planning out the final use of the accession, the number of replicate tests and the number of seeds to be used for each test, as well as monitoring of the population's seed productivity. The results of each test are registered on a specific record card (v.13.8) which is then attached with the seed cleaning record card (v.13.9) and all the other relevant documentation sheets for the accessions.

6.4 Fleshy fruits

Following a preliminary cleaning from any debris debris, the pulp is manually and/or mechanically removed from fleshy fruits, under a stream of water and preferably within 48 hours of collection. The aim is to limit fungal rot and any fermentative processes that can reduce the germination capacity of seeds and compromise viability. In cases where, because of time constraints, it is not possible to remove the pulp, the material must be temporarily stored in a refrigerator at a temperature range of 0^{o}C to 5^{o}C. If, at the moment of collection, the fruits are severely dehydrated, previous to pulp removal, they are immersed in water for a period varying from a few hours to several days, with the aim of facilitating cleaning and separation. At this stage seeds should only contain very small amounts of impurities. When this is not the case, the seeds must be given a second wash in a container full of water, followed by a more selective and intensive manual cleanup so that where possible all fleshy residues are removed. The seeds extracted from fruits are then drained by removing excess water, and are dried for a period of between one to seven days according to the seed type and environmental conditions. Following drying one proceeds with the manual elimination of inert residues present and to dehydration, using the same procedures outlined in the manual (v.6.9) for non-fleshy fruits. When quantities for processing are quite substantial, one can make use of mechanical de-pulping or de-husking equipment.

6.5 Post-maturation

Post-maturation refers to a process of physiological maturity that one observes in seeds and fruits after collection. Post-maturation is necessary for immature seeds to acquire the capability to germinate (Schmidt *et* Jøker, 2001). It is a known fact that at the moment of collection, despite careful observation, it is frequent to collect seeds at different stages of maturity. To ensure a homogenous sample, the post-maturation period allows those seeds which are still in their phase of development to mature to their final stages. To achieve this the material is temporarily retained within plastic, cartoon, aluminium or stainless steel bowls (fig. 15) for a period usually ranging from a few weeks up to a maximum of a month depending on the *taxon* (always by first removing the pulp from fleshy fruits). The ambient temperature must be kept below 20^{o}C and relative humidity below 40%. If the storage period of the seeds is longer than a month it is beneficial to lower the temperature further. The risk is to accelerate the aging process of the sample, giving rise to ambiguities in the interpretation of germination tests and a possible reduction in the conservation period. In this phase, the stored material is cleaned from impurities (dead wood, leaves and soil and other detritus), but is not

manipulated further. In this way, seeds do not dehydrate rapidly but on the basis of the conditions of the collection and the fruit type one can calculate the approximate date in which germplasm can be handled. The material which must be uniformly distributed over the entire surface of the container, as previously explained, is stirred around every 2-3 days to ensure uniformity of the treatment and favour a better aeration and consequent dehydration. Care must also be taken to cover the containers with very fine gauze to prevent contamination by seeds from other accessions.

Figure 15 - Seed batches in post-maturation in the premises of the Germplasm Bank of Sardegna (BG-SAR). (photo: L. Podda)

6.6 Cleaning and processing

If the seeds have the required characteristics, a small quantity is removed from the main batch, and cleaned again with the aim of testing the percentage germination potential and the potential of the material collected. The procedure for further processing of the seed batch is considered valid if the percentage germination is above 50%, excluding cases where there are difficulties for finding germplasm in sufficient quantities, in the case where population is at risk of extinction or when the natural germination of the species is naturally very low. The results of germination tests for newly collected material are not always comparable with tests executed on dehydrated/conserved material, in that at the moment of collection not all the seeds are at the same stage of maturation (e.g. *Fabaceae*) and this necessitates a period of post-maturation. The quality of a newly admitted batch can be a useful alternative to seed germination test both through direct observations (colour, size, weight, floatation tests, presence of parasites), and via the execution of tests for viability such as the “seed sectioning” test in those cases where this has not been done at the time of collection. The seeds are cleaned from residual impurities, resinous residues, empty or aborted seeds, seeds affected by insects and/or other damage which therefore cannot be conserved. The necessary operations at this phase of processing can be executed manually, mechanically or using both modalities.

6.6.1 Manual extraction

In most cases, the use of mechanical de-pulping techniques damages the seeds, leads to deterioration of the seed integuments and exposes them to fungal infections. Manual intervention, although being particularly wasteful in terms of time spent is retained as necessary to separate and disarticulate fruits or infructescences. An operator, can use a wooden stopper (covered at the base by a layer of plastic material), on which a force is applied at more or less a perpendicular angle to work plane so as to grind and separate

the seeds from the flowering and fructiferous organs. The seeds can then be separated from the chaff using interchangeable sieves of different mesh size (fig. 16). When this technique cannot be used, work must be executed manually using laboratory tools (e.g. pliers, tweezers, pointer, etc.). When the seed is very small and even microscopic (e.g. *Plumbaginaceae*, *Scophulariaceae*, *Orchidaceae*), the use of mechanical tools is inadequate since these are incapable of separating seeds from the minute inflorescences; this renders necessary the use of optical instruments e.g. a magnifying glass and stereomicroscope to release the seeds.

Figure 16- Sieve set. (photo: E. Mattana

6.6.2 Room Temperature Extraction

This technique is utilised for genera of coniferous species such as *Abies*, *Cedrus* and some species of *Pinus*, with characteristic procedures for each species. At the end of post-maturation phase, the bracts of the cones open, and the seeds can be extracted by tapping or use of a simple mechanical shaker. If dealing with a resin-rich species it is necessary to ensure that the resin has dried out completely before sieving. The passage in the sieve produces a mixture composed of winged seeds, stems, branchlets, etc. from which, after removal of the wings one must select the intact seeds. The success of the operation is ensured when the cones are collected from the plants at the moment the bract scales start to come apart naturally (Gorian, 2001). In such cases manual operation is necessary, both for sifting as well as for successive steps.

6.6.3 Warm Extraction

This procedure applies mostly for species of *Cupressus* and *Pinus*, with a few exceptions. This can also be employed for the extraction of seeds of *Fagus* and *Alnus*. The cones, tightly closed and scarcely resinous, whose woody scale show a mechanical response to drying, are placed on a wooden surface for post-maturation. Air currents and an irregular turbulence, facilitate drying. Once this period is over, varying according to species, cones start to open and are ready for warm processing. Treatment is effected in special ovens, whose temperature and heating cycle vary in relation to the species and moisture content of the cones. In order to prevent compromising the seed's viability, temperature should never exceed 50^{o}C (Gorian, *op. cit.*). After the extraction phase, selection and cleaning phases follow, which help to eliminate residual impurities including dust, resinous residues, and empty, aborted or insect-damaged seeds which are therefore not conservable. The necessary operations at this phase of processing can be executed mechanically, manually or using both modalities.

6.6.4 Mechanised cleaning operations

Processing of modest quantities of seed is normally executed with small laboratory machines (Gorian, *op. cit.*). The commoner type select on a gravitational basis (fig. 17), making use of an air current that can be regulated, that separates impurities and empty seeds from the viable seeds, selecting seeds on the basis of size and weight. For each

batch of seeds, cleaning procedure requires a number of cycles proportional to the homogeneity of the material and type of germplasm. By regulating air fluxes, the first cycles eliminate impurities and dust, the next steps being seed selection. However, if these operations are not executed correctly, they can lead to a general genetic impoverishment of the accession with respect to the population of origin, in that there could be loss of those seeds that, although viable, have a weight similar to discarded material. At the end of the procedure, the percentage of the discarded material and the yield of the collection campaign can be calculated relative to every accession. In the case where large quantities of seeds must be processed and there is no need for a high degree of cleanliness, e.g. in the case of batches destined for broadcast sowing, it is possible to make use of industrial grade machinery (fig.18 and 19).

Figure 17- Machine for gravimetric selection of germplasm in use at BG-SAR. (photo: E. Mattana)

Figure 18- Machine with air-flux and a dentate cylinder for the separation of seeds at CNNGF El Serranilo. (photo: A. Prada)

Figure 19- Machine with air-flux for the cleaning of large batches of seeds in use at the Blanc de Llovors Forestals (CIEF). (photo: A. Prada)

6.6.5 Manual or mixed operations

In some cases, automated methods are incapable of giving the desired results, such as when the seeds are very small and of similar dimensions to that of dust or have tissues that are finely shredded or reduced to dust. For this reason, one will have to use a sieve set (fig. 16) with variable mesh sizes from 1cm to 0.1mm, to favour selective elimination of impurities. In the more complex cases, seeds can be separated with the aid of tweezers and other laboratory tools. The combined use of manual and mechanical techniques is often applied for those cases where there is an initial "rough" manual cleaning preceding a mechanical session followed by more refined manual cleaning until the seeds are thoroughly cleaned of all impurities.

6.7 Quantification of accessions and germplasm analysis

Once the grade of purity and state of cleanliness of the material is verified, seeds are counted and the total weight of clean seeds is compared to the average weight of a seed. There are various systems for image analysis (v.10.5) that allow for weight measurements, and number of seeds in a sample without the necessity to count by hand, but for this to be reliable the batch must have no impurities. Concurrently one must execute a series of observations on the germplasm (integuments, endosperm, cotyledon, embryo, etc.) via microscopes, stereomicroscopes and x-ray illuminators (negativoscope) in such a manner as to identify anomalies and characteristics peculiar to the taxonomic unit being analysed. Furthermore the percentage moisture content (% mc) within the seeds must be determined. This is indispensable to identify the timeframe and method for dehydration preceding storage. The seed moisture content is important since it influences the rate of metabolic processes and, consequently, the longevity of seeds. In general, this data (mc %) is obtained according to the ISTA standards and in particular by following the precautions and instruction listed hereunder (IBPGR, 1982).

- Minimise the exposure time of the seeds to ambient conditions in the laboratory.
- Determination of % mc is executed on two replicas from a thoroughly mixed sample.
- For accessions with very humid seeds, a pre-dehydration step may be necessary.
- For some seeds categories (e.g. *Poaceae* and *Fabaceae*) scarification may be needed.
- Containers for desiccation must be glass or metal, with a hermetic/airtight seal to prevent variations in humidity. Before being utilised they must be dried in an oven for an hour at 130^{o}C and placed in a dessicator to cool.
- One must weigh 4.5-5g of seeds as replicas in pre-weighed containers. In the case of limited seeds in the accessions it is sufficient, though less accurate, to use two replicas of 0.5g each.
- Seeds of arboreal species and seeds with high oil content must be dried at low temperature to avoid volatilising the essences: $103^{o}\pm2^{o}$C for 17 ± 1 hours (treatment at low temperatures). Seeds of other species are dried at 130^{o}-133^{0}C for 1 hour (treatment at high temperatures), with exception of maize and other cereals for which the drying period is 4 and 2 hours respectively. Drying must be done in an oven with forced ventilation. After drying, containers are closed, cooled in a dessicator for 30-45 minutes and re-weighed.
- Moisture content (on a humidity basis) is calculated as the loss in weight expressed as a percentage figure to one decimal point. If M_1 is the weight of the containers (with lid), M_2 is the weight of the container and seeds before drying and M_3 is the weight of the container and seeds after drying, mc % is found using the formula:

$$MC\ \% = 100 \times (M_2 - M_3) / (M_2 - M_1)$$

The significance of this parameter varies according to the physiological state of the seed at the time of analysis and this helps to understand whether the seed is ready for storage or must be dehydrated further. Calculation of the humidity present in the seed can be executed by dedicated electronic instruments, known as humidity analysers or thermobalances (fig. 20), that concurrently weigh and dehydrate the sample via computerised controls (Suszka *et al.*, *op. cit.*; ISTA, 2006). Replicas are scarified to

favour loss of water through their integuments and are placed inside the instrument. The temperature that can be chosen by the operator is usually 105°C to avoid vaporisation of organic substances including oils. The instrument weighs and, at the same time heats the sample usually via infra-red rays, a process which is timer-controlled and stops automatically once the decrease in weight becomes stable.

Figure 20 – Examples of electronic humidity analysers or thermobalances, in use at the Sardinia seed bank (BG-SAR) and at the Banc de Llavors Forestals (CIEF) (photo E. Mattana)

6.8 Qualitative tests

Amongst the factors that determine the quality of the seeds, a number are considered significant and include the genetic complement, age and the growth condition to which the mother plant was exposed, the climatic conditions, physiological state of the mother plant during seed formation, stage of seed maturity at the moment of collection, collection technique, processing and methods for storage (Piotto *et al.*, 2001). The quality can be expressed via useful parameters in correlation with the seed's response *in situ* and *ex situ*. Before conservation storage of the seeds, a number of additional parameters must be qualitatively tested including the determination for their germination capacity, viability, as well as other tests that may characterise the genetic makeup of the seed and other physiological aspects that cannot be shown in the germination tests. In this chapter, the text avoids detailing the commoner tests, that are illustrated in "Official methods for seed analysis" (Ministry for Agriculture and Forestry, 1993; v.2.2.1) as well as International Seed Testing Association (ISTA, 2006), but lists the critical aspects as well as the reasons for the various tests. Details of the most well studied parameters and the major tests employed that determine the qualitative characteristic of the seeds, now follow.

6.8.1 Germination capacity

The percentage of germinated seeds (normal and abnormal) indicates the capacity for germination. This represents the most commonly used parameter to evaluate a batch of seeds, but does not express any other qualitative factor. ISTA defines germination as the emergence or development that leads the seeds to reach a stage where it is able to indicate whether it will develop into a normal plant, environmental conditions permitting (ISTA, 2004). Some authors consider germination as the potential development of a seedling via the emergence of a radicle with 1mm set as the minimum radicle length for macroscopic observation. The rate at which radicle emergence occurs

is an important aspect of germination, furnishing important information on seed quality, but is not indicative of the presence of genetically determined problems arising, for example, by phenomena of introgression (e.g. *Orchidaceae*).

6.8.2 Viability

A seed is considered viable when it possesses the morphological, physiological or biochemical characteristics essential for its germination. A decrease in viability is usually accompanied by a reduction of a number of internal parameters including its respiratory capacity, unsaturated fatty acid content, membrane lipids, enzyme activity and mRNA content. Tests to determine viability furnish only an estimate of the seed quality (indicating whether seed is 'alive' or not), but is very fast (24/48 hours) in contrast to the classical germination experiments that require longer periods. Viability must not be confused with capacity to germinate, infact viable but dormant seeds do not necessarily germinate. The following are some of the tests used to determine seed viability.

Tetrazolium test
The Tetrazolium test is a colorimetric test which makes use of a 1% solution of 2,3,5-triphenyltetrazolium chloride or bromide at pH 6.5 - 7.5 (ISTA, 2006), a chemical which is photosensitive, colourless and soluble in water. This solution is taken up by the cells of seed tissues and, acted upon by mitochondrial dehydrogenase enzymes if the tissue is viable, and is modified into an insoluble red compound; know chemically as "formazan". This colorimetric test is used in the event that the viability of a seed batch needs to determined in a short period of time, or when one needs to test seed viability as a follow up to a germination test that gives incomplete results. It is also used when working with *taxa* that show deep dormancy or take a very long time to germinate. The resulting colour at the end of the colorimetric test can be a shade of red for living or viable tissues, while dead parts do not colour up. This test tends to overestimate viability by 10% with respect to the values given by germination tests (Piotto *et al*., 2001).
Indigo carmine
Indigo carmine is a colorimetric test used in some countries as an alternative to the Tetrazolium test both for its cheaper price and relative simple procedure. The test makes use of a dextrose and sodium hydroxide solution. Seeds are freed from their integuments after being left to imbibe in distilled water for 24 hours. Next the embryos are extracted and immersed for 1-2 hours (varies with species) in a 1/2000 solution of indigo carmine at 20^{o}C in the dark; they are then rinsed in water and examined. Dead tissues take a blue colour, while live tissues do not colour up (Suszka *et al*., *op*. *cit*.).

Lugol solution
A solution composed of potassium iodide and iodine is used to stain the tissue. The stain reacts with amide groups giving a sepia blue colouration to the embryo containing amides which is therefore presumed to be viable.

Conductivity test
This is a test that measures seed quality indirectly, by evaluating the integrity of tissue and cellular membranes. When exposed to imbibition of water, seeds with damaged membranes show loss of cellular contents (ions, carbohydrates, etc.) which in turn modify the characteristic of the solution, furnishing a measure of electrical conductivity.

The advantage of this test, which however is used for a limited number of species, is its rapidity and simplicity (Piotto *et al*., 2001).

Fluorescein diacetate test
This is a colorimetric test for a rapid estimation of seed viability. It is also used to determine viability for pollen, roots of arboreal species, meristematic cultures or seeds of *Orchidaceae*. The fluorescein diacetate rapidly penetrates inside viable cells with intact membranes; esterase enzymes transform it into a fluorescent compound that spreads through the cells. A fluorescent microscope, equipped with a special light source and filters is used to quantify viable or damaged tissues and embryos (Piotto *et al*., 2001).

Radiographic analysis
This method furnishes a relatively precise indication on the embryo's development and the seed's stage of maturity, as well as the presence of larvae, pests or pathogens, which may be affecting the seed. Radiography is a non-destructive method that is very useful in cases where the germplasm is limited in quantity or the entity is threatened by extinction. This method is also used for conifer seeds. However, given the high cost of the equipment and precautions required during use, its use is presently limited (Suszka *et al*., *op*. *cit*.; Martin *et al*., 1998; Gudin *et al*., 1992).

Magnetic resonance
In some cases, results obtained with radiography do no reflect accurately the quality of the tissues, especially when the seeds are completely imbibed. Viable imbibed tissues can be confused with non-viable ones, maikng it easier to distinguish them when moisture content is reduced. Magnetic resonance is a non-destructive technique that gives images of protons (H^+) in water and fatty acid chains inside tissues. One can also follow the movement of metabolites, this being particularly useful to evaluate the physiology of the seeds. Using computerised data analysis, one can obtain high resolution images of particular metabolites (e.g. study of the structure and distribution of seed lipids).

6.8.3 Seed vigour test

Seed vigour is defined as the sum total of those properties that determine the level of seed activity and batch's response during germination in a vast range of environments (ISTA, 2004). Vigour cannot be measured via a single parameter because it is a concept that involves several aspects of seed behaviour, including rate and uniformity of germination and seedling development, capacity of seedlings to emerge in unfavourable conditions; the response following storage (in particular the capacity to retain the initial germinability). Vigorous seeds are potentially capable of optimal behaviour in conditions not considered ideal for the species in the sample. Differences in vigour can be manifested by variation in biochemical processes and in reactions during germination (enzyme reactions, respiratory activity, etc.) in the speed and uniformity of emergence of the seedlings, in growth during culture and after establishment and in the capacity to germinate in unfavourable environmental conditions. The vigour potential can condition the growth of adult plants, as well as their fruiting and seed yield. The definition of seed vigour considers anchorage of seedlings, but not eventual dormancy or the genetic makeup of seeds. Tests based on behavioural aspects of the seeds during germination,

for example accelerated aging, the cold-test (Piotto *et al*., 2001) and the conductivity test, are usually employed for the evaluation of specific components of vigour (Piotto *et al*., 2001; Elias *et al*., 2006).

6.9 Dehydration

6.9.1 Tolerance to dehydration and categories of conservation

Seeds can be classified in two principal categories on the basis of their response to dehydration and their behaviour during storage. The first group defined as "Orthodox" or "tolerant to dehydration", consists of those seeds whose storage consideration is substantially a function of their moisture content and the storage temperature. Such seed types can be stored out with no damage at low moisture content (even much lower than those reached in natural conditions); their longevity increases with a decrease in temperature and moisture content which can be calculated using the equation for seed viability (Roberts, 1973; Ellis and Roberts, 1980; Ellis, 1980; Pritchard *et* Dickie, 2003). Most seeds of plants in our latitudes belong to this group (Hong *et al*., 1998). Alterations (tab. 1) that orthodox seeds can undergo during storage are summarised below:

Table 1 Alterations to orthodox seeds during low temperatures storage in relation to water content.

Water content of orthodox seeds (%)	Possible alterations during storage at low temperatures
Inferior to 5	Lipid oxidation
Between 5 and 6	Practically none (ideal level for storage of seeds of most species)
Between 10 and 18	Marked development in activity of cryptogram
Superior to 18	Increase in respiration
Superior to 30	Germination of non-dormant seeds

Tolerance to dehydration is principally linked to properties of the cellular protoplasm. To tolerate dehydration, cellular tissues must be capable of limiting or repairing damage, maintaining proper physiological integrity during the period in which the tissue is dry, and oversee mobilisation of mechanisms necessary for the repair of tissues during the rehydration phase (Black and Pritchard, 2002). Specifically, some mechanisms that permit seed dehydration, relate also to their capacity to simplify intracellular structures (in particular mitochondria); the ability to inhibit metabolic activity; efficiency of anti-oxidant systems; the ability to synthesise protective proteins in cell membranes (LEA proteins); the ability to synthesise hydrophobic proteins that surround lipid bodies and prevent their agglutination during dehydration, as well as the capacity to vitrify substances such as sugars during desiccation (Berjak and Pammenter, 2002).
The second group, the "recalcitrant" seeds, or "dehydration sensitive", consists of those seeds that do not tolerate significant dehydration of the moisture content present at the moment of dissemination (in general ranging from 20 to 70%, but very often between 30 and 50%). Those seeds cannot be conserved with high moisture levels because this promotes germination in short periods and also cannot be kept at temperatures inferior to zero, since this would results in substantial damage to tissues as a result of freezing of water in the seeds. The conservation procedures of these types of seeds is still under development but envisages cryopreservation of the embryos in liquid nitrogen (v.7.13). The embryos represent very small structures resistant to desiccation and relatively

uniform in dimensions and moisture content and also able to survive controlled cyoprotective dehydration. With respect to the above description, positive results have been obtained with species of the genera *Quercus*, *Arthocarpus*, *Calamus*, *Elaeis*, *Hevea*, *Nephelium* and *Shorea*.

The elevated moisture content in recalcitrant seeds is especially evident in seeds of forest species, which are usually of "bulkier" dimensions and larger weight. The percentage of water at the moment of dispersion is a good indicator of its response to conservation, a high level characterises seeds that are difficult to conserve.

Seven percent of the seeds of around 7,000 species in 65 families studied so far, were found to be recalcitrant (Hong *et al*., *op*. *cit*.), but lack of detailed information on various plant species found around the world means this figure is likely to change. In this group, one finds seeds of numerous tropical species (coconut, mango, avocado, cacao, etc.), but there are also important arboreal species of our latitudes (e.g. *Quercus*, *Aesculus*, *Castanea*). Of the seeds that are most sensitive to dehydration one can also include the so-called "viviparous" because these seeds initiate germination when already on the mother plant (or during dispersion), as happens in aquatic plants of great ecological importance (e.g. mangroves). The *Fagaceae*, *Moraceae*, *Sopataceae*, *Lauraceae* are families with a high number of species whose seeds are highly sensitive to dehydration. From the above it follows that recalcitrant seeds do not form soil seed banks as is frequent in orthodox seeds.

A third category is the "intermediate seeds" (Dickie *et* Pritchard, 2002) that consists of those seeds that tolerate dehydration better than recalcitrant seeds, but tolerate it worse with respect to the orthodox types. Once partially dehydrated they do not tolerate low temperature treatment (inferior to O^oC), but cope better when exposed to mild weather (around 15^oC). In general these types of seeds tolerate dehydration to humidity values of 10 to 20% (Hong *et al*., *op*. *cit*.).

It is useful to consider that the identification of the categories described, can aid management of germplasm but are not rigid, given the continuum between orthodox and recalcitrant seed. For example citing the longevity of tomato seeds, they can be conserved for longer than 25 years (at -18^oC and 5% moisture content) while tea plant seeds (*Camellia sinensis* Kuntze) remain viable for 2-8 weeks (Walters, 2004). Despite the general tendency for germplasm banks to harmonise work criteria, the protocols applied for the definition of seed categories are still not sufficiently homogenous. Therefore a species can be considered recalcitrant by some, and orthodox or intermediate by others. For a given species with recalcitrant seeds, it is frequently found that tolerance to desiccation is higher in populations that live in less humid parts of the species distribution range. In some cases, improved techniques applied to the dehydration process, have given a longer storage period for seeds considered recalcitrant (e.g. *Fagus sylvatica* L.).

At a global level, management of genetic resources of species that live in humid tropical zones present the most complex problems. The difficult seed conservation process and limited longevity of the seeds of some species present the major pre-occupation. Knowledge of seeds that perish rapidly was already documented in the VI Century A.D where a Chinese scholar refers to the best method to conserve chestnut (probably *Castanea mollisima* Blume). Since the seventies, good progress has been made in conservation of recalcitrant seeds of some *Fagaceae* of ecological and economic importance in Europe, followed by numerous studies on other seeds that are intolerant to desiccation (Suszka *et al*., *op*. *cit*.; Piotto *et* Amadeo, 2004; Black *et* Pritchard, *op*. *cit*.). On the whole, our present knowledge and available technology do not permit an

accurate conservation and management programme for the genetic resources of highly perishable seeds.

6.9.2 Drying room

The reduction in sample moisture can be achieved via various methods including exposure to dry, well ventilated and shaded environments. Germplasm banks often make use of specialised drying rooms, which although expensive, give optimal results. Material destined for dehydration in a drying room (fig. 22) is arranged on open trays, and using dehumidifiers and air conditioners to maintain the relative humidity values at 10-15% and temperature between 10 and 25°C (FAO/IPGRI, 1994). These parameters are controlled to avoid that the seed integuments crack and/or wrinkle in the drying process. This treatment has a variable duration in relation to the seed type and varies from between 80 to 180 days. It is important that the drying room allows a good air circulation considered sufficient at 10 air cycles per hour (IPGR, 1982). In these conditions, seeds batches are exposed to dehydration inside cardboard boxes, bags made from porous/breathable textiles or trays (fig. 22) and weighed regularly to monitor changes in weight; if W_f is the weight that corresponds to 5±1% of the final moisture content (mc_f), and W_0 is the weight of the seed batch at the start of dehydration, one can determine the dry weight that should be reached at the end of the process, according to the formula (IBPGRI, *op. cit.*):

$$W_f = W_0 \times (100 - mc_0)/(100 - mc_f)$$

where mc_0 = original mc%
and $mc_f = 5 \pm 1\%$
At any moment it is possible to verify the moisture content of the accession (mc_f) with the inverse formula:

$$mc_f = 100 - [\ (W_0 / W_f\ ) \times (100 - mc_0)\ ]$$

with mc_f and W_f = mc% is the weight of the accession at the moment of weighing.

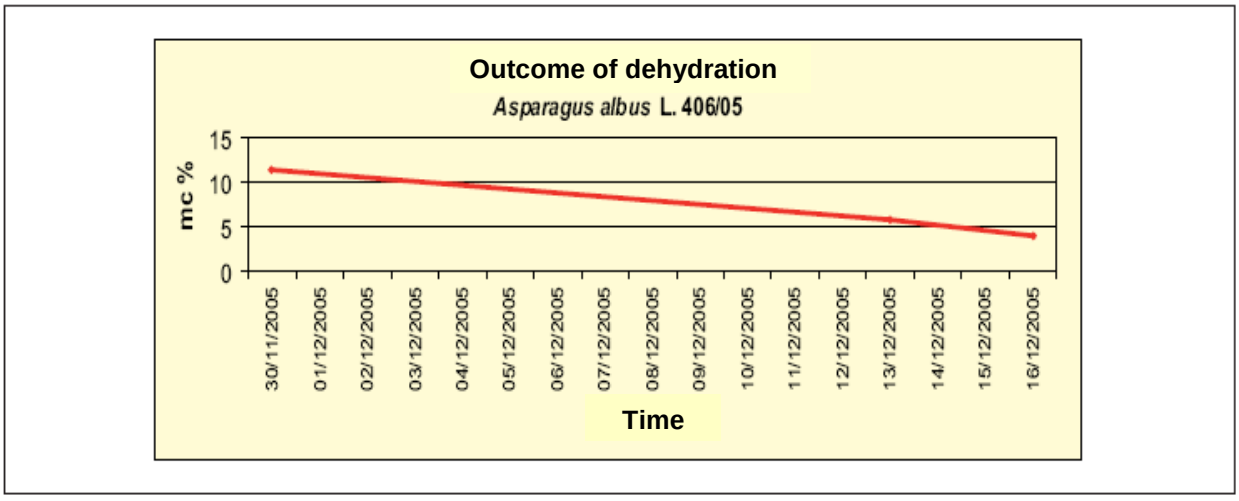


Figure 21- Monitoring of the dehydration of an accession of *Asparagus albus* L. (data: BG-SAR)

Once the moisture content is verified to be between 3.5% (for seeds with high content of oils) and 6.5% (for seeds with low content of oils), where such values of mc% correspond to the equilibrium relative humidity (ERH) of 15% at 15°C (Linington, 2003), seeds are considered ready for long term conservation at low temperatures

(Roberts, 1973; Ellis *et* Roberts, 1980) (fig. 21), usually by freezing at temperatures lower than -18°C (FAO/IPGRI, *op. cit.*). The seeds however, can be also conserved in refrigerated structures at temperatures of between -5° and +5°C.

Figure 22- Structures for seed dehydration at BG-SAR: humidistat with values of the relative humidity and temperature within the chambers, dehumidifier with chemical absorbent and stored material. (photos: E. Mattana)

Recently an alternative method to monitor internal moisture of seeds during dehydration was developed (fig. 23). This method presents an enormous advantage over other methods for the calculation of mc%, with the most important difference of not being destructive. It is based on the determination of parameters defined as "water activity" (wa) measured on a scale of 0 to 1, and represents the equilibrium relative humidity measure (ERH) between water inside the seed and the surrounding environment. Values of aw and mc% are correlated by an isothermal curve (Probert, 2003) and, varies both in relation to seed composition, and temperature. For this reason the exact correlation of the two parameters can be determined only empirically using both values for each seed type. Determination of aw and therefore ERH, represents a sufficiently accurate measure for evaluation, even if indirectly, it represents the water content of the seeds.

Figure 23- Instrumentation for measure of water activity (aw) at the M.A.I.Ch (Crete). (photo: G. Bachetta)

6.9.3 Artificial drying agents

Dehydration of accessions can be achieved via the use of artificial drying agents such as silica gel (fig. 24) that is placed in contact with the seeds in a hermetic/sealed containers. Given its absorption property, the internal humidity of the seed batches is lowered to values guaranteeing its long-term conservation (Probert, *op. cit.*). The quantity of drying agent employed varies according to the seed composition, the quantity of the material and above all the oil content. The general ratio of seeds/gel is considered as 1:1.

Fig 24: Examples of different types of self – indicating gels on the market.

7. Packaging and conservation

Given that the main target is of guaranteeing long term seed storage, it is important to control and monitor moisture content and humidity that represent the most delicate parameters for good seed conservation. In effect, long term storage does not require frequent handling of seed batches; for this reason it is necessary to utilise hermetically sealed transparent containers, to allow easy monitoring of moisture content inside seeds. Control of moisture in containers is often done via an indicator (e.g. silica gel).
It is fundamental to guarantee a perfect seal and the integrity of containers used for long term seed conservation. Several types of containers have been tested to evaluate their efficiency (tab. 2) where details are given with corresponding advantages and disadvantages in the course of storage (Gōmez-Campo, 2001). On the basis of this study, it appears that containers are fundamentally of three types.

Table 2 – Advantages and disadvantages of the various types of containers used in conservation.

CONTAINER TYPE	ADVANTAGES	DISADVANTAGES
Triple layered aluminium bags	Various formats, light, can be resealed, hermetically sealed, occupy little space.	Sample not visible, possibility of damage by external pressure or compression.
Polythene bags	Adaptable format, transparent, light, can be resealed, occupy little space.	Do not last long (polythene become porous), easily perforated by external seed structures, possibility of damage by external pressure or compression.
Glass flasks	Various formats, can be easily closed (on application of pressure), transparent, hermetically sealed.	Heavy, fragile, occupy large spaces.

For long term conservation, transparent glass flasks are preferred with a moisture indicator that changes colour when moisture is above 15%. For very small seeds so as to prevent dispersion of the seed inside the glass container, it is recommended to place them in polythene sachets, which are permeable to air which can then be placed in hermetically sealed glass flasks.
Closure of glass flasks can be achieved using different techniques; the more commonly used involves sealing by a hot oxygen-propane flame (fig. 25) (Gōmez-Campo, *op. cit*.). For this method, a cork septum is introduced in the glass tubes (fig. 25) to confine the germplasm in a reduced volume, and avoiding heat damage during the sealing process and subsequent placement in the final long term cool storage equipment. Inside the tube, a plastic tag is inserting listing data relevant to the accession and its date of creation. This can also be achieved by an adhesive barcode attached to the test-tube's exterior. With the aim of assessing the efficacy of the hermetically sealed tubes, these are inserted in a pneumatically sealed glass jar containing a saturated solution of sodium chloride (NaCl). At ambient temperatures, this solution establishes a high humidity inside the container. After around 4 weeks, it is possible to verify that the silica gel has remained

Figure 25 – Flame-sealed glass vials (photo G. Bacchetta)

colourless and therefore a perfectly maintained hermetic seal of the tubes which guarantees correct conservation of the seeds. Other methods include use of glass vials with a pressurised seal (rubber washer and aluminium casing) or screw. Also commonly used are glass vials with a pressurised seal and rubber washer (fig. 26).
Though use of a hot oxygen-propane flame can guarantee a secure seal, it is an expensive method, both in that the method is a time consuming process as well as the fact that the test-tubes cannot be re-used.
The perfect sealing of containers can be ascertained by testing its seal before storage and, for further guarantees, using the double container storage system. Seeds are inserted in the vials (variable volume, depending on quantity and the seed shape, from 10 to 50ml) sealed hermetically with using a crimper with a rubber seal and aluminium cap. The vials are stacked in the 500 to 1,000ml glass jar provided with a pressure seal. Inside the pot one can place an paper indicator strip for monitoring any eventual changes in humidity within the jars (fig. 26).

Figure 26 – Glass vial closed by a pressure crimping device in use at BG-SAR inside with two gelatine capsules impregnated with self indicating silica microgranules, inserted to monitor the humidity content, and a kilner jar with a self indicating strip to monitor humidity inside the jar (photo E. Mattana).

The closure operations must be executed within the dehydration room, where it is necessary to conserve the various containers (complete with washers and casing), as well as the moisture indicator, in a manner that everything is in equilibrium with the parameters for optimal temperature and optimal humidity in long term conservation.
The most commonly used moisture indicator is self-indicating silica gel granules (fig. 24). The silica gel is capable of adsorbing excess water inside containers, changing colour in the process. Silica gel is available commercially in a variety of forms: granules and perforated sachets. An alternative to the use of self indicating silica gel is the use of humidity indicator cards (HIC) or paper strips having areas impregnated with a solution of silica gel that changes colour depending on percentage of moisture (fig. 26).

7.1 Long term conservation

After hermetically sealing the seeds batches, these must be stored so as to guarantee their conservation with an estimated viability of decades. All this is applicable for seeds that allow dehydration (orthodox seeds). There is no possibility of long term, secure and guaranteed conservation of seeds sensitive to dehydration (recalcitrant seeds). Many experimental techniques have been tried and tested, but the full transposition of these

methods and scientific results in conservation procedures adopted by banks, has not yet occurred.

7.1.1 Freezing

According to the standards of the "International Plant Genetic Resources Institute" (IPGRI) freezing at storage temperatures of -18°C or less (IBPGRI, 1985a) is an efficacious method to prolong seed viability that can be conserved for long periods. Despite all this, it is considered that enzymatic processes within the seeds are not completely inhibited, and a consequent auto-dehydration, although very slow, is unavoidable. To avoid freezing the water found in tissues, especially for voluminous seeds (e.g. *Pancratium maritimum*) it is necessary to wait several months before obtaining the optimal level of humidity that permits the successive freezing stages.
Freezing is done in a type of freezer as those commercial availably or by using specialised freezing equipment. In the first case, costs are kept low, as well as the bulk's space and maintenance of structures can be positioned within the dehydration room (e.g. Lombardy Seed Bank, Trentino Seed Bank). This solution is preferred by banks with small dimensions, because this allows collection security at low costs, the only limitation being the capacity for germplasm storage. Banks of medium and large size, use freezing chambers which are made to measure with more sophisticated and dedicated freezing and controlling systems. Consequently, having resolved the problem of space, these can tackle the natural expansion of the collection in the medium and long term with more tranquillity (fig. 27).

Figure 27 – Interior of the -25°C freezer of the germplasm bank of Sardenia (BG-SAR). Photo C. Pontecorvo)

7.1.2 Lyophilisation (Freeze-drying) and Ultra-dehydration

Lyophilisation refers to a process permitting the dehydration of the seeds to values of between 1 and 3%. This technique is based on studies started in the eighties on the on the long term conservation of pollen aimed at reducing loss of viability after storage (Schoenike *et* By, 1981; Cerceau *et* Chalie, 1986).

In France this technique was developed via the Palynology Laboratory of the National Centre for Scientific Research if the Museum of Natural History in Paris. At the Conservatoire Botanique National Mediterranean (CBNM) of Porquerolles, this technique was started in 1987 where seed batches were periodically subjected to off site lyophilisation; since 1992 the acquisition of a lyophiliser has permitted the use of this technique directly in Porquerolles (fig. 28).

As noted earlier one needs to remember
that water is found in various forms in all living tissues. It is also good to note that the water that is most important in the dehydration process is that distributed in intracellular spaces and cytoplasmic vacuoles.
Since the tissues to be conserved are destined for regeneration, the dimensions of ice crystals play a fundamental role; an accelerated rate of freezing can in fact, lead to simple vitrification of water without deterioration of cellular frameworks. Once freezing is complete, material is dehydrated by placing under a reduced pressure of 10^{-2}atm thus ensuring the transformation of ice to water vapour. Considering the diversity that occurs between seeds, especially those concerning differences in the seed coat/integuments structure, seed dimensions and quantity of water at the moment of collection, it is necessary to identify the ideal methods and timeframe for their lyophilisation. The objective of experimentation with this technique or orthodox seeds is to evaluate period of viability in contrast to other conservation techniques including storage at low temperatures.

Figure 28 – Lyophiliser in use at the CBMN Porquerolles (Photo G. Bacchetta)

The following is a description of the lyophilisation procedure and the protocols in use through the CBNM of Porquerolles. First step is for the collected seeds to be dehydrated according to the classical procedure to favour post-maturation. Following this primary dehydration two methods are applied for their evaluation of moisture content:

- Weighing before and after desiccation in an oven at 104^{o}C for 24 hours in a seed sample. Water lost is expressed as percentage of weight.
- Weighing before and after lyophilisation of seeds in a defined time period (from 30 to 90 hours). The time point at which variation of weight becomes constant, makes it possible to estimate the loss of water corresponding to the content of free water in the seeds. The lyophilisation time reported in the literature is the minimum period favouring this loss of water. Concurrently, a germination test with a sample of lyophilised seeds is effected with the aim of verifying the viability of the batch. Actually the average time employed for lyophilisation is around 24 hours.

Studies effected through the CBNM of Porquerol have been conducted on 140 species belonging to 20 families (*Solanaceae*, *Saxifragaceae*, *Fabaceae*, *Liliaceae*, *Brassiceae*, *Apiaceae*, *Lamiaceae*, *Euphorbiaceae*, *Asteraceae*, *Cyperaceae*, *Poaceae*, etc.) representative of the Mediterranean flora of France.
On the basis of experience gained one can make use the following observations:

- An accelerated dehydration rate can generate a slight lag in the germination process, without damaging the seedling development;
- For several species one notes an improvement in germination of lyophilised seeds stored at ambient temperature with respect to samples that are lyophilised and conserved at +5^{o}C to -20^{o}C, and often seen in seeds with hard and impermeable coats;
- Lyophilisation of seeds identified as “dormant” does not affect germination in any manner;

- Storage of lyophilised batches at ambient temperature (fig. 29) permits a reduction in electricity bills without observing differences in viability with respect to lyophilised batches maintained at +5°C.

This technique opens interesting perspectives for long term conservation of orthodox seeds. At the moment, the CBNM of Porquerolles has 1750 batches of lyophilised seeds for a total of 500 different species. Comparative studies on the viability of lyophilised batches and those conserved at -20°C after 10 years of conservation show small differences in favour of lyophilised batches. This technique, however, is not suitable for the conservation of some species. One of advantages of lyophilisation is that it permits the execution of viability or germination test within the next 48 hours. This initial test, therefore allows immediate identification of any eventual deleterious effects of this technique and one can evaluate, whether it is advisable to employ this process of conservation for the species tested.

Figure 29 – Collection of Lyophilised seed batches at the CBNM Porquerolles (Photo G. Bacchetta)

7.1.3 Cryopreservation in liquid nitrogen

Long term conservation of seeds is favoured by the application of cryopreservation techniques, or the storage of germplasm at the temperature of liquid nitrogen (-196 °C). In these conditions metabolic processes of seeds, in particular enzymatic processes, are completely stopped due to the absence of water in the liquid state. In this manner germplasm viability can potentially be preserved for an infinite period. The efficacy of this technique, shown in laboratory trials on numerous species, has led various researchers to consider cryopreservation as the only technique available capable of long-term conservation and as being reliable in any situation. This technique is indicated in the case of some recalcitrant seeds, of species that use vegetative propagation, of species that are rare, threatened or in danger of extinction, as well biotechnological products of high value such as cell lines for the production of pharmacologic extracts, selectional clones, or genetically modified material (Engelmann, 2004; Gonzalez-Benito, 1998; Havengt *et al*., 2004; Hirano *et al*., 2005; Panis *et al*., 2001).

Theoretical basis for Cryopreservation

The basic principle underlying cryopreservation is that the likelihood of damage during the freezing phase depends on the quantity of free water in the biological system and free water's capacity to crystallise during the process. Cryopreservation of tissues can be used 1f one avoids formation of intracellular ice-crystals that can easily damage the function and integrity of membranes and intracellular organelles. In nature, some species of plants have adapted a system that minimises formation of ice-crystals at temperatures below O°C through synthesis of specific substances (sugars, proline and protein) that lower the freezing point of intracellular liquids enabling them to 'superfreeze' (Atics *et* Nalbantoglu, 2003; Griffith *et* Yeish, 2004). As the temperature of liquid nitrogen drops, formation of ice can be avoided by different mechanisms, the

principal being vitrification. Vitrification refers to a physical process of non-crystalline solidification of water which produces transition from an aqueous solution to an amorphous and vitreous state (Panis *et al*., *op.cit.*).

Cryopreservation in practice

When undertaking a cryopreservation protocol the following phases need active consideration:

a. pre-treatment;
b. cryopreservation;
c. post-freezing recovery

Considering that cryopreservation can be applied to any type of biological material, from seeds to apical meristem, cells, corms, pollen, somatic embryos, etc., specific protocols must be applied in the initial phases, depending on the type of material being conserved.

a. Pre-treatment- consists of manipulation of germplasm before proceeding with cryopreservation. Although it does not improve post-freezing recovery, on the whole it increases survival of the material when applied in combination with other cryopreservation strategies. The more common pre-treatment methods consist of gradual exposure of plant tissue from temperate climates to a regime of cold acclimatisation: application of osmotic agents that reduce water content before freezing or pre-cultivation of tissues on substrates containing 'anti-stress' compounds such as proline and abscisic acid (Benson, 1999).

b. Cryopreservation- as mentioned previously, the presence of water is equally detrimental in both for traditional conservation as well as cryopreservation. For this reason several strategies for cryopreservation are aimed at minimising moisture content of tissues or ensure that water is less disposed to crystal formation before proceeding to exposing the pre-treated tissue in cryovials to liquid nitrogen. The choice of a specific strategy is fundamentally determined by the type of material that must be cryopreserved (cells, corms, apical meristem, somatic and zygotic embryos, and seeds).

- Standard protocols for cryopreservation: This was the first protocol developed for hydrated plant tissues (Withers *et* King, 1980) and is based on an initial, slow cooling phase (at a rate of 0.5 – $2^{o}C\ min^{-1}$) in the presence of a cryoprotective solution often containing dimethylsulfuroxide (DMSO) at a concentration between 5 and 15%. When the material reaches a temperature of -$40^{o}C$ an important requirement is for the intracellular solution to be concentrated sufficiently for vitrification before successive immersion in liquid nitrogen. This method has been however substituted by alternative techniques mostly because of the high cost of instrumentation needed in the cooling programme. However this method is still in use especially in cryopreservation of undifferentiated material, cellular suspensions or corms.
- Dehydration by air: It is a simple method that is easily achieved allowing for reduction of water content of hydrated tissues. Generally samples are dried under a flux of sterile air (in laminar flow); however when using a highly reproducible method, the plant material is introduced in sealable vials containing a predetermined amount of silica gel (Uragami *et al*., 1990). This method is directly applied on orthodox seeds, zygotic embryos, and pollen of numerous species. The methodology of ultra dehydration has been applied on various recalcitrant species including *Quercus robur* L. (Berjak *et al*, 2000).

- Encapsulation/dehydration: This method was developed by Fabre *et* Dereuddre (1990) and represents a variant from dehydration by air, applied on apical meristems and somatic and zygotic embryos. This technique consists of a generation of 'artificial seeds' via encapsulation in spheres of calcium alginate. These are obtained by immersing the plant material in solution of free calcium alginate; successively material and solution are aspirated with help of a pipette and distributed in a 100mM solution containing calcium ions (Ca^{2+}). Ca^{2+}. determine polymerisation of the alginate around the germplasm and in the formation of the alginate capsule. The next step is osmotic dehydration via immersion of the alginate capsule into a 0.75M sucrose solution for 16-72 hours followed by drying under a stream of sterile air in a laminar flow hood, or in silica gel for 3-8 hours to reduce moisture content (Benson, *op. cit.*).
- Vitrification: This is a technique which was used for the first time by Uragami et al (1989), which, as described earlier, consists of a technique for the substitution of intracellular water with a vitrification solution, thus avoiding the possibility of ice needle formation during the vitrification transition. This process is generally initiated with the exposure of the germplasm to a substitute which is enriched with a cryoprotective agent such as 1.2M sorbitol for 1-2 days. Subsequently, the germplasm is immersed in a solution containing the vitrification compound (PSV2) consists of 30% glycerol (v/v), 15% ethylene glycol (v/v), 15% DMSO (v/v) and 0.4M sucrose; and maintained in ice for 20-120 minutes. After the vitrification process, the plant material is immersed in liquid nitrogen. This results in the intra- and extracellular vitrification of cellular components (Benson op. cit, Engleman, *op. cit*). Fifteen years after its publication, this remains the most utilised cryopreservation protocol. Its success is probably attributed to its simplicity, reproducibility and from the fact that it can be applied with good results for a large variety of plant tissue and plant species (Engleman, *op .cit*).

c. Post-freezing recovery: When germplasm is brought back to ambient temperature to re-activate its development, a few precautions must be taken. Generally the germplasm cryovials are extracted from liquid nitrogen, and placed in a water bath at 25-35^{o}C or even left to thaw at ambient temperature (Benson, *op. cit.*). In case the cryopreservation protocol included vitrification, the germplasm must undergo a phase that allows removal of the vitrifying solution once defrosted. It is therefore placed in a 1.2 M sucrose solution and is successively placed in a cool environment for its reactivation.

Application of cryopreservation to germplasm

Cryopreservation can be applied to any type of biological material. The following refers tot eh progress achieved in the cryopreservation field of orthodox and recalcitrant seeds of rare, endemic and threatened plants. Orthodox seeds (v.6.9.1) are characterised by the fact that they undergo a process of dehydration that considerably reduces their moisture content. These seeds do not present any difficulties for their conservation in traditional germplasm banks, so cryopreservation has been applied in the case of seeds with limited longevity and/or seeds of rare and threatened plants. In this case the technique of cryopreservation is simple and does not require much time or any specific requirements; given that the seeds are introduced into the polypropylene cryovials (fig. 30) and these are immersed directly in liquid nitrogen without

Figure 30 – Polypropylene cryovials for cryopreservation. One can note that the plant material (in this case vegetative buds) immersed in a cryoprotective solution before being immersed in liquid nitrogen. (Photo J. Ramirez Luna)

the need for any pre-treatment. This technique has been tried with species of the following families *Asteraceae,Brassicaceae Caryophyllaceae, Cistaceae* and *Scrophulariaceae* (Gonzalez-Benito) , *op. cit.*

Recalcitrant seeds (v.6.9.1) are characterised by a high percentage of humidity in their tissues, by sensitivity to dehydration (that results in loss of viability if it falls below certain levels); hence the difficulty in conserving them. In addition there is considerable sensitivity to low temperatures. However cryopreservation has shown several possibilities to overcome these limitations. The most promising strategy, in this regards consists of working directly on isolated embryos. This is because when the seed is placed in liquid nitrogen as for orthodox seeds, its survival after freezing is practically none (Marzaline *et* Khrisnapillay, 1999). Embryos are very small structures, resistant to desiccation and with relatively uniform characteristics with respect to dimensions and moisture content (Fu *et al*., 1993); therefore they can be placed through a controlled cryoprotective dehydration (realised for example, in sterile conditions under laminar flow) before being introduced in liquid nitrogen, both directly and after progressive cooling. Such experiments have been carried out with good results on various species of *Quercus*, and other genera that include species with recalcitrant seeds and of great importance in temperate and Mediterranean ecosystems (Gonzalez-Benito, *op. cit.*), such as *Arthocarpus*, *Calamus*, *Elaeis*, *Hevea*, *Nephelium* and *Shorea* (Marzaline *et* Khrisnapillay, *op. cit.*).

Another promising technique for the conservation of embryos consists of encapsulation followed by dehydration. In this case encapsulated zygotic embryos are kept for a period of time in a liquid substrate with an elevated concentration of sucrose, after which they are partially dehydrated under laminar flow or by use of silica gel. They are then directly immersed in liquid nitrogen. Some authors underline the particular sensitivity shown by embryos of recalcitrant seeds manifesting a negative response before extraction, an action that precedes their physical separation from the rest of the seeds (Benson *et al*., 1996). This phenomenon has been frequency observed in tropical species, the symptoms being of a rapid oxidation process that primarily involves phenolic compounds. These bring about a gradual blackening both of the embryonic tissue as well as the substrate immediately surrounding the tissues with the resulting inhibition of growth that leads to cell death. In these cases, the dehydration in sucrose followed by drying in a stream of sterile air is the most commonly used technique to avoid the emergence of this deleterious oxidative process. Some authors have also suggested that the addition of activated charcoal in the post-freezing recovery stage of the embryos. Where, this methods having been demonstrated to be very effective to absorb potentially toxic phenolic compounds (Normah et Moezolina, 1996).

The implementation of cryopreservation techniques has played a fundamental role in the progress for conservation of rare or threatened plants. An example is seen in orchid species in danger of extinction such as in the case of *Bletilla striata* Rchb.f. (Hirano *et*

al; opacity). In this case, the immature seeds of *Bletilla*, collected 4 months after pollination, with an average moisture content of 33%, were cryopreserved. This procedure has been realised by applying a pre-treatment and vitrification protocol. The pre-treatment consists of maintaining the collected seeds in a substrate called "New Dogostima" (ND) (Tokuhara and Mii., 1993), solidified on 0.2% Agar, and enriched with 0.3M sucrose. In this condition the seeds are maintained at 25ºC for three days under continuous illumination at intensity of 62.0mMm^{-2}s^{-1}. After this pre-treatment, seeds are exposed to a process vitrification that involves addition of a cryoprotective solution (2M glycerol and 0.4M sucrose on ND substrate) in 2mL cryovials and maintained in this vial for 15 minutes at 25ºC. Next, the solution is removed and the seeds are dehydrated at 0ºC for 2 hours with 2ml PSV2. Vitrification solution that contains 80% glycerol (v/v), 15% ethylene glycol (p/v) and 15% dimethylsulphoxide (p/v) on ND substrate enriched with 0.4M sucrose and adjusted to pH 5.4 (Sakai *et al*., 1990). Afterwards the cryovials containing the vitrified seeds are immersed in liquid nitrogen.

7.2 Active Collections

In addition to the material set aside for long term conservation (the base collection) every bank must guarantee the availability of seeds for short-term uses such as for *in situ* population reinforcement or reintroductions, sowing directly in the field, *ex-situ* conservation such as regeneration, exchanges through *Index Seminum* and for scientific research experiments. Batches of seeds destined for such uses constitute the so-called "active collection". In this case, the seeds are preserved by refrigeration at a temperature between 0 and 10°C without the need for any special freezing equipment.

7.2.1 Seed sowing

Plant propagation is a fundamental process for the conservation of species which reproduce sexually. There is no use for conserving an accession from a given taxonomic unit or seed batch if these do not have the capacity to reproduce the original material. In the field of conservation, plant propagation is carried out to cater for different objectives, for example to reinforce populations during *in situ* conservation, to create new populations in different locations or to regenerate an old population; furthermore, it is often necessary to grow plants of a species for various scientific projects with a number of different objectives.
In this part of the manual, it is not our intention to give an exhaustive description of all the methods and techniques currently used for plant propagation. The aim of this chapter is to summarise the best experiences in cases where there is a limited supply of seeds from small and/or threatened populations.
The most appropriate plant propagation and plant production technique often tends to be species-specific and is often a function of the very diverse requirements of the plants so that specific protocols may need to be devised. Whatever the situation, it is always advisable to use the most suitable substrate and to place the seeds under the most suitable environmental conditions that favour germination. Seeds should be protected from abiotic and biotic agents that may cause harm to the tender plants (eg. adverse climatic conditions, disease, birds, small mammals such as rodents, etc.)

Seed nursery conditions
Considering that seed germination is possibly the most delicate stage in plant development, adequate care must be taken at this stage. For this reason, nursery

conditions should be such that ambient conditions such as temperature, humidity and light can be controlled. The ideal situation would be dedicated seed germination rooms equipped with controls for air temperature and humidity, thermostatically controlled seed beds to maintain temperature, automatic watering systems with adequate drainage and uniform artificial lighting directly over the seeding trays/ containers.
As a general rule the level of environmental controls that can be affected depend on the degree of automation present which can vary from manual to thermostatically to computer controls or combinations of these. When the number of seeds in very small, the use of dedicated germination rooms and cultivation chambers (eg. phytotron) is recommended. In cases where facilities do not included the suggested structures, other equipment such as a thermostatically controlled seed bench in a greenhouse can be used (fig. 31). These devices are a relatively cheap alternative which provide suitable temperature control for numerous seeds ((Jimenez and Caballero is obtained, 1990). In extreme situations, one sows the seeds in the open, giving as much protection as possible from severe weather conditions (e.g. wind, elevated solar radiation, frost).

Figure 31 – Temperature controlled greenhouse with thermostatically controlled benches for l plant multiplication. (photo E. Mattana)

The requirements of every species are different and often very specific: as a genera rule one must try to recreate as close as possible the conditions present in the natural habitat. This means that when working with different species, the seedbeds cannot be placed in the same area of the nursery or greenhouse. As an example in order to seed *Littorea anchusa* Moris, an endemic to the coastal areas of the south-western Sardinia, it is important to position the seedbed under direct sunlight for a number of hours. On the other hand, the seedbed for *Anchusa formosa*, an endemic of the Sulcis mountains, needs shaded positions with low temperatures. This means that for the germination of seeds from different species, it is imperative to select the most suitable position in the nursery or greenhouse according to their requirement and grouping together those seeds that have a common requirement.
As an example it is advisable that the same conditions used to germinate seeds of *Anchusa littorea* be used for entities which are typical of the coastal foreshore such as *Limonium* (even if for this a totally different substrate will be used). For entities where the ecological requirements are not known, it would be advisable to work under laboratory conditions, using the dedicated germination cabinets (Thomson, 1979). In this way the best conditions for the entities under review can be characterised with greater precision and thereby establish the germination protocol (v. 10.2). Once the seedlings are obtained it is not advisable to transfer them into the greenhouse directly. As an intermediate step, it is advisable to maintain under laboratory conditions for 3-4 days is in order to observe their development.

Containers for growing on

There is an immense range of containers on the market that can be used for growing on plant with an infinite variety on the type of material and size; in the nursery one fundamentally works with perforated multi well seed trays or single pots of variable height and volumes. When one is working with small amounts of seed from different taxonomic entities, the necessity arises to allocate a large number of small spaces, for

the various types and sizes of trays, depending on the species being grown. In the majority of cases, perforated open or multiwell plastic trays are used (Vilarnau ET Gonzalez, 1999) serving as a miniature seedbed (fig. 32). This system allows for the germination and growing on of a high number seeds on a relatively small area. One can also find on the market, reusable trays made from flexible plastic which are particularly adapted to those entities whose seedlings grow quickly and do not remain in the nursery for a long time; these trays are easily degraded by ultraviolet rays and their manipulation may be problematic because they are very flexible. These containers come ready perforated for water-drainage with the hole size chosen depending on the species being cultivated.

As an alternative it is advisable to use plastic, the more rigid plastic trays used for food storage, which come in various dimensions, piercing them to the base in order to ensure water-drainage. The advantage of these containers is that they do not deteriorate quickly in the sun, are easy to use thanks to their rigidity and help to optimise available space inasmuch as there is an immense range of dimensions available on the market. In some cases it is the much most practical to use multiwell seed trays. Multiwell trays of small dimensions (40/45 cm^3) have been used for *Limonium* species; the root system often adapts well to these types of containers and if the species concerned is known to have a high rate of germination, one can obtain a seedling in practically all the cavities.

As a general rule it is advisable to use multiwell trays for species or accessories wit high rates of germination and where germination is not spaced out, one can obtain the maximum uniformity of development during the first stages of seed growing with the lease possible number of empty containers.

The depth of the container must also carefully be considered equally when one is working with species that reach a large dimension, like those for arboreal species. These pass a relatively long period in the seed nursery before being transplanted in the final container. In these cases, perforated open trays or multiwell trays at least 18cm high are used. Multiwell trays, which are easy to manipulate and are commonly used for seeds of arboreal species with relatively large seeds, it is advisable to use those from $200cm^3$ for conifers and $300cm^3$ for the single trunk deciduous species (Ruano, 2003). Eventually, if the plant is to remain for a longer time in the nursery, transplanting into larger containers is advisable.

From the very beginning of seedling development it is important to avoid deformations of the rooting apparatus in as much as these tend to be maintained and will create problems of stability in the adult plants; the problem becomes particularly serious in those arboreal species that always need good anchorage. Many containers are currently commercially available and are called “root trainers” especially sued for forest trees. These have grooves, fissures or reliefs into them (e.g. that are embossed in the vertical side of the pot walls) that help avoid the more serious deformation that is the spiral coiling of the roots.

The roots of plants raised in containers that rest directly on the ground or on impermeable plastic surfaces tend to grow out on the plastic creating possibility of trauma and injury especially when plants are moved; the use of elevated beds or benches favours the natural aerial pruning pf the roots and the nest and most symmetrical root formation.

All containers must be disinfected before use and/ or reuse. Generally a solution of sodium hypochlorite in water is used, in which the pots are dipped for at least 30 minutes; followed by rinsing in running water.

Root Substrates

No universal rooting substrate exists since properties are expected to vary according to the species that is seeded. One can however male some general recommendations: the pH of the substrate should be slightly acidic with a spongy nature, and it would have to show good water retention and easy to hydrate, fine webbing, low density, high total porosity and good ability to aeration, stable structure (so that it does not contract or expand exercising pressure on seeds), lacking weed seed infestation, phytotoxic substances and in a addition a low salinity, elevated content of organic substances, low rate of decomposition and sufficient level of growth nutrients (Raymond, 1989). As an example, for *Silene cambessedesii* Boiss. et Reut., the most suitable substrate is composed from a mixture of 70% of siliceous sand with grain size varying between 2 and 3 mm and 30% universal compost and this with respect to one species that lives on coastal sand dunes.

Figure 32 – Seedlings of *Silene diclinis* (Lag.) Lainz, seeded in food plastic trays (photo: M. C. Escribà)

Seed sowing and growing-on practices

Seeding involves the exercise of placing the seeds in the substrate; it can be done using laboratory tweezers or mixing the seed batch with fine sterile sand or talc for better broadcast distribution. The depth of the seeds must be equal to or smaller than double the width of the seed (Besnier, 1989). A system that gives good results involves one that covers the seedbed with a thin layer of sterile sand or vermiculite with a with the objective of preventing the dehydration of the surface and protecting it form the direct incidence of sunlight. After seeding, the surface is lightly tapped to press the seeds into the substrate in order to avoid stagnation or losses of humidity.

Irrigation should always be made with good quality water, clean and with low salinity in order to avoid phytotoxicity problems. It must be ensured that the substrate humidity has to be constant before seeding and this is maintained at saturation point though avoiding excesses that could provoke infections that could prove injurious to radicles as is also advisable to consider mist irrigation; if this is not possible, the trays must be hydrated from the bottom by capillarity, the aim being to avoid above all that drops fall on the seedling especially in species with hairy leaves that retain much humidity and consequently tend to rot.

The optimal temperature for development is variable depending on the taxon; a good temperature is generally considered adequate if it oscillates around 20ºC. however many temperate climate species have marked need for alternations of the temperatures, as happens in end of winter – beginning of spring, before being able to give way to the processes of germination.

Drastic changes in temperature can cause serious damage to germination. Temperatures of around 35°C are considered extreme for the cultivation of plants since at these conditions of temperature the photosynthetic ability diminishes considerably. This fact must be taken into consideration in greenhouses or nurseries. When cultivation is

undertaken in dedicated germination growth rooms, these have to be armed with sensors to give the alarm in cases of extreme temperatures.
For a lot of species the optimum requirements of light for the germination; the light intensities; duration of the photoperiod; etc, are not known. It is a known fact that, when the cotyledonary leaves or the first true leaves appear, the seedlings require light. During this phase of growth excessive shading must be avoided in order to avoid spindly plants. The fertilisation is carried out manually, when the first pair of true leaves has been fully developed and given rise to the second pair. It is not advisable to do this earlier in order to prevent root burns. A conventional fertiliser of type N-P-K 5-4-6 with microelements can be used. Slow release fertilizers can also be added during the preparation of the substrate. This type of fertilization has been employed with optimal results for the cultivation of *Dianthus turolensis* Pau et Pau and *Teucrium dunense* Sennen.
Usually no treatments with herbicides are affected when the seedlings are very small; any infesting weeds can be removed manually taking care not to damage the roots.
Transplanting refers to the process in which the seedling is removed from the seedbed and is placed in a larger container, which can be of various types but is a function of the morphology and the size of the rooting apparatus and the estimated time of cultivation. The optimal moment of transplanting is variable in every species, and must be mage when the seedling is strong enough to tolerate the manipulation. As an example, the seedling of *Carduncellus dianius* Webb, have sufficient consistency and flexibility for being transplanted with only a few true leaves. Conversely the seedbeds of *Ononis tridentata* L. must be transplanted at least 2 or 3cm substrate since root attachment is quite high. The seedlings of other species with true leaves like in the case of *Silene diclinis* (Lag.) Lainz (fig. 32) must be transplanted with 4-6 leaves in order to resume vigorous growth. Some species can remain for along period in the seedbed like in the case of *Teucrium lepicephalum* Pau.
One technique used in order to obtain the seedlings of some species consists of collecting material directly underneath a plant thereby obtaining the natural soil seed bank. Even if this is not a regular practice, it may serve as a way to establish germination protocols quickly, as obtaining seeds that are difficult to collect with the conventional methodologies or in order to acquire seeds in the best stage for their germination. As an example, the case of *Filago mareotica* Delile can be cited, whose seeds are extremely small, are conserved in the ground and germinate only at the moment in which conditions are optimal.
In the Mediterranean climate, in order to limit the deleterious effects of elevated solar radiation during the summery period, it is wise to protect the plants from direct solar radiation using natural shading or structures covered with shading materials of various types and various percentages of light exclusion.
All the processes realised from the preparation for seeding until the final destination of the plant, have to be annotated in an appropriate card (v. 13.14). These activities must be fully annotated with the conditions of growth and the place in which the material is found and other information of interest.

7.2.2 Index Seminum

The material used for the exchanges with the other research agencies via the Index Seminum, comes conserved in plastic tubes sealed with parafilm or in triple layered aluminium envelopes or in plastic material with a minimum of 20 seeds and a humidity

indicator. The agency that receives the germplasm with be informed on the origin, the treatment to which the material has been subjected, the controls carried out and the optimal conditions for germination, through a copy of field and laboratory cards relative to the accession.
The directory of the individual accession available for exchange and their amount (in grams and/or seed number) has to be made available and to be updates periodically, through publication notices, catalogues, web pages, etc. The request for material, usually happens through the compilation of electronic or card request from which are dispatched from the requesting institution. An example of the application forms, one can cite that of the Asociación Ibero-Macaronésica de Jardines Botánicos (http://www.aimjb.org/) and hat for the Bank of the Germoplasma of Sardinia (http://www.ccbsardegna. it/).
An important element of communication and also possible point of collaboration between banks can be represented from the exchange of information on the material demanded; in particular the bank that receives the germplasm would have to transmit a series of relative information to every seed batch received, and in particular: the destination of the received batch (e.g. seed bank, seed collection, living collection, research, etc.); germination percentage, eventual pre-treatments and conditions of germination, number of seedlings obtained); seed viability; eventual doubts on the determination of the taxon.

7.2.3 Duplication of the collection

Another important action to be implemented between institutions involved in ex situ conservation is that involving duplication of collections. In this way once can ensure maximum security or the future availability of the collection, especially in cases where technical faults or other problems can compromise the state of conservation of collections in a seed bank.
A fundamental aspect of the exchanges was that relative to the ownership of the germplasm. The institution that sends the material remains the sole owner of that germplasm and could at any moment ask for its restitution; in cases of in situ conservation requirements; the receiving institution is therefore solely a custodian of the germplasm. The exchange of seed batches is subject to phytosanitary regulation established by the competent national and international authorities (v.5.2.3)

7.3 Methods of pollen conservation

With regards top pollen conservation, it is advisable to store this in small aliquots instead of a single container. This is so as to take solely the quantity needed without interfering with the state of conservation of the entire lot. Furthermore the smaller the quantities stored per aliquot, the more uniform are the storage conditions are expected to be. Theoretically, the pollen of every species has its own ideal storage conditions, but not all pollen grain can be stored using the same techniques. The water and the reserve carbohydrate content are the most important characteristics that influence the way that the pollen should be conserved (Pacini *et* Hesse, 2005). In particular, pollen grains with a high water content like those of the *Poaceae*, present difficulties and do not conserve very well (Barnabas *et* Rajki, *op. cit.*). The following is a list of the most common methods used for pollen conservation and storage:

- Conservation at low temperature and low relative humidity. The containers may be small tubes, the best being plastic eppendorf type which should not be sealed. The tubes are stored inside a desiccator that can be maintained at a temperature of between -20 to +4^{o}C and a relative humidity of less than 10%. To ensure that these conditions are maintained, self indicating silica gel granules are placed inside the desiccator, which is then held according to the temperature regime which has been chosen, either inside a fridge or inside a freezer. The simplicity of this method makes it amongst the most frequently used, allowing for the storage of pollen a maximum period of a few months. However, this method is not recommended for the conservation of cereal pollen or partially hydrated pollen.
- Storage at very low temperatures under vacuum. This method involves freezing the pollen between -60 to -80^{O}C and the gradual incomplete removal of water via its sublimation. After this treatment the pollen can be maintained at a temperature slightly below 0^{o}C. This method allows for the storage of pollen for long periods, even up to several years.
- Conservation at very low temperature (cryopreservation). With this method the water content of the pollen is reduces to a pre-determined value in a cryovials after which the cryovials is immersed and stored in liquid nitrogen. This has been the most frequently used method during the last years, and represents the best available long term storage method. A major difference from the other two preceding methods, is that cryopreservation can be used for the storage of cereal pollen, given that these are first (slowly) dehydrated to 20-40% at 20 ^{o}C.
- Conservation in organic solvents: This method overcomes the problem of having to maintain the pollen at a specific relative humidity and furthermore using this method, the stored pollen can be transported from one place to another without the need of being stored at a low temperature. Before being stored in the most common solvents (acetone, benzene, petroleum ether, xylene and toluene), the pollen must still be dehydrated. One can also use the solvents but these influence the vitality. This method has up to now been used on few species and is not currently in common use.
- Independently of the conservation methos used, the return of the pollen preserved under the aboce mentioned/described methods back to ambient or laboratory conditions. For this reason, if the conservation was done at low temperatures, the thawing must be slow and must include also a slow process of rehydration. The latter part of the process is achieved by exposing the open vials or better still, a watchglass (or a flat surface) over which the pollem has been spread, in a container inside which there is a hugh relativev humidity obtained by having a filter paper wick dipped in water. It is advisable that, whatever the conservation method used, before using the pollen, its viability should be verified (v.4.6.4)

7.4 Conservation of vegetative material

The conservation of vegetative material may be a good method and sometimes the only method for the conservation of a genotype, a taxonomic unit or a population. These techniques are employed on specimens that are not producing seed, or are producing it in very small quantities. It proves very useful when one suspects a reduction of the genetic variability of the offspring with respect to the parent generation (for example in the case of population with dioecious taxa with the number of male individuals greatly reduced), when one wishes to conserve a determined genotype with peculiar

characteristics or populations in which the seeds could have been fertilised from pollen that does not belong to the same individual (introgression with allochtonous species or with genotypes of non-autochtonous or unknown provenance. Furthermore, it is always a good strategy,

that for those species that replicate well by asexual means such as the *Salicaceae* (v.10.3) or for species with recalcitrant seeds. In theory, one can utilise any part of the plant for its vegetative propagation. Keeping in mind the classical methods of plant propagation, the material which is most frequently used are the semi ripe green herbaceous and woody cuttings, roots, rhizomes, tubes and bulbs. The following refers to stages of collection and possibility of conservation of this material up to the power of propagation; the technique used is related to the species, the age of the mother plant, and the substrate available and the conservation techniques. As regards the plants form which the material is derived (mother plants or ortets) for topophysic or cyclophysic reasons, one generally obtains better results when these plants are young or the material is obtained from young offshoots.

Fig 33. Multiplication by cuttings practiced at CBNH in Porquerolles (Photo: G. Bacchetta

Woody cuttings (fig 33) are frequently used for deciduous tree species (e.g. *Salix*, *Populus*, *Tamarix*, *Laburnum*, *Cornus*, *Rosa*, *Ribes*, *Vitis*, etc.). Since these can be stored for an appreciably long period and usually do not require complicated methods for the rooting period. The cuttings are taken during the dormancy period of the plant, usually on wood of the previous growing season. The material is taken from the lower/ basal and middle regions of the tree, using vigorous shoots with moderate internode growth. In any case, the entire branches are collected in the field and the cuttings are then made in the laboratory or the nursery. One must make sure that the sharp and clean secateurs are used to obtain a clean cut. Usually the cut at the lower end is oblique in order to indicate the correct polarity for the cutting especially when it comes to planting these in the appropriate medium. The size of the cutting is highly variable, according to the species, but usually in the 10-20cm range with a diameter of between 0.6 to 2.5cm. Each cutting should have a minimum of two good buds. The cut at the lower end must be made immediately underneath a node and the cut at the top about 2cm from the topmost node. These type of cuttings can be stored under high humidity at temperature varying between 0-5 ^{o}C but precautions must be taken to avoid the proliferation if fungi for those species that root very easily, as for example the *Salicaceae*, one can conserve the material in the same conditions that it was collected in the field and only just before rooting, does one proceed to make the cutting. The material is stored in bundles, ensuring that the polarity of all the branches is maintained i.e. the lower ends are all facing in the same direction.

The woody cuttings of evergreen species usually present major problems for rooting compared to the previous group. They are normally collected at the end of winter beginning of spring or the need of summer depending on the species. In the case of evergreen broadleaves, the leaves are either completely or partly removed to prevent them from drying out. For transpiration, it may be useful to maintain them in a humid environment and to prepare them immediately for their rooting in a controlled

environment. Softwood cutting (e.g. Myrtus, some Rosaceae, Acer) or partly lignified (semi ripe cutting) (e.g. *Eunymus*, *Erica*, *Callima*, *Viburnum*, *Nerium*, *Rosmarinus*, *Santolina*, *Taxus*, *Prunus*, *Ilex*, *Lonicera*, *Rhododendron*) are usually collected in spring and summer, must be protected from the loss of humidity and encouraged o root as quickly as possible in a climatically controlled environment. The length of this type of cutting varied between species, usually these are between 3 to 6 cm long up to a maximum of 10cm.Root cuttings must be collected during the resting period of the plant, usually at the end of winter and beginning of spring. During the collection and transport one must maintain the material under humid conditions, taking care to mark the polarity, as is done for other cutting. Root cutting cannot be stored and they need to be cut, processed and planed out, in the appropriate substrate, as quickly as possible, so that they start rooting without delay. Rooting can be done in a protected and controlled environment, one without protection according to the requirements of the species. Root cuttings propagate well and with relative ease for the species of the genera *Acer*, *Populus*, *Malus*, *Rhus*, *Rubus* and *Ulmus*.

Leaf cuttings or axillary bud leaf cuttings, usually for non-deciduous species, are collected in the field as branches to avoid them drying out since they are very sensitive to this. The leaves selected for the propagation have to be healthy and in active growth, and in the case of axillary bud leaf cutting, these have to be well developed. The cuttings have to be cut, processed and planted in the appropriate substrate so that they can root as rapidly as possible.

In the case of grafts, especially in the broadleaves, the material has to be preferably collected form the central or basal portion from strong branches one year old or less with healthy vegetative buds, whilst avoiding the apical buds and those located at the branch intersections. In *Prunus* species, grafting with buds obtained from the dominant apical or distal branches with robust/ strong buds. In the deciduous broadleaves, the material can be collected at any moment in winter and stored, taking care to avoid desiccation, up to the moment of grafting it. In the case of pines, the material is usually collected at the beginning of spring and must be grafted as quickly as possible; material can also be collected from conifer in summer, but must be grafted immediately in a controlled environment very soon after the collection in the case of scion grafts which are not lignified or only slightly so. For shield budding/grafting, it is important so select well-developed vegetative buds. In the field, the branches are out, the leaves are removed such that part of the petiole to which the graft bud attached is retained. This will aid in the grafting, and also inserted immediately. The time of collection of the material not only depends on the species, but also on the climatic conditions such that in hot areas or inside greenhouses once can use shield budding for active buds (spring) whereas for cold environments one has to use dormant buds taken between July and September. In this type of budding, as in other cases, the material must note be allowed to dry and to this end it must be wrapped in paper or humid cloth during transportation.

The collection of material from bulbous plants also constitutes a particular case. Usually the ideal time coincides with their period of rest, in summer or in winter according to the species, when the epigeal part is completely dry. The bulbs are extracted manually or with the aid of a hoe or other gardening implement. In the laboratory or nursery they are washed, disinfected with fungicide and left to dry on the shade. Afterwards the roots are removed, cleaned from soil debris and the bulbils are repotted. The optimum humidity for the storage of bulbs greatly depends on the species, some do not tolerate drought, especially the scaly bulbs (e.g. *Lilium*) which must be stored in sand, vermiculite, sawdust, soil or any other material which maintain a certain level of humidity. In general, the bulbs that have a protection tunic (e.g.

Narcissus) can store well in paper bags in a cool and well ventilated location, away from extremes of temperature up to the point of their multiplication.

As a preventative measure, it is advisable to treat the vegetative material with a fungicide. In the case of bulbs and woody cuttings the treatment is effected before or during the storage period, whereas for non woody material or roots which generally do not tolerate a long storage period, the treatment is effected at the moment of propagation or during the period of rooting. In all cases, it is extremely important to ensure that all the material is well labelled form the point of collection up to the phase of propagation.

For a detailed description of the different methods of propagation, it is advisable to consult specialised texts such as Hartmann and Kester (1983) and MacDonald (1987). The particular case for the propagation of the *Salicaceae* is treated in greater detail in section 10.3 since the family includes a large number of species which forms part of the riparian vegetation, and some of these, such as *Populus nigra*, are found in habitats under threat and for this reason, their conservation must be considered as a priority objective.

8. Germination

Germination tests are necessary for a correct management of accessions present in a germplasm bank. Such a test can have various aims: it primarily allows for the development of an efficient protocol for the taxon, either for the same or other banks. Secondly, this protocol will also be essential to monitor the quality of the stored seed batches. Another aim for developing the germination protocol of a species is that it allows their cultivation in a nursery where the plants are maintained to complete their phenological cycle.
Sometimes the optimal conditions for seed germination in the laboratory do not correspond to the experimental results and the culture of mass propagated plants in the field. Such discrepancies in the methodology are often due to unforeseen causes, and can depend on variations in temperature, on the photoperiod, and the substrate used, apart from rainfall and relative humidity.

8.1 Definition of germination

From a physiological perspective seed germination is the sum of events initiated with the inbibition of water and the activation of pre-germination metabolic processes followed by the elongation of the radicle and finishes off with the rupture of seed coat.
Under laboratory conditions, germination is defined as a radicle-induced rupture of the seed coat or in the absence of a seed coat, the elongation of the radicle (Côme, 1970).
Under optimal conditions, germination of a viable seed allows the formation of a new seedling and thus moves from a passive to an active life form (Musmarro, 1996). The aim behind laboratory-based seed germination trials as given in the official seed germination protocol (Ministry for Agriculture and Forestry, 1993) is to determine the percentage of seeds capable of producing normal seedlings, and potentially able to develop into normal plants under favourable conditions. This definition coincides with that of the International Seed Testing Association (ISTA) which states that:
'The germination of a seed as part of a laboratory seed germination testing procedure is defined as the emergence and development of a seedling up to a stage where the appearance of the structures present indicate that it is capable of development into a normal independent plant if grown under favourable conditions' (ISTA, 2004).

8.2 Environmental factors affecting germination

The principal factors determining the initiation of the process of germination and successive phases of germination are described in detail below.

8.2.1 Water

Water must be in liquid form; water demands vary form one species to another. There is a limit to quantity of water that must be available which must not be exceeded,

otherwise germination would be inhibited. In such cases the embryo enters into an anoxic state and dies. On the other hand for some seeds, e.g. Aquatic plants and rice, germination only takes place if completely immersed in water. Note that if the integuments are impermeable to water, germination does not take place.
The rehydration of seeds opens up integuments allowing for the growth of the radicle. In nature, water in the soil is not all usable, by its very nature; water is retained by soil colloids. Seeds must therefore utilise a force that helps them to withdraw water from the soil. An increase in osmotic pressure of soil does not favour germination (e.g. Addition of NaCl in the inbibition water can slow down considerably the germination capacity of a species). For this reason, in a germination trial, the use of distilled or demineralised water allows for conditions similar to those in nature.

8.2.2 Temperature

This must be compatible with the requirements of the species, having an effect on the rate of biochemical reactions and hence the rate of germination. A low temperature can even potentially induce secondary dormancy.
Setting up a germination trial at a constant or alternating temperature can give variable results, both for the total number of germinating seeds and the rate (velocity) of the germination process. The optimal germination temperature depends on the species and their geographical origin: some species requiring very low temperature (eg. 5^{o}C for *Tulipa* and *Fagus sylvatica*) other higher temperatures. The difference between optimal temperature can vary with species and the level of dormancy of the seeds.
Finally, the interaction with oxygen, and the absence or presence of light during germination may not be easy to spot and therefore require diligence on the person carrying out the trial to identify the these germination requirement/characteristics of a species.

8.2.3 Oxygen

Oxygen demands vary from one species to another. Only oxygen dissolved in water for inhibition is used by the embryo for its metabolic purposes. This gas has low solubility in water, given that solubility is the inverse proportion to temperature. It is also one of the more difficult parameters to measure (acquiring specific instrumentation) in a laboratory of a germplasm bank. On the whole, it is wise not to ignore the effect of oxygen during trials for developing protocols for an effective germination.

8.2.4 Light

Light favours the germination of the majority of seeds that are defined as positively photosensitive. Others do not germinate except in the dark (eg. Cyclamen) and are therefore negatively photosensitive; others are indifferent.
The mechanism of photosensitivity is the subject of numerous studies by Beskin et Beskin (1998) that led to the discovery of the photoreceptor system involving

phytochromes. The system is based on a pigment localise in the embryo. Photosensitivity can be observed with the use of white light on whole and fresh seeds. This photosensitivity may not be present with dry conservation or at the point where embryos are isolated form integuments, from which one can conclude that photosensitivity is due to the integuments themselves (Côme, *op.cit*.). The influence of light on germination of numerous Mediterranean species was also studied by Thanos (Thanos *et al*., 1991; Thanos et al., 1994; Thanos et Doussi, 1995).

Experimental procedure to determine light sensitivity

The exceptional characteristics of responses controlled by phytochrome is their reversibility: activated at wavelengths at 660nm (red light - RL) and inhibited at wavelengths of 730 nm (far red light FRL also called 'near infra-red' light).

The aim of this trial is to show the reversibility of the induction of germination by light. Lettuce (*Lactuca sativa* L.) achenes have a very marked photosensitivity that has allowed identification of this phenomenon. Therefore, the protocol is formed, elaborated/detailed further as from this experiment.

1. The filters

This setup permits the use of different types of illumination without the need for a dark room. This setup is composed for two filters and a black cloth placed in two concentric tubes (PVC). The first tube (red filter) allows for the creation of red light, superimposition of second tube (blue filter) gives a dark red illumination, the superposition of the black cloth on the two filters give darkness. It is important to verify, before starting the test, the availability of 12 complete setups (each having two filters and black felt cloth).

2. Preparation of Petri dish

Place 2 or 3 sheets of filter paper into each of 12 petri dishes circa 36mm. The filter paper must then be soaked abundantly with distilled water. Count 10 achenes in the lid of each dish. When the 12 lots are ready, place the seeds on the wet filter paper and cover immediately with the two filters and the black felt cloth to ensure that there is no exposure to white light.

3. Exposure of the achenes

Place the petri dishes, covered with the filters and the black felt, under fluorescent light. Two trials with six replicates each are carried out. Effect of the exposure time to RL light: left the dark pieces of cloth from six petri dishes and expose to light with filter in place (FRL illumination). To effect the RC illumination it is sufficient to lift the blue filter for 0, 5, 10, 40 an d60 seconds, not forgetting to replace the filter and the blank cloth at the end of the exposure time.

Effect of exposure time to FRL light. Remove the black cloth from the remaining six petri dishes and expose for 30 minutes with the two filters in place (FRL illumination). Remove the blue filter for 5 minutes (RL illumination) and replace the blue filter for

variable times (0, 2, 4, 10, 30 and 60 minutes) and replace the black cover at the end of the FRL exposure.

4. Germination
Leave the 12 petri dishes covered with the two filters and black cloth for 2-5 days at ambient temperature and count the number of germinated achenes per dish. Repeat the results of the two trials as germination curves:

- Trial 1: Percentage germination as a fraction of the RL exposure time
- Trial 2: Percentage germination as a fraction of the FRL exposure time

8.3 Inhibition of germination: Seed Dormancy

Once it reached morphological maturity, the seed enters into a state of slow metabolic activity/suspended animation; to return back to its active life, the external condition must be favourable but more importantly it must have reached its physiological maturation point. In the majority of cases, the seeds do not have a dormancy period and the morphological and physiological maturities are reached at the same time. Conversely, if the physiological maturity is reached at a late point, the seeds are defined as 'dormant'. Dormancy is therefore the physiological state of the seed or embryo which prevents the seed from germinating despite the presence of good ambient conditions for germination (Côme, *op. cit*).
Several authors have defined the types of dormancy, the best studies are attributed to Baskin and Baskin (1998), to Côme (1970) and Côme and Corbineau (1992), but the classification proposed by Nikolaeva (1969) is being adopted here. Two large dormancy groups are identified, the endogenous and exogenous types. In the first group, the embryo is often implicated, whereas in the second group; some seed structure (e.g. wood endocarps, seed coats, endosperms, etc.) which surround the embryo are implicated in the inhibition of germination. When the cause of non germination is due to the integuments, the term tegumental or seed coat inhibition is used (Côme, *op. cit*). The seed coat inhibitors are primarily composed if aromatic and phenolic substances (especially *Apiaceae*). Very often, the seed coats themselves can produce an inhibitory effect by inhibiting the imbibition of water and gaseous exchange (especially *Fabaceae*) or they inihibit the exit of the radicle (e.g. *Prunus* sp. pl.)
There could also be a possibility of combined endogenous dormancy factors. The specific causes that prevent dormancy, both due to endogenous and exogenous causes and the condition required to remove them are shown in Table 3. If there are more causes that induce dormancy (combination of dormancies), for each and every one of these dormancy factors, specific pre-treatments are needed.

Table 3: Endogenous and exogenous factors and pre-treatments to remove their effects.

TYPE OF DORMANCY		CAUSE	CONDITIONS INTERRUPTING DORMANCY	EXAMPLES
EXOGENOUS DORMANCY	Physical (A_1)	Impermeability of seed coats to water	Scarification	*Astragalus maritimum* Moris *Astragalus verrucosum* Moris *Robinia pseudoacacia* L. *Laburnum anagyroides* Medik. s. l.
	Chemical (A_2)	Presence of inhibitory factors in the pericarp (not frequent) sometimes in the fruit exterior	Removal of the pericarp, in some cases with *dilavamento*	*Ferula lascosii* (Lange in Willk. et. Lange) Willk. *Fraxinus chinensis* Roxb. ssp. *rhyncophylla* (Hance) A. E. Murray *Acer psudoplanatus* L.
	Mechanical (A_3)	Mechanical restriction to growth of the embryo	Removal of the integuments	*Euphorbia graminifolia* Vill. *Alaeagnus angustifolia* L.
ENDOGENOUS DORMANCY	Morphological (B)	Incomplete development of the embryo; appears only in combination with other factors	Estivation	*Acis nicaeensis* (Ardoino) Lleodo, A. P. Davies et M. B. Crespo
	Physiological (C)	Physical mechanisms that inhibit germination		
	Light Physiological (C_1)		Brief vernalisation periods, growth hormone stimulation	*Linaria arcusangeli* Atzei *et* Camarda *Bertula pubescens* Ehrh.
	Intermediate Physiological (C_2)		Long periods of vernalisation, gibberellins	*Nothofagus oblique* (Mirb.) Oerst.
	Deep Physiological (C_3)		Very long vernalisation periods,	*Sorbus ocuparia* L.
	(B + C)		Generally long temperature treatments with alternation of temperatures	Very often in the Rosaceae
	(B + C_3)		Long estivation followed by long vernilisation	*Fraxims excelsior* *Vitex angus- castu*s L. *Paeonia* sp. pl.

If during the germination trial, seeds that have been subjected to a dormancy-breaking pre-treatment (V 8.4) are exposed to bad environmental conditions subsequent to seed sowing (eg. high temperature, anoxia, excess water, etc.) they may activate physiological mechanisms that block germination (Côme et Corbineau, *op. cit*). The result is described as “induced or secondary dormancy”, so described to distinguish from “primary dormancy” (shown at the time of seed dispersal).

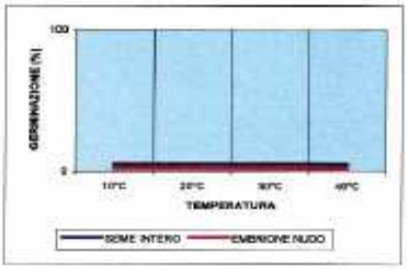
Very often, seeds subjected to secondary dormancy, require cycles of strong changes in temperature as happen at the end of winter – beginning of spring (cold nights, warm days), in order to germinate. In these cases, the (semine Vardive?!) that are found in very warm soil, can induce secondary dormancy and, therefore failure to germinate.

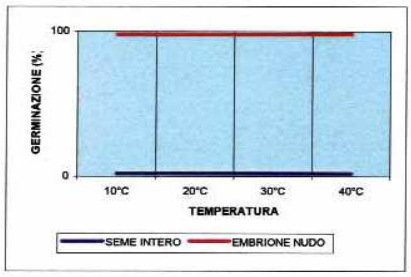
8.3.1 Practical method for the determination of the type of dormancy, for seeds not subjected to dehydration.

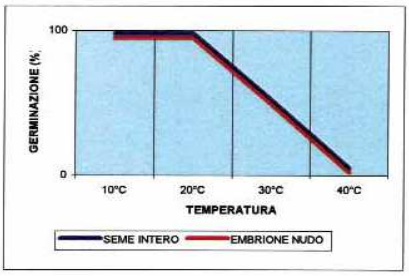
In these cases, where following a series of experimental trials, one continues to obtain very low levels of germination, it is imperative to research the possible cause for these negative results. These can be traced either to the various types of seed dormancy strategies or to a low seed viability for the particular batch under investigation. To determine whether the germplasm under investigation has some form of dormancy, it is possible to proceed using the following steps:

- Carry out the test on fresh material;
- Determine if the seeds are classified as orthodox or recalcitrant (Hong et Ellis, 1996);
- Examine the structure of the seeds (Maroten, 1946) and verify that seeds are well formed;
- Germinate the seeds in the dark, at temperatures spanning the 0ºC - 30ºC range, for the entire seeds and excised embryos, and analyze the results obtained (fig. 34)
- If necessary, evaluate the positive or negative photosensitivity using entire seeds and carry out seed germination tests in the light and at an elevated temperatures (20ºC - 30ºC);
- Verify the presence of phenolic compounds in the seed coats;
- Verify if the cause is due to water impermeability;
- Determine the effect of particular treatments such as cold moist stratification, dry storage and GA3;
- Verify the effect of oxygen.

If, at the end of these tests, the number of germinated seed remains small or below 50% but the tissues are still intact, it would be beneficial to carry out a viability test (eg. Tetrazolium colorimeter assay).
The examples that follow (fig. 34) show the different possibilities for entire seeds and excised embryos subjected to germination trials under a wide temperature range.
The results of these tests may point to the type of dormancy that may be present. Another approach to identify the type of dormancy present in a seed batch or in a species about who germination little is known or published, is described in section 10.2. In this case the eco-physiological requirements for germination are identified by simulating seasonal cycles characteristic of the distribution range of the species of interest.







1 NO DORMANCY.

Both the intact seeds and naked seed embryos give the maximum germination % of all temperatures (eg. *Pancratium maritimum* L .)

2 EMBRYO DORMANCY AT ALL TEMPERATURES

Both the naked embryos and the entire seeds do not germinate at any temperature.

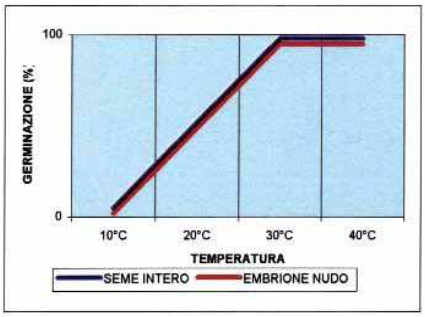
HYPOTHESIS 1: Embryo dormancy that is present at all temperature (eg. *Delphinium pictum* Willd.)
HYPOTHESIS 2: Non viable seeds.

3. NO EMBRYO DORMANCY

HYPOTHESIS 1: Hard seeds coats (eg. Fabaceae)
HYPOTHESIS 2: Positive photosensitivity(eg *Lactuca* sp. pl.)

4. NO SEED COAT INHIBITION. EMBRYO

Fig.34 Behaviour of entire seeds and excised embryos during germination trails carried out to identify the type of dormancy present. (continued)

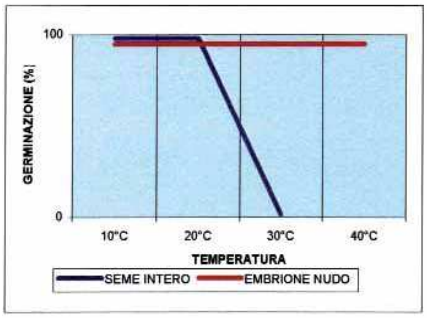


5. NO SEED COAT INHIBITION, EMBRYO DORMANCY THAT MANIFESTS AT LOW TEMPERATURE.

Identical curves show that the seed coat does not obstruct germination, but manifests dormancy at low temperatures.

HYPOTHESIS 1: Species from warm climates (eg. *Chamaerops humilis* L .)

HYPOTHESIS 2: Tropical species.



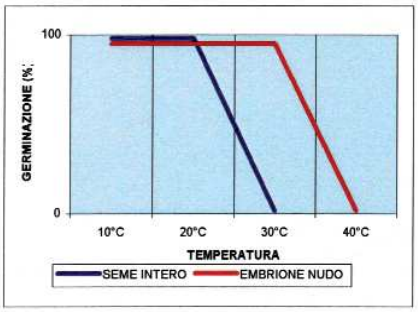
6. NO EMBRYO DORMANCY, SEED COAT INHIBITION THAT MANIFESTS AT ELEVATED TEMPERATURES.

At high temperatures, a germination inhibition is activated at seed coat level.

HYPOTHESIS 1: Species from temp. Climate (eg. *Armeria belgenciensis* Donadilla ex Kerguelern)

HYPOTHESIS 2: Presence of phenolics (eg *Ronya polygama* (desf.) Coincy)

HYPOTHESIS 3:Positive photosensitivity (eg. *Nigella sativa* L.)



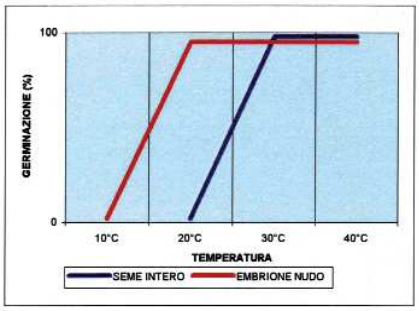
7. EMBRYO DORMANCY THAT MANIFESTS AT HIGH TEMPERATURE.

SEED COAT INHIBITION THAT MANIFESTS AT HIGH TEMPERATURE.

The high temperatures, activate embryo dormancy conditions and seed coat inhibition (at different temperatures)

HYPOTHESIS 1: Temperature climate species (eg. *Arenaria provincialis* Chette et Halliday)

HYPOTHESIS 2: Presence of phenolics (eg *Bellevalia romana* (L.) Reichenb.)



(continued) Fig.34 Behavior of entire seeds and excised embryos during germination trails carried out to identify the type of dormancy present.

8.4 Breaking seed dormancy (pre-treatments)

The term pre-treatment, refers to the sum total of a number of processes, manipulations and other conditioning treatments that proceed seed sowing, all carried out with the objective to bring to the maximum the germination percentage, the velocity and uniformity of the germination (Mezzalira et Piotto, 2003; Piotto,2005) In this regard the terms ‘ pretreatment’ and ‘treatment’ are considered synonymous, though the first term is preferred since it implies a process that is applied ‘before’ germination. In the wider sense, many processes are classified as ‘pre-treatments’ when they act directly on the reduction of dormancy (confettatura??, disinfection, etc.) For different reasons, amongst which physiological dormancy, seed coats tight fitting and impermeable, and preserve of germination inhibitory chemicals (table 3), a considerable number of seeds may not germinate at the end of the germination test, even though they remain viable. To obtain a better germination percentage for the accessions, it is desirable to pre-treat the seeds before initiating the proper germination test. The duration of these pre-treatments must however not be added to the duration period of germination (ISTA,2006)
The official methods for analysis of seeds (Ministry of Agriculture and Forestry, *op. cit.*) accurately indicate the pre-treatment to which seeds of different herbaceous species, shrubs, trees and medicinal herbs must be subjected. In the same way noteworthy institutions such as the *International Seed Testing Association* (ISTA), the *International Plant Genetic Resources Institute* (IPGRI), the *National Resources Conservation Service of the United States Department of Agriculture. The Native Plant Network, The Reforestation, Nursery and Genetic Resources Team, Kew Gardens*, and many other institutions continuously study and update their protocols to optimize the results for their plant propagation or seed testing protocols, including germination, pre-treatments and to remove dormancy. However, for many species, a lot is still not known and in these cases one has to turn to procedures described in sections 8.3.1 and 10.2
Below is a description of the pre-treatments that are most commonly used to remove the dormancy of seeds prior to sowing or seed germination tests. Given the very large variability in the ‘dormancy characteristic’ of a species, the pre-treatment may act positively solely on a section of the seed lot (i.e. on that portion of the seed lot whose requirement are completely satisfied by a given treatment) and as a result create a an artificial selection with the resulting loss of genetic variability.

8.4.1 Cold stratification, vernalisation and pre-chilling

The term pre-chilling (synonymous with vernalisation and cold stratification) refers to the process of exposing dormant seeds to variable temperatures from 2ºC to 5ºC (Côme, *op. cit*) under humid and aerated conditions (unprotected or mixed with a soft substrate) for periods that are characteristic to a species a seed lot. Pre-chilling simulates the action that the cold winter months play on some seeds.
An alternative to cold stratification that is something used and may last several weeks, is the application of large doses of gibberellins (GA3) (IBPGR, 1985)

8.4.2 Warm stratification, aestivation, preheating or warming

The term preheating (synonymous with warm stratification, aestivation and warming) refers to the process of exposing dormant seeds to a temperature that does not exceed 30ºC to 35ºC (generally 15ºC to 20ºC) under humid conditions but with good air circulation for a variable period of time depending on the species. This artificial induced warm stratification substitutes the process that occurs naturally in summer, which almost always proceeds a cold stratification process. When multiple cycles of warm and cold stratification must be applied, the last pre-treatment is always a cold one.

8.4.3 Smoke treatment

To enhance germination of some species associated with habitats that periodically catch fire, over and above a warm pre-treatment, may also require exposure to smoke or similar substitutes (eg. Ericaceae). The treatment was originally started and developed around 1990 by the Kirstenbosch National botanical garden of Claremont, Cape Town (South Africa) The seeds are first enclosed in filter discs that had been previously smoke-treated or saturated with a solution containing extracts that are released naturally during fires of the South African Fymbos (Mediterranean type vegetation). A fixed volume of water is added to the discs and then the seeds are allowed to imbibe for 24 hrs. Other research centers that focus on Mediterranean-type species including ones in California and Australia have developed similar methods that basically try to imitate the effects of fire on germination.

Numerous species present in Mediterranean-type ecosystems respond significantly well to pre-treatments based on smoke. Conversely for Mediterranean basin species, study of this phenomenon has only been started in the last few years (Crosti *et al.* , *op. cit*).The species which are stimulated by smoke, and which above all is considered very specific to the fire, do not necessarily require a specific heat treatment that results from the fire. Indeed, the action of the smoke appears to be more of a chemical nature where as the heat acts more by a physico-mechanical action. The smoke can therefore act directly or indirectly via aqueous solutions or gas reaching the seeds. In those species that are effected by smoke treatment, the response varies accordingly to the concentration of active principle found in the smoke and also according to the exposure time. A chemical belonging to the betunolide group, was recently isolated from smoke, and shown to be active at low concentrations in an analogous way to smoke-induced germination. Furthermore, this chemical stimulates germination of numerous species, even in the dark as happens for Australian Asteraceae species which normally require exposure to light in order to germinate, and this because the smoke chemicals induce effects similar to release of gibberellic acid (Merritt *et al*., 2006).

Very often, seeds that are sensitive to smoke treatment, have seed coats that are equipped with a sub-epidermal layer that, during the seed's dormancy period, allow the absorption of water but prevent intake of key solutes. The action of the smoke modifies this tissue rendering it more permeable and thus favorable to the germination process. The different species apart from differences due to provenance also give highly variable

germination responses, and in this way greatly influence the composition of the post-fire plant community succession. The effect of fire on the vegetation, is particularly complex (Fenner et Thompson, 2005) because of its influence on light, humidity, temperature, pH, space and nutrient availability; all along fire must be considered as of the determining factors that breaks the seed dormancy of species in Mediterranean-type ecosystems.

8.4.4 Scarification

The seeds of some species belonging to families with woody seed coats or hard wood impermeable endocarps (eg. Fabaceae, Cistaceae, Convolvulaceae, Oleaceae, etc) must be subjected to abrasions by mechanical, chemical or physical means to allow them to absorb water.

Scarification may be executed before the initiation of the test, when it is suspected that the treatment may damage the hydrated seeds, or at the end of the treatment and solely for those seeds which are not hydrated. Mechanical scarification involves the piercing, cutting or abrasion with sand paper of the outer seed coat (Fig 35) with the aim of allowing imbibition of water by the seeds. The past of the seed most suited to mechanical scarification is that located at the seed coinciding with the apex of the cotyledons (ISTA, 2006) .Acid scarification involves the immersion of the seeds in 96% sulfuric acid for a variable time period with the intention of making the seed coat thinner. After the treatment the seeds must be copiously rinsed with water before initiating the seed germination test or seed sowing.

Physical scarification essentially consists of immersion of the seeds in boiling water followed by soaking for a period of 12-24 hours with the aim of softening the seed coat and thus favouring imbibition of water. The hot water must be removed from the heat source before adding the seeds. About 10 parts water must be used for each part seed, ensuring that the mass is stirred continuously until the water cools. (Mezzalira et Piotto *op. cit.*). Because the hardness of the seed coat is extremely variable, the physical and chemical scarification processes may exert a selection pressure on the distribution of seeds favoring those with seed coats which are softer compared to those seeds with harder seed coats.

Fig 35 Seeds of *Astragalus maritimus* Moris before (left) and after (right) 24hr soaking in water. The seeds had been manually abraded using sand paper. (Photo E. Mattana)

8.4.5 Removal of seed coats

For some species which have extremely hard seed coats, scarification will still not weaken the seed coat to allow emergence of the radical. In such cases it may be advisable to use a tweezers to remove these seed coats taking care not to damage the embryo.

8.4.6 Removal of the germination inhibitory chemicals.

The presence of inhibitory chemicals within the seed or inside the seed coat may retard or inhibit germination. The presence of these chemicals may be revealed from the formation of coloured patches / streaks on the germination substrate being utilized. The germination inhibitory chemicals may be removed with a pre-washing in water or alcohol (90%) at a temperature of 25ºC followed by drying of the seed before carrying the test. For some Poaceae this can be affected by removal of the seed external structures such as the Lemma and palea (ISTA, 2006). The phenolic substances are after responsible of the inhibition of germination because they act by decreasing the transport of oxygen to the embryo, especially when the temperatures are above 10ºC. Their removal can be effected by repeated washing in water or alcohol, but in this particular instance one must employ germination temperatures which are sufficiently low as to allow oxygen to dissolve in the inbibition water (Côme et Corbineau, *op. cit*). The soaking of seeds in a strong oxidizing agent / e.g.. Hydrogen Peroxide, (bleaching agent) allows the oxidation of the inhibitory chemicals rendering them inefficient (Ogaura et Iurabuchi, 2001) apart from the elimination of some pathogens.

8.5 Some Practical advice[8]

Seed banks are not always sufficiently equipped with the instrumentation and equipment required to run the laboratory efficiently. Under such conditions rapid and efficient determination of the requirements for seed germination of a particular species, cannot be carried out. Despite these limitations, still extremely important to take note of all the conditions and any other type o observations, until the specific aim of accurate compiling and eventually improving, the seed germination protocol. Some combinations of temperatures/ light, temperature/ water, etc , may permit a simplification of the work, but may be difficult or impossible to reproduce in another laboratory that does not have access to the same instrumentation. It is also worth noting that water, temperature, and light play specific roles for each species and that the combination of these factors, if not accurately monitored, may complicate the interpretation of the seed germination results.

[8] These observations arise from over 25 years work experience at the ‘ Banque de Semences del Conservatoire Botanique National Méditerrenéen de Porquerolles’ and are here presented in the form of practical suggestions for those who must operate under limitations imposed by lack of instruments and / or facilities needed for the study of seed germination.

8.5.1 Water

The water quality must be the best possible, and it is therefore recommended to use distilled or reverse osmosis water. If the laboratory is not equipped to have this water quality, one can always use commercial bottled mineral water (with low mineral quantity); this option is preferred to the use of ground or tap water whose overall characteristics are bound to vary over time. One must therefore take note of the name of the mineral water used on the seed germination schedule. Once opened, the water must be preserved 5ºC and used in the shortest time possible. The laboratories must evaluate the convenience and economics of purchasing water against installing a distiller or a reverse osmosis setup.
The use of domestic water purifiers must be avoided in that , to eliminate calcium, nitrates and heavy metals, these units release water containing sodium salts which may negatively effect the germination of some species.
The quality of water used for soaking the filter paper during the seed germination test, must be controlled during the first 48 hours and topped up if one notices that the filter paper is drying up.

8.5.2. Oxygen

This parameter is difficult to control, if one does not use low temperatures that result in a high solubility of oxygen in the inbibition water, or conversely, use high temperatures to reduce its presence.

8.5.3 Temperature

Under ideal conditions, the laboratory of a seed bank should have at its disposal a series of (constant temperatures) incubators that maintain, and have controlled illumination cycles, if not also a heating bed with temperature gradient. Very often all this instrumentation is not easily available.
From a technical and financial point of view, a constant temperature incubator is much more simple to reproduce and allows for tests to be carried out that are also reproducible by third partied. Indeed, the efficiency of the thermal cycles depends on the applied temperatures, the duration of the exposure time at each temperature (dallo scarto tra queste??) and on the time that the incubators take to increase or decrease the temperatures (i.e the reaction time as part of the preset cycle). All this has an effect on the (entita) and speed of germination. It is evident that many variables can result from the single parameter, complicating the analysis of the result of a germination trial.

8.5.4 Light

The duration of the light exposure, the cycles applied, the light intensity, the type of light used, the emission wavelength, the typology of the growth room, the duration at the qualitative level (for the majority of cases this is equal to one year), the availability of the lamp used are all important elements that need to be evaluated and noted.

The reliability of the lamps used, are all important elements to evaluate and note. If one combines light with alternation of temperature it sometimes becomes difficult to reproduce the germination protocol outside the laboratory. Sometimes the duration of the light necessary to induce germination of some seed does not exceed a few minutes. Depending on the case, the requirments for germination in the light or in the dark may be cancelled by the different treatments such as dehydration, decortication or scarification of the seed coat, the use of colder temperatures for seeds that have been inbibed in the dark or warmer temperatures for seeds inbibed in the light. The influence of light on some seeds explains why these, having remained quiscent for a long time in the soil, germinate immediately after being brought to the soil surface following ploughing. Photosensitive seeds sometimes have a better germination after 6-12 months of dry or cold storage.

8.5.5 Hormones and other products

Gibberellins:

Their application is not always necessary even in the light of a presumed dormancy. Furthermore, if one needs to conserve the seedling for multiplication purposes, it may become more difficult to execute due to the presence of the hormones. If the application of gibberellins is necessary for the production of plantlets, one must turn to a suitable culture method, which is very often possible only under highly controlled environmental conditions: suitable illumination for a minimum of 14-24 hrs, temperature cycles with a pronounced adherence between the night and the day period.
Furthermore not all the plants produced using the method can be retained. The application of gibberellins in many cases permits the elimination of dormancy due to positive photosensitivity of the seeds.
The solution of gibberellins prepared in the lab must be stored in a fridge in the dark for a maximum period of 1 week, otherwise on risks not obtaining the desired effect.

Use of other products.

Some fungicide used to suppress infections during germination may induce defects or retardation of growth, same as other pesticide chemicals which have not been tested may bring about developmental defects, retardation of flowering etc. It is therefore generally not advisable to use pesticide treatments (phytoprotective compounds) in seed germination trials.

8.6 Determination and elaboration of protocols

To correctly plan out a germination test, it is recommended to start off with a preliminary bibliographical search, with the intent of obtaining the largest number possible of data and other information o the anatomy, the physiology and the biology of the seeds, if not also on the autoecology of the taxon under study. At the same time, it may be useful to consult, either in specific publication or using web sites, well tied seed germination protocols, even for phytogenetically or ecologically – related taxa. In this

way, it may be possible for the curator of the bank, to set up a specific protocol, selecting the different parameters to be analyzed, the number of replicates to be setup, accordingly to the seed availability and obviously according to the available instrumentation and methodologies.

Several methods exist for the standardization of seed germination protocol trials. (Terry et al. , 2003) ; in any case one must keep in mind that the execution of these procedures is dictated by the resources and instrumentation available at the seed bank (for example the number of incubators equipped with temperature and photoperiod regulators, etc.) and therefore each bank will adopt those protocols that are in line with what is possible to achieve with the resources available.
The 'Banca del Germoplasma della Sardegnia' (BG-SAR, Sardenian Seed Bank), in line with international standards such as IPGRI (1985b) and ISTA (2006) uses the following dichotomous key to decide the best line of action:

1. Preliminary bibliographic search..... 2
2. Consultation with existing germination algorithms and protocols even for similar taxa according to phylogenetic and/or ecological characteristics
    a. A well defined protocol does not exist already..... 3
    b. A well defined protocol already exists..... 7
3. Pretreatments
    a. Estivation (eg.: *Primulaceae*) 4
    b. Vernalization (eg.: *Cistaceae*) 4
    c. Smoke treatment (eg.: *Ericaceae*)..... 4
    d. Scarification (eg.: *Fabaceae*)..... 4
    e. Elimination of germination inhibitors (eg.: *Poaceae*) ……..... 4
4. Imbibition
    a. Seeds not imbibed..... 3
    b. Seeds imbibed 5
5. Seeding
    a. Chemical treatment (KNO3, GA3, etc.)
        i. Darkness and constant temperature
            germination % < 50% 6
            germination % > 50% 7
        ii. Photoperiod and constant temperature
            germination % < 50% 6
            germination % > 50% 7
        iii. Photoperiod and alternate temperature
            germination % < 50% 6
            germination % > 50% 7
    b. Distilled water (no treatment)
        i. Darkness and constant temperature
        germination % < 50% 6
        germination % > 50% 7

ii. Photoperiod and constant temperature
germination % < 50% 6
germination % > 50% 7
iii. Photoperiod and alternate temperature
germination % < 50% 6
germination % > 50% 7
6. Execution of viability testing
a. The results of the germination test are not confirmed (high viability)..... 5
b. The results of the germination test are confirmed (Low viability)..... 7
7. Execution of confirmatory germination test
a. The results are not confirmed…… 5
b. Results are confirmed…… .....**VALIDATION OF THE PROTOCOL**

The number of seeds that may be subjected to analysis varies according to their availability… (If the quantity is sufficiently large one often resorts to carry out destructive investigations), if not applied to different type of protocols. From experience collected over the years at the 'Banca del Germoplasma Della Sardegna', the following scheme is being proposed:

- Number of seeds <500 units = do not carry out seed germination tests
- Number of seeds 500-1000 units = the number of seeds to be analysed is equal to a maximum of10 %
- Number of seeds 1000-5000 units = the number of seeds to be analysed is equal to 10-20%
- Number of seeds >5000 units = the number of seeds to be analysed is equal to a maximum of 20%

However, one must always remember that when working with rare or threatened plat populations, it is often difficult to collect seed lots of around 500 seeds. In these cases, the selection of the number of replicate in a trial must be judiciously studied taking into account a number of consideration, amongst which:

- Level of crucial threat/ loss for the taxon;
- The availability of material of the same taxon coming from other populations
- The availability of accessions for the same population for collections in previous years

The standard germination conditions to which one will subject the seeds of a taxon which is not as yet well studied, contemplates a combination of the following factors:
• temperatures of 5°C [9], 10°C, 15°C, 20°C, 25°C, (in relation also to the geographical area under study);

[9] If the trial at 5°C, does not give positive results, within 1-2 months, in the first trials, the set up can be transferred to higher temperatures, in order to evaluate the necessity of a pre-chilling period. There are however, species, where optimum germination temperatures are close to 5°C (eg. *Fagus sylvatica*)

• photoperiod 12/12 h;
• agar 0.5%-1% or blotting paper (3 sheets)[10];
• gibberellic acid 120 - 800 ppm (IBPGR, 1985b);
• duration of the test - variable from 30 a 60 days with exception;
• one or more Petri dishes whose dimensions depend on the type of seed;
• KNO_3 (0.2% w/v.) (Côme, *op. cit.*; IBPGR, 1985b; ISTA, *op. cit.*).

To allow a correct analysis (including statistical) of the results, it is recommended to make daily observations immediately after the initiation of the test and after that, every two day for the duration of the test.

In those cases of a negative outcome for the germination trial due to a fungal infection, if the trial is repeated, it is advisable to use an antifungal pre-treatment. The antifungal treatment may be affected before the initiation of the test by immersing the material in a solution of commercial bleach (sodium hypochloride – NaOCl) at 1-2% for 5-10 minutes. Alternatively at the point of sowing of the replicates inside Petri-dishes, one can soak the filter discs in a solution of 36% hymexazol, a fungicide that can be utilised both on seeds and substrate (De linan, 2004). It is used by mixing 0.1ml of the product with 500ml of distilled water (Picher ET Campos, *in verbis*)

Fig: 36 – Observations on germination for Astragalus maritimus Moris (photo: F

In those cases where infections appear during the test, one can change the petri-dishes and the filter paper discs, which are washed repeatedly and the seeds are washed with sodium hypochlorite (common bleach).

For seed whose surface are particularly rough and difficult to sterilise (e.g. Astragalus sp. Pl) it is preferred to employ a solution of 'Tween 20' at a concentration of 1%. This compound reduces the surface tension and favours a better contact between the sterilising liquid and the seed coat. After the treatment the seeds must be copiously rinsed under running tap water.

8.7 Analysis of results

[10] These two types of substrate, present different characteristics and are alternately used according to the way in which the test is carried out and the typology of the seeds: agar is to be preferred when there is the possibility of working in a sterile environment or when working with very small seeds or spores (eg: from spores and seeds of the Orchidaceae) conversely large seeds would absorb all the water present in the agar imposing a limiting factor to germination.

The observations effected during germination trials allow for characterisation of results obtained. For this purpose a list of indicators useful for the analysis follows:

8.7.1 Categories of evaluation

While monitoring a germination trial it is possible to observe and note the number of dead and germinated seeds; at the end of the test it is usually possible to identify (v.13.11) the following seed categories (ISTA, *op. cit*.; Ministry for Agriculture and Forestry, *op.cit*.);

- germinated;
- imbibed: seeds that although fresh, vital and imbibing, have not germinated;
- non-imbibed: seeds that have not imbibed (often having very tough integuments that necessitate scarification);
- dead: seeds that are found to be dead after various tests;
- other categories: seeds that do not qualify for the above-named categories, this being especially applicable for empty and infested seeds.

The sum of the percentages of seeds in the different categories must be equal to 100 and the total number must correspond to the number of seeds subjected to the test at the start or the trial. Other distinct classes may be made for seeds that have germinated (ISTA *op. cit.*) as outlined below.

- Germinated seeds with normal seedlings: seedlings are considered normal when they are equipped with all the organs necessary of life of the adult plant. These are subdivided into three sections: intact plants, with slight defects and those with secondary infections.
- Seeds with abnormal seedlings: seeds that although germinated do not produce seedlings which cannot be considered normal. Even in this case three categories are distinguished: damaged seedlings, deformed, and deteriorated[11].

8.7.2 Germination percentage

The germination percentage is calculated for each replicate, it is a relation between the number of germinated seeds and the original number of seeds started off with, multiplied by 100:

$$\frac{\text{Number of Germinated Seeds}}{\text{Total Number of Seeds}} \times 100$$

The final percentage is calculated by taking the average for all the replicates subjected to the same germination condition.

[11] For the evaluation of the seedling, the manual "seedling evaluation" (Don, 2003) published by ISTA may be particularly helpful.

8.7.3 Germination rate ('T50')

The T50 is the parameter that is most frequently used to measure the germination rate. One can calculate the entire number of days that correspond to the time needed to get 50% germination capacity for seed lot (Côme, *op. cit*).
This value can be calculated by linear interpolation using the formula of Coolbear *et al*., (1980), slightly modified according to the definition given by Thanos et Douss (1995):

Using:
$$T_{50} = \frac{[(N/2) - N_1) \times (T_2 - T_1)}{N_2 + N_1} + T_1$$

N = final percentage of germinated seeds
N_1 = percentage of germinated seeds slightly lower than N/2
N_2 = percentage of germinated seed slightly higher than N/2
T_1 = number of days that correspond to N_1
T_2 = number of days that correspond to N_2

The calculation of the T50 is found to be very useful in these cases where the germination period is extremely long (several months) and also assists in verifying the quality of the protocol. It also allows the indirect evaluation of the _____ of the seed lot in that the rate of germination is one indicator of its quality.

8.7.4 Germination delay period

This is defined as the time required (in days) to observe the fruit seed germinating. This does not depend solely on the characteristics of the species, but is an indicator of the aging condition of stored seed lots (when one compares the result obtained using the same germination protocol applied to seeds immediately after they are collected in contrast to seeds that have been in storage for a long time.

8.7.5 Interpretation of germination curves

From the result obtained during the germination test, it is possible to use the different curve typologies for analysis of results. Two types of graphical representations of results (fig 37 and 38) are given below that can be used to analyse, characterise and visualise the data. For these graphs, it is impossible to use T50 instead of the percentage of germination

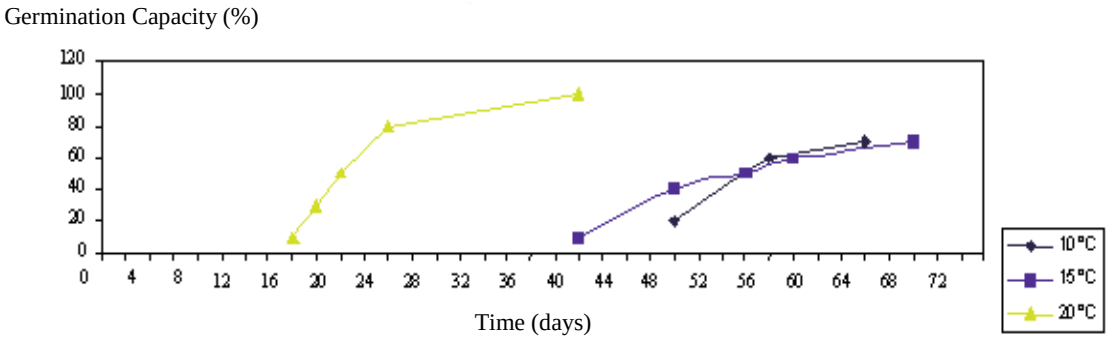


Fig 37 Germination curve for fresh seeds of *Pancratium maritimum* (data: M .Virevaire)
Percentage germination with time

In figure 37 the germination percentage is represented on the y-axis against time (days) on the x-axis. From this, one can deduce that the germination delay in relation to the temperature applied for the test is of 18 days at 20°C, 42 days at 15°C and 48 days at 10°C. The total time for the trial at 20°C was of 42 days and much more for the other temperatures. In terms of the rate and (type ?? entita) of germination, the most effective temperature for this seed lot and the test conditions used (whole seeds, darkness, petri dishes that were humidified using distilled water) is 20°C. This test was carried out on *Pancratium maritimum*, a species that does not show dormancy, is used as a reference guide for germination percentage results obtained for different protocols. They are normally applied using fresh seeds to evaluate the condition of a new accession.
Using this type of graphical representation, one can compare different types of data with the specific aim of evaluating rapidly different protocols, conservation methods, differences in seed collection from diverse populations or for the same populations but with collections made in different years.

Germination percentages in relation to different protocols.
Figure 38 represents a plot of the percentage germination on the y-axis against the germination temperatures tested on the y-axis. This type of Graphical representation allows for the evaluation of the different methods of conservation and hence to determine the optimum germination temperature.
The test utilised for these generalisation have been carried out on a sample taken from a single large collection, both immediately before the storage, after several years of different storage methods. Immediately after collection, the seeds were subjected to trials to evaluate the germination capacity of the seed lot and which served as a reference point for successive tests. For every seed storage method the seeds were

cleaned and mixed with silica gel at the temperatures indicated in the graph, except for those that were stored at laboratory ambient temperature (15-38 °C). The comparison between the curves for the fresh seeds and those for the different storage methods (5°C, freezing and Lyophilisation) demonstrates what germination tests were carried out at the point of collection. The test confirmed that in their majority the seeds had not yet reached physiological maturity. In effect, the stored seeds had undergone a post maturation phase reaching a better homogeneity for successive lots, after their dehydration (having been stored in the cold, at ambient temperature and at 20°C.
In the graph, an optimum germination temperature of 20°C is evident for seeds immediately after collection; from the graph, one also notes that the optimum temperature is in reality equal to 15°C, except for lyophilised seeds that germinate well in a wide range of temperatures.

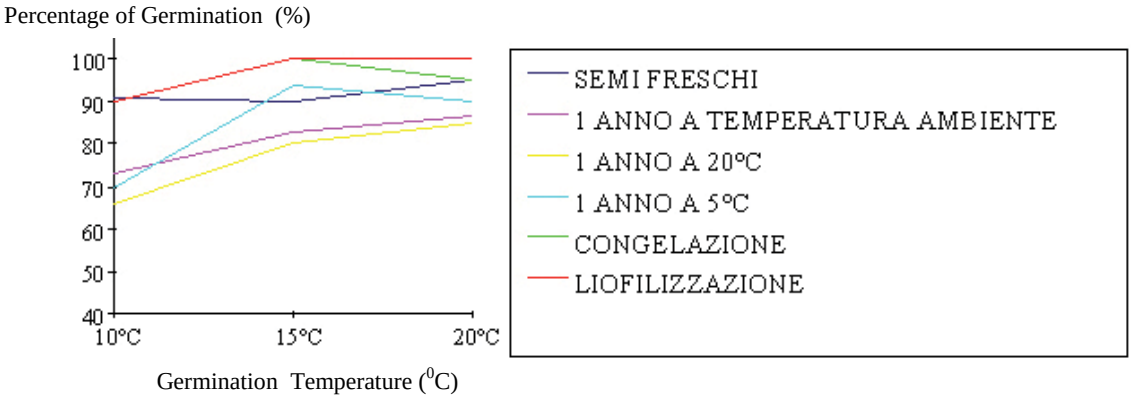


Figure 38: Germination percentages of *Pancratium maritimum* are a function of the storage method and the germination temperature (data: M Virevaire)

The optimal temperatures for germination are therefore between 15°C - 20°C, where one observes a large increase in the percentage germination at lower temperatures. Considering that seeds present a narrower range of optimal temperatures, that is further reduced with increased storage time, and since the seeds diminish their viability with increased storage time, it may be possible to propose a hypothesis on the best storage method. For such a proposal to be considered valid, it will be useful to continue to probe further this study by comparing the seed lots in the same collection with the final aim proposing the best method for the conservation of the species under study.

9. Management of Germplasm collection

9.1 Management of information data and samples

The daily management of the germplasm bank involves the supervision and monitoring of various structures, equipment and variables/parameters. Periodically, inspection of stored batches must be made in order to ensure that perfect hermetic seals are being maintained; to this end it is important to obscure, at frequent intervals, if the humidity indicator shows a discolouration due to an excessive amount of humidity.
Every 5-10 years, depending on the type of germplasm and quantity available, germination tests must be carried out on the stored material (after having re-equilibrated at ambient temperature and humidity for a few days) to test the percentage of viable seeds. When the value percentage viable seeds falls below a certain level (which depends on the initial quantity of the batch and also on the species being considered), it will be necessary to carry out a new collection or with nursery/laboratory regeneration improve the knowledge on the species. As a reference point, the regeneration standards proposed by IPGRI can be utilised; these vary from 65 to 85% and apply for accessions of between 10-20 years stored in active collections (FAO/IPGRI op. cit.).
For the management of a small seed bank, a small register or card filing system is sufficient. When with the passage of time the accessions become much more numerous, it is necessary to use an information management system. The first approach can be made using a index card filing system, but a specific data management software is a precious system to manage accessions. For this reason, mangy seed banks have with time developed dedicated systems, and more specifically, data banks. To cite one example, this is what has been done at the "*Conservatoire Botanique National Mediterranean de Porquerolles*" (CBNMP) (National Mediterranean Botanical Conservatory of Porquerolles) which has developed a management software for its own bank, called VANDA (fig. 39). This system is divided into two parts: one that manages the storage of data and the other that allows retrieval of information using a "multiquery" search system. The software uses a menu system that gives access to sub-menus; the latter allow the opening of a series of cascading windows. The data files support is achieved in the form of base information cards that increase each time that a new accession entry is made. The information cards are composed of the following: place of collection, nomenclature (which includes the biological characteristics of the taxa and information on the legal protection status), bibliography and germination protocols. The accessions generate unique schedules that permit a search of a hierarchic nature, for germination tests and types of treatment.
Taking this experience and what has emerged in the sphere of the Genmedoc project (Fig. 40) as our point of departure, the field and laboratory notes have been developed and are used for the entry of data using a dedicated software. The software allows the archiving, the query search and updating of the system directly through the network. The entire system is protected by a real time data backup safety system, thanks to a virtual server that allows a small number of operators to work simultaneously and to exchange data/interact between them. In effect, the software was tested at the *Banca del Germoplasma della Sardegna* (Sardinian Seed Bank) *and Dipartimento di Botanica di Catania* (Catania Department of Botany).

Fig. 39: A screen print of VANDA, management software package of the seed bank of the National Mediterranean Botanical Conservatory of Porquerolles realised by M. Vicevaire.

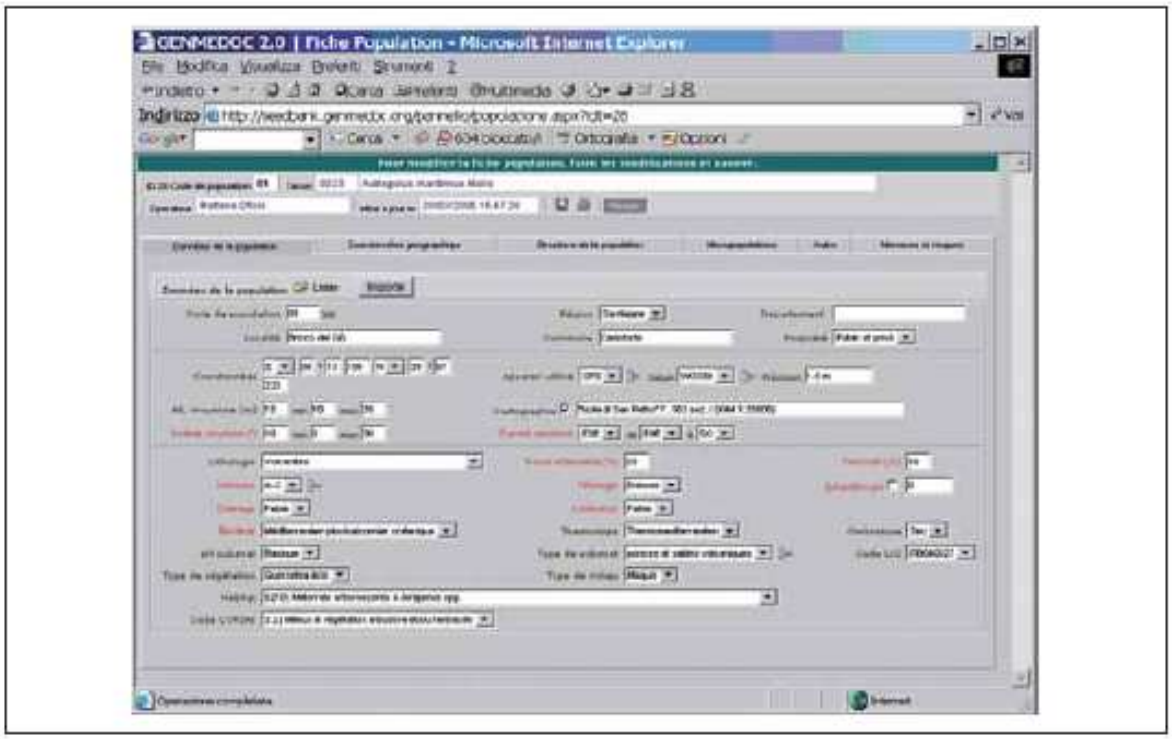


Fig. 40: A screen print of the database of the Interreg IIIB Genmedoc Project.

9.2 Management of Vegetative Material

The procedure relative to the collection and delivery of vegetative propagation material (rhizomes, tubers, bulbs, bulbils, cuttings, etc) to the bank does not depart excessively from the standard procedure described previously. Indeed, every accession must be accompanied by the corresponding collection schedule (v. 13.1) and eventually of any other filled schedule that has been compiled (eg. Phenological data sheet 13.4, etc) and a summary list that contains the name of all the data sheets produced and the information relative to the collectors.

The conservation and multiplication of vegetative material presents substantial differences with respect to the conservation of seeds and for this reason, the adoption of specific protocols is necessary, depending on the type of material consigned to the bank. After having filled the collection data required on the database sheets, one proceeds to verify the quantity and quality of the material produced and any eventual precautions that need to be taken in their management entering this information on the data card. This is especially since in general this is related to material which is more sensitive to manipulation and has the tendency to dehydrate quickly if not maintained under proper environmental laboratory conditions. When necessary one has to identify the most suitable initial treatment (ex. fungicide application, elimination of foliar defects in leafy cuttings or time and condition of storage for the formation of calluses in certain cuttings), and all these procedure details carried out on the material are entered on the data cards. Furthermore, other details such as the number of samples produced from the number of cuttings made and from these, the number that actually survived. Finally, for each sample produced, a code number is assigned and for each the location and techniques used for growing on, must also be indicated (V. 13.13).

10. More In depth Studies

10.1 Guidelines for the collection, storage and sowing of seeds of wild trees and shrubs in Italy

10.1.1 Introduction

If one intends to manage the available natural resources properly, through multiplication of plants by seed, one should take note of two key objectives:

- to maintain or increase biodiversity at the species level, by targeting the propagation of a large number of separate plants if the mechanisms of sexual propagation are known;
- to maintain or increase biodiversity at the genetic level by using techniques that prevent the loss of genetic variability during the breeding of individual species.

With reference to the last point, is important to remember that the seedlings obtained should not lose genetic diversity during the growing stage, one should avoid artificial selections, and must be aware of processes that restrict the variability of genetic traits. The presence of strong heterogeneity is particularly important in the case of plants used for environmental restoration and recovery, while it is less important in short cycle production nurseries. It is not always easy to handle and maintain in the nursery all the biological variability of a species representing its genetic potential, but in many cases, one can minimize the risk of unwanted erosion through the application of appropriate horticultural techniques. Among those deserving special attention, there are the seed sowing operations: namely knowledge of the biological peculiarities of the seed (dormancy, shelf life, etc..), the ideal time of sowing, any pre-treatment required to remove dormancy, and the conditions that may promote or inhibit germination. All the above operations, if conducted properly, are essential to minimise the loss of genetic diversity during the early stages of seed germination and growth of seedlings. The table that is presented below (Table 4) summarises the best indications for the collection, preservation and sowing of seeds of many wild trees and shrubs that grow in Italy. Empty boxes indicate lack of information or specific data for the species under consideration. The part referring to seed collection (see section 4) indicates the season in which this collection is normally performed and it also suggests the time (before the collection) for estimating the size and quality of fruit. As regards seed for storage and propagation (see section 6 and 7) the guidelines refer to the work necessary to obtain and prepare the seeds and the storage temperature at the altitude of seed storage (ie shelf life under controlled conditions). The table contains also the time of sowing, whether the germplasm needs to be subjected to special treatment and the operations to be applied to seeds that show dormancy (see sections 8.3 and 10.2). Also indicated are those species whose seeds have complex dormancy (difficult to remove), species whose seeds tend to germinate at very low temperatures at the end of the vernalisation treatment and species whose seeds tend to regain a state of dormancy (secondary dormancy) when exposed to relatively high temperatures immediately after a pre-treatment applied to remove the primary dormancy.

10.1.2 Notes for use of Table 4

(1) Factors to be considered when proceeding for collection: lists conditions indicating the best time to carry out operation, or situations that can interfere negatively and should be avoided.

(2) Type of processing/treatment of the seed: refers to a set of processes that, starting from the fruits, helps produce seed free of impurities and suitable for planting. The various processing /treatment techniques have been described previously (see section 6.4 and 6.6).

(3) Storage temperature: indicating temperatures normally used in the National Centre for the Study and Conservation of Forest Biodiversity, Peri (VR), Ministry of Agriculture and Forestry, State Forestry Department.

(4) Preservation capacity of the seed: is the seed response to seed storage under controlled conditions. This behaviour classification of the seed is divided into two broad categories: orthodox seeds and recalcitrant seeds. Between the two extremes there is a continuum of conditions, therefore, it may be more precise to classify the seeds as tolerant and non tolerant to dehydration (Piotti and Di Noi, 2003). See also section 6.9 and the glossary.

(5) The species marked with number (5) in the same column (Preservation capacity of the seed) have orthodox seeds whose moisture content needs to fluctuate between 10 and 20% for optimum conservation. In fact these store worse than the typical orthodox seeds, which tolerate smaller moisture contents of between 5 and 7%.

(6) Time of sowing: If not specified, the autumn sowing does not include the pre-treatment of the seed; the spring sowing, however, often requires the use of non-dormant seed that is subjected to pre-treatment before sowing and is ready for germination.

(7) Pre-treatment (if necessary to remove the dormancy): indicates the pre-treatment/s necessary to remove the dormancy, with conditions and duration (see section 8.4). The recommended pre-treatments are basically three types:

- scarification (see section 8.4.4);
- estivation (or warm stratification or preheating or warming) (see section 8.4.2);
- vernalisation (or cold stratification or pre-chilling) (see section 8.4.1).

(**CD**) = indicates species with **complex dormancy**. In the table the term is conventionally applied to those seeds that need dormancy treatments or combinations of longer duration of treatment generally prolonged. The term "complex dormancy" is reported for a species when the dormancy is presumed complicated because it does not respond positively to more commonly used treatments in nurseries. For many species with complex dormancy listed in the table, autumn sowing is shown. This practice is not always followed by germination during the following spring. Very frequently, emergence may in fact occur, during the second or third spring. Autumn sowing, is applied when it is suspected that the dormancy is due to adverse climatic conditions that characterise the season. Can be used when the techniques to facilitate the germination are not known or incubators are not available for this pre-treatment.

(**CTG**) = indicates the seeds of those species that after being subjected to cold stratification, can also germinate even at very low temperatures (**cold temperature germination**). For this reason, when this type of seed is subjected to pre-treatment it must be checked frequently for accumulation of vernalisation cues, especially towards the end of treatment.
(**SD**) = indicates the seeds of those species that, following the removal of primary dormancy (that is present at the time of natural dispersal) through pre-treatment, may remain in a semi-dormant state (**secondary dormancy**) especially where the seed bed is maintained at high temperatures (above +20 ° C) for prolonged periods. Complete germination of dormant seeds of these species is generally favoured by alternating temperatures (cold nights and warm days) in soil, as happens in nature in early spring. The relatively high temperatures at the end of the spring-summer transition may induce secondary dormancy, which stops the germination process completely.

Table 4 - Guidelines for the collection, storage and sowing of seeds of wild trees and shrubs in Italy.

Scientific and vernacular name	Time of fruit formation and growth	Time of collection	Factors to be considered when proceeding for collection (1)	Type of processing/treatment to seeds (2)	Storage Temperature (3)	Preservation capacity (4)	Time of sowing (6)	Pre-treatment (if it is necessary to remove dormancy) (7)
Abies alba Mill. (Abete bianco), *A. cephalonica* Link. (A. greco), *A. nordmanniana* Spach. (A. del Caucaso), *A. pinsapo* Boiss. (A. di Spagna)	summer	Beginning of autumn	Resin deposition on the cones indicates the correct time at which collection can be started	cold treatment	- 7 ° C	orthodox	Autumn sowing pacciamata or spring sowing of vernalised seeds	Vernalisation for 3 – 4 weeks
Acer campestre L. (Acero oppio)	beginning of autumn	Autumn	A brown coloration of the seed indicates the correct time at which seed collection can be started.	cold treatment	+ 2 ° C	See (5) in Note for Instructions on use of the table	Autumn sowing or spring sowing of treated seeds (CD) (GF)	Estivation for 0-8 weeks Followed by vernalisation for 12-24 weeks
Acer monspessulanum L.(Acero minore)	beginning of autumn	autumn	Abundant fruit production does not indicate quality and frequently there is a high level of empty seeds.	cold treatment	+ 2° C	See (5) in Note for Instructions on use of the table	As above	Vernalisation for 8-12 weeks
Acer opalus Mill. (Acero alpino)	autumn	Beginning autumn	as above	cold treatment	+ 2° C	See (5) in Note for Instructions on use of the table	Autumn sowing or spring sowing of treated seeds (GF)	Estivation for 0-12 weeks followed by vernalisation for 4-12 weeks
Acer platanoides L. (Acero riccio)	beginning of autumn	autumn	A brown coloration of the seed indicates the correct time at which seed collection can be started	cold treatment	+ 2° C	See (5) in Note for Instructions on use of the table	As above	Vernalisation for 4-6 weeks
Acer pseudoplatanus L.(Acero di monte)	beginning of autumn	autumn	as above	cold treatment	+ 2° C	See (5) in Note for Instructions on use of the table	as above	Vernalisation for 4-10 weeks
Alnus cordata (Loisel.) Loisel.(Ontano cordato), *A. glutinosa* (L.) Gaertn. (O. comune), *A. incana*(L.) Moench (O. bianco), *A. viridis* (Chaix) DC. (O. verde)	beginning autumn	autumn	che non siano aforti i piccoli pseudostrobili	cold treatment	+ 2° C	orthodox	Sow till end of February or spring with vernalised seeds.	Vernalisation for 4-6 weeks
Amelanchier ovalis Medik.(Foro corvino)	summer	summer	favours the predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing immediately after collection or spring sowing of vernalised seeds.	Vernalisation for 8-12 weeks
Arbutus unedo L. (Corbezzolo)	autumn	autumn	la maturazione è scalare e protratta nel tempo	Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing or spring sowing of vernalised seeds	Vernalisation for 0-8weeks

Scientific and vernacular name	Time of fruit formation and growth	Time of collection	Factors to be considered when proceeding for collection (1)	Type of processing/treatment to seeds (2)	Storage Temperature (3)	Preservation capacity (4)	Time of sowing (6)	Pre-treatment (if it is necessary to remove dormancy) (7)
Berberis vulgaris L. (Crespino)	summer	autumn		Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing or spring sowing of vernalised seeds	Vernalisation for 6-13 weeks (la previa estivation potrebbe essere
Betula pendula Roth(Betulla verrucosa)	summer	end of summer		cold treatment	+ 2° C	orthodox	Autumn sowing or spring sowing of vernalised seeds	Vernalisation for 4-8 weeks
Buxus semforvirens L. (Bosso)	summer	summer		cold treatment	+ 2° C	orthodox	Autumn sowing or spring sowing of vernalised seeds	
Carpinus betulus L.(Carpino bianco)	autumn	autumn		cold treatment	+ 2° C	orthodox	Presenza di dormienza complessa.Semina di end of summer con semi ancora verdi oppure semina primaverile con seme maturo trattato (CD) (GF)	Estivation for 2-8 weeks followed by vernalisation for 12-14 weeks
Carpinus orientalis Mill.(Carpinella)	autumn	autumn		cold treatment	+ 2° C	orthodox	Semina primaverile con seme sottoposto a estivation + Vernalisation(CD) (GF)	Estivation for 3-6 weeks followed by vernalisation for 12-15 weeks
Castanea sativa Mill.(Castagno)	autumn	autumn		cold treatment	+ 2° C	recalcitrant	Autumn sowing oppure primaverile con seme all'aforto, dal momento della raccolta	
Celtis australis L. (Bagolaro)	autumn	autumn		cold treatment	+ 2° C	orthodox	Autumn sowing oppure primaverile con seme vernalizzato (GF)	Vernalisation for 8-12 weeks
Ceratonia siliqua L. (Carrob)	summer	end of summer		cold treatment	+ 2° C	orthodox	Semina primaverile con seme scarificato	Mechanical scarification
Cercis siliquastrum L(Albero di giuda)	summer	end of summer		cold treatment		orthodox.	Semina primaverile seme scarificato (in alcuni casi può essere utile una breve Vernalisationin seguito alla	Scarification
Colutea arborescens L.(Vescicaria)	summer	summer		cold treatment	+ 2° C	orthodox	Semina primaverile con seme scarificato	Mechanical scarification
Coriaria myrtifolia L. (Coriaria, Sommacco provenzale)	autumn	autumn		cold treatment	+ 2° C		Semina primaverile con seme retrattato.Temperature alterne favoriscono la germinazione di seme non dormiente	Application of a solution of gibberellic acid (2,6 x 10-3)
Scientific and vernacular name	**Time of fruit formation and growth**	**Time of collection**	**Factors to be considered when proceeding for collection (1)**	**Type of processing/treatment to seeds (2)**	**Storage Temperature (3)**	**Preservation capacity (4)**	**Time of sowing (6)**	**Pre-treatment (if it is necessary to remove dormancy) (7)**
Cornus mas L. (Corniolo)	summer	end of summer	favours the predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Presenta dormienza molto complessa. Autumn sowing (la	Estivation for16 weeks followed by vernalisation for 4-16 weeks

							germinazione avviene nella seconda primavera) oppure primaverile con seme sottoposto a estivation seguita da Vernalisation; la scarificazione eseguita prima dell'estivation può essere utile (CD)	
Cornus sanguinea L.(Sanguinella)	autumn	autumn	favours the predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing oppure primaverile con seme sottoposto a estivation + Vernalisation; può bastare la sola Vernalisation	Vernalisation for 12-18 weeks (eventually preceded by estivation 0-8 weeks)
Corylus avellana L. (Nocciolo)	end of summer	Beginning autumn	Various types of predation	cold treatment	+ 2° C	sub-orthodox	Il seme non sopporta la disidratazione. Autumn sowing oppure primaverile,in entrambi i casi con nocciole vernalizzate,spesso all'aperto, dal momento della raccolta	Vernalisation
Cotinus coggygria Scop.(Sommacco selvatico)	summer	summer		cold treatment	+ 2° C	orthodox	Semina primaverile con seme dapprima scarificato meccanicamente o chimicamente e poi vernalizzato (CD)	Mechanical or chemical scarification (sulphuric acid (30-45 minutes) followed by 4-8 (or more) weeks of vernalisation depending on provenance
Crataegus sp. pl. (Biancospino)	autumn	autumn		Processing of fleshy fruits	+ 2° C	See (5) in Note for Instructions on use of the table	Semina di fine winter - Beginning primavera con seme sottoposto a estivation + Vernalisation, eventualmente dapprima scarificato (CD)	Estivation for 4-16 weeks followed by vernalisation for 12-20 sett
Scientific and vernacular name	**Time of fruit formation and growth**	**Time of collection**	**Factors to be considered when proceeding for collection (1)**	**Type of processing/treatment to seeds (2)**	**Storage Temperature (3)**	**Preservation capacity (4)**	**Time of sowing (6)**	**Pre-treatment (if it is necessary to remove dormancy) (7)**
Cytisus sp.pl.	end of summer	autumn		cold treatment	+ 2° C	orthodox	Spring sowing of scarified seeds	Mechanical or chemical scarification
Elaeagnus angustifolia L. (Olivagno)	autumn	autumn		Processing of fleshy fruits	+ 2° C	See (5) in Note for Instructions on use of the table	Autumn sowing oppure di fine winter – Beginning primavera con seme	v. epoca di semina

							sottoposto a estivation (in alcuni casi non risulta necessaria)+Vernalisation. Un trattamento alternativo consiste nell'immersione del seme in acqua corrente (+15°C) for 6 giorni seguita da stratificazione fredda per 4 settimane (DS)	
Elaeagnus umbellata Thunb. (Olivagno)	autumn	autumn		Processing of fleshy fruits	+ 2° C	See (5) in Note for Instructions on use of the table	Autumn sowing o di fine winter - Beginning primavera con seme immerso in acqua corrente (+15°C) for 6 giorni e poi vernalizzato for 4 settimane (DS)	v. epoca di semina
Emerus majus Mill.	summer	summer		cold treatment	+ 2° C	orthodox	Semina primaverile con seme scarificatomeccanicamente oppure immerso in acqua calda per 12-14 ore	Mechanical scarification
Erica arborea L. (Erica arborea)	summer	summer		cold treatment	+ 2° C	orthodox	autumn sowing	Many species of Erica respond well to smoke treatment
Evonymus europaeus L. (Fusaria comune)	autumn	autumn		cold treatment	+ 2° C	orthodox	Autumn sowing alternatively beginning of spring with seeds subjected to estivation + Vernalisation.(CD)	Estivation for 8-12 weeks followed by vernalisation for 8-16 weeks
Fagus sylvatica L. (Faggio)	autumn	autumn		cold treatment	+ 2° C	orthodox	Autumn sowing alternatively end of winter - beginning of spring with vernalised seeds. One has to avoid the late spring sowing since high soil temperatures may induce secondary dormancy. (GF) (DS)	Vernalisation for 3-12 weeks (median 8)
Frangula alnus Mill. (Frangola), *F. rupestris* (Scop.) Schur. (F. triestina)	summer	summer *(F. rupestris)*, end of summer	maturazione scalare *(F. alnus)*	Processing of fleshy fruits	+ 2° C	orthodox		

		Beginning autumn *(F. alnus)*						
Fraxinus angustifolia Vahl (Frassino a foglie strette)	autumn	autumn winter		cold treatment	+ 2° C	orthodox	Autumn sowing oppure di fine winter – Beginning primavera con semi sottoposti a pre-trattamenti for rimuovere la dormienza (CD) (GF) (DS)	Possible pre-treatments: estivation (4 weeks) + vernalisation (4-8 weeks) alternatively vernalisation for 8-16 weeks
Fraxinus excelsior L. (Frassino maggiore)	autumn	autumn		cold treatment	+ 2° C	orthodox	Presenta dormienza complessa. Autumn sowing oppure primaverile con seme pretrattato (CD) (GF) (DS)	Estivation (8-16 weeks) + Vernalisation (8-16 weeks)
Fraxinus ornus L. (Orniello)	autumn	autumn		cold treatment	+ 2° C	orthodox	Autumn sowing oppure di fine winter – Beginning primavera con seme sottoposto a pretrattamento	Estivation (2-8 weeks) + Vernalisation (8-15 weeks)
Genista pilosa L. (Ginestra tubercolosa), *G. radiata* (L.) Scop. (G. stellata), *G. tinctoria* L. (G. minore)	summer	summer		cold treatment	+ 2° C	orthodox	Semina primaverile con seme scarificato	Mechanical or chemical scarification (immersion in sulphuric acid for variable time intervals)
Hippophae rhamnoides L. (Olivello spinoso)	summer	end of summer		Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing oppure primaverile con seme vernalizzato	Vernalisation for 4-12 weeks
Ilex aquifolium L. (Agrifoglio)	autumn	winter		Processing of fleshy fruits	+ 2° C	See (5) in Notes for consultation of table	Autumn sowing oppure primaverile con seme pre-trattato (CD)	La dormienza, complessa e legata alla disseminazione ornitocora, non è facile da rimuovere. Si suggeriscono lunghi periodi di estivation (fino a 40 weeks) seguiti da Vernalisation (fino a 24 weeks)
Juglans regia L. (Noce comune)	autumn	autumn		cold treatment	+ 2° C	Sub-orthodox	Il seme non sopporta la disidratazione spinta. Autumn sowing oppure primaverile con	

							seme vernalizzato, generalmente all'aperto, durante tutto l'winter	
Juniperus communis L. (Ginepro comune), *J. oxycedrus* L. subsp. *macrocarpa* (Sibth. *et* Sm.) Neirl. (Ginepro coccolone)	End of summer	autumn	coesistenza nella stessa pianta di frutti di varie età e maturazione al momento della raccolta	Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing oppure di fine winter – Beginning primavera con seme trattato (CD)	Dormienze molto complesse che possono talvolta essere rimosse da estivation seguita da Vernalisation, in alcuni casi può bastare la sola Vernalisation
Laburnum alpinum (Mill.)Bercht. *et* J. Presl (Maggiociondolo di montagna), *L. anagyroides* Medik. (M. comune)	autumn	autumn *(L. alpinum)*, autumn winter *(L. anagyroides)*		cold treatment	+ 2° C	orthodox	Semina primaverile con seme scarificato	Mechanical or chemical scarification
Larix decidua Mill. (Larice europeo)	autumn	winter	si può correre il rischio di raccogliere anche coni vecchi	a caldo	+ 2° C	orthodox	Autumn sowing pacciamata oppure primaverile, preferibilmente con seme vernalizzato	Vernalisation for 3 - 8 weeks
Laurus nobilis L. (Alloro)	autumn	winter	favours the predation by birds	Processing of fleshy fruits	+ 2° C	See (5) in Notes for consultation of table. Many consider these as recalcitrant.	Autumn sowing subito dopo la raccolta (il seme perde rapidamente la vitalità) oppure primaverile con seme vernalizzato durante l'winter	Vernalisation for 8-12 weeks
Ligustrum vulgare L. (Ligustro)	summer	autumn	favours the predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing oppure primaverile con seme vernalizzato	Vernalisation for 4-12 weeks
Lonicera alpigena L. (Madreselva alpina), *Lonicera etrusca* Santi (Caprifoglio etrusco), *Lonicera nigra* L. (Caprifoglio nero), *Lonicera xylosteum* L. (Caprifoglio peloso)	summer	summer *(L. etrusca)*, summerautumn *(L. nigra* e *L. xylosteum)* autumn *(L. alpigena*	favours the predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Non ci sono molte informazioni sulla propagazione for seme; in genere si indica semina autunnale oppure primaverile con seme vernalizzato (CD)	Vernalisation for 12 weeks (sometimes preceded by estivation for 8 weeks)
*Malus sylvestri*s (L.) Mill. (Melo selvatico)	autumn	autumn	favours the predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Semina subito dopo la raccolta oppure primaverile con seme trattato. (DS)	Estivation (2-4 weeks) + Vernalisation (12-16 weeks)

Mespilus germanica L. (Nespolo)	End of summer	autumn	favours the predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Non ci sono molte informazioni sulla propagazione for seme. Semina subito dopo la raccolta oppure primaverile con seme trattato. (DS)	Estivation + Vernalisation
Myrtus communis L. (Mirto, Mortella)	End of summer	autumn	favours the predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Semina tardo autunnale oppure primaverile con seme vernalizzato	Vernalisation for 3-6 weeks
Morus alba L. (Gelso comune), *M. nigra* L. (G. nero)	primavera	fine primavera	Various types of predation	Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing, previa immersione in acqua fredda for 3-4 giorni, oppure primaverile con seme vernalizzato	Vernalisation for 4-8 weeks
Ostrya carpinifolia Scop. (Carpino nero)	end of summer	autumn winter		cold treatment	+ 2° C	orthodox	Semina a fine winter - Beginning primavera con seme sottoposto a estivation + Vernalisation (CD) (GF) (DS)	Estivation for4-8 weeks followed by vernalisation for 16-20
Paliurus spina-christi Mill. (Marruca)	end of summer	autumn		cold treatment	+ 2° C	orthodox	Autumn sowing oppure primaverile con seme vernalizzato	Vernalisation for 10-20 weeks
Phillyrea angustifolia L., (Fillirea) *P. latifolia* L. (Lilatro)	Beginning autumn	autumn	favours the predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing oppure primaverile, in entrambi i casi è meglio impiegare seme scarificato	Mechanical or chemical scarification (sulphuric acid for 30 minutes)
Picea abies (L.) H. Karst. (Abete rosso)	Beginning autumn	autumn		a caldo	+ 2° C	orthodox	Semina primaverile con seme dapprima immerso in acqua fredda for 24-48 ore oppure vernalizzato	Vernalisation for 2-3 weeks
Genere *Pinus*	summer (autumn for *P. nigra* e *P. sylvestris)*	Da dic. a giu. *P. halepensis* Da nov. a maggio *P. pinea* Da ott. a giugno *P. pinaster* summer *P. mugo*, autumn *P.*		cold treatment	+ 2° C	orthodox	For i pini mediterranei semina primaverile senza pretrattamenti, per gli altri semina primaverile con seme vernalizzato for 4-10 weeks	

		cembra e *P. nigra*, aut.-winter *P. sylvestris*						
Pistacia lentiscus L. (Lentisco)	end of summer	autumn		Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing oppure primaverile con seme vernalizzato (2-3 settimane). In alternativa semina primaverile con seme scarificato meccanicamente	Vernalisation o scarificazione (v. Epoca di semina)
Pistacia terebinthus L. (Terebinto)	end of summer	autumn	In some years the production of Vano ???empty/non viable??? seeds is very high.	cold treatment	+ 2° C	orthodox	Autumn sowing oppure primaverile con seme vernalizzato	Vernalisation for 12 weeks
Platanus orientalis L. (Platano orientale)	summer	autumn		cold treatment	+ 2° C	orthodox	Semina subito dopo la raccolta (winter) oppure primaverile con seme vernalizzato	Vernalisation for 6-8 weeks
Prunus amygdalus Stokes (Mandorlo selvatico), *P. avium* L. (Ciliegio selvatico), *P. brigantina* Vill. (Pruno del delfinato), *P. cerasifer* Ehrh. (Ciliegio-susino), *P. cerasus* L. (Marasca), *P. laurocerasus* L. (Lauroceraso), *P. mahaleb* L. (Ciliegio canino), *P. padus* L. (Pado), *P. spinosa* L. (Prugnolo)			primavera (summer for *P. spinosa*) summer for tutti tranne *P. mahaleb* (Beginning summer) e *P. spinosa* (fine summer-autumn)	possono essere predati dall'avifauna, in particolar modo *P. avium* e *P. mahaleb* lavorazione frutti carnosi	+ 2° C	orthodox	Semina di fine winter - Beginning primavera (la germinazione è favorita dall'alternanza giornaliera di temperature del terreno) con seme sottoposto a pretrattamento for rimuovere la dormienza (DS)	Estivation (2-6 weeks) + Vernalisation (4-18 weeks), varia con la specie. For *P. avium* si suggerisce vernal. 6 weeks + estiv. 2 weeks + vernal. 2 weeks + estiv. 2 weeks + vernal. 12 weeks; la germinazione è favorita da forti alternanze diarie di temperature (3°C la notte, 20°c il giorno)
Pyrus spinosa Forssk. (Pero mandorlo), *P. pyraster* Medik. (P. selvatico)	autumn	autumn	favours predation by birds	Processing of fleshy fruits	+ 2° C	orthodox	Semina di fine winter - Beginning primavera (la germinazione è favorita dall'alternanza giornaliera di temperature del terreno) con seme sottoposto a pretrattamento per rimuovere la dormienza (CD) (DS)	Estivation (2-4 weeks) + Vernalisation (12-18 weeks)

Quercus sp. pl.	end of summer	autumn		cold treatment	+ 2° C	recalcitrant	Il seme non sopporta la disidratazione. Semina autunnale subito dopo la raccolta oppure primaverile con seme vernalizzato, generalmente all'aperto, dal momento della raccolta	
Rhamnus sp. pl.	summer	in general end of summer - Beginning autumn	Various types of predation. In some years the production of Vano ???empty/non viable??? seeds is very high.	Processing of fleshy fruits	+ 2° C	orthodox	Rhamnus species show a rather complex dormancy pattern that can vary with different years and provenance. Autumn sowing or spring for pretreated seeds. (CD)	For *Rhamnus alpinus* 12-16 weeks of vernalisation are recommended
Rosa sp. pl.	end of summer	autumn		Processing of fleshy fruits	+ 2° C	orthodox	Sow end of winter, beginning spring for seeds subjected to estivation + Vernalisation. Addition of a compost starter to the substrate helps to accelerate the treatment time since the microbes act by degrading the fleshy endocarp. The treatment is not always effective. (CD) (GF) (DS)	Estivation (8-24 weeks) + Vernalisation (8-24 weeks)
Ruscus aculeatus L. (Pungitopo)	winter	Winter-spring		Processing of fleshy fruits	+ 2° C	orthodox	La specie mostra una dormienza molto complessa e a tutt'oggi non si conoscono metodi veramente efficaci for stimolare la germinazione. Semina primaverile con seme sottoposto a estivation + Vernalisation (anche for più cicli) (CD)	Estivation (4-8 weeks) + Vernalisation (8-12 weeks)
Sambucus sp. pl.	summer	summer	favours predation by birds (in particular *S. nigra*)	Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing con seme non trattato oppure primaverile con seme trattato	For *S. nigra* vernalisation (8-9 weeks), gradisce temp. di 20°C per geminare For *S. racemosa* Vernalisation

								(12-24 weeks) preferisce temperature alternate for germinare
Sorbus sp. pl.	summer	end of summer - autumn*(S. aria, S. domestica), autumn (S. aucuparia, S. torminalis)*	varie predazioni (in particolare su *S. aucuparia* e *S. torminalis*)	Processing of fleshy fruits	+ 2° C	orthodox	Semina subito dopo la raccolta oppure a fine winter – Beginning primavera (l'alternanza giornaliera di temperature favorisce la germinazione mentre le temperature costanti elevate inducono dormienza secondaria) con seme sottoposto a estivation + Vernalisation (o alla sola Vernalisation) (CD) (DS)	Estivation (0-4 weeks) + Vernalisation (12-16 weeks)
Spartium junceum L. (Ginestra odorosa)	summer	summer autumn		cold treatment	+ 2° C	orthodox	Semina primaverile con seme scarificato	Scarificazione
Staphylea pinnata L. (Bossolo)	autumn	autumn		cold treatment	+ 2° C	orthodox	Semina subito dopo la raccolta oppure primaverile con seme sottoposto a estivation + Vernalisation (CD)	Estivation (12 weeks) + Vernalisation (12 weeks)
Taxus baccata L. (Tasso comune)	end of summer	end of summer Beginning autumn		Processing of fleshy fruits	+ 2° C	See (5) in Notes for consultation of table.	Autumn sowing (la germinazione avviene durante la 2a primavera) oppure primaverile con seme sottoposto a trattamento (non sempre efficace) (CD)	Estivation (12-28 weeks) + Vernalisation (8-16 weeks)
Estivation (12-28 weeks) + Vernalisation (8-16 weeks)	autumn	autumno fine autumn		cold treatment	+ 2° C	See (5) in Notes for consultation of table.	Presenta dormienza complessa. Se non si impiega seme trattato, la germinazione si protrae for 3 anni. Semina primaverile con seme trattato (estivation + Vernalisation) (CD) (GF)	Estivation (16 weeks) + Vernalisation (14-18 weeks)
Ulmus sp. pl.	primavera	primavera		cold treatment	+ 2° C	In nature these lose their viability rapidly. Difficult to conserve.	I semi di olmo non hanno dormienza. Semina immediatamente dopo la raccolta (primavera)	

Viburnum sp. pl.	Summer (*V. lantana* e *V. opulus*), autumn (*V. tinus*)	end of summer autumn		Processing of fleshy fruits	+ 2° C	orthodox	Autumn sowing oppure primaverile con semesottoposto a estivation+Vernalisation (CD) (GF)	v. epoca di semina

10.2 How are the ecophysiological requirements for germination determined?

To conserve and manage stored germplasm properly, one should strive to learn more on the strategies that species use for their reproduction. Sexual propagation is of particular interest because it ensures maximum genetic diversity and in this context, the natural ecophysiological requirements that the seeds must meet in order to germinate.

When sometimes it becomes indispensable to restore habitats of special importance for future generations, especially those involving rare endemic species threatened with extinction, it is not unusual to find that their propagation requirements are still unknown.
An important condition that must be established if one decides to carry out artificial propagation of threatened plants or plants of special interest is the existence or otherwise of dormancy (see section 8.3) at the moment of seed dispersal. It is good to remember that the seeds from wild species in cold temperate regions of the world have adapted to these demanding environments by stay dormant in the ground during winter or during a summer and winter, which conditions remove the dormancy naturally and allow germination.
The unknowns can in many cases, be clarified by studies that tend to shed light on the characteristics of the reproductive cycle of a given species and relationships with specific environmental conditions that cause germination (ecophysiology of germination). This type of approach to the scientific investigation of propagation was started in the U.S. in the 1990s and procedures have been progressively improved (Baskin and Baskin, 1998; Piotti and Crosti, 2005). Fresh seeds (recently dispersed or recently) should be used in these studies because the seed in medium to long term storage may sometimes change their characteristics. It is important to understand whether the storage imparts some kind of change in the physiology of seeds (eg induction or removal of dormancy).

For a preliminary indication on the type of dormancy that characterises the seeds of the species under study, it might be helpful to take into account the way the plant of interest disseminates its seeds. Knowing this is useful to realise factors like seed perishability, and the type of dormancy of the species, especially if the information can be supplemented with the observation of the season in which most dispersed seeds germinate. From this observation, one can look for affinities in responses from the behaviour of known species. Some examples of dormancy linked to dispersal (but not necessarily caused by it) may offer explanations:

- many species that live close to river or lakeside environments (eg *Populus, Salix, Ulmus*, etc..). Dissemination in spring and generally produce non-dormant seeds that germinate immediately (but are difficult to maintain alive in storage);
- the seeds of species disseminated in autumn that germinate during the following spring with a dormancy that is generally removed with a period of moist cold treatment (as happens when they are subjected to these natural conditions in winter);

- the seeds contained in brightly coloured or transparent fruits that are frequently ingested and re-dispersed in the autumn-winter period by birds or small mammals, show very complex dormancy which is often difficult to remove (eg *Cornus, Crataegus, Ilex, Viburnum*, etc..);
- seeds scattered in late spring-summer, germinate during the second spring after the dispersion, generally show morpho-physiological dormancy where warm-humid conditions (summer), followed by cold-wet periods (winter) are required to enable germination (eg. Many *Rosaceae*).
- the seeds that are dispersed in spring or summer and germinate in the autumn or the following winter, show a dormancy removable by a dry heat (summer or fire) (eg, many *Cistaceae*).

The methodology developed in United States by Baskin and Baskin (2003) to identify the requirements for the seeds to germinate is relatively simple (though limited data is available from scientific equipment) and sufficiently plastic as to be adapted to particular the characteristics of different climates.

Initially, these tests were carried out to monitor phenological stages that happen to naturally dispersed seeds immediately after their dissemination in their natural environments, with measures to prevent disturbance and damage from predation. During these experiments it was extremely important to confine the seeds in bags of non-woven fabric and place them in small metal cages (to keep a better record of the places of deposition and to prevent predation). Periodically, the material was recovered and observed to determine the progress of germination. From these experiments, a similar methodology was subsequently developed, but carried out in controlled environments (thermostatic cabinets) that reduced influence of undetermined external conditions. They provided the experimenter the chance to maintain stable conditions in order to determine the temperature or the sequence of temperatures necessary to remove the dormancy in species whose ecophysiology of germination was unknown. The thermostatic cabinets or other heat-controlled environments used in these germination tests, were set at a range of temperatures that either remained constant throughout or alternated daily, simulating the thermal conditions of the air in different seasons of the year typical of the region of interest. Generally two sequences were provided for (Table 5) which, although based on the same succession of seasons, either started with a "winter" or with "summer." The pre-soaked seeds were sown and exposed to the treatments in parallel and monitored until germination occurred. Depending on the availability of seeds, the test could also be conducted in the dark and/or with a predetermined photoperiod.

The duration of the photoperiod should be defined by the researcher but is generally between 8 and 14 hours daily. The light is in place for the hot phase of the thermal cycle, or at any part of the day when there is no alternating daily temperatures (for example during the winter conditions at a constant 5 °C). For each of the thermal regimes used in the tests, independent replicate trials are expected to be made where the repetitions are

always conducted under the same conditions of temperature and light throughout the trial (witnesses). The seeds that do not germinate within 30-40 days when subjected to a given thermal regime, are considered dormant.

The researcher will adjust the temperature of thermal cycles to approach as closely as possible to those registered in the natural range area of the species under study. With regards to limitations in the availability of equipment especially thermostatically-regulated cabinets, some seasons may be eliminated: for example, requirements simulating initiation of spring and end of autumn although one has to have the foresight to extend by another four weeks the corresponding periods in late spring and early autumn (Table 5). This system therefore operates at only three different thermal regimes, which can be conducted in a single cabinet where cycles are set in sequence, or in three separate cabinets, each with a fixed cycle, to which the seeds should be transferred once they complete the seasonal simulation periods. If one chooses to duplicate the experiment in the dark, there is no need for more temperature-controlled cabinets since the seed germination container can be simply wrapped in aluminium foil. The count of germinated seeds is held weekly or more frequently as necessary, in the case of dark treatment in the counting should take place under red light and not the visible spectrum.

Table 5 - Scheme for setting up trials aimed at determining the temperature and cycle temperatures required to remove dormancy where the peculiarities of this nature are not known genetic (as amended by Baskin and Baskin, 2003).

Duration of treatment (weeks)	**Parallel sessions of heat cycles**		**Replicates**			
	4 replicates of 25 seeds (A)	**4 replicates of 25 seeds (B)**	**4 replicates 25 seeds (C)**	**4 replicates 25 seeds (D)**	**4 replicates 25 seeds (E)**	**4 replicates 25 seeds (F)**
12	5 °C winter	25/15 °C summer	5 °C	15/16 °C	20/10 °C	25/15 °C
4	15/16 °C start of spring	20/10 °C start of autumn	5 °C	15/16 °C	20/10 °C	25/15 °C
4	20/10 °C end of spring	15/16 °C end of autumn	5 °C	15/16 °C	20/10 °C	25/15 °C
12	25/15 °C summer	5 °C winter	5 °C	15/16 °C	20/10 °C	25/15 °C
4	20/10 °C start of autumn	15/16 °C start of spring**	5 °C	15/16 °C	20/10 °C	25/15 °C
4	15/16 °C end of autumn	20/10 °C end of spring**	5 °C	15/16 °C	20/10 °C	25/15 °C
12	5 °C winter	25/15 °C summer	5 °C	15/16 °C	20/10 °C	25/15 °C
4	15/16 °C start of spring*	20/10 °C start of autumn	5 °C	15/16 °C	20/10 °C	25/15 °C
4	20/10 °C end of spring*	15/16 °C end of autumn	5 °C	15/16 °C	20/10 °C	25/15 °C
12	25/15 °C summer	5 °C winter	5 °C	15/16 °C	20/10 °C	25/15 °C
4	20/10 °C start of autumn	15/16 °C start of spring	5 °C	15/16 °C	20/10 °C	25/15 °C
4	15/16 °C end of autumn	20/10 °C end of spring	5 °C	15/16 °C	20/10 °C	25/15 °C
↓	↓	↓	↓	↓	↓	↓

10.2.1 Interpretation of results

If the species only needs cold wet winter to remove the dormancy and higher temperatures to germinate, the seeds that start with the cold phase (column A) will germinate in the period that simulates following season (spring), while the seeds of column B will germinate during their fifth or sixth phase (these also represent the spring season), only after have been subjected to a treatment that simulates a winter period. No germination should be observed in the controls kept at constant temperature. A variation on this classic case could be demonstrated for those seeds that are able to germinate at very low temperatures once their cold requirements have been met, in this case, germination could be observed at the end of the first stage of column A and the fourth phase of column B, both at low temperatures, or after a certain period at 5 ° C constant temperature (column C).

The seeds that need, moist heat (summer) + cold wet (winter) in successive sequences to remove their dormancy, will germinate during the second spring when subjected to the sequence provided in column A (Table 5, shown with an asterisk). This is the norm in nature for many Rosaceae and the European Ash (*Fraxinus excelsior* L.). If instead, we start with the warm phase (column B), germination is observed just when the requirements for warm-cold-damp + wet conditions are met (Table 5, shown with two asterisks). For eventualities of this type of treatment in the future, one must keep in mind that for seeds that have this type of dormancy (morpho-physiological), there is a need for them to move progressively through the first warm phase to complete development of the embryo, and only if the embryo has reached maturity will the cold phase be effectively in terms of physiology.

It is obvious that a number of parallel procedures, in which successive thermal treatments are concurrently started with different seasons (winter and summer), will get answers faster. The results of these studies may need to be further explored to determine, for example, the appropriate duration of thermal cycles. In the same way they can help to optimize the quantity and simultaneity of germination, and the identification of the optimum temperature for germination that can be applied after the removal of dormancy. The ideal temperature for germination is sometimes lower than might be expected, especially in temperate and Mediterranean environments where cold spells (autumn) are possible during the most humid part of the year and are therefore the ideal time for germination of many species.

The methodology for identifying the ecophysiological needs of germination just described allows one to obtain fairly precise information generally within 12 and 14 months, but the adjustments to be made to the procedure are numerous and remain at the discretion of the researcher, taking into account the availability of seeds, the type, quantity and capacity of thermostatically-controlled chambers and the accuracy of the information that we claim to achieve.

10.3 Collection, storage and management of Salicaceae germplasm

Poplars and willows are easily propagated both by seeds as well as by vegetative propagation, but it is the especially easy propagation by woody cuttings that over time have influenced the genetic improvement programs and development of cultivation techniques. One often forgets the fact that farmers are also able to identify and collect vegetative propagating material from the best genotypes growing wild along the river banks, and to grow them successfully. As for all agricultural and forestry species, even in Poplar cultivation sustainable management of genetic resources and genetic progress in the long term can only be ensured through the conservation of high biodiversity and promoting the gene recombination following sexual reproduction (Bisoffi et al., 1999).

Figure 41 - Seed Orchards of Populus nigra. (Photo: L. Vietti)

The genetic variability can be maintained both through the protection of natural habitats (primary genetic reserves or natural units of conservation *in situ*), or through the establishment of secondary field gene banks (*ex situ* collections) (Fig. 41). In general, given the high variability in the genus *Populus*, the main objective should be to encourage the evolution of different species through the protection of natural populations. Part of the existing genetic variability in wild formations, once identified, collected and characterised, can be effectively kept maintaining genotypes in clonal archives and orchard collections; they are activities which by their construction and management, require the availability of agricultural land and considerable human and financial resources, but that alone is not sufficient to ensure optimal management of genetic resources, including phytosanitary risks to which the plants may be exposed. A cheaper but not an alternative form of conservation of germplasm, but that integrates well with traditional methods of conservation, is the creation of pollen and seed banks and tissue cultures in vitro. This method makes it much easier for transfer of genetic resources between different institutions, limiting the problems that occur with the exchange of cuttings, rooted or other similar plant material. Among the different species of poplar in Europe, one of particularly importance is *Populus nigra* L. (black poplar). In some areas of the middle-lower course of the Danube, *in situ* coordinated projects have been activated for this species, but it is a generic protection program for river ecosystems, with specific measures for the species that is considered at risk of disappearing in much of Western Europe, including Italy. Still lack of information on the species exists, including

a complete inventory of the location of natural formations, which are essential prerequisites to start a systematic type of conservation, though now difficult to apply on a large scale due to the extreme fragmentation and alteration of fluvial ecosystems (Cagelli, 1998). Being a pioneer species in river environments and also of strategic importance to the breeding programs at the international level, the establishment of secondary genetic resources, has never as in this case become of fundamental importance to the conservation of a species. The *Populus nigra* Network which forms part of the 'European Forest Genetic Resources Program' (http://www.ipgri.cgiar.org/networks/euforgen//euf_home.asp) has coordinated several initiatives to create collections of germplasm of known origin and identity in different European countries, including a core collection that includes genotypes representative of the distribution range of the species and a specific database that collects information on over 3300 genotypes maintained in 20 countries and is in support of activities (Vietti and White, 2005). Thanks to the availability of knowledge about the conservation strategies that can be employed (Lefevre et al., 2001), a "dynamic conservation strategy" was launched for the black poplar in some countries like Italy (Vietti et Chiarabaglio, 2004) and Belgium (Vanden Broeck et al., 2002). It is an active form of long-term action consisting of setting up of artificial units for *in situ* conservation (Fig. 41) in sites suitable for natural regeneration. These units are equivalent to seed orchards and are characterised by high genetic variability. The philosophy behind the project is simple: through sexual reproduction and the evolution of complex genes in response to climatic or biotic environmental changes, survival of the species in the river environments of origin, may be ensured.

10.3.1 Vegetative propagation

Vegetative propagation in plants is usually undertaken during vegetative rest period rather than during the growing season. The best time to collect the material to be used for the production of woody cuttings is towards the end the winter (February-March). For this purpose, one year old suckers or branches are usually rooted, which can be easily produced by cuttings of standard length (about 20 cm) with a good number of lateral buds and particularly suited to transplantation with mechanical means. Where the multiplication must begin from adult trees, 3-4 years old branches are generally taken, possibly by cutting the most vigorous branches at the top of the crown; the cuttings produced should have the maximum length possible (about one meter) and most importantly have latent buds at the base of lateral branches or immediately below the ring which separates the growth of two successive years. These are then planted manually where they are buried for at least 2/3 of their length.

In most species of Poplar and Willow, most of the characteristics of the clones remain unaltered from those of the parent plants: these include their rooting ability, habit, growth, form and vigour; and hence the rooting of cuttings in nurseries ensures that

plants obtained retain the characteristics of growth and development of the mother plants. With regards to some the morphological and physiological characters of the clones, some intraclonalal variability is possible when compared to the characteristics of the parent tree; this is especially related to the age adult trees, the edaphic conditions in which the cuttings were grown and the proportion of the crown from which the cuttings for multiplication were taken (Frison, 1996).

Given that the Salicaceae are dioecious plants, when collections of germplasm is to be made for vegetative propagation of the plant, one must ensure an equitable share of vegetative material from plants of both sexes (Landis et al., 2004).

The material for propagation (branches or suckers) and the cuttings produced can be stored for 1-2 months in cold storage at temperatures of between -2 °C and +4 °C, closed in nylon bags, or for large quantities of material, in boxes protected with jute bags to prevent excessive dehydration. Sometimes there is a chemical treatment with dithiocarbamates. When the need arises to ship materials that require long times for customs clearance and delivery, it is a good idea to protect the ends of cuttings with special putty, wax, or simply wrap the cuttings in plastic bags sealed under vacuum.

The rooting potential of cuttings is mainly dependent on genetic factors which vary from species to species, but is also influenced by morphological, physiological and environmental factors, often concurrently interacting with each other. Rooting generally tends to decline with increasing age of the material used for propagation. Prior to implantation it is a good practice to hydrate the material by immersing in water for a period of 10-15 days depending on the initial state of hydration and especially when during the growing season there was prolonged water shortage.

In the case of *Populus nigra*, cuttings root very easily and when the starting material is two or more years of age, one should always prepare cuttings of greater length (30-50 cm) compared to the standard 20 cm. *Populus deltoides* Marshall (American Black Poplar) shows a much lower rooting capability compared to that of *P. nigra*, with significant differences between different genotypes; these are particularly low in individuals with relatively long growing seasons and involving little woody tissue deposition and consequently a high predisposition to dehydration. Most of the hybrids of *Populus x canadensis* Mönch, Poplar clones commonly referred to by the term "Euramerican" because they were obtained from crosses between female *P. deltoides* and male of *P. nigra*, are generally characterised by a better good rooting capacity, a characteristic that is inherited from the male parents. The greater or lesser rooting capacity strongly affects the establishment of nursery material in Poplar cultivation. For this reason, during the establishment of a Poplar wood, to minimise the time between clearing and replanting of poplar rooted plantlets, for the euramerican clones these are normally done in winter (November-February), while for *P. deltoides* clones or phenotypically similar species, the planting is usually done in the later period (February-March). This will also help to limit excessive dehydration of the material after the

transplanting. In *Populus alba* L. (White Poplar) the rooting ability and cutting establishment varies between genotypes, but again the results can be improved by increasing the length of the cuttings (30-50 cm). In contrast to the above, there are very limited possibilities of propagation by cuttings in the case of *Populus tremula* L., *Populus tremuloides* Michaux and *Populus grandidentata* Michaux, a phenomenon probably linked to the absence of root primordia on the branches. The possibility of vegetative propagation of *Populus tremula* is essentially limited to the use of sucker shoots that can be easily root at the junction close to the soil surface. The sucker shoots can also be used successfully for green cuttings in summer (June-July), in which case the cuttings, formed from a short section of new shoot with leaves (a few inches) and bearing a piece of root, is propagated under controlled conditions of temperature and humidity, on sterile substrate with the use of auxin growth hormones. For other minor species of poplar, the rooting ability is highly variable from species to species. *Populus euphratica* Olivier, *P. lasiocarpa* Olivier, *P. heterophylla* L. generally present considerable difficulty to root. In contrast, some American species such as *P. trichocarpa* T & G and *P. balsamifera* L., and other typical Asian species such as *P. laurifolia* Ledebour, *P. maximowiczii* A. Henry, *P. koreana* Rehder, *P. simoii* Carrière and *P. yunnanensis* Dode can be easily propagate due to the good rooting capacity of cuttings (Frison, *op. cit*.).

Figure 42 – Approach grafting. (Photo: L. Cagelli)

The grafting technique may represent a valid alternative for poplar clones that are difficult to propagate by cuttings. This use is commercially limited almost exclusively to specimens of *P. tremula*, which are usually approach (inarching) or bud grafted, using one year old plants of the same species or of white poplar. This approach grafting technique has long been successfully practiced for scientific and experimental purpose (Fig. 42). This type of grafting is carried out during the growing season (August), grafting the scions (flowering branches taken from adult female trees) onto the seedling rootstocks (seedlings raised in pots, usually using the clone I - 214, *P. x canadensis*) (Fig. 43) usually for controlled pollination the following spring. To induce early flowering, the English double cleft grafting technique could be used. Micropropagation *in vitro* procedures are used for the medium-term storage of clones that have been characterised for a particular commercial interest (Lubrano, 1992). When compared to the vegetative propagation by cuttings, this technique allows greater speed of propagation, the possibility of preservation of material for long periods and furthermore, it can be used to exchange material without incurring issues relating to plant

health. Following promising results obtained with other woody species, one variant of the cryopreservation technique – vitrification, has recently been tested on Poplar. The technique of vitrification, followed by direct immersion in liquid nitrogen (-196 °C), has been applied to apical meristems or embryonic tissues where a high survival rate was obtained in *P. alba* (82%), a satisfactory survival rate in *P. canescens* Sm. (54%), and quite poor in *P. nigra* (22%) (Lambardi, 2002).

Figura 43 – Collection of flowering branches with stamens nearing pollen dispersal (Photo C. Lioia).

10.3.2 Sexual propagation

High genetic variability is an essential requirement for the good management of germplasm and even more important when it comes to the implementation of programs for genetic improvement. For this reason, if seed exchanges allow for sampling from base populations characterised by wide genetic variability, the exchange of pollen allows for the immediate creation of crosses with female subjects of particular interest and to plan in advance crossings for programs that have already been launched. Substantial research has been carried out to assess the viability and germination of pollen and seed, to find the optimal conditions necessary for long-term storage and also to identify the influence of different factors that can adversely affect germination and genetic structure over time.

10.3.3 Collection and conservation of seeds

Under the natural climatic conditions of the Padana Plains (Northern Italy) flowering usually starts towards the beginning of March. In a controlled environment (eg greenhouse) flowering of material collected in the field can be triggered in advance during the entire period of vegetative rest. The pace in the appearance of catkins increases if the material is collected towards the end of winter, especially if branches bearing flowers are conditioned in advance at +4 ° C for a period of about one month. The best seed

Figure 44 – Black poplar seeds. These are circa 3mm long. (Photo L. Cagelli).

producers are adults that have been growing in isolation. In the case of Poplar, this is able to flower and produce seed as early as 5-10 years, sometimes, in conditions of stress, even earlier. The seeds (Fig. 44) are very small and a gram contains on average 1000 seeds though weight and volume vary widely from species to species: values range from 442,000 - 3,300,000 seeds per kg in the case of *P. Deltoides*; 1,000,000 - 1,100,000 seeds per kg for *P. nigra* and 1,600,000 - 1,800,000 seeds per kg for *P. alba*, reaching values of 5,900,000 – 19,700,000 in the case of *P. tremula* (Piotti, 1992; et Piotti Us, 2001). As for the trees of *Salix alba* L., the seeds are usually much smaller, with on average 12,000,000 -15,000,000 seeds per kg in this case.

The collection of seeds should be done as close to the time of the natural dispersal as possible, ie during the opening of the capsules; the latter providing seeds with low viability if the capsules are harvested too early. In any case, the period between the harvest and the start of seed conservation must be as short as possible. In order to prevent possible damage, the fruit must be spread in a thin layer and left to dry at room temperature (1-2 days) in order to collect and separate the seeds from the cottony fluff in the space of one week.

Figure 45 Removal of the cottony fluff in Poplar (Photo Ligia).

The removal of the fluff (fig. 45) from the seed lots of *P. nigra* and *P. deltoides* can be done with good results using a jet of compressed air and a series of sieves with meshes of 1.6 mm.

The germination is generally high (80% - 90%), but may worsen considerably in just 3-4 weeks, especially if the seeds are left exposed to the air even for a few days after the dehiscence of the capsules. Poplar seeds also germinate very quickly in favorable conditions, if the seed is fresh germination can occur in only 6-12 hours. A quick test for germination proposed by Simak (1980). (Fig. 46) may be performed by placing the seeds (100 for three replicates) on filter paper discs soaked with deionised water and placed in Petri dishes at +25 °C, and evaluating

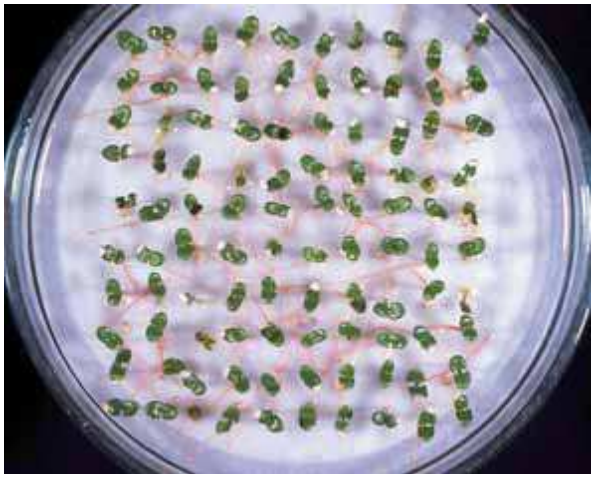


Figure 46 - Germination tests on seeds of Popolus nigra (Photo: L. Cagelli).

the germination 7-10 days after the initiation of the test. To determine with greater certainty the germination tests results it is also possible to use a colorimetric test to assess the viability of the seed batch (Fig. 47).
Although generally the epicotyle emerges from the testa with a certain speed, it should be noted that even apparently normal seeds you can get a high number of abnormal germinelli, despite considerable variability between species and between species and genotypes of the same species. For sowing consistent containers are used generally alveolar 20 cm3 capacity filled with peat substrate and arranged in air conditioned greenhouses at 18 ° - 20 °C. In these conditions, the emergence of seedlings is completed in approximately one week (Cason, in verbis) .
There are many factors influencing the germination time: the time of collection, the time elapsed between harvest and the beginning of storage, the moisture content of the seed and the temperature of storage. Dehydration is a very important factor for the purpose of conservation: the seeds can be stored successfully for several years at low temperatures but only if the water content is first reduced prior values of around 5-8%. The level of hydration can be determined quickly by dehydration with an infrared ray thermobalance a small quantity of seeds (about 200 mg). In the case of seeds produced by *P. nigra* dehydration sometimes occurs naturally without any need for treatment. The moisture content should be reduced gradually, a good way to reach the optimal values of hydration is to leave the seeds in a good air flow for a period of 2-5 days at +20 ° C or better, or by putting the material in an oven at a temperature of 35 ° C for about 10-30 minutes, depending on the initial content of water. With regard to conservation, the material can be placed in small containers such as tubes or better still, packed in vacuum sealed bags. The temperature of storage is a very important factor: a + 4 ° C it is not even possible to preserve the germination capacity for one year. In the case of seed lots of *P. deltoides*, *P. nigra* and *P.x canadensis*, the best results were obtained with temperatures between -18 °C and -40 °C (Cagelli, 1997). Given that at this range of temperatures significant differences in germination have never been observed, the temperature of -18 °C, which is easily reached in a normal freezer, can be considered optimal for preserving the viability of seed lots in the long term. Under these conditions some of the seed of the species mentioned above have maintained good germination (40-50%) for a period of 10 years. Prior to reuse after a long period of storage, it is essential to allow for a gradual transition from very low temperatures to values of around +20 °C; the rehydration should also occur gradually, to prevent a rapid imbibition of water that can cause irreversible damage.

10.3.4 Collection and conservation of pollen

As for seeds, the viability of pollen is influenced by several factors: the time and method of collection, the period between the beginning of collection storage, moisture content and temperature of storage. While the quality of seed can be easily verified by

germination tests, the test of the viability of pollen in Salicaceae is more difficult. There is as yet insufficient information on the correlation between pollen viability and its fertilisation capacity. This last feature is evaluated through the amount of seed produced by the fertilised female structures: according to data from preliminary experiments it seems that, even when using lots of pollen with low viability, one can still get relatively good seed production.

The branches bearing male flower can be harvested throughout winter; the largest amount of pollen, however, is obtained from branches collected in the vicinity of sprouting buds, which can be stored in jars with water (Fig. 48), and maintained n a controlled environment (greenhouse) at a temperature of about +20 ° C and 70% relative humidity.

The collection of pollen can be done in two ways: directly from the anthers at the time of natural dehiscence or from catkin collected at the end of their development and left for 24 hours on sieves at a temperature of about +25 ° C and around 40% relative humidity value. The germination is high immediately after harvest, but may decrease rapidly to zero after a period of one week at +4 °C in silica gel. One of the tests most frequently used to assess the germination of pollen *in vitro* is that proposed by Brewbacker and Kwack (1963). This consists in the preparation of an agar nutrient substrate (KNO_3 0.1 g / l; $CaNO_3$ 0.3 g / l, H_3BO_3 0.1 g / l, $MgSO_4$ $7H_2O$ 0.2 g / l, sucrose 100 g / l for *P. deltoides*, 200 g / l for *P. nigra*), on which pollen grains are allowed to germinate. Germination (Fig. 49) is evaluated at intervals of 12 and 24 hours by measuring the length of the pollen tube. Another quick method is the tetrazolium test (Fig. 47)

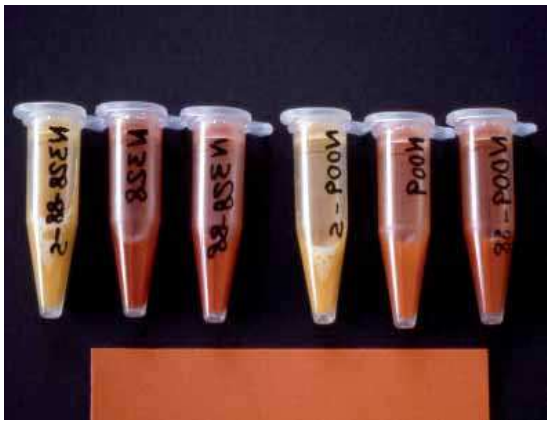


Fig 47. Colorimetric test for Popolus nigra pollen viability based on TTC (photo: L. Cagelli).

Fig 48. Collection of pollen (photo: L. Cagelli).

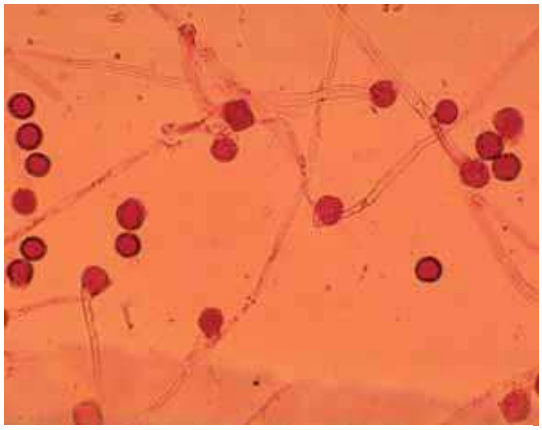


Fig 49. Germination of pollen (photo: L. Cagelli).

which permits evaluation of the viability of pollen according to the intensity of staining taken from pollen grains. Those which react positively to this test have a colour ranging from light pink to pink, the colour reaction begins after about thirty minutes and reaches a maximum within one hour (Rajora et Zuffi, 1986). The moisture content of pollen granules varies greatly between different species and different genotypes of the same species and range between 10% and 80%. As regards the conservation of pollen over time, the water content seems to be the key factor. Before putting the material in storage it is necessary to reduce the water content to values below 10%. In general, a period of 2 hours over silica gel at + 4 °C is usually sufficient to lower humidity values to around 7-10%, both in the case of pollen of *P. nigra* and *P. deltoides*. However, a drying period of about 12 hours at 4 °C is the safest and most commonly used technique for the management of lots of pollen with highly variable moisture content. Again, the water content can be estimated by dehydrating samples of pollen (about 120 mg) using an infrared ray thermobalance (destructive method). After dehydration, the pollen can be stored efficiently in a temperature range of -18 °C and - 40 °C for at least a year. Further studies will be needed to determine water content and optimum temperatures for the conservation of pollen for extended periods of time. At the time of writing, based on tests conducted during artificial crossings, lots of pollen stored for a period of 5 years in a freezer at -40 °C have been used successfully both in their fertilising capacity and also for the number and quality of seed produced. The only important precaution to be followed is to employ a gradual rehydration process on the material before use. For the pollen stored for short periods exposure to high humidity (60-70%) for 1-2 hours at about + 20 °C is sufficient. For pollen rehydration after a long storage period, it is important to ensure a smooth transition in temperature; the hydration is carried out first for a period of 1 hour at +4 °C and then for 2 hours at +20 °C (Stanton and Villar, 1996).

10.4 An example of a demographic study: The AFA project in Spain.

An example of the methodology adopted for the study of aging populations and population dynamics is taken from the draft AFA (Atlas Flora Amenazada), recently developed in Spain (Albert et al., 2003). The project, promoted and financed by the Spanish Ministry of Environment, with the involvement of over a hundred expert, was established with the main objective to analyse the state of conservation of the flora of Spain and to facilitate the optimal management and storage of germplasm. In the first year of the project (2000) the experts worked to update and standardise data on plants on the basis of categories established by the International Union for the Conservation of Nature (IUCN), until the publication of Spanish Red Data Book for threatened flora (Bañares et al., 2003). During 2001-2002 the analysis of field species was carried out with the aim to standardise the work of all operational units, as well as the definition of the methodology for data collecting for demographic surveys. This has allowed the development of a methodology manual. In particular the study of population dynamics

started to take into account long term parameters including, data on the size of individuals, the production of seeds, number of germinating seedlings and the number of those that survive. This has involved long term monitoring of each sample using a considering number of specimens representative of the population. A study of this type can be applied, albeit with some difficulty, to perennial plants (eg geophytes during their vegetative rest stage), but not annual species for which it becomes necessary to analyse the seed bank of soil. In the case of populations with a limited number of individuals, as in the case of some endemics, it is important to monitor in the population census all the individuals present. If studies on populations with extensive and wide distribution are implemented, it becomes essential to identify test areas (permanent quadrants), of variable size depending on the taxon investigated, which are representative of the population and of all the habitats where this species is found. The number, location and size of the quadrants must be related to the permanent population size and ecological distribution range of the species. For example:

- small and homogeneous populations (less than 3000 individuals): the field size must include at least 10% of these individuals and should analyse all, regardless of size or stage of development. When it is obvious that there are different microhabitats present, it is preferable to select the most significant parcels, after first having assessed each of them.
- large and homogenous populations: 2 randomly identified plots, each with at least 5% of total individuals of the population and with up to 300 individuals each. If the population is not homogeneous it is best to select 2-4 parcels, each selected parcel located in a different microhabitat.

The shape of the plot is preferably square or rectangular, if the topography of the site allows. The 4 corners should be marked with stakes, while the sides should be delimited using string or rods. If this form cannot be accommodated, it is important to make an extremely detailed diagram of the shape of the plot and put in measurements in order to calculate the total area and therefore the density of the plants; if necessary, one can also identify the different areas into which the parcel is subdivided such that these include all the 300 individuals. In special situations (cliffs or rocky outcrops), one can use paint to mark out the areas. It is important to prepare a monograph for every parcel of land on the site, with plans, photos, GPS references and other information necessary to give a clear identification of the site and to ensure that anyone can get to the site at any point in time.

10.4.1 Studies of individuals

In order to follow the growth, survival and regeneration of populations it is necessary to identify all individuals present in the parcel, in addition to those that are recruited naturally over time, giving each and every individual an alphanumeric identifier.

It is essential to carry out this census achieve during the flowering-fruiting period in order to include all members of population, especially those that are small and may therefore go unnoticed; this is important so that one can collect at the same time, data on further growth, survival and the biology of reproductive individuals. This survey should be repeated every year, at roughly the same time of the year (with maximum deviations from year to year of one month) in order to obtain homogeneous and comparable data. For annual plants there is no need to mark individual plants, except where one wants to carry out phenological studies on the flowering and/or fruiting of the species. For other species the method of identification of individuals depends on the size of the adult, the habit and the type of habitat these inhabit. Individuals may be identified in various ways in the field, for example by:

- poles of wood or galvanized iron (about 7 cm long) stuck close to each individual, with a metal ring/tag on which the identification code is imprinted;
- metal or plastic labels fixed to the trunk or branches of the individual;
- small flags placed near the individuals showing the identification code.

After having delineated the parcel of land, one can proceed to mark the position of individuals, in the following ways:

1. Mapping on clear plastic sheeting: The map showing the outline of the parcel of land, is covered with a plastic transparency and simultaneously either the outline of the individual is draw, their position indicate as a point. The plastic sheets must have a certain thickness (about 0.5 mm), be transparent and easy to handle ($1m^2$), each sheet will carry a marking code that identifies the exact position in relation to the plot (row-column system) so that one can relocate these in exactly the same point in subsequent monitoring. Where an individual is on the borderline between different sheets, one has to represent on each lamina only the corresponding part of the individual, which can be measured by combining the different sheets involved. Using this methodology it is not necessary to label the different individuals.
2. Mapping using quadrats: The parcel is divided into several squares, using string or ropes tied to stakes stuck to the ground, and each individual is first identified to a specific square number (row-column system) and, its location inside, on the basis of coordinates;
3. Mapping through metal structures: This is very useful for small individuals which are very close together;
4. Mapping via GPS with an instant differential correction system: Each individual is identified through the GPS coordinates; the system is very useful in the case of trees or shrubs of large dimensions.

In grazed areas, it is possible that the characteristics of the land parcel are changed due to the presence of grazing animals, and for that reason a use of a dual identification system is recommended for the relocation of the plants in the following years. In such situations it is advisable to paint the top of the four corner identification posts for the land parcel;

these should be substantially or almost completely buried, their position located by using a measuring wheel with compass directions, metric distances and orientations with respect to three fixed points appropriately identified (eg, large rocks, boulders, cliff faces, etc..).

10.4.2 Data to be collected for each land parcel

Size of individuals: there is no single methodology, given the great variability in plant characteristics. As a guideline one can identify some general principles, which of course must be evaluated and modified from time to time and according to the circumstances when working in the field. In general, for low chamaeophytes or plants with a basal rosette, it may be useful to measure the largest dimension and the dimension perpendicular to it. In the case of ascending chamaeophytes and nanophanerophytes, the most effective measurement is the total height and the diameter of the trunk at the point of contact with the ground. For phanerophytes the easiest parameter to be measured is the diameter of the trunk at breast height (or DBH) and possibly the maximum height. Another simple method for estimating the size of the plants is to count the number of leaves and/or take measurement of their length and/or width. In the case of annual plants, unless there is an extraordinary feature (example larger growth due to over fertilisation), it is not necessary to measure their size since this will be consistent with that of similar plants growing on an annual basis.

As regards the identification of vital stages, 3 stages are identified:

- seedlings: individuals germinated during the current season, identifiable because they still retain the cotyledons,
- saplings or vegetative individuals without cotyledons and reproductive structures;
- breeding adults or reproducers: individuals with reproductive structures.

Where the characteristics of the seedlings cannot be linked to a known species, this data cannot be collected in the first year. In order to ensure correct identification in the following year it will be necessary to collect a sample of seeds at the end of the fruiting season and sowing these under controlled conditions in the laboratory. Having identified the seedlings in the following years the data can be used to identify the rest of individuals in the field or, if this is not possible, any other way that still allows their monitoring in the following years. An alphanumeric identification must be assigned to the different seedlings that grow each year.

10.4.3 Fruit production by the plants

To obtain this data it is necessary to count or estimate the total number of fruits per individual in the plot and to estimate also the average number of seeds per fruit. The number of fruit varies greatly depending on the species, but also because of

environmental conditions. If no data are available from the literature, during the first year it will be necessary to monitor the population during the flowering and fruiting period, at least 3 times. It important to ensure that the reproductive structures remain on the plant until maturity or that a part of the fruit or a scar remains which permits counting at a later time, both if the flowering-fruiting is simultaneous or staggered on the individual or in the population. All this data can be used to estimate the total production of fruits and therefore seeds, where this cannot be counted directly from the plant. In some cases it will be possible to count the total number of fruits produced every single plant of the plot directly. When one cannot make a direct count, one may have two possible scenarios:

1. Species with hermaphroditic flowers and monoecious species: if the number of flowers per plant structures (buds, flowers and / or fruit, according to the phenological stage) is higher than 100, you can proceed to an estimate in the following ways:
   - For species with homogeneous production in the individual, one can count the fruits in half of the plant and multiply the figure by 2 or count the fruits in a third of the plant and multiplying by 3;
   - if the production is mixed, i.e. with variable amounts in different parts of the plant, the data can be obtained by multiplying the total number of inflorescences by the average number of fruits per inflorescence.
2. Dioecious species: here one must specify the sex of the plant and one can then proceed to use the process listed in section 1only for the female specimens.

10.4.4 Other data

In the case where there are clear signs of the presence of herbivores, parasitism, predation and various forms of pest, one must take note the type of damage present (see section 13.5) and this is quantified as a percentage of the whole plant or by a numerical scale from 0 (no part of the plant is affected) to 4 (> 75% of the plant is affected by the phenomenon).

10.4.5 Other studies to be conducted

- Average number of seeds per fruit: collect from trees outside the study plot, 1-2 fruits from at least 30 different individuals and, in the laboratory, count the seeds per fruit (see section13.8 and 13.9).
- Estimate the rate of vegetative reproduction: for those species that reproduce asexually, identify individuals which are the result of vegetative propagation and assign an alphanumeric code with respect to the parent plant??. It is imperative to identify also the type of vegetative propagation involved, which can be deduced by uprooting some individuals and checking how they are united to each other. This

study can be implemented with plants grown in a greenhouse or through other material from *ex situ* collections.

- Soil seed bank: for the study of the population dynamics of annual species we must study the germination potential the seeds stored in the soil (seed bank). At least 50 samples of the top 3 cm of soil (each collected from an area of at least 200 cm^2) need to be taken from the parcel to the laboratory for seed germination. The soil is then monitored for seedlings formed during a period of not less than 2 months. If the seeds are easy to find one can count these directly, avoiding the need for germination tests in laboratory (see section 13.11).

10.5 Image analysis: a useful tool for the characterisation of accessions' colorimetric and morphometric parameters

The germplasm can be characterised by qualitative parameters such as shape, size and colour. These parameters are difficult to measure; in fact some can only be an estimate and not an exact and objective measure. The size is usually estimated manually which is a time consuming process. The colour is measured by visual comparison to colour standards in special photographic reference tables, which can be traced back to the corresponding RYB (Red, Yellow, Blue) and HBS (Hue, Brightness, Saturation) (Fagundez et Izco, 2003). This method is subjective and not repeatable, two operators can assign different colours to the same sample and the same operator can assign different colours at different times. Moreover, often persons are unable to estimate small differences between individual seeds within a sample. Even the shape is estimated visually, and it is not possible to obtain objective values. The limits set out above regarding the morpho-colorimetric study of the seeds, can be largely overcome with real and objective measures of both dimensions related to the shape and colour. Indeed, new technology-based image analysis methods are now available which are non-destructive and rapid (Venora et Grillo, 2006; Venora et al., 2006).

Here's an example of application of the technology analysis 'image for the morpho-colorimetric characterisation of the seeds and subsequent classification. The validity of this application has been found on different bean and lentil crops accessions (Venora et Grillo, op. Cit.) (Venora et al., op. cit.). The images of the seeds that need to be analysed are acquired through a flatbed scanner which is properly standardised (Fig. 50 and 51), either successively or together. The images are subsequently processed through a Macro (Fig. 52-58) using KS400 software system (Image Analysis - Zeiss), specially developed for this type of work with which it is possible to measure the following parameters:

- area;
- perimeter;
- maximum diameter;
- minimum diameter;

- form factor;
- roundness factor;
- relationship between diameters.

The form factor is calculated according to the following formula:

$$Form\ Factor = \frac{4 \cdot \pi \cdot Area}{Perimeter^2}$$

Values for this factor range between 0 and 1, where 1 indicates perfect homogeneity of form.

Roundness factor is the value given by the following formula:

$$Roundness\ Factor = \frac{4 \cdot Area}{\pi \cdot Max\ diameter^2}$$

Values for this factor range from 0 to 1, where 1 indicates equal diameters or perfect circularity.

The ratio of diameters (m/M, Ferret ratio) is the value of the aspect ratio, ie the ratio between the diameter smaller and more using the following formula:

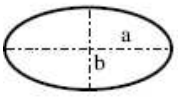
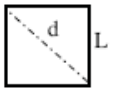
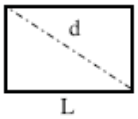
$$Ferret\ ratio = \frac{Minimum\ diameter}{Maximum\ diameter}$$

In this case the values vary from 0 to 1, where 1 indicates that the two diameters are the same.

Table 6 gives some examples of the values of these three factors on four different geometric shapes.

As for the colour parameters the R, Y, B and H, B, S (standard colour manuals) values show respectively the average value of the Red, Yellow, Blue channels and the Hue, Brightness and Saturation of the object under examination and are expressed in gray scale with values ranging from 0 (black) to 255 (white).

Table 6 - Parameters morphometry. (source: G. Venora)

	Form Factor	**Roundness factor**	**Ferret Ratio**
 r = x	1	1	1
 a = 6 b = 4	0.67	0.67	0.67
 L = 2 d = 2.828	0.785	0.64	0.71
 L = 5 l = 2 d = 5.385	0.16	0.44	0.37

If the seeds' integument is textured, bears ornaments or raised surface features it is possible to determine the average area of the textured (ornaments) surface, the number of textured spots per unit area and the percentage of the seed coat covered by these textures. The area of such texture is a parameter that indicates the total surface texture of each seed, expressed in mm^2. As for the colour parameters in the presence of such texture, one has to distinguish the colour of the background (colour % > 50) and the colour of the textures or other raised surfaces.

For each image, the raw data is entered as part of a calculation formula in an excel spreadsheet, and can either be considered as intrinsic characteristics of the seeds, or used in the creation of special statistical classifications (Venora et Grillo, *op. cit.*; Venora et al., *op. cit.*).

The following is the recommended sequence of images taken, which are then used to perform the colorimetric and morphometric measurements of the seed. As an example, images of *Astragalus verrucosus* whose seeds are without ornaments, has been used.

Figure 50 - Original image. This image can be acquired by a remote scanner and subsequently processed using an image analyser. (Photo: G. Venora)

Figure 51 - Standardised Image. This will enable you to obtain results for the overlapping color, using any type of scanner. (Photo: G. Venora)

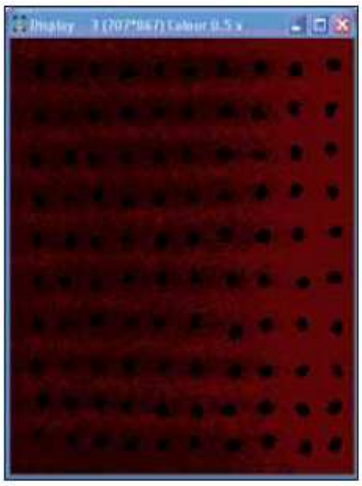


Figure 52 - Contrasted image. Serves to delineate clearly the contours of the objects that should be measured. (photo: G. Venora)

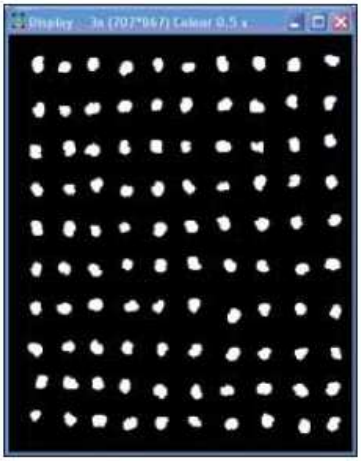


Figure 53 - Image segmented. And 'the result of the separation of the objects to be measured from the background and represents the image that will serve as a footprint to allow the system to distinguish which are the objects of interest. (photo: G. Venora).

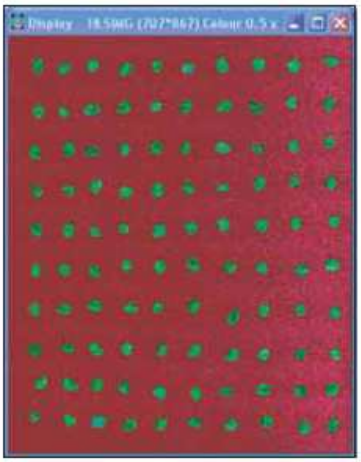


Figure 54 - HLS image. Created from the standard to convert the colors from the RGB (Red, Green, Blue) model to HLS (Hue, Lightness, Saturation) to obtain an image which can measure three other colorimetric parameters. (Photo: G . Venora)

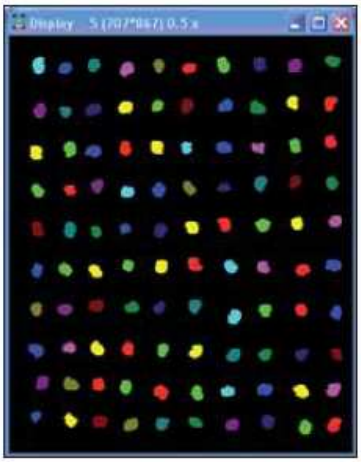


Figure 55 - Image with objects identified. This image has the simple purpose of visually ascertain that all items are to be analyzed separately and that two or more items are not covered by the system as a single seed (the subject). (photo: G. Venora)

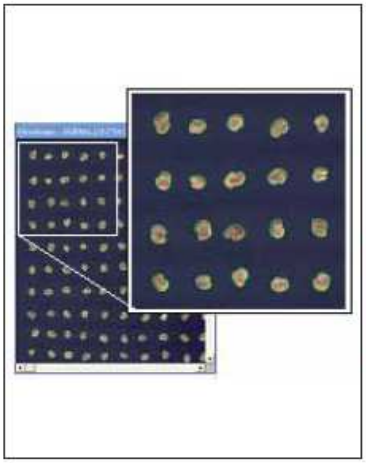


Figure 56 - Image for the measurement of morphometric parameters. Allows the interactive selection of objects which make the measures of shape and size. (Photo: G. Venora).

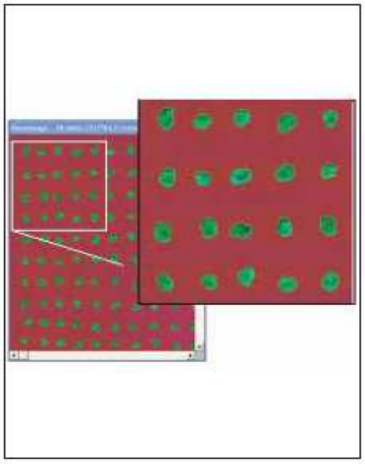


Figure 57 - Image size. Allows you to interactively select objects which perform the colorimetric measures. (photo: G. Venora).

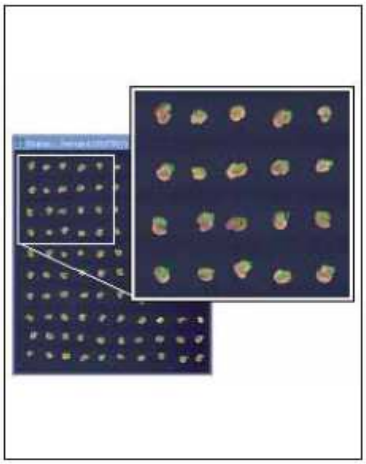


Figure 58 - Picture numbered. This is the image that is saved at the end of the analysis, imprinted with the numbers indicating the order that was followed by the system in the measurement of individual objects. This enables the correlation of other characteristics of the seed (germination - viability) with those measured by image analysis. (photo: G. Venora).

10.6 Fruit and seed dispersion: the influence of the carrier and seed form

On the basis of external structure of the seeds or fruits, Werker (1997) identified several strategies for active/passive dispersal as outlined below.

10.6.1 Anemochory - wind dispersal

Wind dispersion of fruit and seeds is characterised by light seeds, often equipped with appendages which increases the surface area. This dispersal mechanism is typical of many families, particularly the *Apiaceae, Asteraceae, Orchidaceae, Poaceae* and *Scrophulariaceae*. The appendices may be of various types:

- hair covering all or part of the surface of the seed (eg, *Platanus, Nerium oleander*, etc.);
- simple or plumose/feathery hairs forming a crown or a tuft of hair, called a pappus (eg *Taraxacum, Crepis, Sonchus, Hieracium*);
- plumose/feathery tail (eg, *Clematis, Anemone*, etc..)
- wings (eg *Bignonia, Tilia, Fraxinus, Acer*, etc.).

10.6.2 Hydrochory - water dispersal

Many seeds and fruits of aquatic species float on water and are dragged by the current (eg, *Posidonia oceanica, Nuphar*, etc..). Others, which belong to non-aquatic species (eg, *Anchusa formosa, Pancratium maritimum*), can be transported by rain water, rivers or tidal stream and may even be dispersed at considerable distances.

10.6.3 Autochory – self dispersal

Autochory is a type of dispersion in which the dispersal comes though mechanisms originating from the plant itself. The method of passive dispersion through which the fruits or seeds fall near the mother plant as a result of being dragged down by gravity is called barochory. The seeds so dispersed, may eventually be eaten by animals, which shall in this way, affect the dispersion though their faeces (diplochory). The geochory species do not disperse their seeds, but instead bury their fruit, thus ensuring their preservation in place (eg *Morisio monanthos* (Viv.) Ascherson, *Arachis hypogaea* L., *Trifolium subterraneum* L.). The maturation and drying of some fruits is accompanied by launching of their seeds some distance away from the mother plant (balistochory) (eg *Cytisus scoparius* (L.) Link, *Acanthus mollis* L., *Cardamine*, *Geranium*, etc..). In *Hura crepitans* L. the fruit explodes in 18 pieces with such violence that the seeds of 1 cm thick, are projected up to 25 m. In *Mercurialis annua* L. the seeds are also launched at an average distance of 15 cm (Smooth and Pacini, 1997). The dissemination range of seeds through this mechanism is much more limited than that afforded by dispersion through wind, water or animals.

10.6.4 Zoocoria – animal dispersal

Zoochory is the dispersal of fruit and seeds by animals where the animal plays an active role (mainly through endozoochory) or a passive role (epizoochory).

In endozoochory, the seeds are always contained within fleshy fruits or false fruits, eaten by (frugiverous or) fructivorous animals, (e.g. *Ficus carica* L., *Fragaria vesca* L., *Pyrus, Malus, Phoenix*, etc..), and expelled with the faeces. These seeds usually germinate readily, as their integument are softened and partly acted upon by the gastric juices as they move through the digestive system.
Epizoochory is achieved for all those dry seeds fitted with hooked bristles or macro- or microscopic size. The hooks allow attachment of the seeds to the hair or feathers of animals (eg, *Xanthium strumarium* L. *Agrostemma githago* L ., etc..). In addition, several animals simply carrying the seeds over a certain distance attached to their feet. The dispersion of seeds by ants deserves a separate treatment, since their method of dispersion does not involve either endozoochory or epizoochory. It is true that these hymenoptera carry a large number of seeds between their mandibles, particularly those fitted with oil-rich appendages on which they feed (eg, *Euphorbia*). This type of dispersion of diasporas is called myrmecochory.

10.6.5 Elaiosomes and the dispersion of seeds in Mediterranean plants

Dispersion

In the seeds of some dried fruits one notes the presence of an appendage that is not involved in germination, but whose main function is to attract animals involved in their dispersion more commonly ants, rarely birds (Smooth et al., 1996). This appendix has been called "elaiosome" by Sernarder (1906) because it contains lipids, but it is also called also caruncle, etc. However, regardless of the name and its anatomical origin, its function is primarily to facilitate the ecological dispersal of seeds by ants that are a rewarded by an edible elaiosome (Smooth *et al., Op. Cit.*). The elaiosomes are like caps located at one end of the seed, always bearing a colour which contrasts clearly with the rest of the seed which is usually dark brown or black. They are made of dead cells whose cytoplasm is transformed into lipid reserves. The elaiosomes are tender and easily removed, both by the ants and in experimental investigations. The elaiosomes reserve commonly consists of lipids, and only in some cases does it also contain proteins, such as *Cirsium arvense* (L.) Scop., *Cytisus scoparius*, *Euphorbia cyparissias* L., *Centaurea parlatoris* Heldr. or starch as in *Euphorbia lathyris* L., *Lamium purpureum* L. and *Tussilago farfara* L. In *Centaurea jacea* L the reward for the dispersers consists entirely of protein so that this is a case where these structures cannot be called elaiosomes (Smooth *et al., op. cit.*; Viegi et al., 2003). It is interesting to note that measurement of the energy content of the elaiosome is usually equivalent to a third of the seed's energy regardless of systematic group of the seed (Smooth *et al., op. cit.*).

Mediterranean plants whose dry fruits have seeds that bear elaiosomes include species of the following genera: *Euphorbia, Viola, Chelidonium, Corydalis, Melampyrum, Trillium, Vulpia* (Beattie and Lyons, 1975, Aaron and Wilcock, 1994; Lisci et al., op . cit.). Elaiosomes are also found in the achenes of *Asteraceae* such *Sylibum marianum* (L.) Gaertn., in some species of the genus *Centaurea* and seeds in fleshy fruits (eg *Rhamnus alaternus* L.). The elaiosomes may be a constant feature for a given systematic group, such as the Euphorbiaceae, or an occasional occurrence present in a genus with many species such as *Centaurea* (Viegi et al., op. cit.).

Function of the elaiosomes

Regardless of the dispersion, which is the primary function, the elaiosomes may also indirectly help to accomplish as assist in one or more of the following functions:

- help prevent seed predation by other animals because the ants collect the seed from the soil surface immediately they are dispersed from the parent plant;
- seeds deposited by ants inside their nest are protected from fire and high temperatures;
- help avoid intraspecific competition - seeds are removed away from proximity of parent plant that produced them thus reducing chance of competing for nutrients;
- the cell walls of elaiosomes may be thick and contain pectin which are molecules that can absorb and retain water and therefore essential during the initiation process of germination (Bianchini and Pacini, 1996)
- thanks to these thick-walled structures the elaiosomes may be involved in both the dehydration of the seed before dispersion, and in rehydration before germination (Smooth et al., op. cit.; Bianchini and Pacini, *op. cit.*).
- may help regulate the dormancy of the seed, as in *Mercurialis annua*, *Euphorbia cyparissias* and *Calendula arvensis* L. the seed only germinates when the elaiosome is naturally or experimentally removed (Pacini, 1990; Lisci et al., op. cit.).

Elaisomes and diplochory

Seeds with elaiosomes are often diplochorous, which have a dispersion process that occurs in two stages. Aronne and Wilcock (1994) have shown that in the first place *Rhamnus alaternus* fleshy fruits are dispersed by birds and then, after defecation, thanks to elaiosomes that are not damaged during the journey within the digestive system, they are further dispersed by ants.

Pacini (1990) and Smooth et Pacini (1997) show that in *Mercurialis annua* seeds were first launched by a catapult mechanism located in the walls of the fruit, even up to distances of 130 cm and then they are collected by ants that carry them away more commonly to a radius of six meters, rarely more (Smooth et Pacini, *op. cit.).*

Viegi et al. (2003) have however shown that achenes in the genus *Centaurea* may or not have the elaiosomes and pappus depending on the species. When pappus and elaiosomes are present, the length of the pappus hair varies depending on the species, allowing for dispersion to different distances. In any case, the dispersion by the wind always precedes that by ants. In species where only the elaiosome or pappus is present, it follows that there is only one type of dispersion mechanism. The same authors have noted that the presence-absence of elaiosomes does not follow the systematic classification of the genus, confirming that these structures have a more ecological significance for the species rather than systematic and evolutionary significance. The distances at which the ants can disperse the seeds, depends on the ant species; there are different types of social hymenoptera which differ widely in the levels of their sociality. There are ants belonging to species with low levels of social organisation which eat elaiosomes immediately they find the seed, thus dispersing the seed only a little distance away from the place where it was harvested. There are other ants, with a higher level of social organisation, which carry the seeds to their nest, and in this case the elaiosome is removed and the seed thrown out of the nest with the pile of waste matter. Finally there is the matter of *Messori structor*, a Mediterranean ant species that lives in man-made environments and that once it carries the seeds into the nest, removes the elaiosomes, deposits the seeds in separate compartments according to the plant species. It has been suggested that this mechanism of "systematic conservation" serves to eventually consume the interior of the seed which is full of reserves, once the hard integument has softened by the action of bacteria and fungi (Pacini, 1990). Most seeds, however, manage to escape this "abduction" because of the agricultural practices of ploughing and hoeing. In conclusion we can say that the seeds with elaiosomes are classified as orthodox (Baskin and Baskin, 1998), that are dispersed with low water content and with a slowed-down metabolism. This is because the elaiosomes ensure dispersion, but since this may take a variable time which may even be quite long, the presence of elaiosomes is not compatible with recalcitrant seeds that are difficult to survive a long time after dispersal.

10.7 Soil seed bank

Ecologists and biologists now agree on the important role that soil seed banks in the maintenance of ecological and genetic biodiversity (at the species level) in plant populations and communities (Gross, 1990). The study of the soil seed bank, in which one can show the ability of plants to create depositories of buried viable seeds, helps to better define the autoecology of the species, to estimate the resilience capacity of the community, to deepen further the study of the vegetation dynamics and to obtain information to support the planning of management activities (Cerabolini et al., 2003). The formation of a soil seed bank is a strategy common in plants that grow in areas subject to both natural and anthropogenic disturbance. These soil seed banks generally contain more seeds of annuals than perennials plants, more seeds of broad-leaved

herbaceous (annual and perennial) plants than grasses, many seeds of legumes, and many seeds of weedy species specialised for rapid colonisation of disturbed sites (Miller, 2000). Based on the period of persistence in the seed bank, two seed bank groups are identified:

- transitional banks: the seeds remain viable in the soil for about a year;
- persistent banks (or long duration): the seeds remain viable for a long time.

The latter group are those that contribute most to the regeneration of plant biocenosis that have been destroyed or degraded (Thompson, 1993). Many Australian and South African species adapted to survive periodic fire, as well as some Mediterranean conifers, form seed banks in woody fruits that open after fire (serotinous fruit). The types of plants that form seed banks in areas normally subject to fires in northern U.S. are set in the following scheme (Table 7):

Table 7 – Soil seed bank typologies in North American terrains subject to periodic fires (Miller, 2000)

Species Group	Transitory Banks	Persistent or transitional banks		
		In the soil	In the canopy (fruits)	Germination stimulated by fire
Conifer (trees)	X		X	
Evergreen broadleaves (trees)	X			
Deciduous (trees)	X			
Shrubs	X	X		X
Herbaceous with medium to large leaves (annulas)	X	X		X
Herbaceous with medium to large leaves (perennials)	X	X		X
Grasses	X			

The procedure for the study of the soil seed bank consists in first taking samples of soil, storing these in plastic bags, taking care to record the relevant information for the later identification of the seeds present. At the germplasm bank, one proceeds to identify carefully the seeds contained in the sample. The study can be conducted by manually picking or by aspirating with the aid of mechanical suction equipment the seeds from the ground, identifying and counting these, or by placing the soil samples in controlled environments to promote the germination of the seeds it contains. The method of germination requires that the sample, previously placed in a controlled environment, to be distributed (thickness 1 cm) in trays of glass after removing any dead remains of plants (eg branches, leaves, etc..), any small organisms and small stones present (Roberts, 1981). The sample is then watered and periodically checked for any seedlings that germinate gradually.

Alternatively, the samples can be placed in the germination chamber; this option may not allow the germination of dormant seeds that require specific pre-treatment to germinate. This solution is therefore usable only if the protocol for germination of the taxonomic units being investigated is well known.

The difficulty of the application of certain methodologies has led to development of a fast and definitive method, which allows one to predict the presence/persistence of seeds under investigation in the soil. This is done by using information collected via a morphometric investigation of the seed, in this case on the form and size (Thompson et

al., 1993). The method is based on the observation that persistent seeds are generally small and round, while the non-persistent seeds are large, flattened or elongated. The size and shape are in fact the parameters that influence the seed's ability to bury itself in the ground. It has been shown that where seeds are buried, the predation rates are lower than those who that remain on the soil surface (Hulme, 1994) and that predation is considered the main factor that determines the residence time of the seed in the soil (Hulme, 1998). Researchers have only recently begun to consider the study of the soil seed bank in the demographic analysis of plant populations, and this is probably because the data emerging from the soil seed bank (viable seeds and germination rate) are often are difficult to compare with data on seedlings and adult plants.

The characteristics of soil seed banks are crucial for the structure, dynamics and spatio-temporal distribution of plant biocenosis in general and the Mediterranean in particular (Parker et al., 1989, Ortega et al., 1997, Peco *et al*., 1998). Variations in the dynamics of banks is reflected in the composition, distribution and dominance of species (Parker and Kelly, 1989). However, a direct correlation between the vegetation and soil seed bank is not always demonstrated (Thompson, 1986; Leck, 1989; Rice, 1989) because some species reproduce both by seed and by asexual means.

11. Glossary

12. USEFUL ADDRESSES

12.1 Network for the Italian Germplasm Banks for *Ex Situ* conservation of Italian wild flora (RIBES)

Banca del Germoplasma CODRAMediterranea S. r. l.
CODRA Mediterranea S. r. l.
Centro operativo per la difesa e il recupero ambientale
C. da Sciffra
85010 Pignola (PZ)

Banca del germoplasma del CNR di Bari
Istituto di Genetica Vegetale IGV,
Consiglio Nazionale delle Ricerche
Via G. Amendola, 165/ A
70126 Bari (BA)

Banca del germoplasma del Molise
Dipartimento di Scienze e Tecnologie dell'Ambiente e del Territorio
Università degli Studi del Molise
Via Mazzini, 8
86170 Isernia

Banca del germoplasma dell'Orto Botanico di Catania
Dipartimento di Botanica
Università degli Studi di Catania
Via A. Longo, 19
95125 Catania (CT)

Banca del germoplasma dell'Orto Botanico di Padova
Centro di Ateneo Orto Botanico
Università degli Studi di Padova
Via Orto Botanico, 15
35123 Padova (PD)

Banca del germoplasma dell'Orto Botanico di Palermo
Dipartimento di Scienze Botaniche
Università degli Studi di Palermo
Via Archirafi, 38
90123 Palermo (PA)

Banca del germoplasma dell'Orto Botanico di Pisa
Orto Botanico, Dipartimento di Scienze Botaniche,
Università degli Studi di Pisa
Via Luca Ghini, 5
56126 Pisa (PI)

Banca del germoplasma dell'Orto Botanico di Roma
Orto Botanico, Dipartimento di Biologia Vegetale
Università degli Studi di Roma "La Sapienza"
Via Cristina di Svezia, 24
00165 Roma

Banca del germoplasma dell'Orto Botanico di Viterbo
Centro Interdipartimentale dell'Orto Botanico
Università degli Studi della Tuscia
Strada Santa Caterina s. n. c.,
01100 Viterbo (VT)

Banca del germoplasma della Majella
Parco Nazionale della Majella,
67030 Campo di Giove (AQ)

Banca del Germoplasma della Sardegna (BG-SAR)
Centro Conservazione Biodiversità (CCB)
Dipartimento di Scienze Botaniche
Università degli Studi di Cagliari
Viale Sant'Ignazio da Laconi, 13
09123 Cagliari (CA)

Banca del Germoplasma dell'Appennino Centrale
Centro Ricerche Floristiche dell'Appennino (C. R. F. A.)
c/o Parco Nazionale del Gran Sasso e Monti della Laga
S. Colombo - via prov. le km 4,2
67021 Barisciano (AQ)

Banca del germoplasma delle Alpi Sud-Occidentali
CBV – Centro per la Biodiversità Vegetale
Ente Gestione Parchi e Riserve Naturali Cuneesi
Via Santa Anna, 34
12013 Chiusa Pesio (CN)

Banca del germoplasma per la conservazione delle specie anfiadriatiche
Centro Orto Botanico Interdipartimentale di Servizi
Università Politecnica delle Marche
Via Brecce Bianche s. n.
60131 Ancona (AN)

Banca di germoplasma del Mediterraneo ®, ONLUS
Via Pietro Floridia, 2
90129 Palermo (PA)

Banche del Germoplasma Livornesi
Museo di Storia Naturale del Mediterraneo e Conservatorio
delle specie vegetali di Rosignano
c/o Provincia di Livorno,
P. zza del Municipio 4
57128 Livorno (LI)

Laboratorio per la conservazione della diversità vegetale ligure
Centro universitario di Servizi Giardini Botanici Hanbury
Università degli Studi di Genova
Corso Montecarlo, 43, La Mortola
18039 Ventimiglia (IM)

Lombardy Seed Bank (LSB)
c/o Dipartimento di Ecologia del Territorio e degli Ambienti Terrestri
Università degli Studi di Pavia
Via S. Epifanio, 14
27100 Pavia (PV)

Trentino Seed Bank (TSB)
Museo tridentino di scienze naturali
Via Calepina, 14
38100 Trento (TN)

12.2 Fédération Conservatoires Botaniques Nationaux Français (FCBN)

Fédération Conservatoires Botaniques Nationaux Français
Keramenez
29470 Plougastel-Daoulas (France)

Conservatoire Botanique National Alpin
Domaine de Charance
05000 Gap (France)

Conservatoire Botanique National de Bailleul
Hameau de Haendries
59270 Bailleul (France)

Conservatoire Botanique National du Bassin Parisien
61, rue Buffon
75005 Paris (France)

Conservatoire Botanique National de Brest
52, allée du Bot
29200 Brest (France)

Conservatoire Botanique National de Mascarin
RD 12, Domaine des Colimaçons
97436 Saint-Leu, Ile de la Réunion (France)

Conservatoire Botanique National du Massif Central
Le Bourg
43230 Chavaniac – Lafayette (France)

Conservatoire Botanique National Méditerranéen de Porquerolles
Parc National de Port Cros
Castel Sainte Claire
83418 Hyères cedex (France)

Conservatoire Botanique National de Midi-Pyrénées
Vallon de Salut, BP 315
65203 Bagnères-de-Bigorre cedex (France)

Conservatoire National des plantes médicinales, aromatiques et industrielles
Route de Nemours
91490 Milly-la-Forêt (France)

Conservatoire Botanique de Franche-Comté
Porte Rivotte
25000 BESANCON (France)

Mission Conservatoire Botanique Aquitaine, Poitou/Charentes
Direction de l'Environnement, Conseil Général de la Gironde, Esplanade Charles De Gaulle
33074 Bordeaux cedex (France)

Mission Conservatoire Botanique des Antilles Françaises
Antenne de la Martinique, Parc Floral, BP 4033
97254 Fort-de-France (Antilles Françaises) Antenne de la Guadeloupe

Museum National d'Histoire Naturelle
57, rue Cuvier
75005 Paris (France)

12.3 Red Española de Bancos de Germoplasma de Plantas Silvestres (REDBAG)

Banco de germoplasma del Jardín Botánico de Córdoba
Avda. de Linneo s/n
14004 Córdoba (Spain)

Banc de germoplasma del Jardí Botànic de la Universitat de València

C/ Quart, 80 46008 Valencia (Spain)
Banco de germoplasma del Jardín Botánico Canario Viera y Clavijo
Apartado de Correos 14 de Tafira Alta
35017 Las Palmas de Gran Canaria (Spain)

Banco de germoplasma del Real Jardín Botánico de Madrid
Plaza de Murillo, 2,
28014 Madrid (Spain)

Banc de llavors del Jardí Botánic Marimurtra
Passeig Carles Faust, 9
Apartat Correus 112
17300 Blanes – Girona (Spain)

Banc de llavors del Jardí Botànic de Sóller
Crta. Palma - Sóller, km. 30,5
Apartado de correos 44.
07100 Sóller (Spain)

Banco de semillas del Real Jardín Botánico Juan Carlos I
Universidad de Alcalá de Henares – Comunidad de Madrid

Ciudad Residencial Universitaria, Bloque A3-p.7. Campus de la Universidad de Alcalá.
28805 Alcalá de Henares (Spain)

12.4 Germplasm Banks and International Centres for Plant Conservation

Seed Conservation Department
Royal Botanic Gardens
Kew, Wakehurst place
RH176TN Ardingly, Haywards Heath (England, UK)

Seed Room, Kirstenbosch NBG
Private Bag X7 CLAREMONT,
Cape Town 7735 (South Africa)

Centro Agronómico Tropical de Investigación y Enseñanza CATIE
Banco de semillas forestales Turrialba, Costa Rica

Banc de germoplasma del Jardí Botànic de Barcelona
C/ Dr. Font i Quer, 2. Parc de Montjuic.
08038 Barcelona (Spain)

Banc de llavors forestals – Generalitat Valenciana
Avda. Comarques del Pais Valencià, 114

46930 Quart de Poblet (Spain)
Centro Nacional de Mejora Forestal El Serranillo
Dirección General para la Biodiversidad - Ministerio de Medio Ambiente
Ctra. de Fontanar, Km.2 Apdo. de Correos, 249
19080 Guadalajara (Spain)

National Center for Genetic Resources Preservation
Department of Agriculture, Agriculture Research Services.
1111 South Mason
Fort Collins, Colorado 80521-4500 (USA)

Institut des Régions Arides
Laboratoire d'Ecologie Pastorale
4119 Médenine (Tunisie)

Instituto Universitario de Investigación CIBIO
(Centro Iberoamericano de la Biodiversidad)
Universidad de Alicante
30008 Alicante (Spain)

Mediterranean Agronomic Institute of Chania (MAICh)
Adresse: B.P. 85
73100 Chania – Crete (Greece)

12.5 Websites

http://www.apat.it
APAT - Agenzia per la protezione dell'ambiente e per i servizi tecnici

http://www.ars.usda.gov/is/AR/
Agricultural Research Service

http://www.aosaseed.com
Association of Seed Analysts (AOSA)

http://www.agraria.org/coltivazioniforestali.htm
Atlante delle piante forestali

http://www.anbg.gov.au/anpc/
Australian Network for Plant Conservation

http://www.tufts.edu/~cchester/biodiversity.html
Biodiversity links

http://digilander.libero.it/alberiitaliani/boschi.htm

Boschi Italiani
http://www.bgci.org.uk/
Botanic Gardens Conservation International (BGCI)

http://www.rbg.ca/cbcn/
Canadian Botanical Conservation Network

http://www.centerforplantconservation.org/
Center for Plant Conservation

http://www.ccb-sardegna.it
Centro Conservazione Biodiversità – Università di Cagliari

http://www.lib.berkeley.edu/EART/vegmaps.html
Checklist of online vegetation and plant distribution maps

http://www.compagniadelleforeste.it/
Compagnia delle Foreste

http://www.portcrosparcnational.fr/conservatoire/
Conservatoire Botanique National Méditerranéen de Porquerolles

http://www.cbn-alpin.org
Conservatoire Botanique National Alpin de Gap-Charance

http://www.cbnbrest.fr/
Conservatoire Botanique National de Brest

http://inpn.mnhn.fr/cbnbp_new/
Conservatoire Botanique National Bassin Parisien

http://www.cbnbl.org/
Conservatoire Botanique National de Bailleul

http://www.corpoforestale.it/wai/serviziattivita/CITES/index.html
Convenzione di Washington C.I.T.E.S.

http://www.corpoforestale.it/wai/index.html
Corpo Forestale dello Stato

http://www.dfsc.dk/
Danida Forest Seed Centre

http://botit.botany.wisc.edu/images/veg/
Distribuzione di piante in base all'habitat

http://www.bioplatform.info/
European Platform for Biodiversity

http://www.ecnc.nl/doc/ecnc/saxifrag/euroflor.html
European Centre for Nature Conservation

http://www.fs.fed.us/database/feis/
Fire Effect Information System

http://www.fao.org
Food and agriculture organization of the United Nations

http://forests.org/links/
Forest Conservation Links

http://www.forestscience.info
Forest Science Database

http://germoplasma.arsia.toscana.it/Germo/home.htm
Il germoplasma della Toscana

http://www.cibio.org
Instituto Universitario de Investigación CIBIO (Centro Iberoamericano de la Biodiversidad). Universidad de Alicante (España)

http://www.itis.usda.gov
Integrated Taxonomic Information System

http://www.ippc.int
International Phytosanitary Portal (IPP): the official web site for the International Plant Protection Convention (IPPC)

http://www.ipgri.org
International Plant Genetic Resources Institute

http://www.ipni.org/index.html
International Plant Names Index

http://seedtest.org/en/home.html
International Seed Testing Association (ISTA)

http://www.usd.edu/isss/
International Society for Seed Science

http://www.iucn.org/

International Union for the Conservation of Nature (IUCN)
http://www.botany.net/IDB/botany.html
Internet Directory for Botany

http://www.jardibotanic.pcn.es
Jardí Botànic de Barcelona – Spain

http://www.step.es/jardcan/
Jardín Botánico Canario Viera y Clavijo – Spain

http://www.jardinbotanicodecordoba.com/
Jardín Botánico de Córdoba – Spain

http://www.jardibotanicdesoller.org
Jardí Botànic de Sóller – Spain

http://www.jardibotanic.org
Jardí Botànic de la Universitat de València – Spain

http:/www.jbotanicmarimurtra.org
Jardí Botánic Marimurtra – Spain

http://www.forgen.net/main/bds.asp
Libro nazionale dei boschi da seme

http://www.ecologie.gouv.fr
Ministère de l'Ecologie et du Développement Durable

http://www.mnhn.fr
Museum national d'Histoire naturelle

http://www.nsl.fs.fed.us
National Seed Laboratory (USA)

http://www.nativeseeds.org/
Native Seed/SEARCH – USA

http://www.atl.cfs.nrcan.gc.ca/seedcentre/seed-center-e.htm
National Tree Seed Centre (Canada)

http://www.nativeplantnetwork.org/network/general.asp
Native Plants Propagation Protocol Database

http://www.nature.nps.gov/biology/endangeredspecies/
Natural Parks Service, Endangered Species

http://www.cof.orst.edu/coops/ntc/ntc.htm
Nursery Technology Cooperative

http://plants.usda.gov/
Plants database, USDA Natural Resources Conservation Service

http://www.fs.fed.us/database/feis/plants/
Plant Species Life Form

http://www.ricercaforestale.it/riselvitalia/BIODIVERSITA/Riselvitalia1.1/sottoprogetto_1.1.htm
Progetto Riselvitalia, produzione materiale forestale di propagazione

http://www.rjbalcala.com
Real Jardín Botánico Juan Carlos I – Spain

http://www.rjb.csic.es
Real Jardín Botánico de Madrid – Spain

http://www.rngr.net/
Reforestation, Nursery and Genetic Resources

http://www.reteortibotanicilombardia.it/
Rete degli Orti Botanici della Lombardia

http://www.rbgkew.org.uk
Royal Botanic Gardens, Kew

http://www.rbgkew.org.uk/data/sid
Royal Botanic Gardens, Seed information Database

http://www.sanbi.org/products/seeds.htm
Seed Room, Kirstenbosch National Botanical Garden

http://www.genmedoc.org/
Sito ufficiale del Progetto Interreg III B "Genmedoc": Création d'un réseau d'un centre de conservation du matériel génétique de la flore des régions méditerranéennes de l'espace MEDOCC

http://www.granicoltura.it
Stazione Sperimentale di Granicoltura per la Sicilia

http://www.ensconet.com
The "European Native Seed COnservation NETwork"

http://www.nerium.net/plantaeuropa/main.htm
The Global Strategy for Plant Conservation in Europe
http://www.rngr.net/Publications/ttsm
Tropical Tree Seed Manual

http://www.seedcentre.nl/
Wageningen Seed Centre

http://www.calflora.org/index0.html
Wild Plants in California

http://www.nsl.fs.fed.us/wpsm/
Woody Plant Seed Manual

http://stort.unep-wcmc.org/imaps/gb2002/book/viewer.htm
World Atlas of Biodiversity

http://www.populus.it/xilo.php
XILOGLOS - Glossario multilingue dei termini utilizzati in tecnologia del legno

http://redlist.org
2003 IUCN Red List of Threatened Species

12.6 Specialised Scientific Journals

Crop Science
The Crop Science Society of America, Inc. Madison, WI. USA
http://crop.scijournals.org/

Journal of New Seeds
The Haworth Press, Inc. Binghamton, NY. USA
http://www.haworthpress.com/

Native Plants Journal
USDA Forest Service, SRS
1221 South Main Street
Moscow, Idaho 83843–4211, USA
http://www.nativeplantnetwork.org/journal/

Plant Physiology
http://www.plantphysiol.org

Seed Abstracts
CAB International, Wallingford, UK
http://www.cabi-publishing.org/

Seed Info
Official Newsletter of the WANASeed Network, ICARDA, Aleppo, Syria
http://www.icarda.cgiar.org

Seed Science and Technology
Proceedings of the International Seed Testing Association (ISTA)
http://www.seedtest.org

Seed Science Research
CAB International, Wallingford, UK
http://www.cabi-publishing.org/
http: //hort.cabweb.org/

Seed Technology
Association of Seed Analysts, INC (AOSA). Las Cruces, NM. USA
http://www.aosaseed.com/

Seed Testing International
ISTANews Bullettin
http://www.seedtest.org

Semillero América Latina
Bollettino elettronico mensile pubblicato da New Forests Project, Washington DC 20003 USA
http://www.newforestsproject.com

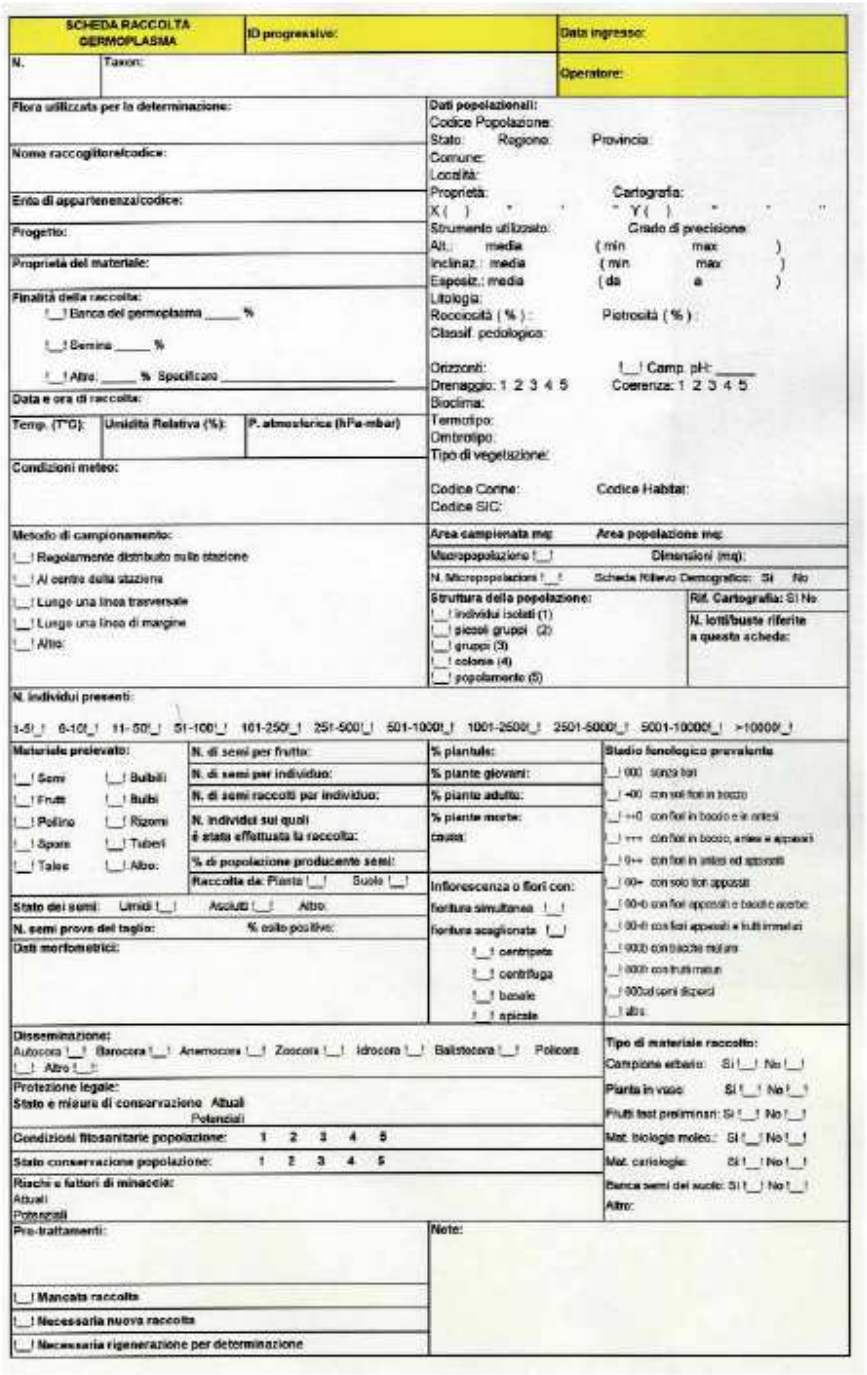
Cryobiology
Society for Cryobiology
http://www.elsevier.com/wps/find/journaldescription.cws_home/622814/description#description

Cryoletters
The Royal Veterinary College, Royal College Street, London NW1 0TU, UK
http://www.cryoletters.org/

13. RECORD CARDS AND DOCUMENTATION/DATA SHEETS

For the compilation of record cards and data sheets referred to in this manual the following reference texts have been consulted: Albert *et al.*, 2003; Badescu, 1997; Brullo *et al*., 1996; Braun-Blanquet, 1951; CORINE, 1993; Flynn *et al*., 2004; Gardin *et al.*, 2002; Hong *et al*., 1998; IPGRI, 1982 e 1985a; ISTA, 2006; Marion *et* George, 2001; Martin, 1946; Martin *et* Barkley, 1961; Mossa *et al*., 2004; Pinna, 1977; Pignatti, 1982; Pignatti, 1995; Raunkiaer, 1934; Rivas-Martínez *et al*., 2002; Royal Botanic Gardens Kew; Soil Survey Staff - USDA, 1998; Thomsen *et* Kiklev, 2000; Ubaldi, 2003; Zangheri, 1942.

13.1 Germplasm collection record card



SCHEDA RACCOLTA GERMOPLASMA | ID progressivo: | Data ingresso:

N. | Taxon: | Operatore:

Flora utilizzata per la determinazione:

Nome raccoglitore/codice:

Ente di appartenenza/codice:

Progetto:

Proprietà del materiale:

Finalità della raccolta:
!__! Banca del germoplasma _____ %
!__! Semina _____ %
!__! Altro: _____ % Specificare _____

Data e ora di raccolta:

Temp. (T°G): | Umidità Relativa (%): | P. atmosferica (hPa-mbar)

Condizioni meteo:

Dati popolazionali:
Codice Popolazione:
Stato: Regione: Provincia:
Comune:
Località:
Proprietà: Cartografia:
X( ) ' ' " Y( ) ' ' "
Strumento utilizzato: Grado di precisione
Alt.: media (min max)
Inclinaz.: media (min max)
Esposiz.: media (da a)
Litologia:
Rocciosità (%): Pietrosità (%):
Classif. pedologica:

Orizzonti: !__! Comp. pH: _____
Drenaggio: 1 2 3 4 5 Coerenza: 1 2 3 4 5
Bioclima:
Termotipo:
Ombrotipo:
Tipo di vegetazione:

Codice Corine: Codice Habitat:
Codice SIC:

Metodo di campionamento:
!__! Regolarmente distribuito sulla stazione
!__! Al centro della stazione
!__! Lungo una linea trasversale
!__! Lungo una linea di margine
!__! Altro:

Area campionata mq: Area popolazione mq:
Macropopolazione !__! Dimensioni (mq):
N. Micropopolazioni !__! Scheda Rilievo Demografico: Si No
Struttura della popolazione:
!__! individui isolati (1)
!__! piccoli gruppi (2)
!__! gruppi (3)
!__! colonie (4)
!__! popolamento (5)
Rif. Cartografia: Si No
N. lotti/buste riferite a questa scheda:

N. individui presenti:
1-5!__! 6-10!__! 11- 50!__! 51-100!__! 101-250!__! 251-500!__! 501-1000!__! 1001-2500!__! 2501-5000!__! 5001-10000!__! >10000!__!

Materiale prelevato:
!__! Semi !__! Bulbili
!__! Frutti !__! Bulbi
!__! Polline !__! Rizomi
!__! Spore !__! Tuberi
!__! Talee !__! Altro:

N. di semi per frutto:
N. di semi per individuo:
N. di semi raccolti per individuo:
N. individui sui quali è stata effettuata la raccolta:
% di popolazione producente semi:
Raccolta da: Pianta !__! Suolo !__!

% piantule:
% piante giovani:
% piante adulte:
% piante morte:
causa:

Stadio fenologico prevalente
!__! 000 senza fiori
!__! +00 con soli fiori in bocciolo
!__! ++0 con fiori in bocciolo e in antesi
!__! +++ con fiori in bocciolo, antesi e appassiti
!__! 0++ con fiori in antesi ed appassiti
!__! 00+ con solo fiori appassiti
!__! 00+0 con fiori appassiti e bacche acerbe
!__! 00+0 con fiori appassiti e frutti immaturi
!__! 0000 con bacche mature
!__! 0000 con frutti maturi
!__! 0000 semi dispersi
!__! altro

Stato dei semi: Umidi !__! Asciutti !__! Altro:
N. semi prove del taglio: % esito positivo:
Dati morfometrici:

Infiorescenza o fiori con:
fioritura simultanea !__!
fioritura scalarmente !__!
!__! centripeta
!__! centrifuga
!__! basale
!__! apicale

Disseminazione:
Autocora !__! Barocora !__! Anemocora !__! Zoocora !__! Idrocora !__! Balistocora !__! Policora !__! Altro !__!:

Tipo di materiale raccolto:
Campione erbario: Si !__! No !__!
Pianta in vaso: Si !__! No !__!
Frutti test preliminari: Si !__! No !__!
Mat. biologia molec.: Si !__! No !__!
Mat. cariologia: Si !__! No !__!
Banca semi del suolo: Si !__! No !__!
Altro:

Protezione legale:
Stato e misure di conservazione Attuali
Potenziali

Condizioni fitosanitarie popolazione: 1 2 3 4 5

Stato conservazione popolazione: 1 2 3 4 5

Rischi e fattori di minaccia:
Attuali
Potenziali

Pre-trattamenti:

Note:

!__! Mancata raccolta
!__! Necessaria nuova raccolta
!__! Necessaria rigenerazione per determinazione

13.2 Phenological data card

SCHEDA RILEVAMENTO FENOLOGICO | ID progressivo: | Data ingresso:

N. | Taxon: | Operatore:

Rilevatore: | Data:

N. giorno calendario fenologico

Dati popolazionali ed ecologici:

Codice Popolazione:

Stato: Regione: Provincia: Comune: Località:

Foglio IGM:

N ° ' " E ° ' "

Strumento utilizzato: Precisione del dato:

Alt.: media (min max)

Inclinaz.: media (min max) Esposiz.: media (da a)

Litologia:

Rocciosità (%): Pietrosità (%):

Classif. pedologica:

Orizzonti: |__| Camp. pH: _____ Drenaggio: 1 2 3 4 5 Coerenza: 1 2 3 4 5

Bioclima:

Termotipo: Ombrotipo:

Tipo di vegetazione:

Codice Corine: Codice Habitat:

Tipo corologico:

DATI BIOLOGICI

Forma _____ Sottoforma _____

|__| Monocarpica
|__| Pluricarpica

|__| Caducifoglia
|__| Semicaducifoglia
|__| Sempreverde

Stati del ciclo vegetativo
|__| (NC)
|__| (Ut)

Durata

FASE VEGETATIVA

Gemme: |__| Assenti |__| Presenti: |__| Abbozzate |__| Poco sviluppate |__| Completamente formate

Colore _____ Forma _____

Larghezza _____ Lunghezza _____

Struttura/Disegno _____

Germogli: |__| Assenti |__| Presenti: |__| scarsi |__| poco numerosi |__| numerosi

Altezza media _____
Altezza min _____
Altezza max _____

Foglie: |__| Assenti |__| Presenti: |__| immature |__| mature |__| senescenti

FIORITURA

% individui fioriti _____

|__| Assente
|__| Presente: |__| simultanee |__| scalarizzata |__| centripeta |__| centrifuga |__| basale |__| apicale

|__| ___ % +00 solo fiori in bocciolo
|__| ___ % ++0 fiori in bocciolo e in antesi
|__| ___ % +++ fiori in bocciolo, in antesi ed appassiti
|__| ___ % 0++ fiori in antesi ed appassiti
|__| ___ % 00+ solo fiori appassiti

Scapi fiorali: |__| Assenti |__| Presenti: |__| eretti |__| ferali

FRUTTIFICAZIONE

|__| fiori e frutti contemporanei

|__| Assente
|__| Presente: |__| frutti immaturi ___ % |__| frutti maturi ___ % |__| frutti deiscenti ___ %

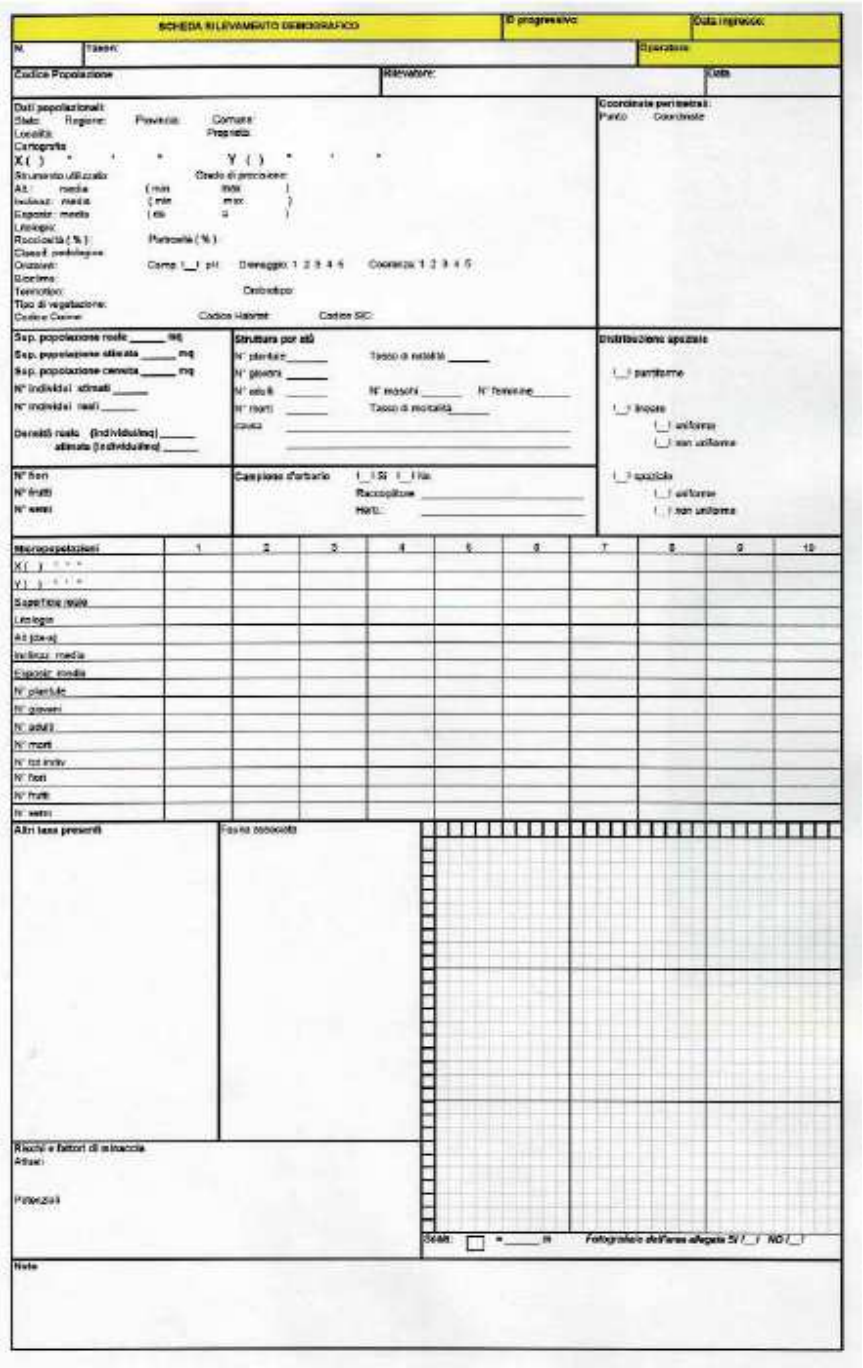
|__| semi assenti
|__| semi presenti: |__| vuoti |__| pieni

Disseminazione: |__| Autocora |__| Barocora |__| Anemocora |__| Zoocora |__| Idrocora |__| Balistocora |__| Policora |__| Altro: _____

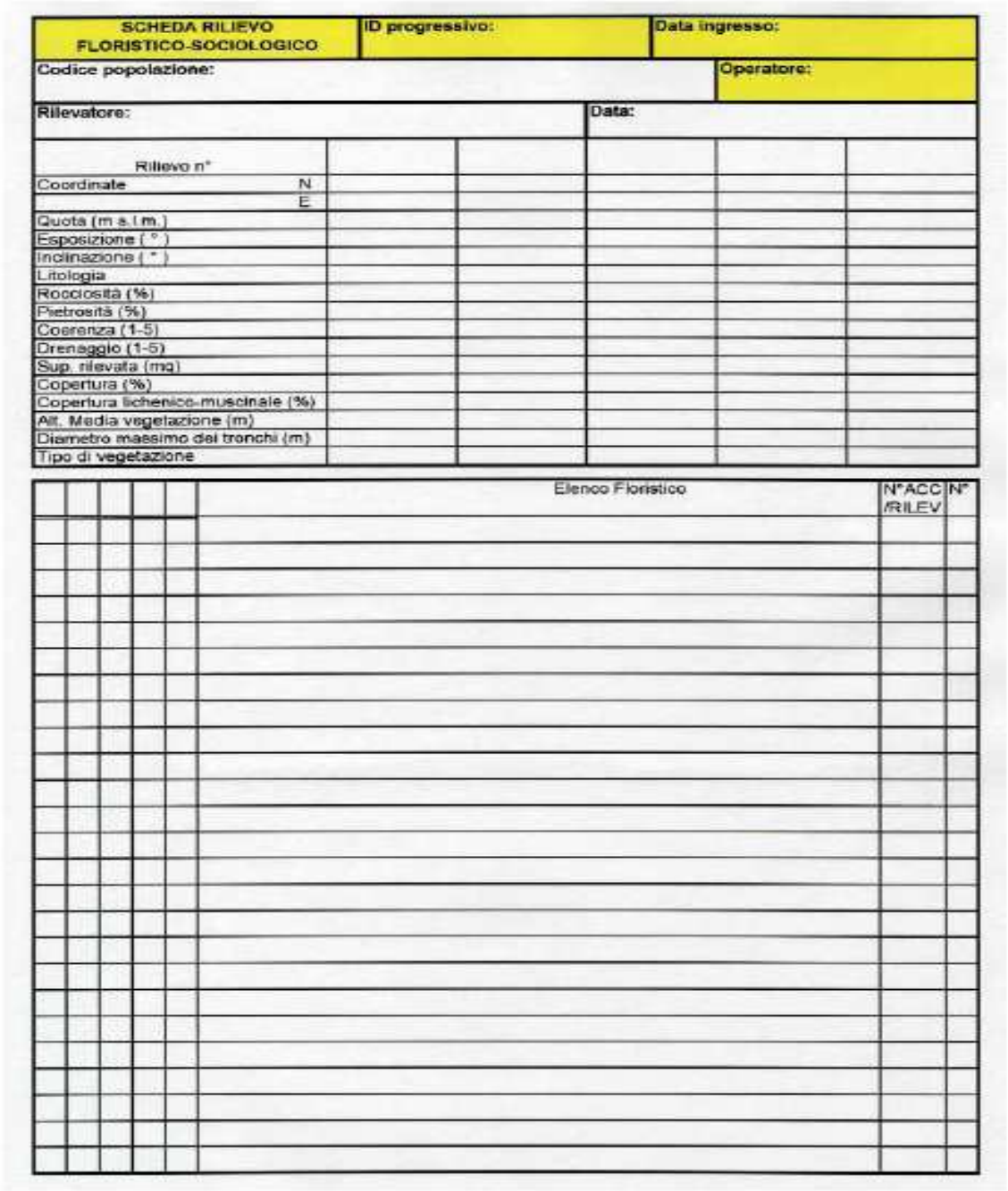
Fattori biotici e abiotici di disturbo/minaccia:

NOTE

13.3 Demographic data card



13.4 Phyto-sociological data card



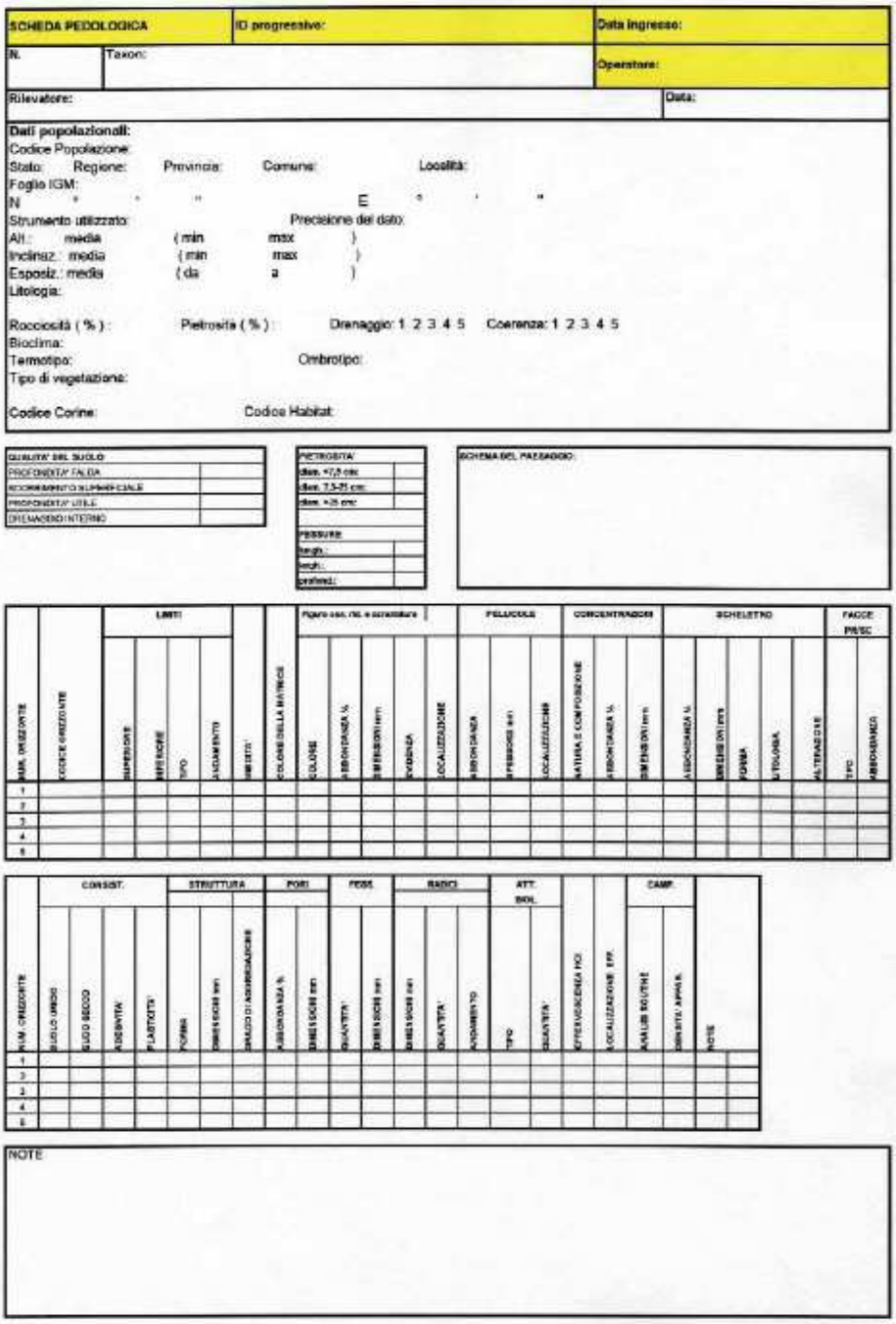
SCHEDA RILIEVO FLORISTICO-SOCIOLOGICO	ID progressivo:	Data ingresso:
Codice popolazione:		Operatore:
Rilevatore:		Data:

Rilievo n°					
Coordinate	N				
	E				
Quota (m s.l.m.)					
Esposizione (°)					
Inclinazione (°)					
Litologia					
Rocciosità (%)					
Pietrosità (%)					
Coerenza (1-5)					
Drenaggio (1-5)					
Sup. rilevata (mq)					
Copertura (%)					
Copertura lichenico-muscinale (%)					
Alt. Media vegetazione (m)					
Diametro massimo dei tronchi (m)					
Tipo di vegetazione					

					Elenco Floristico	N°ACC /RILEV	N°

13.5 Associated fauna card

13.6 *Meteorological-climatic studies*



SCHEDA PEDOLOGICA | ID progressivo: | Data ingresso:

N. | Taxon: | Operatore:

Rilevatore: | Data:

Dati popolazionali:

Codice Popolazione:

Stato: Regione: Provincia: Comune: Località:

Foglio IGM:

N ° ' " E ° ' "

Strumento utilizzato: Precisione del dato:

Alt.: media (min max)

Inclinaz.: media (min max)

Esposiz.: media (da a)

Litologia:

Rocciosità (%): Pietrosità (%): Drenaggio: 1 2 3 4 5 Coerenza: 1 2 3 4 5

Bioclima:

Termotipo: Ombrotipo:

Tipo di vegetazione:

Codice Corine: Codice Habitat:

QUALITA' DEL SUOLO

PROFONDITA' FALDA

ACCUMULO SUPERFICIALE

PROFONDITA' UTILE

DRENAGGIO INTERNO

PIETROSITA'

diam. >7,5 cm:

diam. 2,5-75 cm:

diam. <2,5 cm:

FESSURE

largh.:

lungh.:

profond.:

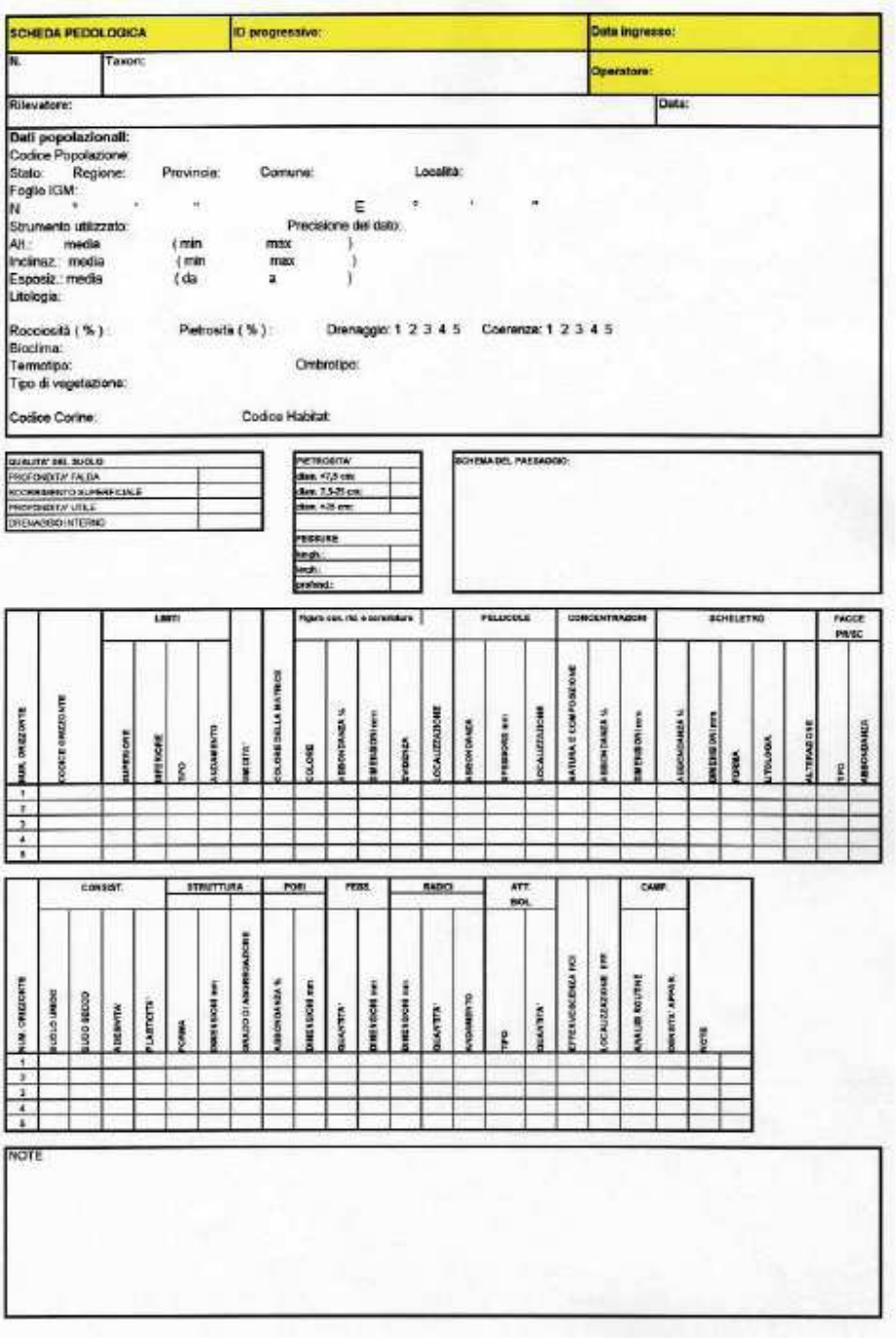
SCHEMA DEL PAESAGGIO:

NUM. ORIZZONTE	CODICE ORIZZONTE	LIMITI: SUPERIORE	LIMITI: INFERIORE	LIMITI: TIPO	LIMITI: LINEAMENTO	LIMITI: OMOLOGHI	COLORE DELLA MATRICE	Figure pedologiche e accidentali: COLORE	Figure pedologiche e accidentali: ABBONDANZA %	Figure pedologiche e accidentali: DIMENSIONI mm	Figure pedologiche e accidentali: EVIDENZA	Figure pedologiche e accidentali: LOCALIZZAZIONE	PELLICOLE: ABBONDANZA	PELLICOLE: SPESSORE mm	PELLICOLE: LOCALIZZAZIONE	CONCENTRAZIONI: NATURA E COMPOSIZIONE	CONCENTRAZIONI: ABBONDANZA %	CONCENTRAZIONI: DIMENSIONI mm	SCHELETRO: ABBONDANZA %	SCHELETRO: DIMENSIONI cm	SCHELETRO: FORMA	SCHELETRO: LITOLOGIA	SCHELETRO: ALTERAZIONE	FACCE PED/SC: TIPO	FACCE PED/SC: ABBONDANZA
1																										
2																										
3																										
4																										
5																										

NUM. ORIZZONTE	CONSIST.: SUOLO UMIDO	CONSIST.: SUOLO SECCO	CONSIST.: ADESIVITA'	CONSIST.: PLASTICITA'	STRUTTURA: FORMA	STRUTTURA: DIMENSIONI mm	STRUTTURA: GRADO DI ARRANGIAMENTO	PORI: ABBONDANZA %	PORI: DIMENSIONI mm	RADICI: QUANTITA'	RADICI: DIMENSIONI mm	RADICI: QUANTITA'	RADICI: ANDAMENTO	ATT. BIOL.: TIPO	ATT. BIOL.: QUANTITA'	EFFERVESCENZA HCl	LOCALIZZAZIONE EFF.	CAMP.: ANALISI SOLUTIVE	CAMP.: DENSITA' APPARATI	NOTE	
1																					
2																					
3																					
4																					
5																					

NOTE

13.7 Pedological data card



SCHEDA PEDOLOGICA | ID progressivo: | Data ingresso:

N. | Taxon: | Operatore:

Rilevatore: | Data:

Dati popolazionali:

Codice Popolazione:

Stato: Regione: Provincia: Comune: Località:

Foglio IGM:

N ° ' " E ° ' "

Strumento utilizzato: Precisione del dato:

Alt.: media (min max)

Inclinaz.: media (min max)

Esposiz.: media (da a)

Litologia:

Rocciosità (%): Pietrosità (%): Drenaggio: 1 2 3 4 5 Coerenza: 1 2 3 4 5

Bioclima:

Termotipo: Ombrotipo:

Tipo di vegetazione:

Codice Corine: Codice Habitat:

QUALITA' DEL SUOLO	
PROFONDITA' FALDA	
ACCORGIMENTO SUPERFICIALE	
PROFONDITA' UTILE	
DRENAGGIO INTERNO	

PIETROSITA'	
diam. <7,5 cm	
diam. 7,5-25 cm	
diam. >25 cm	
PRESSURE	
length:	
length:	
prahed.:	

SCHEMA DEL PAESAGGIO:

NUM. ORDINATIVO	CODICE ORIGINATIVO	LIMITI					COLORE DELLA MATRICE	Figure con. rid. e concrezioni					PELLICOLE			CONCENTRAZIONI			SCHELETRO					FACCE PR/SC	
		SUPERFICIE	DIFFUSIONE	TIPO	ANDAMENTO	SPESSORE		COLORE	ABBONDANZA %	DIMENSIONI mm	EVIDENZA	LOCALIZZAZIONE	ABBONDANZA	DIMENSIONI mm	LOCALIZZAZIONE	NATURA E COMPOSIZIONE	ABBONDANZA %	DIMENSIONI mm	ASSORTIMENTO	DIMENSIONI mm	FORMA	LITOLOGIA	ALTERAZIONE	TIPO	ABBONDANZA
1																									
2																									
3																									
4																									
5																									

NUM. ORDINATIVO	CONSIST.				STRUTTURA			PORI		FESS.		RADICI			ATT. BIOL.			CAMP.			
	SUOLO UMIDO	SUOLO SECCO	ADESIVITA'	PLASTICITA'	FORMA	DIMENSIONI mm	GRADO DI ARMONIZZAZIONE	ABBONDANZA %	DIMENSIONI mm	QUANTITA'	DIMENSIONI mm	DIMENSIONI mm	QUANTITA'	ACCRESCIMENTO	TIPO	QUANTITA'	EFFERVESCENZA HCl	LOCALIZZAZIONE EFF.	ANALISI ROUTINE	GRADO APPROF.	NOTE
1																					
2																					
3																					
4																					
5																					

NOTE

13.8 Initial tests schedule

SCHEDA PULIZIA e CONSERVAZIONE GERMOPLASMA

ID progressivo:

Data ingresso:

N.

Taxon

Operatore:

Precauzioni da adottare nel maneggiare il materiale:

N. semi prova del taglio:

% esito positivo

Categorie di risposta alla conservazione

|__| Ortodosso
|__| Intermedio
|__| Recalcitrante

Fonte:

Test iniziali:

Peso dei frutti Peso medio _____ g

N° frutti/Kg. _____

Volume dei frutti

N° medio frutti/litro _____

N° medio semi per frutto: _____

N° di frutti contati _____

Peso medio fresco seme: ___ g

Peso fresco totale ____ g

Prime pulizie (frutti carnosi): |__| Manuale |__| Meccanica |__| Chimica Data: ___/___/_____

Trattamento:

Quarantena: Data inizio: ___/___/_____

T. ambiente (15-20°C) _____ °C

Umidità relativa <6% _____ %

Note:

Postmaturazione:

Data inizio ___/___/_____

Data finale ___/___/_____

Durata____ gg

T. ambiente (15-20°C) _____ °C

Umidità r. <40% _____ %

Prova di Germinazione in ingresso:

|__| SI Data inizio ___/___/_____ Data finale ___/___/_____ % di germinazione: _____ %

|__| NO Motivo:

Pulizia: |__| Manuale |__| Meccanica |__| Chimica Data ___/___/_____

Trattamento:

Contenuto in olii _____ %

Fonte

Contenuto in proteine _____ %

Fonte

MC iniziale _____ %

Metodo

Peso medio seme (mg)

Peso totale accessione (g)

N° semi puliti

Diametro medio medio (mm):

Disidratazione

Data iniziale ___/___/_____

Data finale ___/___/_____

Durata____ gg

Temperatura (15-20°C) ____ °C

MC% finale (3,5-6,5 %) ____ %

Metodo:

Prova di tenuta dei contenitori e delle guarnizioni:

Data iniziale ___/___/_____ Tipo di contenitore: _____

Data finale ___/___/_____ Tipo di guarnizione: _____

Condizioni:

Temperatura: _____ °C

Umidità: _____ %

Risultati: _____

Campioni prodotti:

Codice campione	Data di chiusura	Prova di Tenuta	Data stoccaggio	Ubicazione:

Test qualitativi eseguiti:

Vitalità:

|__| Test di germinazione (TG)

|__| Prova del tetrazolio (TTC)

|__| Prova di conducibilità (PC)

|__| Prova con diacetato di fluoresceina (FDF)

|__| Indigo Carmin (IC)

|__| Risonanza magnetica (RM)

|__| Raggi X (RX)

|__| Altro (AA):

|__| Vigore e Performance (VP):

Note:

Responsabile:

PAGINA 1

13.9 Seed cleaning and storage protocol A

SCHEDA PULIZIA e CONSERVAZIONE GERMOPLASMA

ID progressivo:

Data Ingresso:

N.

Taxon

Operatore:

Precauzioni da adottare nel maneggiare il materiale:

N. semi prove del taglio:

% esito positivo:

Categorie di risposta alla conservazione:

|__| Ortodosso
|__| Intermedio
|__| Recalcitrante

Fonte:

Test iniziali:

Peso dei frutti Peso medio _____ g

N.° frutti/Kg. _____

Volume dei frutti

N° medio frutti/litro _____

N° medio semi per frutto: _____

N° di frutti contati _____

Peso medio fresco seme: ___ g

Peso fresco totale ____ g

Prima pulizia (frutti carnosi): |__| Manuale |__| Meccanica |__| Chimica Data: __/__/_____

Trattamento:

Quarantena: Data iniziale: __/__/_____

T. ambiente (18-20°C) _____ °C

Umidità relativa: circa _____ %

Note:

Postmaturazione:

Data iniziale: __/__/_____

Data finale: __/__/_____

Durata____ gg

T. ambiente (18-20°C) _____ °C

Umidità relativa: circa _____ %

Prova di Germinazione in ingresso:

|__| SI Data iniziale: __/__/_____ Data finale: __/__/_____ % di germinazione _____ %

|__| NO Motivo:

Pulizia: |__| Manuale |__| Meccanica |__| Chimica Data: __/__/_____

Trattamento:

Contenuto in oli: _____ %

Tecnica

Contenuto in proteine: _____ %

Tecnica

MC iniziale: _____ %

Metodo

Peso medio seme (mg)

Peso totale accessione (g)

N° semi puliti

Diametro medio medio (mm):

Deidratazione

Data inizio: __/__/_____

Data finale: __/__/_____

Durata____ gg

Temperatura: (15-20°C) ____ °C

MC% finale: (3,5-6,5 %) ____ %

Metodo

Prova di tenuta dei contenitori e delle guarnizioni:

Data iniziale: __/__/_____ Tipo di contenitore: _____

Data finale: __/__/_____ Tipo di guarnizione: _____

Condizioni:

Temperatura _____ °C

Umidità: _____ %

Risultati: _____

Campioni prodotti:

Codice campione	Data di chiusura	Prova di Tenuta	Data stoccaggio	Ubicazione

Test qualitativi eseguiti:

Vitalità:

|__| Test di germinazione (TG)

|__| Prova del tetrazolio (TTC)

|__| Prova di conducibilità (PC)

|__| Prova con diacetato di fluoresceina (FDF)

|__| Indigo Carmine (IC)

|__| Risonanza magnetica (RM)

|__| Raggi X (RX)

|__| Altro (AA)

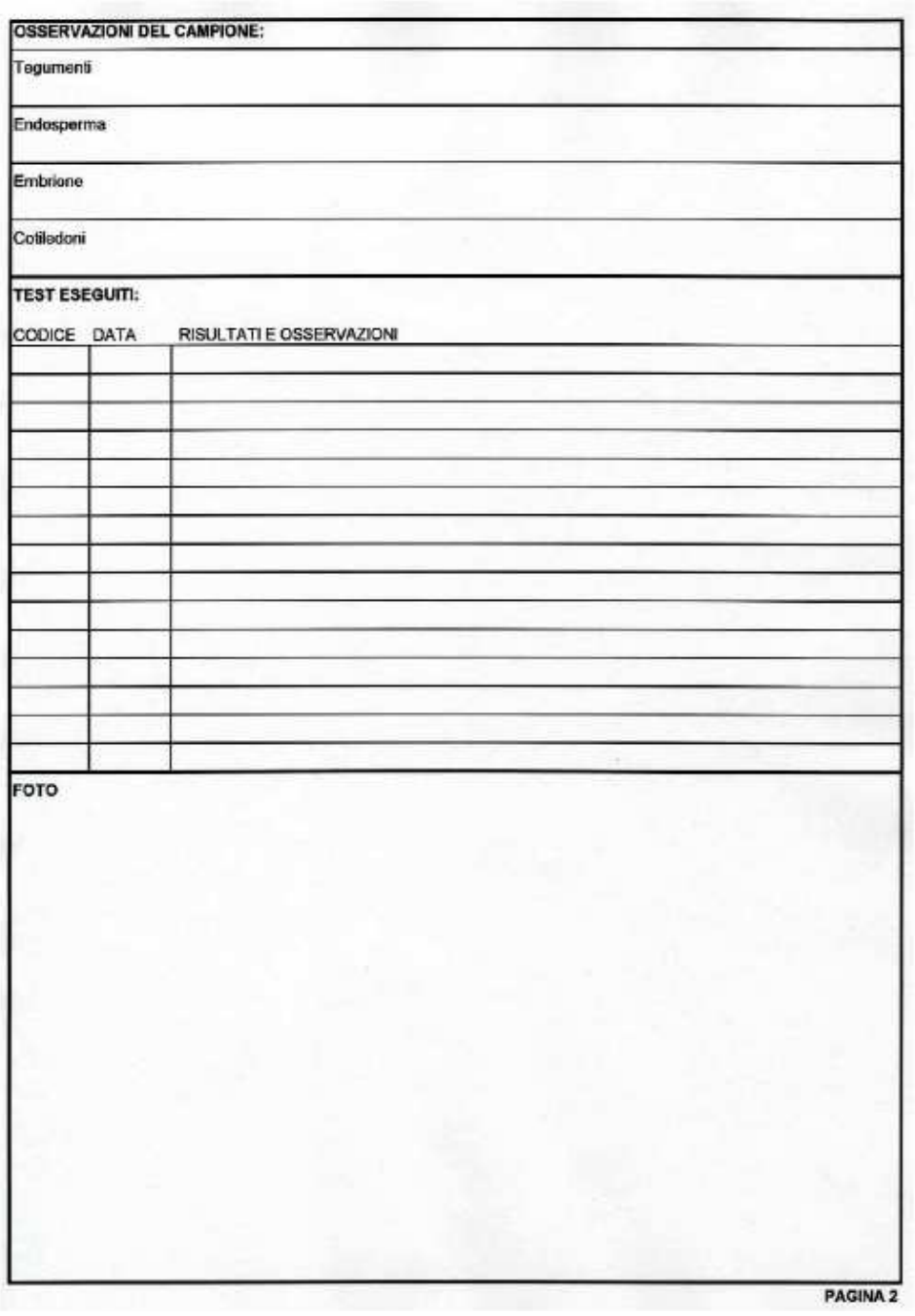
?__| Vigore o Performance (VP):

Note:

Responsabile:

PAGINA 1

Seed cleaning and storage protocol B

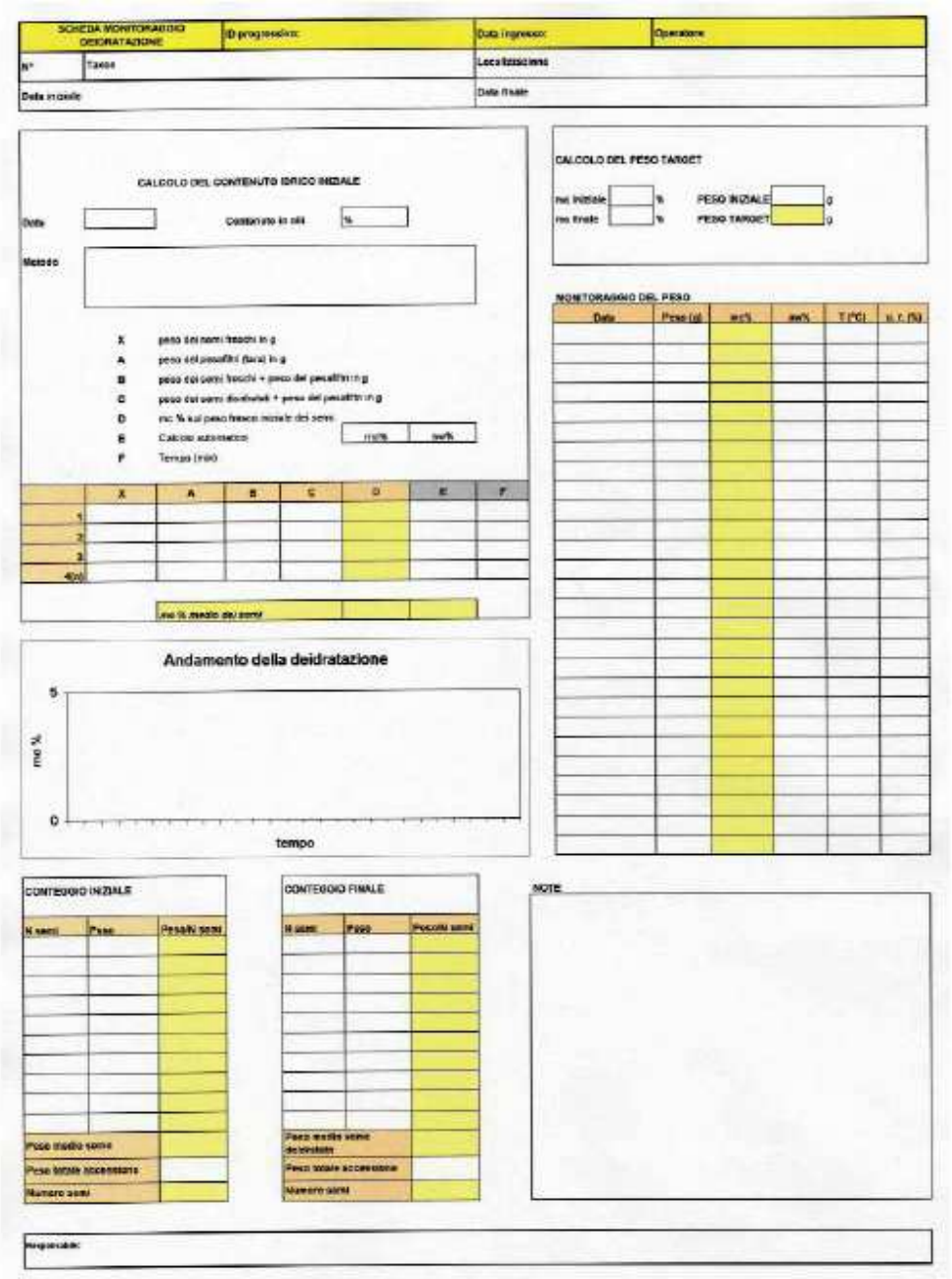


OSSERVAZIONI DEL CAMPIONE:		
Tegumenti		
Endosperma		
Embrione		
Cotiledoni		
TEST ESEGUITI:		
CODICE	DATA	RISULTATI E OSSERVAZIONI

FOTO

PAGINA 2

13. 10 Seed dehydration schedule



13.10 Germination test A

SCHEDA PROVA DI GERMINAZIONE		ID progressivo:	Data ingresso:
N.	Taxon	Operatore:	
Codice test di germinazione:		Data iniziale e finale della prova:	
Periodicità test: _____ anni prossimo test: ___/___/_____		dal ___/___/_____	al ___/___/_____

Provenienza del materiale: !__! T amb. (T °C) ___ !__! camera fredda (T °C) ___ !__! congelatore (T °C) ___

!__! Altro ______________________ Data di raccolta:

Tipo di germoplasma:

Pretrattamenti	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Pre-chilling (T°C)																
Pre-chilling (gg)																

Test condotto in sterilità !__! Metodo:

Condizioni di germinazione	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
N. semi per replica:																
Temperatura (°C) :																
Umidità (%):																
Fotoperiodo (h):																
Carta filtro																
Agar:																
GA3 (ppm)																
KNO_3 (%)																

Osservazioni e andamento della germinazione

Data	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
GERMINATI																
IMBIBITI																
NON IMBIBITI																
MORTI																
ALTRE CATEGORIE																
vuoti																
infestati																
% DI GERMINAZIONE																

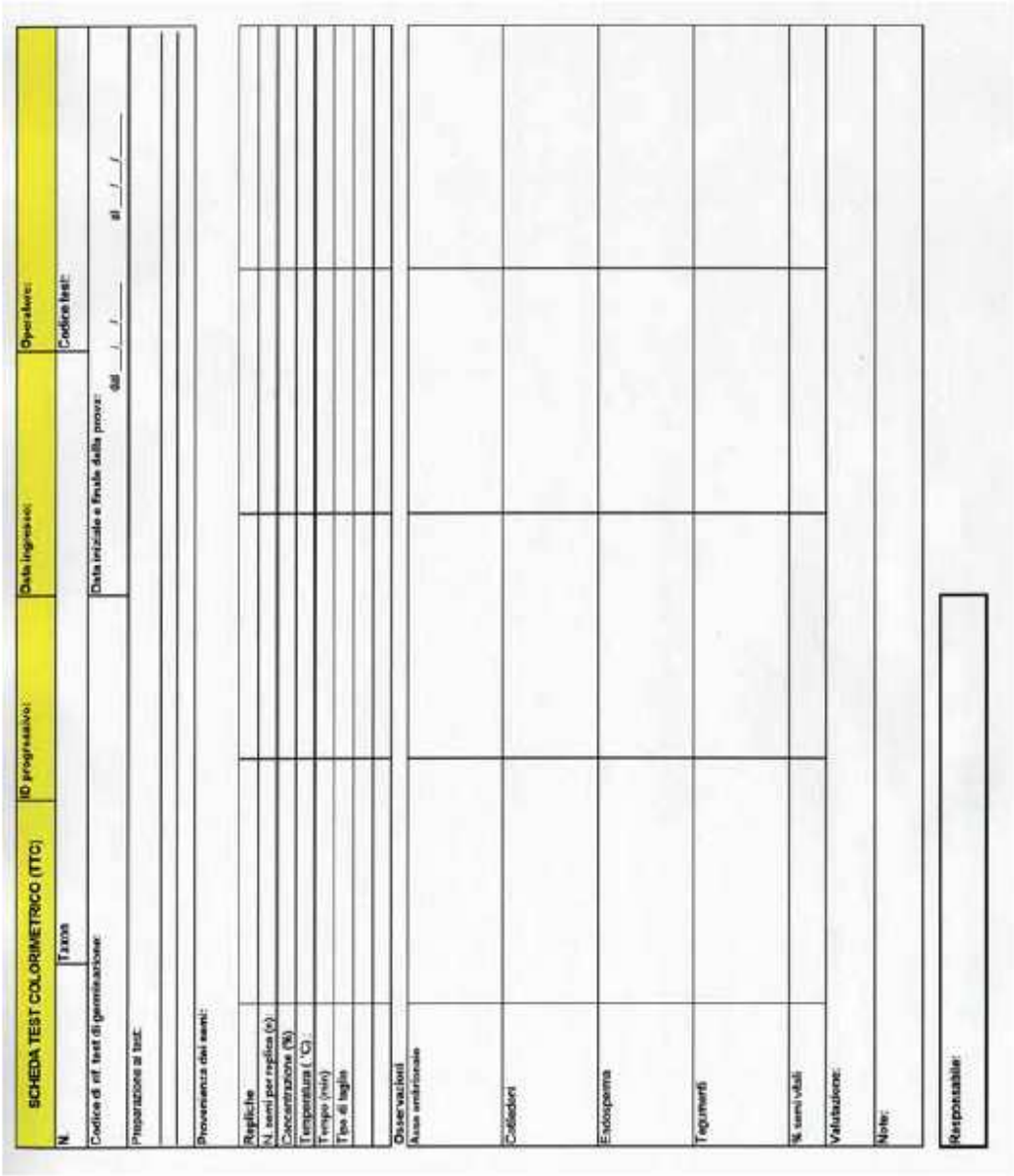
% MEDIA DI GERMINAZIONE		RITARDO DI GERMINAZIONE (gg)		T50 (gg)	
Responsabile:					PAGINA 1

Germination test B

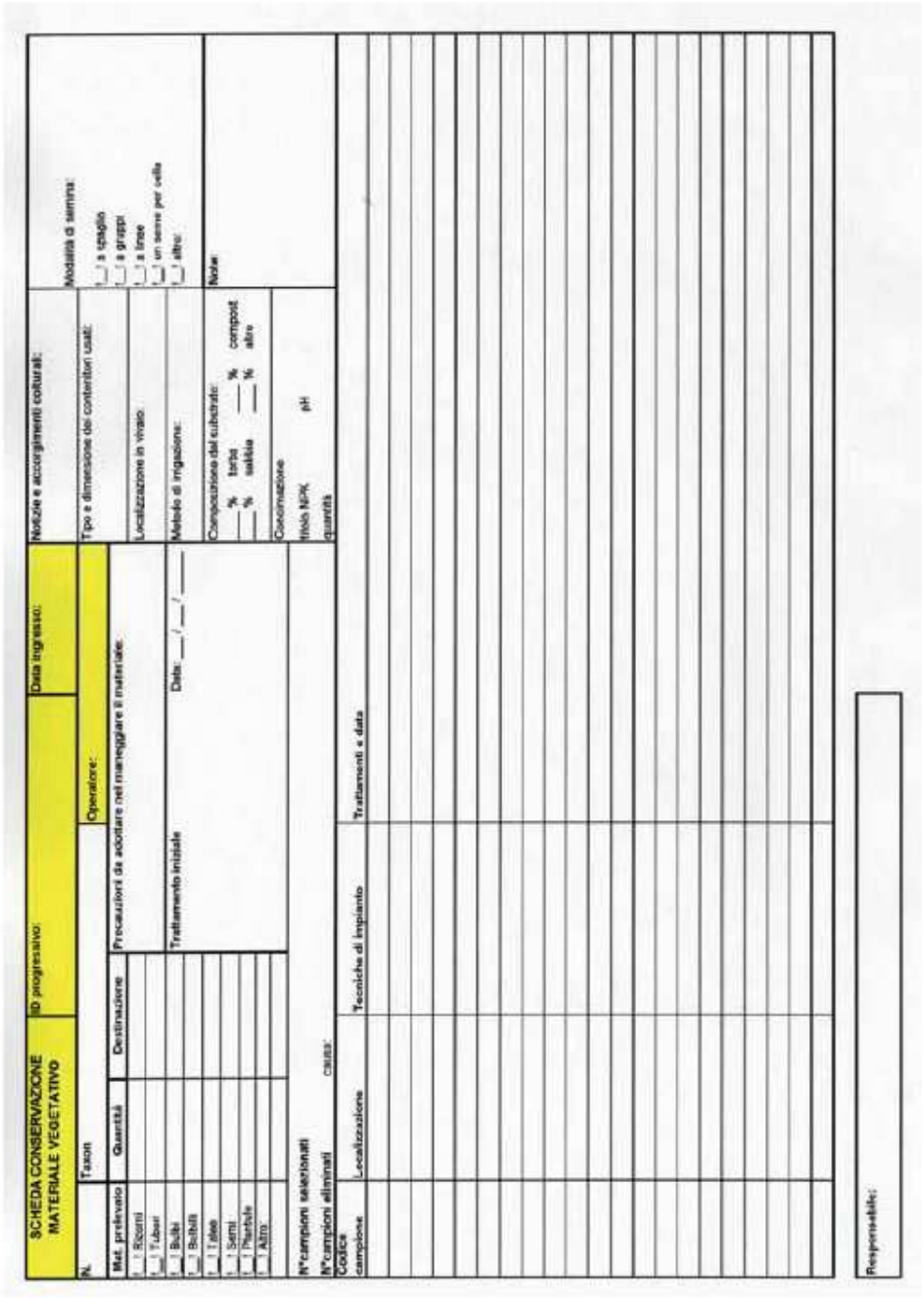
Osservazioni e andamento della germinazione	
DATA	NOTE OPERAZIONI TRATTAMENTI
Note:	

PAGINA 2

13.11 Colorimetric test



13.12 Management of vegetative material



SCHEDA CONSERVAZIONE MATERIALE VEGETATIVO

ID progressivo:

Data ingresso:

RL

Taxon

Operatore:

Mat. prelevato: Ricami, Tuberi, Bulbi, Bulbilli, Talee, Semi, Plantule, Altro

Quantità

Destinazione

Precauzioni da adottare nel maneggiare il materiale:

Trattamento iniziale

Data: __/__/____

N°campioni selezionati

N°campioni eliminati

Causa:

Codice campione

Localizzazione

Tecniche di impianto

Trattamenti e data

Notizie e accorgimenti colturali:

Tipo e dimensione dei contenitori usati:

Localizzazione in vivaio:

Metodo di irrigazione:

Concimazione:

titolo NPK

pH

quantità

Composizione del substrato:

___% torba ___% compost

___% sabbia ___% altro

Modalità di semina:

__ a spaglio

__ a gruppi

__ a linee

__ un seme per cella

__ altro:

Note:

Responsabile:

13.13 Seed collection management

SCHEDA DI GESTIONE DELLA SEMINA		ID progressivo:	Data ingresso:
N.	Taxon	CODICE DELLA SEMINA:	Operatori:
FASE DEL PRETRATTAMENTO		FASE DEL TRAVASO	FASE DI CRESCITA
N° di semi o peso (g)		Data inizio/fine	Data inizio/fine
Data inizio/fine	Tipo di pretrattamento	N° di semi o peso (g)	N° di piante
		Data di inizio della emergenza delle plantule	N° di plantule travasate
		Condizioni	Condizioni
		Substrato	Substrato
		Contenitore	Contenitore
		Temperatura e umidità	Temperatura e umidità
		Altro	Altro
		Localizzazione	Localizzazione
Responsabile del pretrattamento		Responsabile del seminato	Responsabile del travaso
			Responsabile della fase di crescita

Data	Controlli/Trattamenti

Osservazioni

Conteggio finale e data	

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