**Tópicos y perspectivas de la investigación científica sobre el mejoramiento genético de especies perennes en INIFAP**

**Topics and perspectives of scientific research on genetic improvement of perennial species in INIFAP**

Miguel Ángel Vallejo Reyna¹*, José Vidal Cob Uicab²

**Resumen**
El aumento de requerimientos de bienes y servicios, la degradación de los ecosistemas y el cambio climático conminan a la comunidad científica a buscar alternativas eficientes en el uso y manejo de los recursos forestales maderables y no maderables, así como de otras plantas perennes como los frutales. Por ello, invertir en el progreso de los sistemas agroforestales y el desarrollo de la agroecología es una actividad indispensable. A diferencia de las plantas anuales, en las que las principales técnicas de fitomejoramiento implican hibridación y retrocruzas, en las especies de ciclo de vida largo se deben emplear otros métodos cuya finalidad sea aumentar y mejorar las cualidades útiles para la sociedad. De esta forma se podrán maximizar los beneficios que se obtienen de estas especies y minimizar el impacto ecológico y ambiental. México es un país que por tradición ha dado mayor importancia a la agricultura sobre la forestación y la silvicultura, actividades que hoy día son muy importantes para mitigar los efectos de cambio climático y la conservación de los ecosistemas; sin embargo, instituciones como el Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP) han realizado esfuerzos que buscan contribuir al desarrollo del campo mexicano a través del mejoramiento genético de plantas perennes. El presente texto busca resaltar algunos puntos importantes en cuanto a la historia, las técnicas y algunas experiencias del INIFAP relacionadas a este tema, tan relevante para el bienestar de las sociedades rurales y la conservación de la biodiversidad.

**Palabras clave:** Cultivo de tejidos in vitro, especies forestales, marcadores moleculares, mejoramiento genético, propagación vegetativa, selección.

**Abstract**
The increase in demands for goods and services, the degradation of ecosystems and climate change, compels the scientific community to seek efficient alternatives in the use and management of timber and non-timber forest resources, as well as other perennial plants such as fruit trees. For this reason, investing in the progress of agroforestry systems and the development of agroecology is an indispensable activity. Unlike annual plants in which the main breeding techniques involve hybridization and backcrosses, in species with a long life cycle, other methods are necessary to increase and improve the useful qualities to society. In this way, the benefits that are obtained from these species can be maximized and the ecological and environmental impact minimized. In Mexico traditionally greater importance has been given to agriculture over afforestation and forestry, activities that today are very important to mitigate the effects of climate change and for the conservation of ecosystems. However, some institutions such as INIFAP have made efforts that contribute to the development of the Mexican countryside through the genetic improvement of evergreen plants. This text seeks to highlight some important points regarding the history, techniques and some experiences of INIFAP related to this matter so relevant for the well-being of rural societies and the protection of biodiversity.

**Key words:** In vitro tissue culture, forest species, molecular markers, genetic improvement, vegetative propagation, selection.

Fecha de recepción/Recepción date: 3 de septiembre de 2020
Fecha de aceptación/Acceptance date: 16 de agosto de 2021


*Autor por correspondencia; correo-e: vallejo.miguel@inifap.gob.mx
Introduction

The diversification and artificial domestication of plants is a continuous activity carried out by humanity from the Neolithic period to the present day. It is a mutualistic process of coevolution where humans have managed to establish close relationships and benefits with plant species (Abbo and Gopher, 2020). From different geographical centers of origin (Vavilov, 2009), ancient societies developed mechanisms for selecting the phyto-diversity from their surroundings to satisfy their need for food, clothing and housing (Harris, 1967). This knowledge of the environment and the close bond established with plants and animals contributed, in part, to a profound transformation of the human perception of nature, social order, and economy (Abbo and Gopher, 2020).

Plant domestication is closely tied to the origin of agriculture, in particular of some members of the Poaceae family that include the most important agricultural crops such as rice, corn and wheat. However, the origin of the management practices carried out in timber and non-timber forest species has received little attention. However, trees and other non-tree perennials (mainly fruit trees) have been subject to domestication for at least 6 000 years, with a considerable increase in the number of cultivated species in the last 3 000 years ago, which coincides with the first techniques of vegetative propagation through cuttings and grafts (Meyer et al., 2012).

The overexploitation of medieval forests in Europe compelled some villages, agricultural associations, monasteries and cities, to draft the first concepts and regulations aimed at keeping forests in good condition and avoiding their devastation (Schmithüsen, 2013). Despite this, the industrial development of the second half of the 18th century provoked a considerable loss of forested areas in Europe. Hence, during the early 19th century Germany, France, Norway, Denmark and Russia established the first forestry schools and academies. These sought to introduce research and scientific principles to the extraction of wood, and to implement them in an orderly and sustainable way, with emphasis on the importance of maintaining the soil associated to forestry production (Schmithüsen, 2013).
With the advent of genetics, in the second half of the 20th century, a genuine interest began in the international community of forest scientists to understand the basic principles that govern the phenotypic characteristics of tree species. This made systematic classification possible, as well as understanding the ecological and population dynamics that affect their evolution, and assimilating biochemical and physiological processes with developmental implications. Likewise, the first quantitative genetics and genetic improvement studies carried out the selection of outstanding individuals in order to monitor the inheritance of valuable traits and theoretically estimate additive and non-additive genetic variations (Libby et al., 1969).

In Mexico, formal research on forestry issues began with the decree creating the Mexican Institute of Forestry Research in 1932 (Urbina, 2017) and, later, with the creation of the National Institute of Forestry Research (INIF) and the Postgraduate College (Colpos). It is within these institutions that the inclination to implement modern genetic concepts in the forest sector began in the late 1950’s (SAGARPA, 2017; Urbina, 2017). During the following decades, the first forestry programs for the management and improvement of germplasm in the INIF experimental fields were consolidated, together with the creation of seed banks, and the Forest Herbarium. Later, with the merger of INIF into the current Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), the forest germplasm and biotechnology laboratory of the Centro Nacional de Investigación Disciplinaria en Conservación y Mejoramiento de Ecosistemas Forestales (Cenid Comef) was created. Avant-garde research lines were promoted, which, together with conventional management, strengthen public programs for forest improvement and conservation of the country's genetic resources (Vallejo et al., 2019).

Continuously linked to the National Development Plan (Presidencia de la República, 2019), INIFAP's research programs in forest genetic improvement seek to meet the commitments for the intergenerational conservation of phylogenetic resources and contribute to the socioeconomic well-being of rural communities in the different Mexican states. The main lines of research focus on population genetic diversity and
structure; these aim to preserve the evolutionary potential of forest resources as part of the bio-cultural heritage of rural economic units, and to influence efficient use. Furthermore, they seek to generate science that contributes to the understanding of the biochemical, molecular, genetic, physiological and ecological processes that shape the phenotypic variability of perennial plant species.

**Conventional forest genetic improvement**

Conventional forest genetic improvement is based on the selection of individuals with outstanding and desirable phenotypic characteristics that are part of a given population when they are in their reproductive stage. For the selection process to be effective, the existence of genetic variation within its range is essential, as it is essential to identify that the trait of interest is heritable and has socioeconomic value. These characteristics must have the potential to increase the productivity of timber or non-timber goods (resins, metabolites, fruits, etc.) obtained from forest resources or to respond favorably to adverse conditions of biotic or abiotic stress and, in this way, keep its evolutionary potential (Zobel and Talbert, 1988).

In line with the above, the data obtained from the selection of individuals from a given population, offer a rigorous description of the phenotypic plasticity. However, this information is insufficient and, as genetic improvement programs progress, it becomes necessary to use assessment methodologies that allow the estimation of genetic parameters (Torres, 2000). Thus, the phenotypic information is structured in databases and is related to its geographical origin to define the provenance of the germplasm. Subsequently, seeds are collected (NMX-AA-169-SCFI-2016) and provenance tests are established, that is, the planting of genotypes with different geographical origin in a common site with homogeneous physiographic and environmental characteristics to evaluate their behavior over time (Callaham, 1964). In addition, forest geneticists carry out progeny tests (the field evaluation of individuals descended from selected trees) in order to predict with statistical tools the genetic values of the parents based on the performance of their descendants (White and Hodge, 1989).
The aim of provenance and progeny trials with respect to the improvement of forest species is to obtain genetic gain. That is, to have a superior performance of the evaluated phenotypic characteristic concerning the average performance in the natural population. This requires the use of outstanding genotypes, the quantification of phenotypic variables, and the estimation of the heritability of the characters (Flores et al., 2014). However, despite the successful cases of different forest plantations in obtaining genetic gain, conventional genetic improvement has limitations; among them, the existence of biases in the estimation of the genotype, since the expression of the phenotype is affected not only by genetic variation but also by the environment and the effects of dominance and epistasis (White et al., 2007). On the other hand, provenance and progeny trials in trees involve a significant investment in economic resources, logistics, and very long selection cycle times before achieving significant results (Lynch and Walsh, 1998). For this reason, it has been necessary to create efficient selection methods in order to reduce time, costs and to estimate heritability and genetic gain more accurately.

Early selection is a type of indirect selection in which multiple traits, that manifest in premature ages, are quantified and correlated with a characteristic of economic interest observed only in mature stages of the individual (Vargas-Hernandez and Adams, 1992; Wu, 1998; White et al., 2007). However, this selection technique poses two main challenges: 1) Defining the optimal age for selection of the next generation, and 2) Implementing the use of early assessment data to improve selection. For this, it is necessary to create robust correlation matrices to calculate the selection index with greater fidelity (Burdon, 1989).

Starting in the 1960s, the first germplasm collections in coniferous species in Mexico were established. In 1963, the INIF initiated a program for the establishment of seed areas for Pinus spp., Abies spp. and Pseudotsuga spp.; although it was not until 1985, with the creation of the Centro de Genética Forestal A. C. (Center for Forest Genetics A. C.), that the efforts to improve forest genetics increased significantly. At the same time, the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias
(INIFAP), resulting from the merger in 1985 of the National Institutes for Forest Research (INIF), Agriculture (INIA) and Livestock (INIP), resumed research and actions aimed at this purpose, with the program of genetic improvement of woody species of commercial value. In 1993, INIFAP implemented activities to collect germplasm of red cedar (*Cedrela odorata* L.), mahogany (*Swietenia macrophylla* King), *sac-chacá* (*Dendropanax arboreus* (L.) Decne. et Planch.), and boxwood (*Cordia alliodora* Ruiz & Pav.), in order to obtain improved lines of species with high productive potential for central and southern Veracruz State (Sánchez *et al.*, 2003).

In addition, with the financial support of some state governments, producer associations and private companies, forest genetic improvement programs began in several states in the north and center of the country. Unfortunately, after a few years of operation, most of these programs were canceled temporarily or permanently, due to lack of financing and association with the productive sector. This situation prevented efforts from resulting in orchards that could provide genetically improved seed for use in intensive reforestation efforts or commercial plantations (FAO, 2012).

On the other hand, with the creation of the National Forest Commission (Conafor) in 2001, specific actions focused on the production of forest germplasm of known and genetically improved origin were established. Simultaneously, actions initiated to create a National Forest Genetic Resources System that focused on integrating and promoting forest genetic improvement and production (FAO, 2012).

These genetic improvement actions have been concentrated in few species of the following genera: *Pinus, Cupressus, Taxus, Eucalyptus, Gmelina, Hevea, Cedrela* and *Swietenia*; based upon the economic value of the production of sawn wood, many of them are already being assessed for their possible commercial and environmental use (FAO, 2012). However, most commercial forest plantations in Mexico are small and do not have genetic improvement programs. Therefore, the results in the progress of the improvement activities show a different degree of success in each of the species involved and with different perspectives of long-term permanence (Conafor, 2009).
Selection assisted by molecular markers

A different way of selecting superior genotypes is through the evaluation of genetic information derived directly from the study of hereditary material contained in deoxyribonucleic acids (DNA) and ribonucleic acids (RNA). Unlike the variation found in phenotypes, DNA variability within or between populations is not affected by environmental conditions, but is the result of evolutionary forces that have influenced the adaptation of the species to its area of distribution (Francia et al., 2005). The development of the polymerase chain reaction (PCR) advanced the study and understanding of the main molecule behind the inheritance of characters (Bartlett and Stirling, 2003). The PCR technique allows the use of DNA markers (nuclear, chloroplastic or mitochondrial) to quantify the genetic diversity in genetic improvement programs in perennial species (Porth and El-Kassaby, 2014).

For example, in the Tecomán Experimental Field of INIFAP, with the collaboration of the Colegio de Postgraduados, SSR (single sequence repeats) markers for the identification of Mexican lemon hybrids have been implemented (Carrillo-Medrano et al., 2018). SSRs are non-randomly distributed regions throughout the genome, characterized by being tandem repeats of nucleotide sets (Carneiro et al., 2016). In their study, Carrillo-Medrano et al. (2018) were able to successfully identify and differentiate 13 crosses of Mexican lemon with other varieties using four molecular markers. The above, is part of the INIFAP-Tecomán Mexican lemon genetic improvement program that searches for genotypes with greater resistance to diseases.

On the other hand, some researchers from INIFAP's Uruapan-CIR-Pac Experimental Field developed a protocol to describe the genetic diversity of Pinus pseudostrobus Lindl. From the seed area of the hydrological region (RH) Lerma-Santiago (Orozco et al., 2010). This RH is one of the most complex in the country since it has a great diversity of hydrological, geographical, economic, ethnic and historical characteristics, in addition to its abundant population. Therefore, obtaining quality germplasm is essential for management and reforestation plans. The AFLPs (Amplified Fragment Length Polymorphism) obtained by Orozco et al. (2010) are the first step for a seed
quality assessment program since these molecular markers detect multiple *loci* and allow estimating different parameters of population genetics.

Another interesting example from INIFAP regarding the use of molecular markers in the forestry sector was the genotyping of the rubber tree (*Hevea brasiliensis* (Willd. Ex A. Juss.) Müll. Arg.), by means of a high-resolution dissociation analysis (HRM) (Ortiz *et al*., 2017). This automated analysis allows the differentiation, with low cost and great precision, of the existing variation between two or more polymorphic sequences. In the study, carried out by the Cenid Comef Forest Biotechnology Laboratory, three genetic markers allowed the differentiation of ten clonal lines, a research further enriched by the morphological characterization of the clones. The precedent is relevant for the improvement and selection of the rubber crop, because it provides certainty during the production of the plant in the nursery and the establishment of multiplication gardens and commercial plantations. It also facilitates the selection of plants based on their susceptibility or tolerance to pests and diseases, as well as the preservation of their germplasm (Ortiz *et al*., 2017).

The common denominator of the three previous examples is the use of the PCR technique to identify some genetic characteristics typical of different varieties or populations of individuals. However, these techniques are often complicated when trying to make a more detailed association between genetic and phenotypic variations. This is due to three limitations: 1) The amount of molecular markers is small in relation to the size of the genome, which implies a significant information bias; 2) Much of the variation is located in unknown or non-coding regions, which prevents the understanding of the genes and physiological mechanisms involved in the phenotype; and 3) In doing studies that involve many populations and a large number of individuals it becomes expensive and difficult to operate and analyze. To counteract the above, in recent years the use of next-generation massive sequencing technologies and bioinformatics tools has been promoted to accelerate the domestication process of trees (Harfouche *et al*., 2012; Isik, 2014).
An example of the above is the study of the Mexican lemon variety of INIFAP in response to the infection of *Candidatus Liberibacter asiaticus*, which causes the Yellow Dragon disease (also called *Huanglongbing* or HLB) that is responsible for huge economic losses in citrus plantations worldwide. By means of messenger RNA sequencing (RNA-seq), 41,522 transcripts were obtained. This data set represents the first approach to the use of the RNA-seq technique for the Mexican lemon and reveals valuable information for future breeding programs, and will be an essential tool to facilitate the design of new molecular markers to diagnose and study the HLB disease (Arce-Leal *et al.*, 2020).

*Vaccinium macrocarpon* Aiton (large cranberry) is a small perennial shrub that belongs to the Ericaceae family. It is an example of a non-timber forest resource that, together with other berries, has become one of the most important agricultural/forest products in the country due to its exportation value. Diaz-Garcia and collaborators (2020) from the *Pabellón* Experimental Field of INIFAP performed a genotyping by sequencing analysis to identify the domestication status of this species. This research used 121 native (wild) and 111 selected commercial accessions. As a result, more than 21,000 single nucleotide polymorphisms (SNPs) were detected throughout its ten chromosomes. Analysis of the genetic data revealed a clear directional and gradual transition from wild to cultivated forms. However, the authors also mentioned that the recurrent introgression of wild germplasm into commercial cultivars and the reduced number of breeding cycles has slowed down the genetic differentiation between wild and cultivated large cranberry materials. This shows that cutting-edge innovations are required to accelerate the genetic improvement process in species with long life cycles (Diaz-Garcia *et al.*, 2020).
Vegetative propagation

As mentioned above, domestication of trees and other long-lived perennials (including bananas and palms) occurred at a later period than most cultivated annual species. Few trees have undergone this process for more than 4 000 years and, in most cases, only during the last 2 000 years (Meyer et al., 2012). The origin of tree cultivation is closely related to the development and management of vegetative propagation techniques, in particular cuttings and grafts (Meyer et al., 2012). This type of plant reproduction makes it possible to multiply, in a practical way, a genotype/phenotype of particular interest and is an essential part of forestry practices (Libby and Rauter, 1984). INIFAP has contributed to the development of asexual propagation, characterization and establishment of clonal orchards of tropical and fruit trees. Researchers from the forest area in the states of Veracruz and Quintana Roo have achieved the standardization of propagation protocols by grafting of red cedar (Cedrela odorata) and mahogany (Swietenia macrophylla) for the establishment of clonal banks and asexual seed orchards. This has been carried out at the El Palmar Experimental Field, Veracruz and at the San Felipe Bacalar Experimental Site, Quintana Roo ((Sánchez et al., 2003; Curti-Díaz et al., 2012; Salazar-García et al., 2016; Ortiz et al., 2017; Avendaño-Arrazate and Cueto-Moreno, 2018; Sampayo-Maldonado et al., 2019; Cob et al., 2020).

On the other hand, in vitro culture also allows the multiplication of selected material. This technique, founded on the totipotency of the plant cell, allows the regeneration of a complete individual from a fragment of the plant. In the Forest Biotechnology Laboratory of Cenid Comef of INIFAP, has developed several in vitro culture protocols for forest species. An example of the above is the propagation for mahogany (S. macrophylla) through in vitro germination, proliferation of axillary buds and callus, that can be used for the regeneration of complete plants, as well as for the development of research on biochemical routes and the production of natural metabolites with defense potential against pests (Wegier et al., 2013).
Furthermore, the Mexican avocado (*Persea americana* Mill. var. *drymifolia*) which belongs to INIFAP germplasm bank, has been successfully reproduced in Murashige y Skoog (MS) basal medium. The developed protocol generated acclimatized plants under greenhouse conditions. This multiplication under laboratory conditions allows the production of plants resistant to an adverse environment such as drought, flood, salinity or the absence of pathogens (Cortés-Rodríguez *et al*., 2011).

Micropropagation can also be useful to induce genetic variability that does not exist in nature. The use of ionizing radiation such as X-rays, gamma rays and neutrons, or the use of chemical mutagens, produce changes in the genetic material. When this technique is applied to *in vitro* grown vegetative material subjected to a certain selection pressure (for example salinity or drought), new variants with improved characteristics can be obtained (Ahloowalia and Maluszynski, 2001). In this way, in collaboration with the National Institute for Nuclear Research, INIFAP forest biotechnology researchers have generated a proof of concept to obtain mutants of *Psudotsuga menziesii* (Mirb.) Franco through irradiation with different doses of gamma rays. This work represents the first effort of INIFAP for the artificial induction of genetic variability in a conifer (Castillo-Martínez *et al*., 2018).

**Genetic engineering**

The genetic improvement of tree species by means of recombinant DNA technology is still incipient. The combination of modern biotechnologies such as DNA synthesis, vertical transfer of genetic material, CRISPR-based editing and next-generation high-throughput sequencing, increasingly allow precise manipulation of genes and genomes (Simon *et al*., 2019). In recent years, the development of efficient protocols for the precise editing of tree genomes has begun and is expected to be a powerful tool to accelerate the genetic improvement process in species with a long life cycle (Van Zeijl *et al*., 2018; Fan *et al*., 2020). Although in Mexico there are no published studies involving the genetic manipulation of forest species, there is an enormous potential of both natural and human resources that can sustain scientific exploration
and should not be neglected as the basis for its own homegrown technologies (Vallejo et al., 2019). However, research carried out in this regard must be founded on human and environmental biosafety guidelines, local and national needs, and the characteristics of the species to be studied, so that they can be accepted by society and be successful in solving a very particular problem in the Mexican forest sector.

**Conclusions and Perspectives**

Forests and tropical forests are biological systems that harbor great diversity and sustain complex ecological dynamics that provide, locally and globally, several environmental services and goods that allow the sustainability of rural and urban human settlements. Within these ecosystems, trees have a significant social, ecological, economic and spiritual value; furthermore, these perennial plants with a long life cycle cover around 30% of the emerged lands. Its sustainable use is a demand in today's society, defended, mainly, by the younger generations, who urge to address the challenge from the different areas of social and biological knowledge.

The current overpopulation and environmental crisis requires more and better innovations in forestry practices. Megadiverse countries, as well as centers of origin of domestication and diversification of plants, such as Mexico, demand the application of scientific knowledge and state-of-the-art technologies that favor the sustainable use of forest goods and services; that their use be socially, culturally and ecologically responsible as well as economically viable. Thus, it is urgent to give greater importance to the issue of forest genetic improvement, as an operational tool in support of forest plantation programs, and to optimize results. In this sense, Mexico has great opportunities for the implementation of genetic improvement programs due to the great diversity of ecosystems and perennial species for human use. If the forest genetic improvement programs are carried out in an orderly and well-structured manner according to the species of study and circumstances of each region and, on the other hand, successful links are established between academia and the rural economic units, it will be possible to move towards a circular economy that benefits the entire society.
Throughout its 35 years of existence, INIFAP has maintained forest genetics as its research topic, providing important information on the evolution, conservation, management and sustainability of natural forests and of forest and fruit plantations. However, these efforts are limited by the lack of mid and long-term programs and support for the modernization of the field and laboratory infrastructure. Thus, despite some isolated efforts, it has not been possible to consolidate a robust institutional program that manages to link INIFAP effectively with other national and international institutions where research is more advanced.

At present, there are examples -of woody species- in foreign countries that use forefront tools in molecular biology to define associations between the genotype and the phenotype (Valenzuela et al., 2021), the characterization of the genotype and physiology (Ye et al., 2019), and the detection of important genes for stress tolerance (Gong et al., 2018). In Mexico, there are research groups of recognized prestige that analyze, through ecological genomics and traditional genetics, the diversity of forest genetic resources with the aim to preserve and develop predictive models of phenotypic traits that may be applicable to programs of sustainable forest management (Wehenkel et al., 2017; Arenas et al., 2021). It is for these reasons, that INIFAP should support and strengthen programs for the improvement of forest ecosystems, take advantage of the experience of its researchers, laboratory infrastructure and experimental fields, to become a national benchmark for the forest sector in Mexico by contributing to the solution of the environmental and social challenges of the 21st century.

**Acknowledgments**

This review aims to commemorate and acknowledge the efforts of the research staff who throughout the history of INIFAP have shown interest, commitment and experience in order to contribute to the conservation and genetic improvement of forest species in Mexico. We are grateful to the federal and state governments for the financial support to carry out some research projects.
Conflict of interest

The authors declare that they have no conflict of interest related to the content of this text.

Contribution by author

Miguel Ángel Vallejo Reyna: preparation of the sections related to the use of biotechnological tools in forest genetic improvement and the editing of the manuscript; José Vidal Cob Uicab: preparation of the section related to conventional genetic improvement. Both authors worked equally to achieve the final review and approval of the content.

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