The Reorganisation of European Hazelnut Genetic Resources in the SAFENUT (AGRI GEN RES) Project

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Abstract

During the period 2007-2010, the European Project SAFENUT: "Safeguard of almond and hazelnut genetic resources from traditional uses to modern agro-industrial opportunities", has been carried out in Europe, by researchers from six European countries and the participation of 11 different partners. Several aspects referred to *Corylus avellana* L., were tackled: the survey of local national and European hazelnut collections and *on farm* recovery of "ecotypes"; the evaluation of plant material (morphological, biochemical and molecular assessment); the creation of a hazelnut core collection; the analysis of ecological, economic and socio-cultural aspects related to sustainable production and traditional knowledge; and, finally, the establishment of the SAFENUT website and database. With the aim to harmonise the morphological evaluations, specific descriptors were elaborated for the characterization of genetic materials, both in the permanent collection and in new selections. Three hundred six hazelnut accessions were analyzed at 10 SSR loci in order to verify the genetic authenticity. The main cultivars of each country were evaluated for fatty acids, tocopherols, phenolic compounds, mineral and protein contents. In the light of the above, the multivariate analysis of the entire data allowed the creation of the hazelnut core collection. The recovery of traditional knowledge was undertaken by different activities: questionnaires were elaborated following the interviews of students and their parents in all Partner countries; a survey on the festivals related to this species was carried out and a publication was released. Furthermore, 63 filled questionnaires addressed to farmers offered the opportunity to compare problems, technical practices and biodiversity at the European level. Finally, the SAFENUT Database, a web interface available at the address: hppt://www.safenut.net, has been set up in order to provide users driven on-line search-queries, across multi-trait data based on germplasm evaluation. The European SAFENUT experience has allowed the realization of an efficient working group, able to draw out the authentic significance of

genetic resources, not only working on the unique genetic background offered by genetic resources, but also on the cultural meaning that they represent.

INTRODUCTION

An exhaustive study of the preservation and utilization of European hazelnut (*Corylus avellana* L.) genetic resources were carried out during three years (2007-2010) in the frame of SAFENUT Agri Gen Res action (http://safenut.casaccia.enea.it). Six European countries and 11 partners have been involved in this project (Table 1). The European SAFENUT Action represents a resource strategy for re-organizing and sharing, in a more efficient manner the hazelnut genetic resources by upgrading the knowledge on his value as well as the precious cultural meaning related to traditional and historical uses of people who conserved and, in some cases, improved them. Hazelnut is a commodity of international economic importance, high variability in morphological, agronomical and commercial characters in this species, their excellent nutritional and nutraceutical properties make its consumption indicated for the human health.

The main objective of the work was to increase the knowledge of the European hazelnut (*C. avellana* L.) in order to enhance its characterization, preservation and utilization. Five different activities were proposed in this project: the survey of local, national and European *Corylus avellana* L. collections and on farm recovery "Ecotypes"; the morphological, phenological, molecular and biochemical (fatty acids, phenolics and mineral composition) plant material characterization; the creation of a core collection, representative of the maximum genetic diversity of this species; the analysis of ecological, economic and socio-cultural aspects related to sustainable production and traditional knowledge and, finally, the establishment of the SAFENUT website and database. The main results obtained during the tree years of the project, are briefly presented in this work.

MATERIALS AND METHODS

Survey of local, national and European *Corylus avellana* L. collections and on farm recovery "Ecotypes"

Two different actions were carried out referring to plant material: gathering the informations on hazelnut accessions maintained in European collections, and the recovery of hazelnut "Ecotypes". A list of all the accessions maintained in the 13 European collections (one collection in France and Greece, two collections in Slovenia and Spain, three collections in Portugal and four collections in Italy) was established. The information was revised accurately to verify the trueness to type of different accessions, detect possible erroneous spelling in any of the cultivars or references and remove synonyms. A survey to avoid the extinction of hazelnut genetic diversity existing in most European areas was also performed. In winter, wood from selected material was collected by different partners and was sent to IRTA-Mas de Bover to be propagated by grafting, following the methodology proposed by Lagerstedt (1981).

Morphological, phenological, molecular and biochemical plant material characterization

A chart with the specific descriptors for hazelnut, following current hazelnut descriptors (Thompson et al., 1978; UPOV, 1979), was harmonized and standardized among partners to characterize hazelnut material including: general characteristics (cultivar, synonyms, origin and areas of growing), tree traits (10 characters), flowering traits (6 characters) and nut and kernel traits (32 characters). The main cultivars of each country (Table 2) were characterized according to this chart, each year of the project. For the description of material surveyed a less specific chart was used. Additionally, nuts from a set of 7 reference cultivars ('Castanyera', 'Gironell', Merveille de Bollwiller', 'Negret', 'Pauetet', 'Tonda di Giffoni' and 'Tonda Gentile delle Langhe'), were sampled at different countries' collections in the second and third year and analyzed for morphological and chemical traits, to assess the influence of the environment on the genotype performance.

Hazelnut material from different collections (young leaves, in spring and/or immature catkins in September-October) were collected and sent to UNITO and ENEA (Italy) to identify the accessions by molecular markers. A set of 10 selected SSR loci (CaT-B107, CaT-B501, Cat-B502, CaT-B503, CaT-B504, CaT-B505, CaT-B507, CaT-B508, CaC-B020, and CaC-B028) was used to fingerprint hazelnut material. DNA was extracted using the procedure by Thomas et al. (1993) with modifications. PCR amplifications and SSR analysis were performed following the procedure described by Boccacci et al. (2006). Results of the run were then analyzed with Gene Mapper software and alleles were designated by their size in base pairs (bp).

Biochemical analyses were focused on the nutritional and nutraceutical aspects: vit. E, fatty acids, fat content; phenolics; mineral content (K, Ca, Mg, P) and total protein content. The hazelnut oil was extracted from the samples and the tocopherol analysis was performed using the method illustrated by Kodad et al. (2006). It was used a liquid-liquid extraction followed by HRGC using a capillary column RTX 2330 (30 m x mm I.D.). All analyses were carried out on 2 replications per each cultivar; the calibration curve for quantitative analysis was calculated using high purity standard for Gas Chromatography and HPLC (SIGMA). After calibration, the extraction and analysis methods were tested using a reference matrix containing tocopherols and fatty acids (baby food NIST certified). Analysis were carried out in CRAB (Italy). Phenolic compounds in raw kernels were analyzed at BF (Slovenia). Forty inshell nuts per accession, dried according standard procedure with 12% of humidity were collected at different locations in early autumn each year. Before being analyzed, they were stored in cold room, at 10ºC. Sample preparation and determination of polyphenolics content were carried out according to Solar et al. (2009) and Jakopic et al. (2010). The analysis of seven wide-spread cultivars was repeated during two years. The study on mineral nutrient and protein content was carried out in the Pomology Institute, NAGREF (Greece). The methodology for the determination on mineral nutrient: A K, Ca, P, Mg and protein content is described by Bacchetta et al. (2009).

Creation of a core collection, representative of the maximum genetic diversity of this species

The hazelnut material to be included in the "Core collection", defined by Brown (1989), as a selected and smaller collection representative of species' genetic diversity, was

identified of the basis of molecular, morphological and biochemical characterization of the main hazelnut cultivars analyzed in the project.

Analysis of ecological, economic and socio-cultural aspects related to sustainable production and traditional knowledge

The survey on existing exhibitions of hazelnut was carried out looking for the opportunities to expose nuts (pomologic exhibitions) and / or their products (processed nuts) as well as to celebrate hazelnuts as trees. The most used methods were Internet, and also, inquiry data from Municipalities or Professional Organizations of the different countries involved in the project. A questionnaire about the knowledge and traditional hazelnut uses, including 13 main questions, addressed to students was elaborated. The same questionnaire was then proposed to their parents and grandparents, to gather more information. This methodology allowed collecting data from a large platform. The involved schools were from Greece, Italy, Portugal, Slovenia and Spain.

Establishment of the SAFENUT website and database

The data base (DB) characteristics and the conceptual model of the SAFENUT DB, was defined. At the same time, queries and descriptors were defined to be included for each record of this DB. The conceptual model of the SAFENUT DB was defined in collaboration with Spazio Verde s.r.l. and discussed among partners.

RESULTS AND DISCUSSION

All the results obtained in this project, can be found in www.safenut.net. In the following paragraphs, only those most relevant are presented.

Survey of local, national and European *Corylus avellana* L. collections and on farm recovery "Ecotypes"

An initial list of 291 hazelnut cultivars was gathered from material existing in the 13 hazelnut collections studied. The morphological and molecular characterization of the accessions allowed to detect some homonymous and synonymous cultivars and spelling mistakes in the analysed germplasm. Finally, a list of 202 cultivars and 58 selections was elaborated. Cultivars come from the following countries: Albania (1 cultivar), Balkan area (2 cvs.), Belgium (1 cv.), England (12 cvs.), France (8 cvs.), Germany (6 cvs.), Greece (1 cv.), Hungary (1 cv.), Italy (51 cvs.), Netherlands (1 cv.), Portugal (3 cvs.), Romania (4 cvs.), Slovenia (3 cvs.), Spain (84 cvs.), Turkey (7 cvs.) and USA (9 cvs.). Nine cultivars from unknown origin were listed. Most of this material is present in different European collections (Rovira et al., 2011).

An important hazelnut survey was carried out in different European areas: Drama, Sfendami and Pieiria (Greece), Basilicata, Latium, Marche, Piemonte, Sicilia, Liguria and Sardinia (Italy), Felgueiras, Viseu, Moimenta and Miho (Portugal), Cresnejevec, Tepanje, Sentjur pri Celju and Vrhpec (Slovenia) and Asturias (Spain). This material was grafted at IRTA-Mas de Bover. Some of this material is still in nursery, but most of them is already planted in collections, in order to continue their study and performance in collection .

Morphological, phenological, molecular and biochemical plant material characterization

During the study the material of each collection was accurately observed and morphologically characterized. Material surveyed, including a total of 140 minor endangered cultivars, landraces and ecotypes found, was also characterized. Results showed the great genetic variability present in *Corylus avellana* L.

A total of 306 accessions, 114 local landraces and 191 accessions from the European hazelnut collections, were characterized by molecular markers (SSRs). The 10 SSR loci used in this study identified 78 unique genotypes among the 192 accessions with cultivar names sampled in collection fields, due to several duplications and cases of synonymy or misnaming. Referring to landraces surveyed in each country, genetic data showed several cases of genotypic identity among landraces and between landraces and cultivars, but new genotypes were also identified. New local gene pools were identified, such us in Italian regions (Liguria in North-east Italy and Latium in Central Italy).

At the end of the project a total of 101 hazelnut cultivars were evaluated for fatty acids content: for most of them the analysis were replicated each year. The oil content with mean value of 57% varied widely among cultivars and ranged from 46.95% to 65.20%. The main fatty acid found in hazelnut were oleic (mean 80.77%) and linoleic (mean 1.38%), in agreement with literature (Alasalvar et al., 2009; Xu and Hanna, 2010). Oil was poor in saturated fatty acids, represented by palmitic (mean 5.90%) and stearic (mean 2.49%). The mean value of linolenic was 0.11 %. The alpha tocopherol content varied from 108.25 ppm ('Trenet' cv. from Spain) to 360.69 ppm ('Pauetet' cv. from France). The results obtained in the 7 reference cultivars, showed that the samples grouped together for sites of origin indicating an effect of environment on the total oil content.

Twenty phenolic compounds were determined and quantified in hazelnut kernels of 118 accessions, during the three years of the project. Among the phenolics which were found in the kernels using HPLC-PDA, procyanidin trimer-1 and myricetin-3-*O*-rhamnoside were the most abundant in all cultivars. In general, more flavonoids than phenolic acids were detected using HPLC-MS. The concentration of single phenolic compound was strongly affected by the cultivar. Gallic acid, catechin and myricetin-3-*O*-rhamnoside were shown as the most cultivars dependent. Gallic acid and catechin have already been identified in the hazelnut (Yurtas et al., 2000; Jakopic et al., 2010), while myricetin-3-*O*-rhamnoside was firstly determined in the hazelnut kernels. There were five cultivars ('Del Rosso', 'Camponica', 'no 11', from Italy; 'Cosford' from France and 'Castanyera' from Spain), with sum of 13 phenols between 200 and 225 mg/kg. On their opposite, six cultivars ('Barrettona', 'Tonda Bianca', Nociara', 'no 5', from Italy; 'Grifoll' from Spain' and 'Potami' from Greece) had less than 60 mg/kg phenolic compounds in their kernels. Considering seven reference cultivars, results showed significant differences among each other with regard to their phenolic constitution. The influence of the growing site was the most evident in 'Negret', and the most stable phenolic constitution was found in 'Pauetet'.

Referring the study of mineral nutrients, a total of 92 samples coming from different European hazelnut collections were analyzed, for two or three years, together with the seven wide-spread hazelnut cultivars. A great genetic variation was found in all mineral nutrient and protein contents (Table 3). Values obtained in mineral nutrient and protein contents were

in the range of those found by Açkurt et al. (1999), Ozdemir et al. (2004) and Silva et al. (2005). Results suggest that growing and environmental parameters may have significant effects on hazelnut mineral contents.

Creation of a core collection, representative of the maximum genetic diversity of this species

The results obtained in material characterization indicate a large range of variation among cultivars from several traits and compounds of technological and/or nutraceutical interest. Regarding morphological traits, 20 cultivars have been selected to be a part of the hazelnut core collection. This material has been chosen due to their genetic diversity presented in different characters. Concerning phenolic contents, 7 accessions were proposed: 'Camponica' (ENEA), 'Castanyera' (IRTA), 'Del Rosso' (UNITO), 'Da Veiga' (UTAD) and 'Cosford' (ANPN), as sources with the highest summary content of 13 phenolic compounds; 'T/16' (BF) as a high summary content and good source of epicatecin, and 'Pauetet' as the most stable cultivar with regard to the content of 13 phenolics and production area. On the basis of mineral nutrient and total protein contents, 5 cultivars were proposed: 'Longue d'Espagne' (ANPN) was among the highest in total mineral content, 'Casina' (ANPN) was among the highest in total mineral content and particularly high K content; 'Pauetet (ANPN) was among the highest total mineral and particularly high Mg content, 'T/10' and 'Pellicola Bianca' (BF) having the highest total protein content. On the basis of fatty acids and tocopherol contents, the following cultivars can be indicated for the inclusion in the core collection: 'Comun', 'Da Veiga', 'Culplà', Morell', 'Negret', 'Extra Giaghli', 'Palaz', Istrska okroplodna leska', 'Tonda Gentile Romana', 'Tonda Gentille delle Langue', 'Tonda di Giffoni', 'Ada', 'Barretona', 'Tonda Bianca', 'Nocchione' and 'Ricia di Talanico'. Further studies are need to make the group more representative of the hazelnut genetic pool.

Analysis of ecological, economic and socio-cultural aspects related to sustainable production and traditional knowledge

The survey realized, 63 hazelnut orchards, and the questionnaires answered filled by 1115 students, 506 parents and 576 grandparents clearly showed that hazelnut not only have an agronomic value but also represents a symbol of history, art folklore and food, closely related to the territory and to the culture of the population. A booklet on "Hazelnut and Almond European Festivals" was edited in 2009. Twenty-six hazelnut festivals have been recorded and included several traditional recipes.

Establishment of the SAFENUT website and database

Morphological specific descriptions of 65 hazelnut cultivars have been published and they are available on the database. The morphological, biochemical and molecular data of the cultivars analyzed during the project as well as the list of new hazelnut ecotypes and their morphological evaluation has been produced and can be found at: http://www.safenut.net.

CONCLUSIONS

During the three years of SAFENUT project a great effort has been made by all partners, to obtain the maximum results concerning characterization and traditional knowledge of European hazelnut material. Data obtained, and presented in the data base, are useful both for the scientific and industrial sector. The main interest for this crop comes from the producers' world (farmers) and from a part of the chemical industry which deals with some active principles that can almost exclusively be found in some of these accessions.

More specific studies should be conducted to define the core collection which is a prerequisite for the acquirement data of quantitative traits such as the resistance to biotical and abiotical stresses.

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Table 1. Partners involved in hazelnut studies in the frame of SAFENUT Project.

Country	Partner
France	ANPN (Association Nationale de Producteurs de Noisette)
Greece	NAGREF (National Agricultural Research Foundation- Pomology Institute) NAGREF-ISPOT (National Agricultural Reseatch Foundation-Institute of Olive Trees and Subtropical Plants)
Italy	ENEA* (Ente per le Nuove Tecnologie, l'Energia e l'Ambiente) CRA (Centro di Ricerca per la Frutticultura, Roma) UNITO (Universita degli studi di Torino) CRAB (Conzorcio di Recerca Applicate alla Biotecnologia)
Portugal	UTAD (Universidade de Trás-os-Montes e Alto Douro)
Slovenia	BF (Biotehniška fakulteta, Univerza v Ljubljani)
Spain	IRTA (Institut de Recerca i Tecnologia Agroalimentàries, Catalunya) SERIDA (Servicio Regional de Investigación y Desarrollo Agroalimentario, Asturias)

*Coordinator Partner

Table 2. Traditional cultivars of countries involved in SAFENUT, characterised by morphological and phenological traits during the three years of the project.

Coutry	Cultivars
France	'Bergeri', 'Corabel', Cosford', 'Feriale, 'Fertile de Coutard', 'Ferwiler', 'Gunslebert', 'Imperatrice Eugenie', 'Longue d'Espagne', 'Merveille de Bollwiller', 'Rotblaftrige Lambernuss'
Italy	'Camponica', 'Riccia di Talanico', 'Tonda Gentile Romana', 'Nocchione', 'Tonda di Giffoni', 'Barretona', 'Barcelona', (P1), 'Tonda Gentile delle Langue', 'Tonda di Biglini'
Portugal	'Grada de Viseu', 'Da veiga', 'Comun', Provence, 'Molar'
Slovenia	'Istrska dolgloplodna leska', 'Istrska okrogloplodna leska'
Spain	'Casina', 'Castanyera', 'Culplà', 'Gironell', 'Grifoll', 'Morell', 'Negret N-9', 'Pauetet', 'Segorbe', 'Vermellet'

Table 3. Mineral content variability found in the 92 hazelnut accessions analysed.

Mineral nutrients	Range (mg/100^{-1} DW)	Cultivars presenting higher valuest
K	440.5 - 829	'Merveille de Bolwiler', 'Casina', 'Feriale', 'Karydaty'
Ca	238.0 - 552.0	'Vermellet', 'Potami'
P	172.0 - 460.9	'Extra Giaghli', 'Palaz', 'Tombul'
Mg	121.3 - 211.0	'Común', 'Nostrale'
Protein	4.8 - 19.7	'T/1', 'Tombul', 'Pellicola Bianca', 'Barrettona', 'Merveille de Bolwiler'