UNIVERSITY OF MOLISE

Department of Biosciences and Territory

DOCTORAL THESIS

“BIOLOGY AND APPLIED SCIENCES”

Curriculum BIOLOGY

XXXVI cycle

“Recovery, characterization and conservation of plant agrobiodiversity of Alto Medio Sannio inner area”

S.S.D. BIO/01

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Academic year 2023/2024

“Recovery, characterization and conservation of plant agrobiodiversity of Alto Medio Sannio inner area”

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Dedication

To zio Peppe

Abstract

Agrobiodiversity underpins ecological functions, sustainable development, food security and climate change resilience, contributing to the overall ecosystems' health and human well-being. A key component of agrobiodiversity is represented by landraces, plant genetic resources adapted to restricted marginal rural environments and associated with a set of traditional cultural practices and knowledge, which constitute a gene pool of unexplored variability, useful for biodiversity enrichment, climate change mitigation and human health promotion. However, landraces are increasingly threatened with extinction by replacement with improved commercial cultivars released by the industrial agriculture system, therefore active conservation strategies are necessary to maintain these critical resources.

The aim of this doctoral thesis was to identify possible development paths linked to the territory, to the agrobiodiversity and to the sustainability in the marginalized areas based on the recovery/preservation, characterization and valorisation of plant local resources. The inner area of "Alto Medio Sannio" was selected as case study planning different complementary and consecutive research activities. Firstly, a recovery activity of all typical legume landraces and of the associated traditional knowledge was conducted throughout the area of study. The collected local germplasm was stored ex situ in Molise Germplasm Bank and a comprehensive characterization activity – paper I, II and III – was performed on the most representative common bean (*P. vulgaris* L.) landraces.

In detail, in the paper I (Falcione et al. 2022), a multi-level characterization approach allowed to explore the diversity among seven Italian common bean landrace populations (Ciliegino, San Michele Rosso, Monachella, Mascherino, Pinto, Tuvagliedda Rossa and Suocera e Nuora). Results of the work showed that populations were separated in two main groups of seed morpho-colorimetric features, while phaseolin characterization indicated that all the populations belong to Andean gene pool, displaying four typical Andean phaseolin isoforms. The ISSR molecular markers data provided important insights on the genetic variability and phylogenetic relationships among Italian common bean landrace populations, unveiling genetic similarities among "Alto Medio Sannio" common bean landraces and populations coming from Tuscany and Basilicata region. The frequent seed exchange practice occurring among farmers, following the transhumance pathways along the Apennine regions, could have influenced the genetic structure of the common bean populations. Results of the short-term salinity and osmotic stress response showed morphological and biochemical changes at organ level strictly related to the genotype and the stress typology, with a higher impact of salinity over osmotic stress, and allowed to identify common bean genotypes with marked "climate smart traits" (Monachella, Ciliegino and Tuvagliedda Rossa) due to increased amount of stress protective antioxidants (chlorophyll and carotenoids). Furthermore, experimental data reported a positive correlation between proline level and salinity stress sensitivity, suggesting the potential use of this osmolyte as a biochemical marker for a large-scale screening of salt sensitive bean landraces.

In the paper II (Falcione et al. 2024), a complementary characterization approach allowed to identify three Italian common bean landrace populations (Ciliegino, San Michele Rosso and Fagiolo d'Acqua) as a valuable source of diversity and bioactive chemical features. Results of this study showed seed morphological, genetic and phaseolin diversity among the three common bean populations. NMR seed metabolomic profiles revealed the presence of a vast number of metabolites, ranging from amino acids, sugars, organic acids, nucleosides, and other miscellaneous compounds. Furthermore, the low

levels of organic acids (lactic and pipecolic acid) found in Ciliegino seeds may have played a suppressive role in colon cancer cell proliferation, while the high levels of stress protective metabolites (citric acid, alanine, leucine and methionine) reported in San Michele Rosso may have contributed to counteract mid/long term salinity stress, both at seed and plant level. Data also showed that the stress mitigating compounds such as proline, sugars and carotenoids were not correlated with oxidative stress level (MDA) detected in leaf of all the three common bean populations, however, the structural reorganization in the main pigment protein complexes contributed to increase the photosynthesis efficiency of San Michele Rosso plants under salinity conditions.

In paper III (*in preparation*), a MS-based metabolomics has been performed to deep investigate the seed metabolome of two Italian red common bean landrace populations, Ciliegino and San Michele Rosso, and one from Spain, Anguiano bean. The preliminary results indicated that the populations present distinct metabolomic profiles, being separated in three distinct groups in the PCA and PLS-DA, with a higher accumulation of specific chemical categories, such as organic acids in Ciliegino and Anguiano bean and flavonoids in San Michele Rosso. These molecules could act as potential biomarkers of populations' distinctiveness and differentiation (metabolomic fingerprints). In a forward-looking vision of this work, the completion of the annotation process of the metabolite reference set will allow to define the chemical classes enriched in each genotype. Furthermore, a more in-depth analysis of these metabolites, in term of the corresponding biological significance and the possible involvement in the environmental stress tolerance, will enable to determine the local resources value in regard to nutritional, therapeutic and/or the abiotic/biotic stress resilience potential.

Taken together, the data of this thesis enabled to recognize local landraces as a reservoir of diversity, stress adaptive traits and valuable bioactive compounds, with possible beneficial effects for human health and for facing the challenges of the increasing climate variability. The conducted experimental activity provided clear evidence for the establishment of adequate conservation strategies to preserve common bean landraces and for supporting the socio-economic revitalization of the marginalized inland rural areas where these local resources are confined − all in full accordance with some "place-based" strategies proposed by the public policy National Strategy for Inner Areas (Italian SNAI), which plans to promote territorial development and economic and social regeneration of these fragile Italian territories.

Table of contents

List of publications

This thesis is based on the work contained in the following papers:

I. **A Multi-Level Approach as a Powerful Tool to Identify and Characterize Some Italian Autochthonous Common Bean (*Phaseolus vulgaris* L.) Landraces under a Changing Environment**

   Martina Falcione, Melissa Simiele, Alessandra Renella, Gabriella Stefania Scippa, Paolo Di Martino and Dalila Trupiano.
   (*2022*) *Plants*, 11: 2790.
   https://doi.org/10.3390/plants11202790

II. **Building the Resilience of Marginal Rural Areas Using a Complementary Characterization Approach: Possible Beneficial Health Effects and Stress Tolerance of Italian Common Bean (*Phaseolus vulgaris* L.) Landraces**

   Martina Falcione, Alessandra Renella, Vadym Samukha, Mayra Colardo, Melissa Simiele, Gabriella Stefania Scippa, Marco Segatto and Dalila Trupiano.
   (*2024*) *Diversity,* 16(4): 240.
   https://doi.org/10.3390/d16040240

III. **Metabolite Profiles of Common Bean (*Phaseolus vulgaris* L.) Landraces under the Spotlight of Mass Spectrometry-based Metabolomics**

   Manuscript in preparation

Other contributions

This list includes author’s contributions not related to the thesis work.

I. **Progetto Demetra. Buone pratiche per un’agricoltura sostenibile e multifunzionale.**

Lima Giuseppe, Mastronardi Luigi, Stanisci Angela, De Francesco Maria Carla, De Curtis Filippo, Del Grosso Carmine, Di Brita Aldo, Di Cristofaro Marco, Falcione Martina, Huamani Cahuas Claudia Fiorella, Marchese Lucia, Marino Stefano, Palmieri Davide, Pastorini Angelo Paolo, Renella Alessandra, Travaglini Tania, Persichilli Christian, Pilla Fabio, Sciarretta Andrea, Scippa Gabriella Stefania, Trupiano Dalila.
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1 Introduction

The Convention on Biological Diversity (CBD) defines the biodiversity as "the variability among living organisms from all sources including terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems" (Feest et al. 2010). Biodiversity plays an important role in sustaining human lives, by contributing directly (through provisioning, regulating, and cultural ecosystem services) and indirectly (through supporting ecosystem services) to many constituents of human well-being, such as security, basic material for a good life, health, good social relations, and freedom of choice and action. For this reason, beneficial effects of biodiversity affect multiple aspects of human life, going beyond to its purely contribution to provide material welfare and livelihood, and they are strictly dependent on healthy and well-functioning natural ecosystems able to provide adequate flows of ecosystem services (Millenium Ecosystem Assessment 2005).

Agricultural biodiversity, or agrobiodiversity, is a subset of biodiversity referring to genetic resources relevant to food and agriculture and to all the components of biological diversity that constitute the agricultural ecosystems. It represents the result of natural selection processes and century-old efforts of breeders and farmers, selecting, breeding and developing genetic resources and appropriate production systems and methods, able to sustain productivity and livelihood (FAO 1999; Allen et al. 2014).

Agrobiodiversity encompasses the variety and variability of animals, plants and micro-organisms that are necessary for sustaining the key functions, the structure and the processes of agro-ecosystems (FAO 1999). It exists at several levels, from the different ecosystems in which people raise crops and livestock, through the different varieties and breeds of the species, to the genetic variability within each variety or breed (Martinez-Juarez et al. 2015).

Agrobiodiversity comprises many types of biological resources tied to agriculture, including:

- domesticated crops and wild plants including trees (used for food and other products such as timber, fibre, fuel and medicine), domestic and wild animals (used for food, fibre, hides, furs, power and manure), and wild and cultivated fish and other aquatic animals (used for food or feed), within field, forest, rangeland and aquatic ecosystems;
- non-harvested species within agroecosystems, which support agricultural production and food provision, including soil micro-organisms, pollinators (bees and other insects) and beneficial predators;

- non-harvested species in the wider environment, which support the functioning of agroecosystems (agricultural, pastoral, forest and aquatic ecosystems) such as trees, field borders and fallow lands, able to sustain functions such as the water, soil nutrient and carbon cycles, and to provide shade and windbreaks, watershed and wildlife protection (FAO 2004; Thrupp 2004; FAO 2018).

Agricultural biodiversity is developed and actively managed by farmers, herders and fishers, therefore human activity is fundamental to shape and conserve this type of biodiversity, as many components of agrobiodiversity would not survive without this human interference. It represents the result of the interaction between the environment, genetic resources, management systems and practices used by culturally diverse people. As a consequence, traditional and cultural knowledge developed by local communities over ages can be considered as integral parts of agrobiodiversity (FAO 2004).

Traditional knowledge is defined as any indigenous knowledge, innovation, practice, custom or tradition that people in a given community have developed over time and continue to develop. Such knowledge is orally passed on from generation to generation and concerns plant and animal genetic resources as well as the quality of soil and water, which provide the basis for both the productivity and adaptability of agricultural systems (FAO 2004; Baul and McDonald 2014). Traditional knowledge is based and tested over centuries of experience and use and it is adapted to the local socioeconomic culture and environment. Moreover, this knowledge is dynamic and continuously changing, forming the basis of agroecosystems sustainability and resilience under a changing environment (Agnoletti and Santoro 2022).

Based on these premises, agricultural biodiversity, with its complex nature and multi-level diversity, provides economic, social and ecological benefits for humankind since it is central to human livelihood, global nutrition security, sustainable food systems, ecosystems and environmental health and climate change resilience, allowing to develop dynamic and flexible responses to the continuously evolving environments (Chaudhary et al. 2020).

For these reason, agrobiodiversity can significantly contribute in achieving several Sustainable Development Goals (SDGs) set by Agenda 2030 of United Nations, including “No poverty” (SDG1), “Zero hunger” (SDG2), “Good health and well-being” (SDG3), “Responsible consumption and production” (SDG12), “Climate action” (SDG13), “Life below water” (SDG14) and “Life on land” (SDG15) (https://sdgs.un.org/goals) (Blicharska et al. 2019; Bisht et al. 2020) (Fig. 1).

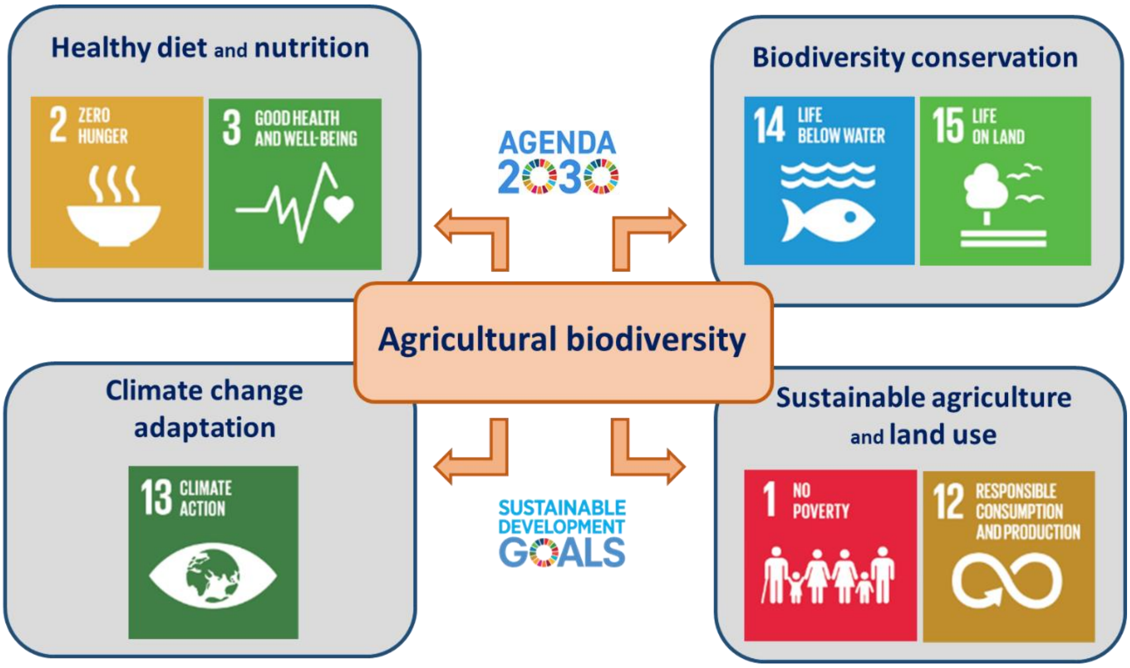


Figure 1. Agricultural biodiversity contributes to multiple sustainability dimensions and Sustainable Development Goals (SDGs) set by Agenda 2030 of the United Nations (Image produced by Martina Falcione).

However, in the last decades, bio- and agrobiodiversity are challenged by several anthropogenic factors, including climate change, habitat transformation, nutrient cycling alteration, pollution, over-exploitation of biological resources, change in land use and in consumption patterns, which are causing serious repercussions on nutrition and livelihood security, ecosystem and environmental health and on long-term sustainability of human well-being (Chaudhary et al. 2020).

The planetary boundaries of bio- and agrobiodiversity intactness have been already overshot and ecosystems, species, wild populations, local varieties, breeds of domesticated plants and animals are shrinking, deteriorating or vanishing at an unprecedented rate (Banks-Leite et al. 2020). A large number of animal and plant genetic resources are on the verge of extinction while many of them have been already extirpated from the agricultural systems before their full utilization was made, leading to the erosion of important gene pools and associated knowledge (Ruiz-Gonzalez and Vicente 2023).

To reverse the progressive decline of biodiversity and its genetic resources, two main conservation approaches could be implemented: in situ and ex situ conservation.

In situ conservation consists in the conservation of ecosystems and natural habitats and in the maintenance and recovery of populations of species in their natural surroundings and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties. This latter is called on-farm conservation and is part of wider in situ conservation. It involves the maintenance of landraces by farmers with traditional agricultural systems in fields and gardens (Maxted and Kell 2009; Marfil et al. 2015).

On the other hand, ex situ conservation refers to the maintenance of populations outside their native habitat; for plant genetic resources, this results in the conservation of plant material mainly in germplasm banks and botanical gardens (Quazi et al. 2021).

Unlike ex situ conservation, where the evolutionary and adaptive potential of populations are frozen due to the removal from their natural context, in situ conservation allows the continuous adaptation of species and landraces within their natural habitat, ensuring the preservation of the genetic variability during the dynamic interaction of target populations with biotic and abiotic factors of the changing environment (Galluzzi et al. 2010).

However, the adoption of a complementary strategy that incorporates both ex situ and in situ approaches is considered an ideal scenario to effectively conserve the gene pool of the target species (Marfil et al. 2015)

1.1 Plant landraces

Plant agrobiodiversity comprises all the plant genetic resources with actual or potential value for food and agriculture. These include cultivars, landraces (also called “farmer varieties,” “local varieties” “traditional varieties” or “primitive varieties”), crop wild relatives, ecotypes and genetic stocks. All these resources contribute to reach the world food security and nutrition, increase farming resilience and maintain sustainable biodiversity (Borromeo 2012; Salgotra and Chauhan 2023). A key component of these plant genetic resources is represented by landraces, which are defined as genetically variable and distinct populations of cultivated plants, lacking formal crop improvement and characterized by a specific adaptation to the environmental conditions of the area of cultivation and by a close association with traditional uses, knowledge, habits, dialects, and celebrations of the people who developed and continue to grow them (Pacicco et al. 2018; Canella et al. 2022).

Local landraces represent a source of unique traits derived from many years of adaptation to the original territory, enable to enrich biodiversity and maintain and stabilize agroecosystems in a sustainable way to make it functional. The continued cycles of local planting, harvesting and farmer selection performed over time resulted in highly adapted resources to local agro-environmental conditions and practices (Villa et al. 2005; Azeez et al. 2018). Furthermore, landraces host considerable high genetic variability between and within populations, constituting a valuable reservoir of genetic diversity that is interesting for future breeding programs, as well as for the development of new agricultural systems and new products. The cultivation of these resources in restricted geographical area allowed to establish a tight bond with traditions, customs knowledge, habits, and history of the territory of origin, representing an integral part of the local cultural heritage and contributing to build the local cultural identity (Marone et al. 2021).

Landraces are mostly grown by elderly farmers, running small farms or home gardens, using traditional agricultural practices developed over decades, even centuries, based on long experience, proven traditions and know-hows, and limited external energy inputs (Negri 2003).

Besides being adapted to specific agro-climatic conditions, these genetically variable germplasm also possess high adaptive capacity to thrive under harsh environments, displaying high resilience and providing provisioning services under non-optimal farming conditions, representing a trusted source to achieve yield stability and food security (Ficiciyan et al. 2018).

Due to their adaptability to unfavourable conditions, these resources are generally cultivated in marginal areas, remote and isolated territories presenting many environmental constraints. In these areas, traditional forms of agriculture are still being practised, and landraces constitute an important source of profitability for agricultural activities and economic development of these rural territories (Ahmadzai et al. 2022).

Furthermore, local varieties are characterized by a wide variability of chemical composition, representing a treasure-trove of bioactive compounds with multiple beneficial effects for human health and for adaptation to environmental constraints. The chemical and nutritional composition of landraces, as well as the unique organoleptic properties characterizing these resources, is determined by the long-time interaction between the genotype and the environment (Berni et al. 2018; Sans et al. 2018). Indeed, unlike the modern varieties, local landraces present peculiar phytochemical profiles, and besides showing higher amount of the most important contributors to flavour (sugars, acids, free amino acids and volatiles), they are also enriched of health-promoting compounds, which could play a key role in human wellness by supplying an enhanced nutrition.

Several Turkish lentil (*Lens culinaris* Medik.) landraces showed higher contents of minerals and oligoelements, such as Cu, Fe, Mn and Zn compared to the commercial varieties, as well as common bean (*Phaseolus vulgaris* L.) landraces showed higher levels of Se, Zn, with a significant increase also in linoleic acid content (Karaköy et al. 2012; Celmeli et al. 2018).

Moreover, higher content of tocopherol, an important plant antioxidant compound playing effective roles in abiotic stresses response and in prevention and reversal of various human diseases (Hasanuzzaman et al. 2014), was observed in the seeds of seven landraces of soybean (*Glicine max*

L.) in comparison with the modern cultivars developed by cross-breeding (Medina-Lozano et al. 2021). Furthermore, significantly higher levels of lutein, the most abundant xanthophyll in the photosynthetic apparatus of higher plants with a high antioxidant activity and diversified beneficial role in eye disorders, cancer, cardiovascular diseases and other human systemic conditions (Buscemi et al. 2018), were also found in three Italian landraces of carrot, especially in the Tiggiano Yellow-Purple landrace (Scarano et al. 2018).

Also, Jakobek et al. (2020) and Raigon et al. (2008) reported higher concentration of phenolic compounds, in particular flavanols and phenolic acids, in apple and eggplant (*Solanum melongena* L.) landraces compared to modern varieties, suggesting the potential role of these compound as antioxidant and anti-inflammatory in many crucial biological processes in plant and in human chronic diseases, such cancer, diabetes and cardiovascular and neurodegenerative diseases (Kumar and Goel 2019).

In many cases, compounds with nutraceutical potential are also involved in plant defence mechanisms against biotic and abiotic stress (Berni et al. 2018). Indeed, landraces are found to synthesize a myriad of secondary/specialized metabolites (SMs), which, besides acting as health-promoting compounds with potential positive effects on human health, they also play a crucial role in the interaction with the environment and in plant stress mechanisms (Elisa et al. 2022; De Rossi et al. 2023). Although SMs are not directly involved in the physiological processes of plant growth and development, they are essential for plant survival under stressful conditions, representing important components of the plant defence response (Alberghini et al. 2022). These natural products are volatile, semi-volatile and non-volatile compounds produced from common building blocks deriving from primary metabolic pathways, which can be ascribed to three major groups considering their biosynthetic pathways: nitrogen-containing compounds (cyanogenic glycosides, alkaloids and glucosinolates), phenolic compounds (phenylpropanoids) and terpenes (isoprenoids) (Balestrini et al. 2021).

These metabolites can be responsible of the major adaptation capacity to harsh pedo-climatic conditions and environmental pressures performed by local landraces compared to their commercial counterparts (De Rossi et al. 2023). Indeed, better adaptation of landraces to environmental cues were reported in many studies. Several Spanish barley landraces outperformed modern cultivars at low-productivity sites by reporting higher grain yield (Yahiaoui et al. 2014), as well as high grain yield, early phenology and lodging resistance were displayed by wheat landraces in semiarid and Mediterranean sites under low and high irrigation regime compared to modern cultivars (Frankin et al. 2021).

Moreover, Xiahong et al. (2011) asserted that altitude gradient did not affect agronomic characteristics of several Chinese landraces of rice, such as plant height, panicle length and number of effective panicles, whereas these agronomic traits significantly fluctuated in the modern varieties. Salinity stress slightly reduce or even increase fruit number in two Spanish tomato landraces compared to the commercial variety "Moneymaker" and, also, soluble solid contents, an important fruit quality indicator, was significantly higher in both landraces compared to "Moneymaker" under salt stress. Furthermore, ripe fruits of one of the two tomato landraces, were found enriched in lycopene, a noncyclic carotenoid acting as potent ROS scavenger under oxidative stress, both under control and salinity conditions (Massaretto et al. 2018). On the other hand, higher level of rust resistance in oat landraces in comparison to the modern varieties was reported by Sanchez-Martin et al. (2016), while the Italian landrace chickpea "Longano" was found unaffected by the widespread fungal pathogen *Fusarium oxysporum* f. sp. *ciceris* (Fierro et al. 2019).

Saponins, a heterogeneous class of molecules chemically referred to triterpene and steroid glycosides, are widely distributed in plant species and, besides encouraging plant immunity against a wide range of insect pests, pathogens and other herbivores, they impart bitter flavour to food plants, reduce the bioavailability of nutrients and negatively affect protein digestibility by inhibiting various digestive enzymes (trypsin and chymotrypsin) (Hussain et al. 2019).

Tannins, phenolic compounds of high molecular weight ranging from 500 to more than 3000 Da, are closely associated with plant defence mechanisms towards herbivores and insects. They are also known to interfere with dietary iron absorption and with digestion, by inhibiting the activities of trypsin, chemotrypsin, amylase and lipase, as well as phytic acid (Thakur et al. 2019).

Moreover, alkaloids, one of the largest groups of plant secondary metabolites, can act as defence compounds in plants, being efficient against pathogens and predators due to their toxicity; however, they are considered to be anti-nutrients because of their action on the nervous system, disrupting or inappropriately augmenting electrochemical transmission (Matsuura and Fett-Neto 2017).

Thus, although several plant specialized metabolites are largely recognized as defensive molecules against environmental stress, some of them, such as saponins, tannins, phytic acids, and alkaloids, also display undesirable antinutritional, anti-palatability or toxicity properties (Corso et al. 2020).

However, the levels of the anti-nutritional factors in most of food crops have been reduced probably through the selection process operated by farmers during the domestication, mainly oriented towards the elimination of the harmful/unwanted compounds in cultivated plants (Ku et al. 2020).
Therefore, achieving an appropriate equilibrium between reducing the anti-nutritional factors in plants/landraces and preserving the organoleptic qualities and the secondary metabolites' beneficial effects for human health and for the plant adaptation to environmental constraints represents a crucial aspect (El Houssni et al. 2024).

Indeed, local landraces, with their high adaptation to extreme and uncertain climatic conditions, together with their nutraceutical potential and ability to produce moderate but stable yields under low-input farming systems, constitute important natural resources with excellent climate change resilience, able to cope with the current global climate crisis, improve human health and promote world food security and sustainable development (Durodola 2019).

1.2 Marginalized inner areas as hotspot of agrobiodiversity

During the last decades, Italian territory have experienced rapid changes in land use and in social development models, leading to a massive expansion of urban settlements, primarily in flat, coastland and most accessible districts. This process had severe repercussions on the upland and mountainous areas located in Italian inland territories, which became excluded from the main national socio-economic development trajectories and underwent to a progressive process of marginalization and abandonment (Sallustio et al. 2018; Bertini et al. 2022).

These peripheral and marginal lands, known as inner areas, represent fragile territories, distant from the main centres of basic services supply, characterized by demographic decline and ageing process of the population, lack of accessibility to essential services and infrastructures, low socio-economic connectivity, and modest territorial competitiveness (Bertini et al. 2022) (https://www.agenziacoesione.gov.it/strategia-nazionale-aree-interne/?lang=en). These territories are formed by small or micro villages, mostly concentrated in Alpine and Apennine regions, with a strong rural identity and serious constraints for agricultural activities due to the harsh pedo-climatic conditions (slope and erosion phenomena) and the hydrogeological instability (Sechi et al. 2020; Bertini et al. 2022).

Although these areas suffer a state of decline and abandonment, they dispense exceptional agro-silvo-pastoral and cultural resources, including impressive landscapes, wild and agricultural biodiversity, ancestral knowledge, and strong cultural and social values, resulting from the long co-existence between humans and territory and able to provide many ecosystem services (Agnoletti and Santoro 2022).

In these remote territories, the presence of the valuable biocultural heritage, intended as the inter-connected combination of knowledge, biological diversity, landscapes and culture, could act as a driving force to activate a social, economic and cultural regeneration process, able to create new income opportunities, increase territorial attractiveness and promote a more equitable and balanced development between urban/"core" and peripheral areas (Scaramuzzi et al. 2023).

In this perspective, National Strategy for Inner Areas (Italian SNAI) is an innovative policy, launched by the Italian Government in 2012, aimed at promoting territorial development and cohesion to counteract marginalisation and demographic decline within "inner areas" throughout the country. In order to reach this goal, this policy relies on the implementation of tailored and "place-based" strategies to address inner areas' real needs, including the promotion and valorisation of local resources, such as local landraces, which could enhance the socio-economic development through territorial distinctiveness (Duglio et al. 2023) (https://www.agenziacoesione.gov.it/strategia-nazionale-aree-interne/?lang=en).

SNAI recognizes in Italy a total of 72 inner areas, covering more than 50% of the national surface and hosting 52% of Italian municipalities and 22% of its population.
Among them, "Alto Medio Sannio" is situated in mountainous and hilly areas of Molise region, where most of its territory is covered by rural areas (98%) and agricultural lands (63%), which comprises 33 different municipalities (Fig. 2)
(https://agriculture.ec.europa.eu/system/files/2023-04/rdp-factsheet-italy-molise_en.pdf;
https://www.agenziacoesione.gov.it/strategia-nazionale-aree-interne/?lang=en).
According to Boccardo (2017), the territory of Molise is endowed with a century-old history of agro-silvo-pastoral tradition, and cultivation of many autochthonous crops, including fruit, olive and grapevine has always represented the backbone of its rural economy, playing a major role in households' food and local communities income security.

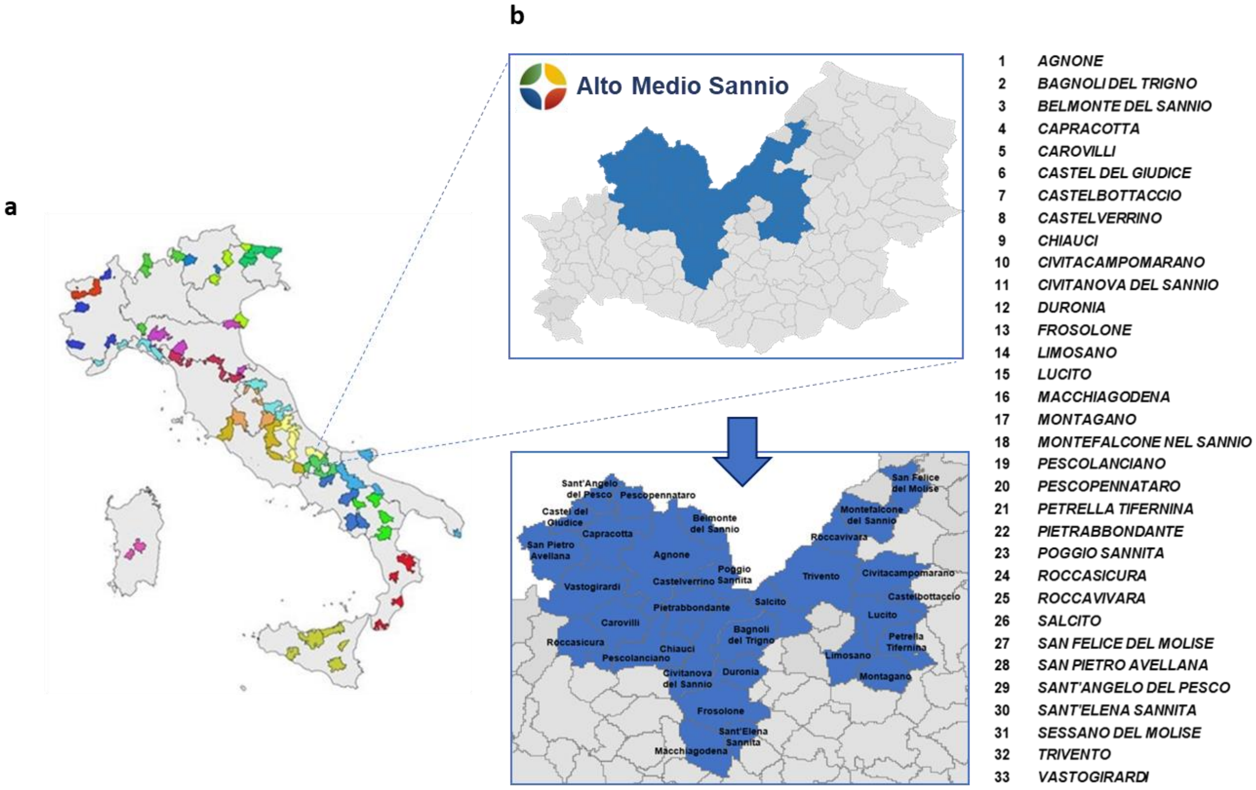


Figure 2. Italian inner areas. The 72 inner areas, recognized by the National Strategy for Inner Areas (SNAI), located throughout the Italian territory (a); "Alto Medio Sannio" inner area situated in Molise region with the list of its 33 municipalities (adapted from https://www.agenziacoesione.gov.it/strategia-nazionale-aree-interne/?lang=en) (b).

In this context, due to the presence of drove roads that served as vital natural connecting elements among several Apennine regions in previous centuries, the "Alto Medio Sannio" area was once inhabited by ancient populations primarily devoted to sheep farming, which gave rise to the first "transhumance society". In detail, this area is characterized by a large variability of natural landscapes, including high mountains, river valleys, hilly and high-altitude agricultural lands and pastures, and by a wide orographic diversity in a limited geographic area, ranging from 300 to 1730 m. a.s.l., which represents the highest peak of the territory. Valuable natural, cultural and historical resources enriched the area, along with many niche agri-food products, which, however, are not sufficiently valorised. In the last 40 years, "Alto Medio Sannio" underwent a progressive process of depopulation, leading to an increased level of the elderly population, which reached the highest percentage (27.7%) compared to the corresponding regional (23.5%) and national (21.2%) average reported in inner areas. Agnone is the official headquarter of the inner area and, along with Frosolone and Trivento, represent the most populated municipality, which dispense the majority of the basic services for the entire area. Agriculture and handicraft are regarded as the most profitable sectors of the local economy, which suffer from a lack of generational change (https://www.altomediosannio.it/download/molise-alto-medio-sannio-strategia/; http://old2018.agenziacoesione.gov.it/opencms/export/sites/dps/it/documentazione/Aree_interne/STRATEGIE_DI_AREA/Bozza_della_strategia/Bozza_strategia_Agnone.pdf).

This area, as most of peripheral regions and inner areas located in Mediterranean countries, often host many traditional agricultural settings, characterized by the presence of small-scale farms, mainly relying on the propagation of local resources with limited external energy inputs, and where the cultivation of local landraces by old farmers represent the primary source of subsistence due to their wide adaptability to the unstable climatic and soil conditions characterizing these territories (Lioi et al. 2005; Guiomar et al. 2018). From this perspective, landraces are considered suitable for the development of useful materials for sustainable agriculture. Indeed, the high resilience to environmental constraints displayed by landraces allows to guarantee higher provisioning services under non-optimal farming conditions and to ensure stable yield, food and livelihood security to local communities in these vulnerable areas (Montesano et al. 2012).
Furthermore, consumers tend to be attracted to food products that are differentiated from others by traits, qualities, or geographical origin. Therefore researchers, consumers, and policymakers are paying increased attention to traditional landraces. In this context, the assessment of the existing landraces diversity represents the first step of a characterization process focused on the preservation and possible exploitation of endangered plant genetic resources (Stagnati et al. 2022). Indeed, it is also fundamental to establish landraces value in terms of environment and human health “services”, both essential to translating them into competitive products and a prerequisite for envision appropriate strategies for their on-farm conservation.

Thus, the characterization, conservation, and valorisation of local resources dispensed by these marginalized inner areas, including local landraces, could successfully attain SNAI territorial cohesion objectives aimed at slowing down and reversing inner areas marginalization process, contributing to promote social, economic and cultural revitalization of these valuable, but too often neglected and abandoned, territories. Although the paramount importance, this locally-adapted germplasm is currently experiencing a progressive loss of genetic diversity, leading to the process of genetic erosion (Thanopoulos et al. 2024).

1.3 Genetic erosion of landraces

The term “genetic erosion” was coined for the first time by Food and Agriculture Organization of the United Nations (FAO) to describe the dramatic loss of genetic diversity affecting plant genetic resources. Genetic erosion has been defined as “the loss of genetic diversity, in a particular location and over a particular period of time, including the loss of individual genes, and the loss of particular combinations of genes such as those manifested in landraces or varieties. It is thus a function of change of genetic diversity over time” (Mathur 2011). The process of genetic erosion in plant genetic resources can happen at three different levels: at crop level, as an impoverishment in the assemblage of crop species used in agriculture, at the level of varieties (landraces and cultivars) of a specific crop, also known as varietal erosion, and at the level of alleles/genes (Wouw et al. 2010).

According to FAO, of the 25 000 known edible plant species, only 7 000 have been used in the history of humanity to meet food needs and no more than 150-200 are commercially cultivated. Furthermore, only three cultivated species (rice, maize, and wheat) account for fully 60% of the human energy intake (Padulosi et al. 2013). As a consequence, the world's food supply relies on a very small number of plant species, excluding many valuable plant genetic resources from agricultural production systems, which therefore becomes neglected, underutilized and more vulnerable to genetic erosion.

The crop genetic diversity found at the present time resulted from several important events occurred during the crop history (Fig. 3). Wouw et al. (2010) highlighted three major evolutionary bottlenecks which led to an enormous reduction in the genetic diversity of cultivated plants:

1) The first one occurred during the domestication process from the wild ancestors into the domesticated forms, as only a subset of the diversity in the progenitors found its way into the domesticated species (*domestication bottleneck*);
2) The second one happened during the dispersal of the crop, as only a few individuals became disseminated around the world (*dispersal bottleneck*);
3) The third one, occurred with the massive utilization of the modern cultivars that is causing the progressive landraces replacement and the genetic erosion (*modernization bottleneck).*

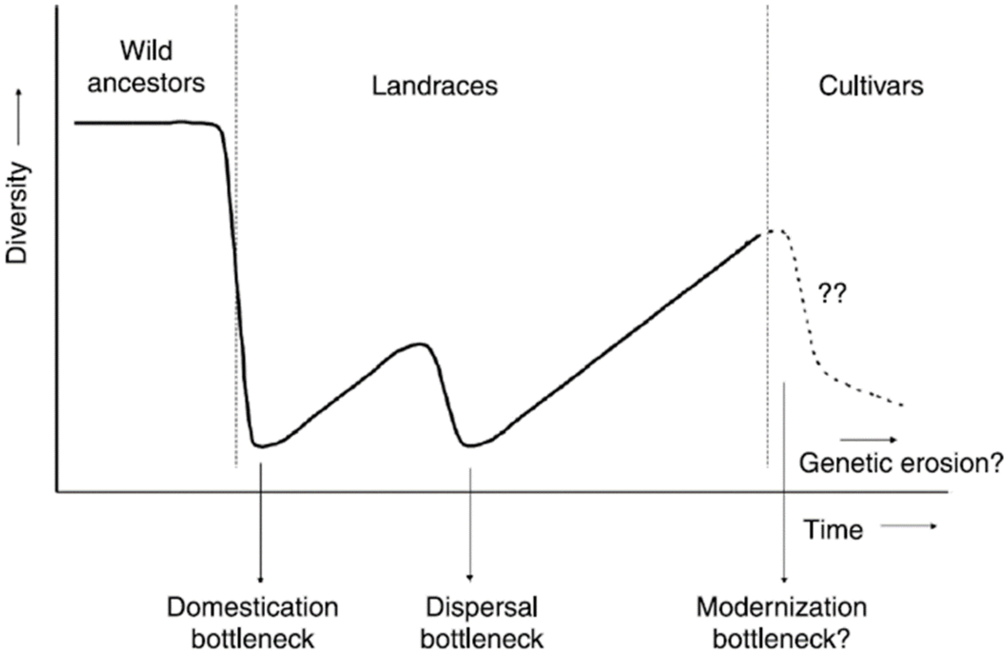


Figure 3. Trend of crop diversity from the wild ancestors until modern cultivars [Figure from Wouw et al. (2010)]

At the present time, the main cause of the genetic erosion in landraces is the replacement of these local varieties by improved and high-yielded modern cultivars (Singh et al. 2017), which are bred to be genetically uniform in order to maximize the production ability under those inputs that make the environment best suited for these crops (irrigation and fertilization) (Vetelainen et al. 2009).

Around 1950-1960, in order to address the challenge for feeding a growing global population and to generate economic stability and secure political alliances, the Green Revolution promoted the introduction of new modern breeding cultivars and the associated high-input agronomic practices, resulting in a gradually replacement of crop landraces and in the destruction of the habitats of their wild relatives (Khoury et al. 2022; Pathirana and Carimi 2022).

The higher provisioning services, such as crop yield, and the rapid, extensive and mechanized farming systems connected with the cultivation of modern varieties were preferred by farmers, inducing the abandonment of landraces cultivation, which were deemed to be old fashioned and unattractive in comparison to modern and exportable crops produced in much simpler production systems (Padulosi et al. 2013).

However, it is not just the widespread adoption of modern varieties that is regarded as the current cause of genetic erosion in landraces; indeed, the roots underlying this complex phenomenon are tied to several environmental, social and economic factors.

Genetic erosion is also caused by the urbanization and the massive migration of populations from rural areas to bigger urban centres, which resulted in abandonment of farmlands and marginal territories, with a consequent disappearance of these resources. Furthermore, changes in food preferences of a growing urban population and an increasing market and consumer demand for standardized products can be added to the drivers of the progressive loss of landraces (Wouw et al. 2010).

Other contributing factors are the ageing of rural populations, the lack of inter-generational transmission of traditional knowledge and agricultural skills and the low interest among young people towards landraces, and more in general towards traditional agriculture. Indeed, traditional farming practices applied to landraces, such as seed harvesting, cleaning, conditioning, and storing, are considered time-consuming from the young workers, which are more prone to get access to modern seed supply systems and to use rapid mechanized agricultural techniques (Negri 2003; Marone et al. 2021).

Moreover, climate change and environmental degradation resulted in change in cropping patterns, promoting the progressive use of uniform monocultures with great use of irrigation and chemical fertilizers, as well as natural disasters and protracted human conflicts induced large-scale displacement of farming communities, leading to the loss of all the traditional crop genetic resources used by the farmers involved (Pathirana and Carimi 2022).

The disappearance of landraces not only means local genetic erosion but also “local cultural erosion”, by which both biological and cultural evolution is hampered (Vetelainen et al. 2009). Indeed, traditional knowledge associated with local landraces represent important models of environmental resilience and sustainability, besides providing cultural, symbolic importance and identity for local communities (Agnoletti and Santoro 2022).

Many studies reported the wide-spread genetic erosion affecting local landraces in several geographic areas.

A drastic reduction of traditional maize landraces in the state of Morelos, Mexico, during a 50-year period (1967-2017) was reported by McLean-Rodriguez et al. (2019). After the introduction of improved maize cultivars, of the 93 Mexican maize landraces accessions first collected in the 1967 and stored at International Maize and Wheat Improvement Center (CIMMYT) Maize Germplasm Bank, only 14 were conserved by local families under constant cultivation, while the remaining were definitively lost.

Similar trends were found for Kam Sweet Rice (KSR), a Chinese rice landrace that has been cultivated for thousands of years in Guizhou, Hunan and Guangxi Provinces of China, and particularly appreciated by local communities for its unique qualities, including well glutinous features, strong resistance to diseases, pests and to adverse abiotic conditions. Field research interviews conducted among farmers demonstrated that the KSR local varieties decreased more than 50% in the investigated villages compared to the past 10–20 years (Wang et al. 2018).

Moreover, Gadissa et al. (2021) assessed the extent of on-farm diversity and genetic erosion of barley landraces in Bale highlands, Ethiopia. Results showed that, from the total of 25 barley landraces recalled by farmers during the field survey, only 11 are grown at present time and the remaining 14 were totally eroded from the districts where they had previously been cultivated, reporting a high genetic erosion rate (56.0%).

Furthermore, significant decline in the number of landraces cultivated was reported also in Northwest Syria between 1999 and 2019: forage, legume, cereal and vegetable landraces varieties were sharply decreased by 67, 52, 50 and 48%, respectively, mostly due to the competition with commercial hybrids and the falling market demand (Darvish et al. 2022).

Considering the dramatic process of genetic erosion which is hampering the landraces diversity, effective conservation strategies and characterization activities should be implemented in order to realize the full potential of these endangered genetic resources, which can significantly affect the landrace added value, promote their maintenance on the territory and prevent their definitive loss (Thanopoulos et al. 2024).

1.4 Characterization and valorisation of germplasm diversity

Crop plants are characterized by an amazingly great diversity of morphologic, genetic and biochemical traits, resulted by the continuous selection practiced by farmers to meet their basic needs and cultural beliefs and by the adaptation process to the geographic, climatic and edaphic factors of the cultivation sites (Zengele 2018). An adequate knowledge of the existing diversity among plant genetic resources is crucial for their effective management, conservation, and utilization (Mondini et al. 2009).

In particular, for local landraces, performing efficient characterization activities should be considered a high priority in order to explore the wide diversity retained by these neglected and untapped resources, providing precious information which can be helpful to: I) promote the survival of these endangered resources; II) define priorities for conservation programs; III) broaden the genetic base of crop improvement for the development of the “next generation” varieties (Scippa et al. 2008; Kyratzis et al. 2019).

Furthermore, it is a matter of primary importance to produce “quality marks” for landrace resources, which could guarantee long-term conservation through their continued use.
A full characterization of landrace germplasm represents the first step to obtain community recognition and denomination awarded by the European Union, such as “Denomination of Protected Origin” (DOP), “Indication of Protected Origin” (IGP) and/or other specific attestations, which could definitely help in increasing the direct valorisation of landrace products in local markets by adding value, which, in turn, makes landrace cultivation attractive and promote their continued in situ maintenance (Mennella et al. 2003; Raggi et al. 2020).

Considering the goal of the biodiversity preservation, germplasm resources can be characterized at distinct levels and by using different analytical approaches.

1.4.1 Morphological, biochemical, and genetic characterization

The morphological characterization represents the first step for an efficient utilization of genetic resources and allows the assessment of the phenotypic features that contribute to the entire diversity in a germplasm collection (Boutsika et al. 2021). The analysis of highly heritable and internationally accepted descriptors, defined by the International Plant Genetic Resources Institute (IPGRI) and by the Union for the Protection of new Varieties of Plants (UPOV), is an important and commonly used practice for germplasm identification, resulting to be highly informative and allowing an efficient discrimination among accessions (Athinodorou et al. 2021; Marzario et al. 2023). However, difficulties in making these measurements objective and repeatable are evident.

In the last decades, electronics and computer science have created possibilities to replace human visual assessment and reduce the potential subjectivity in phenotypic data acquisition (Venora et al. 2007). Since then, significant increase in computer-assisted image analysis applications has been highlighted in the plant biology research field. Morphological characterization through automatised image analysis represents a reliable, time-effective, and non-destructive method to investigate the diversity among plant species, providing accurate, repeatable, and objective measurements (Lo Bianco et al. 2015). Indeed, several studies proved the importance of using software-assisted image analysis for the morphological characterization of cultivated species, such as *Cucumis melo* L. and *Malus domestica* (Suckow) Borkh. (Sabato et al. 2015; Sau et al. 2019), and for the identification of unknown plant seed material in archeobotany (Orrù et al. 2012; Ucchesu et al. 2017). Moreover, this powerful approach has been successfully applied for the identification and discrimination of legume landraces (*Lens culinaris* Medik., *Phaseolus vulgaris* L., *Pisum sativum* L.), by evaluating several seed morphometric and colorimetric features (Venora et al. 2007; Venora et al. 2009; Smykalova et al. 2011).

However, a diversity analysis solely based on morphological evaluation of germplasm is biased due to the environmental influence on phenotypic traits, which could invalidate the objectivity and reliability of the analysis (Figàs et al. 2018; Stavridou et al. 2021).

In addition to morphological diversity analysis based on phenotypic features, biochemical marker analysis represents an effective alternative marker technique used to assess diversity among germplasm collections (Singh et al. 2023).

Before the DNA revolution, biochemical markers were the first molecular tools to be used to evaluate the degree of genetic variability and species relationships. Besides being fast and cost-effective, the use of biochemical marker requires only small amounts of biological material, and it involves the investigation of enzymatic (isoenzymes and alloenzymes) and non-enzymatic proteins (storage proteins) (Mondini et al. 2009; Zahoor et al. 2022). Biochemical markers can unveil the polymorphisms of amino acid sequences of specific proteins as well as indirectly detect polymorphisms of the nucleotide sequences of the genes from which they are translated. Alternatively, some protein variations may appear due to post translational modifications. As a result, electrophoresis-based protein banding patterns provide important information regarding the genetic similarities and differences within and between different species, proving the usefulness of this method to assess genotypes' variability (Kumar et al. 2018; Singh et al. 2023).

In particular, phaseolin, the major seed storage protein of common bean (*Phaseolus vulgaris* L.), could be used as a biochemical marker to evaluate common bean populations diversity and to identify the belonging gene pool (Silva et al. 2010).

This trimeric glycosylated protein belongs to the 7S vicilin class and accounts for the 50% of the total seed proteome. Its molecular mass ranges from 45 to 51 KDa and it is encoded by a gene family of 6-10 tightly linked sequences organized on chromosome 7. In detail, members of the phaseolin gene family can be subdivided into two different gene subfamilies named α and β, encoding for α- and β-type polypeptides, respectively. Phaseolin exhibits polymorphisms in its sequence, resulting in a variation of its electrophoretic pattern. Consequently, the genetic variability of phaseolin sequence makes this protein a valuable and informative biochemical marker in studies on bean genetic diversity and evolution (Mariniello et al. 2007; López-Pedrouso et al. 2014).

In detail, phaseolin variation revealed that common bean diversity is organized into two main eco-geographical gene pools: the Mesoamerican (from Mexico to Colombia) and the Andean (from Perù to Argentina) gene pool. Indeed, these two gene pools show distinct phaseolin types named after the genotypes in which they were first discovered: common bean accessions from Mesoamerica exhibit "S" (Sanilac), "M" (Middle America), or "B" (Boyaca) phaseolin types, while Andean beans present "T" (Tendergreen), "C" (Contender), "H" (Huevo de Huanchaco), "A" (Ayacucho), "J" (Jujuy) or "I" (Inca) phaseolin banding patterns (De La Fuente et al. 2012).

Based on this assumption, phaseolin represents a reliable biochemical marker to assess the genetic differentiation and structure of common bean populations, proving its useful application in studies of domestication and dispersion of bean varieties (Nikolić et al. 2007).

However, biochemical markers are able to screen only a very small expressed genome portion and sometimes, within cultivated species or varieties, their polymorphism is rather low (Kumar et al. 2012; Kumar et al. 2018).

The limitations associated with morphological and biochemical markers are overcome by molecular markers, which reveal differences at the genotypic level without being influenced by the environment. Furthermore, analysis can be performed using DNA from any growth stage (Kumar et al. 2012). Molecular markers have revolutionized and modernized the ability to characterize plant genetic resources, proving to be effective and reliable tools to assess genetic variation within as well as genetic differentiation among populations, playing critical roles in evaluation of germplasm collections and in phylogeny, species evolution and molecular plant breeding studies (Barcaccia 2010).

Currently, a wide range of different molecular markers as are used for the analysis of plant genetic diversity and population structure, which can be classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. However, in the last decades, due to the advancements in molecular techniques and to the high-speed of the PCR analysis, the use of PCR based markers increased tremendously (Idrees and Irshad 2014).

Among the PCR-based methods, ISSR (inter simple sequence repeats), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), SSR (simple sequence repeats) and SNP (single nucleotide polymorphism) are regarded as the most widely used genetic markers. However, ISSR markers represent a simple and quick method that overcomes most of the disadvantages of the other techniques, such as the high cost of AFLP, low reproducibility of RAPD and prior information to develop specific primers for SSR polymorphisms (Henareh et al. 2016). In particular, ISSR molecular markers have been extensively used to investigate the genetic biodiversity and structure of many common bean germplasm collections constituted from few to tens of accessions (Galván et al. 2003; Svetleva et al. 2006; Marotti et al. 2007; Pena et al. 2014). This marker involves the amplification of DNA sequence between adjacent and inversely oriented microsatellites and usually show high polymorphism. Furthermore, abundance of microsatellites in genomes and their hypervariability among individuals of the same as well as different species ensures the utility of ISSR markers to obtain a reliable estimation of germplasm genetic diversity and effectively assist conservation strategies (Grover and Sharma 2016).

1.4.2 Characterization of plant stress response under a changing environment

In present context of climate crisis, assessing the environmental stress response profile of plant genetic resources represents an important prerequisite to identify potential genotypes with stress tolerant traits which can withstand the diverse agroecological conditions and easily adapt to future climate scenarios, prioritizing the conservation strategies for this next generation germplasm (Dwivedi et al. 2016).

Indeed, during the last two decades, the world has experienced an abrupt climate change, mostly caused by anthropogenic pressures, which increasingly threaten the viability and resilience of natural ecosystems (Malhi et al. 2020). Mediterranean basin has been identified as one of the most vulnerable regions to climate change globally and, in particular, salinity and osmotic stresses currently represent the most common environmental factors that hampers biodiversity and agrobiodiversity of this area (Kim et al. 2019; Alzahib et al. 2021). Indeed, in the Mediterranean area, 25% of cropland is affected by moderate-to-high salinization, mostly due to land desertification and improper irrigation practice, causing an excessive accumulation of salts on soil surface (Tomaz et al. 2020).

Stress conditions induce morphological, physiological and biochemical alterations in plants (Zia et al. 2021). A wide range of parameters could be evaluated in order to deeply understand the mechanisms underlying plant stress response patterns. Among them, indicators of plant growth and developmental (e.g. germination percentage and accumulation of biomass) represent the most prevalent features taken into account to evaluate plants stress response. Indeed, salinity and osmotic stress can cause irreversible damage to photosynthetic apparatus due to several factors, including excessive accumulation of ions (Na^+ and Cl^-) in tissues, decreased CO_2 supply due to osmotic stress-induced hydrostatic stomatal closure, ultrastructural changes in chloroplasts, damages to the oxygen-evolving complex (OEC), limited electron flow from PSII to PSI and impaired chlorophyll biosynthesis or accelerated degradation (Hnilickova et al. 2021; Vineeth et al. 2023). As a consequence, all these processes have severe repercussions on biomass accumulation, plant growth and production at all life stages. Also seed germination, the first stage of the plant's cycle and a critical phase for crop yield and establishment, is hampered by salt and osmotic stress. The excess of toxic ions (Na^+ and Cl^-) and the associated osmotic stress disrupt key biochemical processes, energy production, respiration and the nutrient and hormone balances resulting in reduced, delayed, and sometimes inhibited seed germination and early-stage plantlet establishment (Hmissi et al. 2023).

The ability of plants to maintain an appropriate water status is crucial for their growth and survival in water-deficient environments (Yan et al. 2023). In this regard, Relative Water Content (RWC) is an important criterion of plant water status, and its evaluation could provide indications regarding the balance between absorbed water by plant and consumed through transpiration (Soltys-Kalina et al. 2016). Changes in water potentials, caused by salinity and osmotic stress, could generate disruptions in normal cellular activities and accumulation of compatible osmolytes, such as proline, glycine betaine and soluble sugars in plant cells, which can regulate osmotic pressure, resulting in decreasing of the cell osmotic potential and thus in maintenance of water absorption and cell turgor pressure under stress conditions (Ranganayakulu et al. 2013; Behzadi Rad et al. 2021). One osmolyte that has received much attention is proline; its accumulation was reported in many plant species under diverse abiotic stress conditions and its benefits might outweigh its role in osmotic adjustment due to its ability to stabilize macromolecules structures and to scavenge reactive oxygen species (Signorelli et al. 2014). Likewise, sugars, besides playing a multifaceted role in plant growth and development (e.g. germination, photosynthesis, and flowering senescence processes), they represent essential components of abiotic stress tolerance in plants. Indeed, their accumulation has been widely reported as a response to abiotic stresses in plants, being involved in stress signalling transmitting systems, osmoprotection and regulation of the equilibrium between the generation and scavenging of reactive oxygen species (ROS) (Sami et al. 2016). Indeed, overproduction of reactive oxygen species (ROS) is frequently reported in stress conditions, causing high rate of lipid peroxidation in the cell with increased levels of malondialdehyde (MDA), which is considered to be one of the final products of membrane peroxidation and a reliable marker of oxidative stress and membrane damage (Hasanuzzaman et al. 2020).

Therefore, the joint analysis of several plant morpho-physiological indicators under stress conditions could provide a comprehensive interpretation of plant stress response patterns, obtaining important insights regarding the potential adaptability of these resources to climatic variables.

1.4.3 "-omics" characterization approaches

During the last decades, the rapid advances have made high-throughput technologies available for the study of biological systems, laying the foundation for the development of the so-called "-omics" era (D'Argenio 2018). In particular, the current upsurge in omics-based technologies have transformed the traditional toolkit for the biodiversity characterization (De León et al. 2023), thus, many "omics" fields have been proposed by researchers to characterize the huge and complex diversity of germplasm.

The development of the Next Generation Sequencing (NGS) technologies has allowed the massive production of whole genome/transcriptome sequence data, which in turn enables a more in-depth exploration and characterization of plant genetic resources diversity (Lee et al. 2014). According to the methodologies applied and the outcomes to achieve, the field of genomics (the complete set of genetic material within an organism) can be subdivided into a number of areas: structural genomics, which encompasses the creation of high-resolution genetic and physical maps; comparative genomics, which involves the comparison of two or more genomes to uncover both their similarities and differences; functional genomics, which involves the functional characterization of genes and their interactions with other genes in a regulatory network (Kamali and Singh 2023). Furthermore, the term "genoplasmics" has been coined in order to indicate a new cross-disciplinary research field that seeks to apply the principles and techniques of genomics to germplasm research. In detail, analysis of the genomes of plant genetic resources represents a key tool to evaluate the diversity and relationships among collections, also providing novel insights into candidate genes associated to environmental stress tolerance (Henry 2014; Jia et al. 2017).

Also, transcriptomics, encompassing the comprehensive analysis of all RNAs transcribed by a cell or tissue, including both coding and non-coding RNA at a specific functional state, plays a crucial role in germplasm characterization by providing valuable insights into whole-genome expression patterns. The NGS-based RNA sequencing (RNA-Seq) profoundly impacted the gene expression profiling, enabling the detection and quantification of known, novel and less abundant transcripts (Kamali and Singh 2023). This innovative approach allows to simultaneously identify mutations, sequence variations, and alternative splicing variants, as well as it provides an overall view of the dynamic changes in gene expression in response to different environmental stimuli, allowing to profile the differentially expressed genes (DEGs) under different physiological conditions, discovery novel genes associated with stress signalling/mitigation and offer potential traits for improving stress tolerance in germplasm collections (Pan et al. 2020; Miao et al. 2021).

Other important components of "omics" sciences are represented by plant epigenomics, phenomics, metabolomics and proteomics, which have increasingly become the main stream in plant biology research and in germplasm characterization activities.

Epigenomics is defined as modifications in gene expression that are not associated with changes in DNA sequence but with certain epigenetic mechanisms including cytosine DNA methylation, histone modifications, and small RNA-mediated methylation, able to alter chromatin structure and eventually modulate gene expression (Chandana et al. 2022). There is emerging evidence that epigenetic variants of functional and/or agronomic importance exist in many plant genetic resources' gene pools (Varotto et al. 2022). Epigenetic variations have emerged as important regulatory components of several aspect of plant physiology, being involved in the control of plant developmental processes and participating in shaping phenotypic plasticity to the environment. Indeed, several studies indicated that modification of DNA methylation patterns may facilitate ecological adaptation and might represent a means for plant organisms to cope with environmental stress. Therefore, deepening our understanding into the epigenetic mechanisms and the epigenetic diversity involved in plant adaptation can be valuable in exploring plant climate resilience and also in supporting future agricultural production challenges (Gallusci et al. 2017).

Phenomics constitutes a multidisciplinary field that enables the comprehensive characterization of phenotypic, agronomic and physiological properties of crops by using robust high-throughput tools for data capture and processing, such as innovative sensor- or image-based instruments (Nguyen and Norton 2020).

Metabolomics represents one of the fastest developing branches of the "-omics" approaches, which involves the simultaneous analysis of all metabolites in a given biological system through sophisticated analytical platforms (Marchev et al. 2021). The performance of metabolomic approach depend on the methodologies and instruments used to comprehensively identify and measure each metabolite (Patel et al. 2021). Indeed, due to the high diversity of metabolites in terms of molecular weight, chemical stability, solubility, and polarity, there is no ideal method to allow the extraction and detection of the entire cellular metabolome. Conventional extraction methods (maceration, soxhlet, etc.) require polar solvents, such as ethanol, methanol, water, methanol–water mixtures to extract mostly polar components, while non-polar ones, such as chloroform, ethyl acetate or hexane, are used to extract all lipophilic components (Duportet et al. 2012). In recent years, these methods have been replaced by advanced techniques (ultra-sonication, microwave, and supercritical methods), which have several advantages including reduced use of solvents and extraction times (Zengin et al. 2019; Bitwell et al. 2023).

Also, the recent developments in chromatographic separation technologies, able to facilitate analysis of a broader range of metabolites with different physiological properties, have helped to reach the simultaneously detection of several metabolites through the application of more than one analytical technique (McCullagh and Probert 2024). Spectroscopic and hyphenated analytical techniques are generally used for metabolomics analysis, such as liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectrometry (MS) and can be performed applying two different approaches: untargeted metabolite profiling (related to metabolic fingerprinting), allowing the global detection of all metabolites in a biological sample, and targeted metabolite profiling, focussing the investigation on predetermined groups of specific compounds (Marchev et al. 2021). In detail, the analysis of mostly volatile organic compounds and lipids is carried out using GC–MS, while the majority of semi-polar metabolites are analysed using LC–MS (Zeki et al. 2020). On the other hand, besides HILIC (Hydrophilic Interaction Liquid Chromatography) is now well established as the LC mode most applied for separating polar metabolites, these chemical compounds, together with ionic metabolites, remain some of most difficult to characterise (Lioupi et al. 2023). This novel technique has emerged as a scientific breakthrough of plant sciences due to its holistic and comprehensive view of the chemical composition of plant tissues and cells, striking a shift from the conventional phytochemistry or biochemical estimations of only selected core metabolites (Barh et al. 2015).

Plants are well known as factories producing plenty of structurally and functionally different natural compounds, the number of which is estimated to range from 200 000 to 1 000 000. These metabolites play pivotal roles in mediating plant environmental interactions and stress adaptation, besides holding health-promoting attributes with potential beneficial roles in the treatment of several human diseases (Sun et al. 2021; Wu et al. 2023). Metabolomics, with its new and advanced approach, could allow to uncover and explore the extensive plant phytochemical variability, leading to the identification of robust biomarkers with different potential applications, enable to assist the germplasm diversity characterization and establish its nutritional quality and/or environmental stress adaptive skills, allowing to set up appropriate valorisation strategies to promote its use (Marchev et al. 2021; Girelli and Angilè 2023).

Indeed, metabolomics represent a powerful tool for the characterization of germplasm collections since it allows the assessment of metabolomic fingerprint for each sample tested, investigating the phylogenetic relationships among the taxa. It is also able to differentiate plant species varieties within the same genus as well as distinguish different cultivars/landraces of the same plant species through the identification of unique compounds, which act as potential chemotaxonomic markers for plant individual discrimination (Yuk et al. 2013; Fayek et al. 2021).

As examples, this approach has been successfully applied to differentiate 63 *Urtica* accessions (Farag et al. 2013), several important medicinal herbs belonging to *Echinacea* spp. (Frédérich et al. 2010), also it was able to shed further light on the intricate intraspecific relationships within *Cucumis melo* (Moing et al. 2020) and proved a significant divergence between wild and cultivated accessions of watermelon and maize (Xu et al. 2019; Yuan et al. 2021).

More recently, the analysis of plant metabolomic fingerprint has been applied also for the characterization of germplasm according to its geographical origin, with extensive application in the field of food traceability and authentication (Cassago et al. 2021). Indeed, the large diversity of primary and specialized secondary metabolites supplied by plant genetic material makes these resources highly amenable to the search for chemical unique identifiers and metabolic markers of food authenticity (Erban et al. 2019).

Therefore, it protect consumers and honest producers from food frauds, assessing also the commercial value of the plant genetic material endowed with characteristic nutritional and sensory attributes, strongly linked to their geographical origin as well as traditional know-hows (Kang et al. 2008; Longobardi et al. 2017; Llorach et al. 2019). Thus, metabolomics has turned into a fast and reliable analytical tool to estimate the germplasm nutritional quality and the stress adaptive traits, elucidating its vast chemical arsenals with multiple functional properties, including health-promoting molecules and stress defense-related compounds, which could be used as a foundation to incentivize their valorisation activity (Carrera et al. 2021; Hernández-Guerrero et al. 2021).

In this context, numerous metabolomics-based investigations revealed the presence of a vast numbers of nutraceutical and health-promoting compounds in plant genetic resources, proving to be valuable source of key functional components (Darwish et al. 2022; Ali et al. 2023; Anju et al. 2024). Likewise, metabolomic approach has been applied to elucidate the seed chemical profile of several common bean (*P. vulgaris* L.) varieties, revealing the presence of important and health-beneficial metabolites, including pipecolic acid (Pip) and GABA (Gamma-Aminobutyrate), which act as a chemopreventive compound and regulator of blood pressure, pain and anxiety, respectively, and omega-3 and omega-6 unsaturated fatty acids, exhibiting renowned anti-inflammatory activity

(Samukha et al. 2024) . Moreover, higher accumulation of saponins, exerting radical scavenging, anti-mutagenic activity and prevention of colon cancer proliferation (Bulut et al. 2023), as well as increased amount of syringic acid (phenolic compound), with antioxidant, antimicrobial and anti-inflammatory properties (Subramani et al. 2022), were detected by metabolomic analysis in common bean seeds.

Considering that environmental stress conditions cause innumerable transformations in plant metabolisms, leading to the accumulation of specialized metabolites, metabolomics could unveil or shed light also on their potential roles in plant stress response mechanisms(Carrera et al. 2021). Indeed, metabolomic studies conducted on several stress-susceptible and stress-tolerant cultivars, besides confirming the proven role of several compounds (e.g. osmoprotectants) in the stress response, they identify new chemical components with a strategic role in the stress mitigation process, enhancing the comprehension of plants' ability to withstand stress conditions and highlighting the promising ability of these plant resources to address climate change adaptations (Carrera et al. 2021; Manickam et al. 2023; Wu et al. 2023).

In common bean (*P. vulgaris* L.), an ultrahigh performance liquid - chromatography tandem mass spectrometry (UPLC-MS) - based metabolomic approach allowed to identify a total of 26 differentially accumulated metabolites between sensitive and tolerant genotypes exposed to terminal drought stress. Among these compounds, pyroglutamic acid, eriodictyol (flavonoid), cyanidin 3-sambubiosid, anthocyanin pigments and xanthine were found highly accumulated in stress tolerant genotypes compared to sensitive cultivars, playing an influential role in drought stress mitigation, as they are reported to enhance photosynthesis, display antioxidant effects and maintain osmotic balance (Subramani et al. 2022).

Metabolomic analysis was also carried out to dissect the chemical profile variations in common bean resistant and susceptible genotypes incited by rust (*Uromyces appendiculatus*), one the main plant pathogen causing substantial crop yield losses in several areas worldwide. This potent approach, beside detecting key metabolites triggered by rust infections in both genotypes, including flavonoids, terpenoids, alkaloids, and lipids, it enabled to highlight specific enriched metabolites in resistant genotypes, such as aconifine, D-sucrose, galangin and rutarin, which act as a defence compound against the rust pathogen (Makhumbila et al. 2023).

Moreover, metabolomic analysis uncovered the role of several metabolites in common bean cultivars exposed to cold stress. In detail, methionine and several flavonoids, such as naringenin-7-O-glucoside, were clearly upregulated in cold-tolerant common bean samples under chilling stress, compared to the cold-sensitive counterparts, suggesting their pivotal role in alleviating the stress-induced detrimental effects (Yang et al. 2023).

Along with metabolomics, proteomics has hugely contributed to explore the germplasm diversity, representing a potent tool that complements the biodiversity analysis of plant genetic resources (Ialicicco et al. 2012). It provides a snapshot of the complete set proteins present within a defined biological setting at a given time and under given conditions, giving access to cellular metabolism at the level of proteins and allowing to obtain significant insights on biochemical properties, functional roles and structural changes, such as post-translational modifications (PTMs), of proteins during plant growth and in response to internal and external stimuli (Eldakak et al. 2013; Vailati-Riboni et al. 2017; Smolikova et al. 2020).

This technique is known as an efficient method to simultaneously resolve a vast number of proteins and, when coupled with mass spectrometry (MS), it can allow the identification and characterization of selected proteins. More specifically, proteome analysis could be performed by using two different approaches: gel-based and gel-free proteomics. In the gel-based proteomics, two-dimensional polyacrylamide gel electrophoresis (2-DE) allows the resolution and visualization of thousands of different proteins on a single gel, while their detailed identification is performed by using mass-spectrometry (MS). However, this gel-based approach shows some disadvantages, including labour intensiveness, inability to characterize complete proteomes and insensitiveness to low-abundance proteins. Therefore, in recent years, most developmental endeavours have been focused on alternative approaches, such as promising gel-free proteomics, where liquid chromatography (LC) ensures efficient protein separation and advanced MS systems provide more sensitive and accurate protein identification and quantification (Eldakak et al. 2013; Jain et al. 2019).

Currently there is no single method which can provide qualitative and quantitative information of all proteins profiling. Indeed, the progress of proteomics has been driven by the development of new technologies for peptide/protein separation, mass spectrometry analysis, isotope labelling for quantification, and bioinformatics data analysis. Mass spectrometry has emerged as a core tool for large-scale protein analysis.

For example, in shotgun proteomics (bottom-up strategy) complex peptide fractions (generated after protein proteolytic digestion) can be resolved using different fractionation strategies, which offer high-throughput analyses of the proteome of an organelle/cell/tissue/organ and provide a snapshot of the major protein constituents. With a similar procedure, in which different protein or peptide fractionation techniques can be applied prior to MS analysis and database searching, the top-down method measures the intact proteins while the middle-down strategy analyses larger peptides resulted from limited digestion or more selective proteases (Zhang et al. 2013) (Fig. 4).

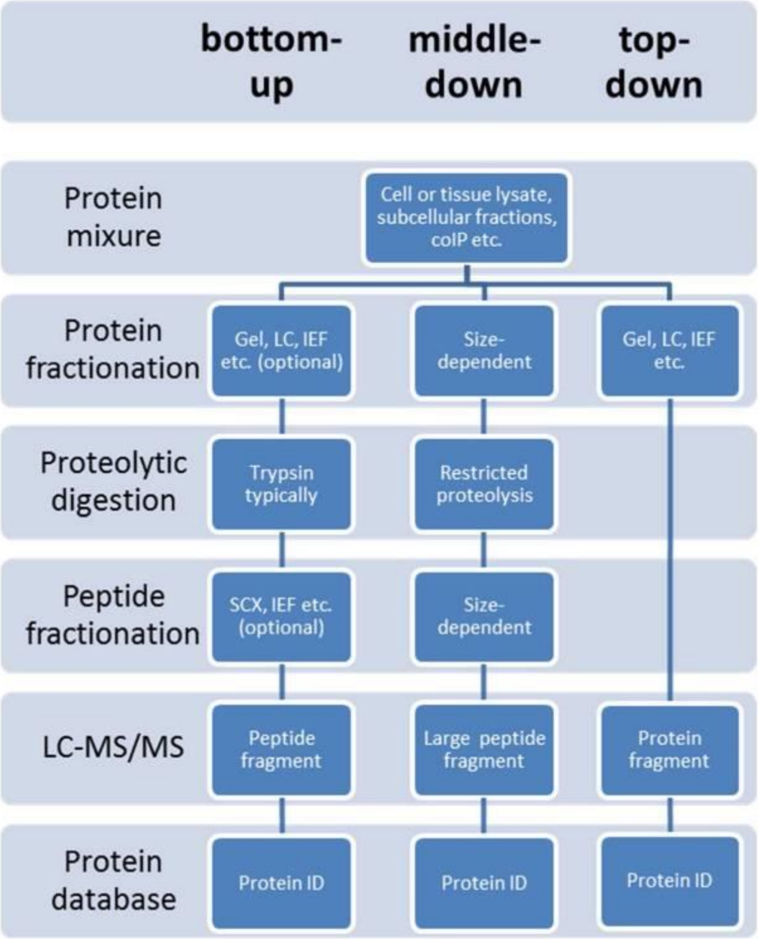


Figure 4. Different gel free proteomic strategies: bottom-up vs. top-down vs. middle-down. The bottom-up approach analyses proteolytic peptides, the top-down method measures the intact proteins, while the middle-down strategy analyses larger peptides resulted from limited digestion or more selective proteases [Figure from Zhang et al. (2013)].

However, all these approaches allow the identification as well as the quantification of differentially expressed proteins, which represents important biomarkers to elucidate adaptive responses to specific macroenvironmental conditions, explore the nutritional value and assess the genetic distance among plant organisms.

Indeed, both abiotic and biotic stresses cause significant alterations in protein expression in plants, and proteomics provides a means for the identification and the comprehension of several novel proteins' role under stress and non-stress conditions, unveiling their functional activity in mediating specific cellular processes (Barkla 2016; Zargar et al. 2017).

Comparative proteomics profiling performed in several plant species under stress conditions revealed numerous differentially expressed proteins among genotypes, leading to the identification of potential candidate proteins involved in stress tolerance mechanisms, which, besides providing vital insights to develop “climate proof crop varieties” in breeding programs, could explain the high added value of these plant genetic resources (Vanhove et al. 2012; Zadražnik et al. 2013; Zhao et al. 2016; Riahi et al. 2019; Jardak et al. 2021).

This is the case of heat shock proteins (HSPs) and late embryogenesis abundant (LEA) protein family), involved in protein folding, defence, desiccation tolerance and osmotic homeostasis, and of expansin and kiwellin, being responsible of cell wall modulation and growth, which were found upregulated in a stress tolerant barley population in salt treatment conditions, suggesting their critical roles in the alleviating the harmful effects of salinity stress (Riahi et al. 2019).

Moreover, proteomic analysis in maize samples reported significantly expression change levels of 135, 35 and 201 proteins under the heat, drought and combined stress conditions, respectively, allowing also to identify proteins exclusively responding to one stress typology or to their combination. Overall, the differential expressed proteins were mainly represented by chaperones and proteases, which played an important role in the adaptive response of maize to heat, drought and combined stress (Zhao et al. 2016).

The leaf proteome analysis of several banana accessions exposed to drought stress revealed a clear difference between control and stressed plants, leading to the identification of differential expressed proteins, including small heat shock proteins (sHSPs) and proteins related in reactive oxygen species (ROS) detoxification (isoflavone reductase like protein, glutathione reductase, cysteine synthase and glutathione transferase), which were found much more abundant in stressed plants (Vanhove et al. 2012).

A proteomic analysis conducted on drought-tolerant and -sensitive common bean cultivars under water deficit highlighted the presence of high amount of differential expressed proteins across the samples. Besides some proteins were found more abundant under drought stress in both cultivars, such as stress ROS scavenging proteins, proteins involved in ATP interconversions [nucleoside diphosphate kinase (NDPK) and ATP synthase] and in protein synthesis (glutamine synthetase, ribosomal proteins, cysteine synthetase and acetohydroxyacid synthase), some others, mainly related to photosynthesis [oxygen evolving enhancer proteins (OEE1 and OEE2) and chlorophyll a/b binding proteins] and glycolysis (enolase) process, were found sharply increased in drought tolerant common bean, suggesting their potential role in drought tolerance mechanisms in this species (Zadražnik et al. 2013).

Moreover, proteomic analyses have undertaken to characterize the diversity of seed proteins at the intra- and interspecific level in several species, detecting biochemical markers which can be useful in assessing genetic variability and in establishing the phylogenetic relationships among organisms. Indeed, proteomic analysis resolved hundreds of protein species across several lentil (*Lens culinaris* L.) landraces and commercial cultivars, revealing 103 protein spots differentially expressed within and between populations. In detail, 24 protein components, namely 7S and 11S globulins, maturase K, P54 protein, probable proteinase, cyclin-like F-box and Bowman–Birk protease inhibitor were found to be essential for population discrimination and were proposed as landrace markers, enabling to evaluate the genetic variability and phylogenetic relationships among the samples (Scippa et al. 2010).

Likewise, this technique emerged as a useful tool to obtain relevant information regarding critical nutritional quality properties of crops, such as the presence of putative allergens (Xu et al. 2007; Akagawa et al. 2007). Indeed, for instance, soybean (*Glycine max* L. Merr.), a valuable source of edible oil and protein for feeding both animals and humans, possesses about 15 proteins recognized by IgEs from soy-sensitive people. However, three proteins, Gly m Bd 60 K, Gly m Bd 30 K, and Gly m Bd 28 K, represent the major seed allergens in soybean sensitive patients. Proteomic analysis allowed to accurately examine the distribution of the major seed allergens in wild, landrace, ancestral, and modern soybean genotypes, representing a crucial tool for identifying and eliminating unwanted compounds and improving the nutritional value of these legume species (Xu et al. 2007).

Moreover, proteomic research also proved its usefulness in achieving a more detailed and comprehensive characterization of the wheat (*Triticum aestivum* L.) protein allergens, identifying four major human IgE-binding antigens [serpin, R-amylase inhibitor, γ-gliadin, and low molecular weight (LMW) glutenin] and assuming a particular significance for food and nutrition topics (Akagawa et al. 2007).

Overall, proteomic approach, due to its multiple applications, enables to dispense an exhaustive molecular-scale evaluation of several aspect of the germplasm diversity, providing valuable inputs for boosting germplasm valorisation activities and relieve the current rate of genetic erosion for several endangered plant genetic resources.

2 Objectives

The present study aims to demonstrate that a complementary characterization approach, combining morphological, genetic and biochemical/metabolomic analyses with stress response-related studies, proves to be an efficient method to explore landrace diversity and identify plant climate-smart distinctive traits in the context of the current climate change. In detail, the idea was to identify/decipher the key features endowed by the plant agrobiodiversity present in the “Alto Medio Sannio” inner area, stimulating the conservation strategies towards this endangered genetic material and, at the same time, providing a possible direction and potential scenarios for enhancing the socio-economic regeneration of the selected marginalized inland area.

To accomplish these objectives, a detailed sequences of research activities were planned.

Firstly, active interventions were employed, focusing on recovery of legume landraces throughout “Alto Medio Sannio” inner area, to immediately limit the ongoing germplasm loss, collecting the germplasm material and all the associated traditional knowledge. These activities were followed by the ex situ conservation of the collected resources in Molise Germplasm Bank (University of Molise).

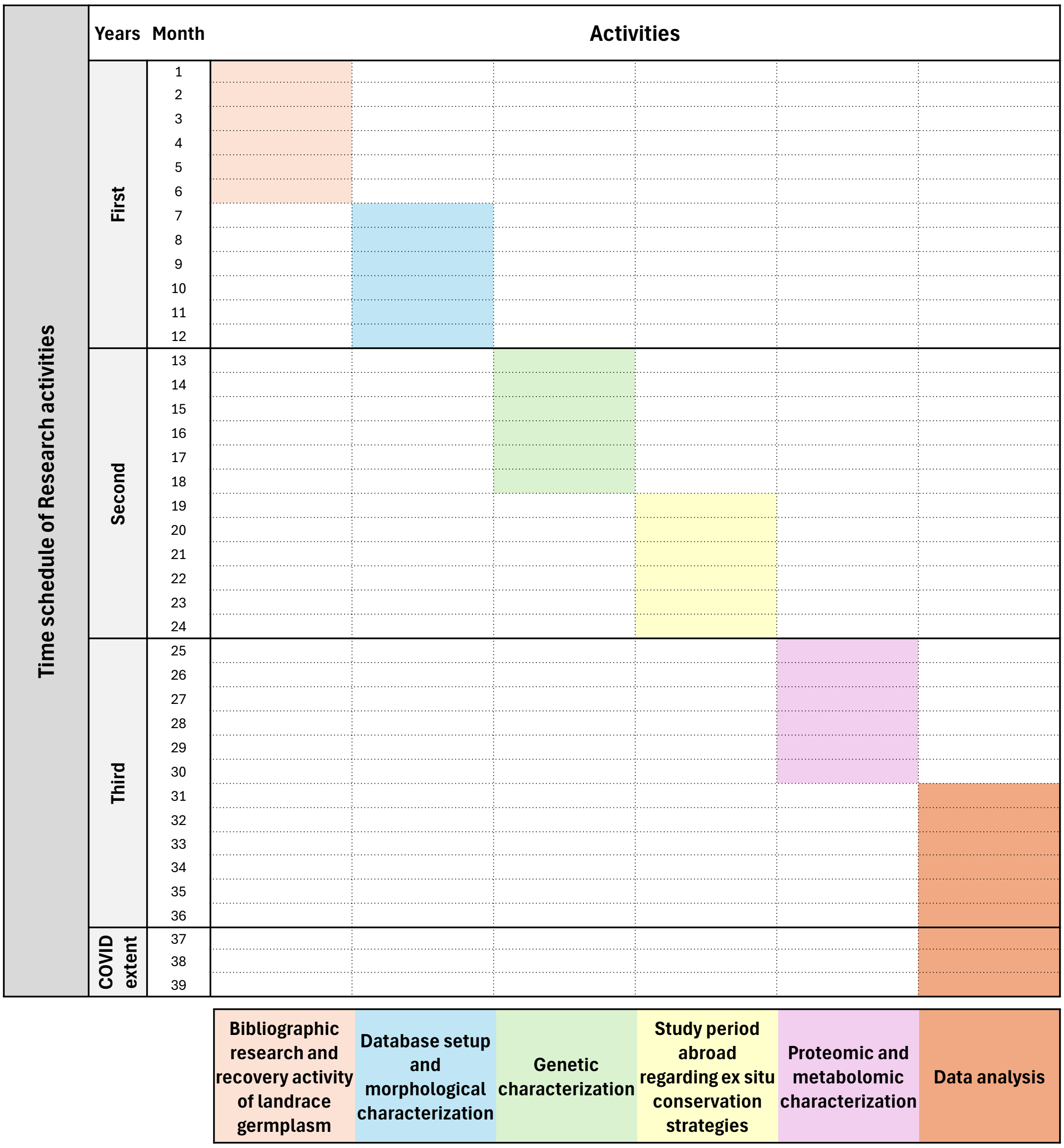
Successively, a multi-level approach, on which the research articles of this doctoral thesis are based (Paper I, II and III-*in preparation*), was used to investigate and characterize the diversity among some common bean (*Phaseolus vulgaris* L.) landraces most representative of “Alto Medio Sannio” inner area, dissecting:

- the seed features, the phaseolin gene pool and the genetic distance among the populations;
- the ability of the populations to counteract salinity and osmotic stress, some of the most alarming abiotic stresses caused by climate change in the Mediterranean region;
- the presence in the populations of specific metabolic fingerprint/valuable bioactive compounds with benefic properties for human wellness or plant stress tolerance.

Overall, the expected results - directly related to increased knowledge on common bean nutritional and stress tolerance attributes - may contribute to fully explore the potential of local plant genetic resources in term of their market attractiveness, pushing the preference of consumers beyond the local community, and, at the same time, encourage farmers to maintenance their on-farm cultivation. Thus, this research not only support the conservation strategies for this endangered locally-adapted germplasm, but could also act as a driving force for the development and the promotion of the Italian inland marginal areas, where these resources are generally confined.

3 Ph.D thesis research activities

The experimental research activity present in this doctoral thesis has been carried out along 3 years (39 months in total: 36 ordinary plus 3 months as COVID extension) in full accordance with the objectives and timeline set by the PhD scholarship associated to the research topic "Aree interne/aree marginalizzate" ("inner areas/marginalized areas") funded by Piano Stralcio "Ricerca e Innovazione" 2015–2017 Asse "Capitale Umano", Fondo per lo Sviluppo e la Coesione (FSC), of the Italian Ministry of Education, University, and Research (MIUR) (Fig. 5).



Time schedule of Research activities

Years	Month	Activities					
First	1	■					
	2	■					
	3	■					
	4	■					
	5	■					
	6	■					
	7		■				
	8		■				
	9		■				
	10		■				
	11		■				
	12		■				
Second	13			■			
	14			■			
	15			■			
	16			■			
	17			■			
	18			■			
	19				■		
	20				■		
	21				■		
	22				■		
	23				■		
	24				■		
Third	25					■	
	26					■	
	27					■	
	28					■	
	29					■	
	30					■	
	31						■
	32						■
	33						■
	34						■
	35						■
	36						■
COVID extent	37						■
	38						■
	39						■
		Bibliographic research and recovery activity of landrace germplasm	Database setup and morphological characterization	Genetic characterization	Study period abroad regarding ex situ conservation strategies	Proteomic and metabolomic characterization	Data analysis

Figure 5. Time schedule of the Ph.D research activities. Plan of the research activities conducted in accordance with the objectives and the timeline of the Ph.D project.

In detail, in compliance with the Italian national policies of territorial cohesion and of plant agrobiodiversity preservation, set by National Strategy for Inner Areas (SNAI) (https://www.agenziacoesione.gov.it/strategia-nazionale-aree-interne/?lang=en) and by National Plan for Agrobiodiversity (PNBA) (MiPAAF 2008), respectively, and in accordance with the global goals of sustainable development set by Agenda 2030 of the United Nations (https://sdgs.un.org/goals), this doctoral thesis has been focused on the plant agrobiodiversity of "Alto Medio Sannio" inner area respecting the Ph.D research project timeline presented and reported below.

During months 1-6, bibliographic research and a recovery activity of landrace germplasm in "Alto Medio Sannio" inner area have been carried out. More specifically, the landrace collecting mission has been performed in collaboration and with the support of the public company "Gruppo di Azione Locale (GAL) Alto Molise" of Agnone (IS), under the supervision of Dr. Mario Di Lorenzo.

Next, the activities of the months 7-12 have been dedicated to the setup of the digital database, containing all the information of the gathered local germplasm, and to the morphological characterization of some selected common bean (*P. vulgaris* L.) landraces collected. Furthermore, in months 13-18, the genetic characterization of these common bean samples has been performed.

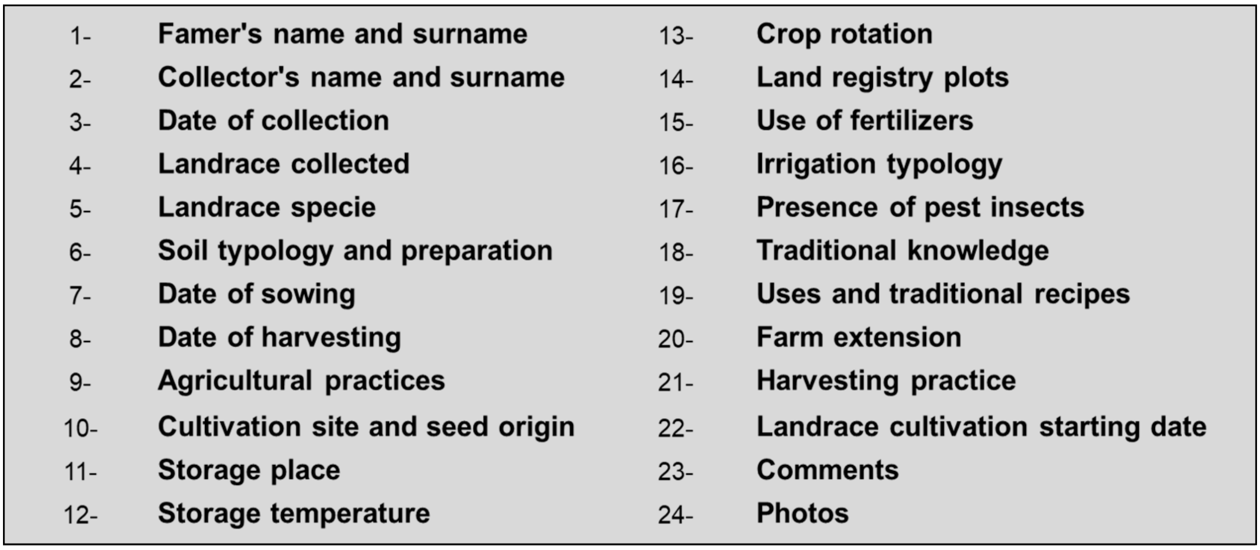
During the months 19-24, the time schedule of the PhD program included a study period abroad (not financed by FSC and MIUR) conducted at the University of Malta, as part of the image-based/karyotyping characterization of some common bean landraces (Annex I).

During the 25-30 months, the project foreseen a proteomic and metabolomic characterization activities, while during the last months of the PhD program (31-36 plus 37-39 months), it has been performed the integrated data analysis. All the research activities and products are described and illustrated in sequence below.

3.1 Bibliographic research and recovery activity

The recovery activity of legume landraces in “Alto Medio Sannio” inner area was conducted in collaboration with “GAL Alto Molise”, a public company situated in Agnone (IS), Italy, aimed at creating a connection with the territorial context of “Alto Medio Sannio” and assisting the landraces collection across the area.

During this activity, on-farm surveys were carried out in the 33 districts of “Alto Medio Sannio” inner area in order to collect germplasm (seeds) of legume landraces from traditional famers, along with important agronomic information and traditional knowledge associated to these local resources. The information was collected performing accurate interviews to famers, by using a technical data sheet defined by the INEA (National Institute of Agricultural Economics) and successively modified by Molise Germplasm Bank (Fig. 6). Name and age of the farmers, farm extension, agricultural practices, and know-hows, as well as peculiar uses, local names and traditions are the main information gathered during the on-farm collection mission.



1- **Famer's name and surname**
2- **Collector's name and surname**
3- **Date of collection**
4- **Landrace collected**
5- **Landrace specie**
6- **Soil typology and preparation**
7- **Date of sowing**
8- **Date of harvesting**
9- **Agricultural practices**
10- **Cultivation site and seed origin**
11- **Storage place**
12- **Storage temperature**
13- **Crop rotation**
14- **Land registry plots**
15- **Use of fertilizers**
16- **Irrigation typology**
17- **Presence of pest insects**
18- **Traditional knowledge**
19- **Uses and traditional recipes**
20- **Farm extension**
21- **Harvesting practice**
22- **Landrace cultivation starting date**
23- **Comments**
24- **Photos**

Figure 6. Structure of the technical data sheet used during the on-farm landraces survey.

At the end of the on-farm survey, 57 local legume landraces accessions were collected from 26 different traditional farmers in 14 districts of “Alto Medio Sannio” inner area (Table 1). In the remaining districts, the presence of traditional farmers and cultivation of legume landraces was not detected, suggesting that, in these territories, the agricultural production of local landraces has probably been lost.

Table 1. List of the legume landrace accessions collected from traditional farmers in several districts of "Alto Medio Sannio" inner area. *P. vulgaris* accessions are indicated as "bean".

Alto Medio Sannio municipality	Farmer	Species	Landrace
1 Agnone	Tonino Scampamorte	*Cicer arietinum*	Chickpea
		Lens culinaris	Lentil
		Phaseolus vulgaris	"Pinto" bean, "Bianco Lungo" bean, "a Ciliegia" bean and "Pinto" bean
2 Capracotta	Luca and Loreto Beniamino ("Alti Sapori Le Miccole" farm)	*Cicer arietinum*	"Ceciolino" chickpea
		Lens culinaris	Lentil
		Phaseolus vulgaris	"Fiocco di Neve" bean and "Ciliegiotto" bean
		Pisum sativum arvense	Forage pea
		Vicia faba	Fava bean
	Giuseppe Di Tanna	*Cicer arietinum*	Chickpea
		Lathyrus sativus	Grass pea
		Lens culinaris	Lentil
		Phaseolus vulgaris	"per Contorno" bean, "Schiacciati" bean, "a Palline" bean and "Borlotto" bean
3 Carovilli	Alberto Di Domenico	*Phaseolus vulgaris*	"D'Acqua" bean
	Michele Parisi	*Phaseolus vulgaris*	"Fagioloni" bean
	Franco Conti	*Phaseolus vulgaris*	"Ciliegino" bean
4 Castel del Giudice	Carmine Valentino Mosesso	*Phaseolus vulgaris*	"della Levatrice" bean
5 Castelverrino	Antonio Pannunzio	*Cicer arietinum*	Chickpea
		Vicia faba minor	Field bean
6 Chiauci	Domenico Colabuono	*Cicer arietinum*	Chickpea
		Lens culinaris	Lentil
		Vicia faba	Fava bean
	Biagio Di Pilla	*Phaseolus vulgaris*	"Pinto" bean and "D'Orto" bean
	Antonio Esposito	*Phaseolus vulgaris*	"della Quarantana" bean
7 Frosolone	Sabrina Colaneri and Nicolina Castagna	*Phaseolus vulgaris*	"Borlotto" bean, "Cannellino" bean and "Tondino" bean
		Cicer arietinum	Chickpea
8 Pescolanciano	Domenico Minichiello	*Cicer arietinum*	Chickpea
9 Pietrabbondante	Enzo Santoro	*Lathyrus sativus*	Grass pea
		Phaseolus vulgaris	"D'Acqua" bean and "Pinto" or "di Castiglione" bean
	Rodolfo Di Carlo	*Phaseolus vulgaris*	"D'Acqua" bean and "Pinto" or "di Castiglione" bean
10 Poggio Sannita	Antonio Mastronardi and Concetta Palombo	*Cicer arietinum*	Chickpea
		Pisum sativum	Pea
11 San Pietro Avellana	Amalia Ricci	*Phaseolus vulgaris*	"Confetto" bean
12 Sant'Angelo del Pesco	Giuseppina Di Paolo and Settimio Casciato ("Mauro Casciato" farm)	*Phaseolus vulgaris*	"Borlotto" bean, "Suocera e Nuora" bean, "di Quaranta Giorni" bean
13 Sant'Elena Sannita	Franco Prezioso	*Phaseolus vulgaris*	"Rosso" bean
	Maria Di Iorio	*Lathyrus sativus*	Grass pea
		Phaseolus vulgaris	"Piattelli" bean
14 Vastogirardi	Lucia Masciotra	*Phaseolus vulgaris*	"Monachella" bean, "Ciliegino" bean and "a Zecca" bean
	Paolo Di Benedetto	*Phaseolus vulgaris*	"Ciliegino" bean

Among all the legume landraces collected in the study area, common bean (*Phaseolus vulgaris* L. – 60%), chickpea (*Cicer arietinum* L. – 14%) and lentil (*Lens culinaris* Medik. – 12%) resulted to be the most frequently found (Fig. 7a). In detail, 34 accessions of common bean (*Phaseolus vulgaris* L.), 8 of chickpea (*Cicer arietinum* L.), 7 of lentil (*Lens culinaris* Medik.), 3 of grass pea (*Lathyrus sativus* L.), 2 of fava bean (*Vicia faba* L.), 1 of field bean (*Vicia faba* L. *minor*), 1 of pea (*Pisum sativum* L.) and 1 of forage pea (*Pisum sativum* L. *arvense*) were found in several municipalities of "Alto Medio Sannio" inner area (Fig. 7b). Among all the municipalities surveyed, Capracotta showed the highest landrace' species diversity (Fig. 7b).

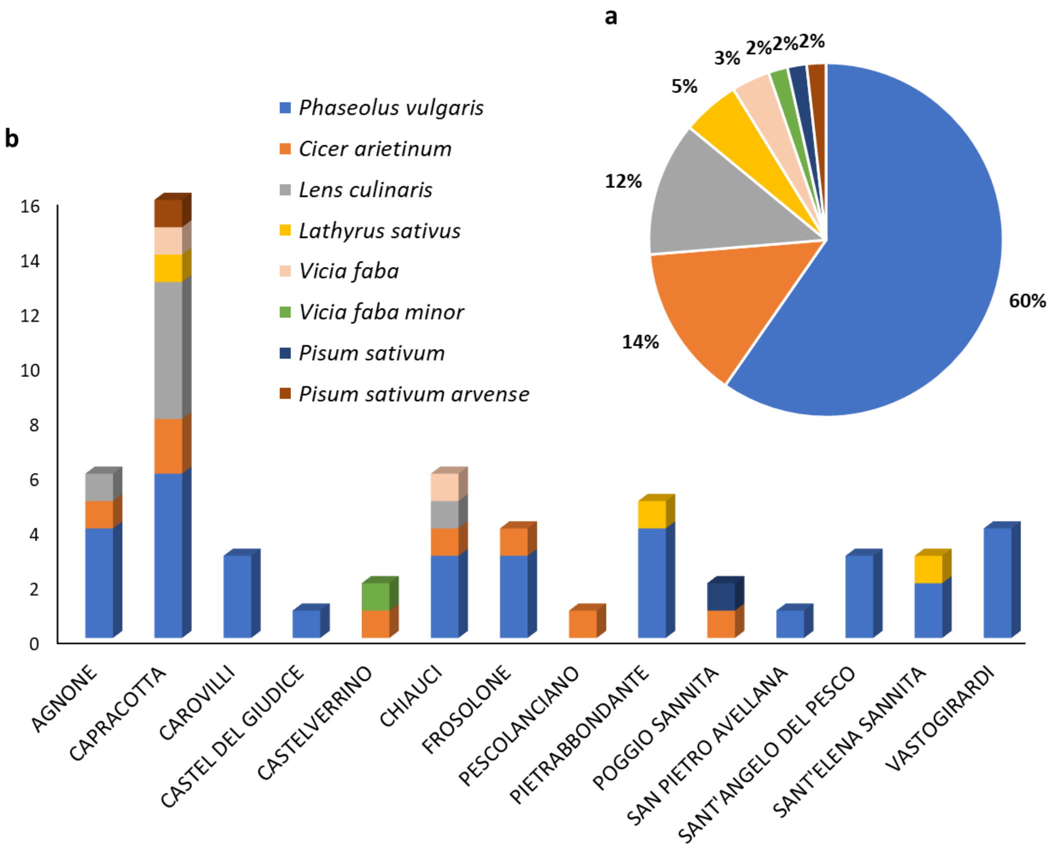


Figure 7. Subdivision of the 34 legume landrace accessions collected according to plant species (a) and to "Alto Medio Sannio" municipalities (b).

All the accessions collected were labelled with a code and registered in a specific digital database, along with the agronomic and traditional knowledge information associated, and are currently stored ex situ in Molise Germplasm Bank.

During the on-farm survey, farmers approached in a friendly way and expressed a great willingness to show their home gardens and share their knowledge to support the conservation of local varieties (Fig. 8). They declared to maintain the on-farm cultivation of landraces for the great devotion to local traditions and for the peculiar organoleptic traits characterizing these resources. Most of the landraces represent an integral part of family tradition and heirloom; indeed, the majority of farmers inherited seed landraces from their parents/relatives, and only a small part acquired them from neighbouring villages. According to their knowledge, all the local landraces collected have been cultivated for at least 50 years, or more, in the farming site.

Paolo Di Benedetto, Vastogirardi **Enzo Santoro**, Pietrabbondante

Lucia Masciotra, Vastogirardi **Carmine Valentino Mosesso**, Castel del Giudice

Figure 8. Some traditional farmers in their home gardens.

The local landraces collected often present original dialect names, which refers to seed colour, shape, time of ripening, local tales and anecdotes, and are frequently used for the preparation of different traditional dishes recipes, proving their strong link with the uses, customs and traditions of the territory of cultivation.

This is the case of common bean landraces “Ciliegino” and “Ciliegiotto” (cherry), “Pinto” (multi-coloured), “Monachella” (nun), “Fiocco di Neve” (snowflake), “a Confetto” (comfit shaped), “Due Facce” (two-sided), “Suocera e Nuora” (mother-in-law and daughter-in-law), “di Quaranta Giorni” or “della Quaratana” (40 days of ripening), “Piattelli” (flat-shaped), “della Levatrice” (of midwife) and “d’Acqua” (of water).

Interestingly, the association of these resources with the territory is so close that, several common bean landrace accessions with similar morpho-colorimetric seed features, are characterized by different vernacular names according to the traditional knowledge of the cultivation sites. This happens, for example, for a small, round, and mottled brown seed coat common bean landrace, which is called "Pinto" in Chiauci and Agnone municipalities, "a Zecca" in Vastogirardi, "di Quaranta Giorni" in Sant'Angelo del Pesco and "Rosso" in Sant'Elena Sannita (Fig. 9).

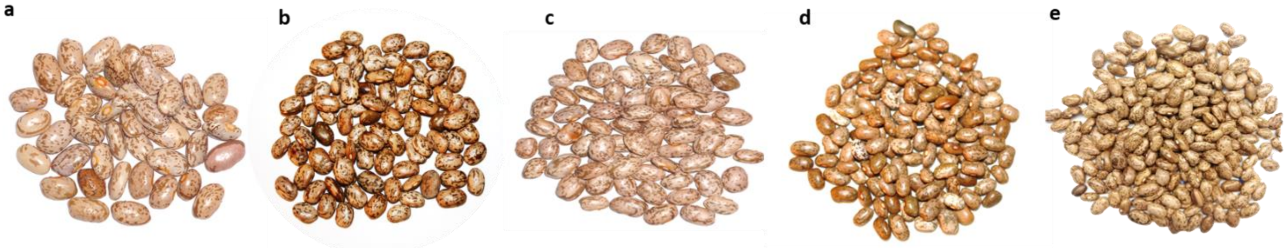


Figure 9. Common bean landrace accessions, with morphological and colorimetric seed similarities, are characterized by distinct dialect names according to the local traditions of the cultivation site. "Pinto" – Chiauci (a); "Pinto" – Agnone (b); "a Zecca" – Vastogirardi (c); "Quaranta Giorni" – Sant'Angelo del Pesco (d); "Rosso" – Sant'Elena Sannita (e).

Cultivation of these local landraces in "Alto Medio Sannio" inner area is primarily maintained by elderly people (age range: 61-80 years old), however, several young farmers (up to 40 years old) are also actively involved in their on-farm conservation (Fig. 10).

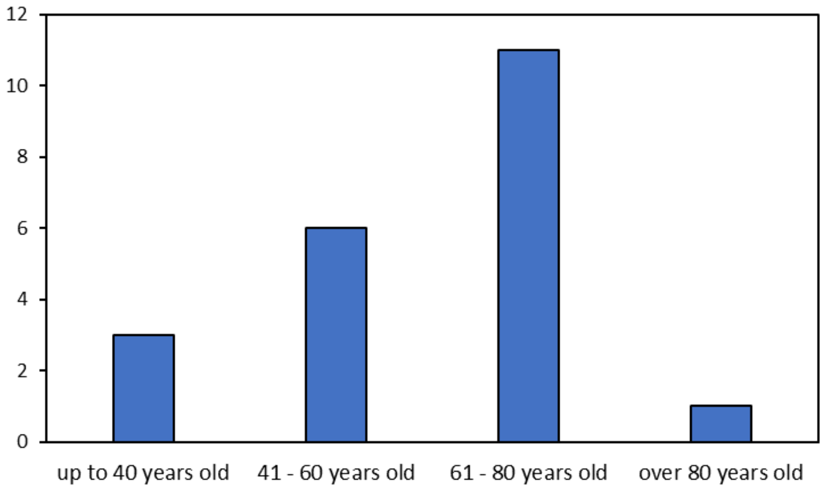


Figure 10. Subdivision of the 26 traditional farmers according to age groups.

Legume landraces collected are mainly cultivated in home gardens with limited dimensions (10-30 m^2) and, only in a few cases, in small scale family-run farms. These local resources usually show medium-low grain yield and are mainly used for family subsistence, while a minor part is sold in small scale markets.

The cultivation of local landraces is carried out by using traditional and low input farming systems, which include the use of mechanical tools for soil preparation and, occasionally, the use of natural fertilizers (manure). On the other hand, the use of chemical fertilizer is very limited. Adoption of crop rotation is frequently reported, which allows to maintain the soil fertility, while the irrigation, by using specific water implant, is performed only during prolonged drought periods. According to traditional beliefs, seed sowing occurs with crescent moon, in absence of Bora wind and in specific days dedicated to several popular saints.

Manual harvesting of pods is usually carried out by growers, following traditional agricultural practices (Fig. 11). The pods are handpicked from the plant and sundried for several days. After drying, seeds are beaten out from the pods using a traditional wooden stick and traditional sieves are used to completely remove plant residues. Seeds are sundried until complete dryness and are generally stored in glass/plastic boxes or in cotton bags, inserting a piece of iron, garlic or bay leaf, which, according to the traditional knowledge, could prevent the attack of pest insects.

Most of landraces show pest resistance, however, a small fraction of them reported green bug, snail and aphids attacks at plant level. On the other hand, seeds are frequently attacked by *Acanthoscelides obtectus*, which often impacts the seed viability.

Figure 11. Some steps of the manual harvesting of pods performed by the traditional farmers.

Considering all the information collected during the legume landraces recovery activity, the long-time cultivation (at least 50 years) of these resources in a restricted geographical area, their deep association with the local territory, also proven by the assignation of dialect and vernacular names, and their cultivation with traditional agricultural practices fulfil all the criteria of "local landraces" defined by the PNBA (MiPAAF 2008) and allow to consider these resources as "autochthonous" of the territory.

In conclusion, analysing the data gathered during the on-farm collection mission throughout the "Alto Medio Sannio" inner area, some common bean accessions showed the highest diversity in terms of distinct seed shape/color features, special end-use qualities, and specific traditional knowledge important for their farming, management, utilization, and conservation compared to other "Alto Medio Sannio" legume landraces. Furthermore, common bean is widely recognized as a rich world resource of biodiversity, playing a key role in reaching multiple dimensions of sustainable development (Uebersax et al. 2023). For all the above reasons and considering also the next exposed in the paragraph below we focused the subsequent research activities on the common bean (*P. vulgaris* L.) species.

3.1.1 The importance of common bean landraces

Mediterranean basin has a long history of legume cultivation, as their use in pastures, soil improvement, dietary supplement, and medicine dates back to the Greek and Roman time (Flint-Hamilton 1999) and are considered to be one of the first crops cultivated by humankind and remained a staple food for many populations all over the world. They represent a nutrient-rich food source, providing proteins, complex carbohydrates, dietary fibre, essential amino acids, minerals, vitamins, and low content of saturated fats (Maphosa and Jideani 2017).

Legumes belong to the large family of Leguminosae or Fabaceae, which encompasses 670-750 genera and 18 000-19 000 different grain, pasture and agroforestry species. They are second only to the Gramineae in their importance to humans and are widely known for their nitrogen-fixing ability (Graham and Vance 2003).

Among the numerous species of legumes, the common bean (*Phaseolus vulgaris* L.) is the most important legume for human nutrition worldwide and represents a valuable source of diversity. It is cultivated in many countries, recognizing the American continent as the world 's largest producer, while in Europe, its cultivation and production is mainly related to countries surrounding the

Mediterranean basin, such as the Iberian Peninsula, Italy, Greece, and the Balkan regions (Catarcione et al. 2023).

This ancient legume crop originated in the Central South America region, where the wild ancestor of *P. vulgaris* probably evolved. Successively, the domestication process from wild beans occurred separately in Mesoamerica and Andean Southern America around 8 000 years ago and resulted in two main different geographical gene pools within the cultivated forms, the Mesoamerican (from Mexico to Colombia) and the Andean (from Perú to Argentina) one, which can be differentiated at morphological, biochemical and genetic level (Fig. 12a) (Paniconi et al. 2010; Castro-Guerrero et al. 2016; Catarcione et al. 2023).

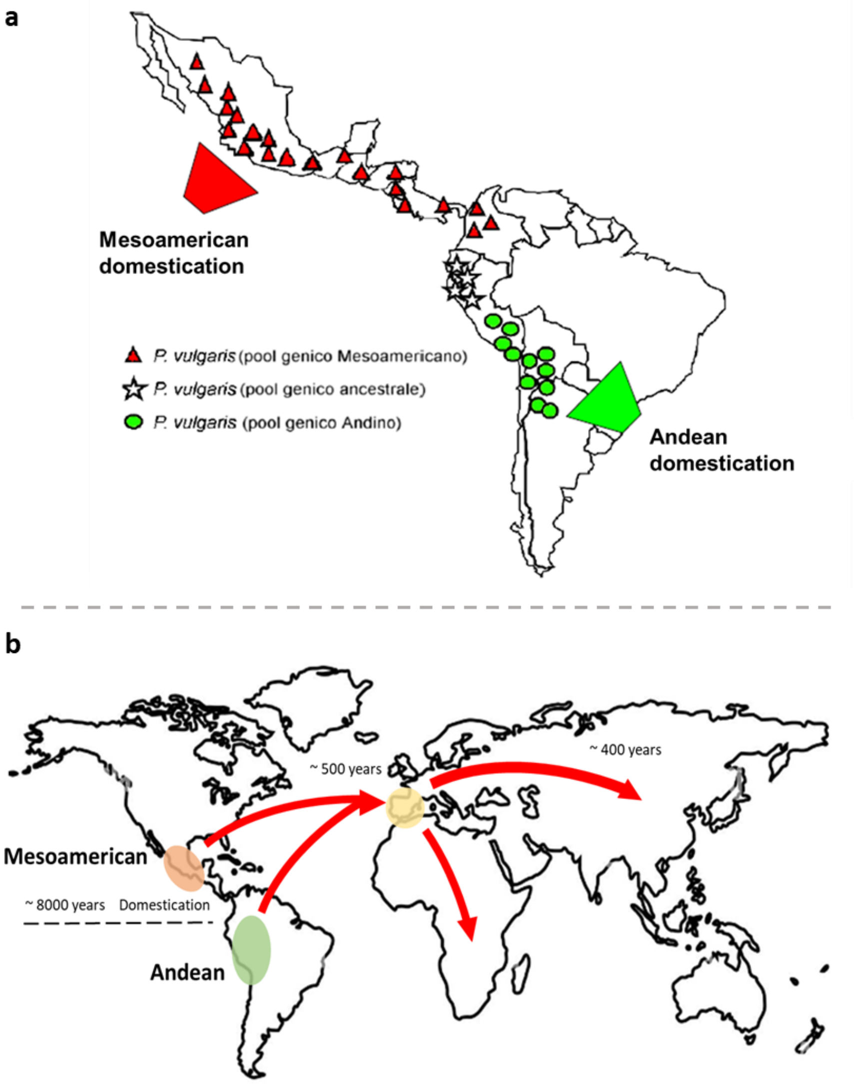


Figure 12. Domestication and global distribution of common bean (*P. vulgaris* L.). Origin of the common bean wild ancestor and independent domestication process in Andean South America and in Mesoamerica [figure adapted from Giardini et al. (2007)] (a); Introduction of common bean from the American continent into Europe and its subsequent distribution worldwide [figure adapted from Castro-Guerrero et al. (2016)] (b).

Common bean was first introduced into Europe during the 16th century by Spanish sailors and traders returning to their native lands and, shortly after, was distributed in Africa and Asia (Paniconi et al. 2010; Castro-Guerrero et al. 2016; Catarcione et al. 2023) (Fig. 12b). In particular, in Italy, according to the first historical documentations, was brought in 1532, when the Spanish Emperor Charles V donated some seeds to the Pope Clemente VII and, after receiving a bag of seeds from the papal's court as compensation, the literate Pietro Valeriano started the cultivation in the Belluno province (Veneto region, Northeastern Italy) (Venora et al. 2009; Piergiovanni and Lioi 2010).

After its first introduction, common bean found in Europe a second centre of diversification: the selective pressure, due to the microclimate adaptation of the cultivation areas, along with the deliberate or unintentional selection carried out by farmers to meet agronomic, aesthetical and organoleptic community preferences, had a huge impact on the evolutionary process of this legume species across the European territories and led to the establishment of a wide array of landraces that have been cultivated for centuries in restricted geographical areas (Scarano et al. 2014; Lo Bianco et al. 2015; Catarcione et al. 2023). The additional variation generated by this adaptive evolutionary process resulted in a huge diversification of common bean landraces in terms of morphological, genetic, agronomical, nutritional, and organoleptic features.

The Italian peninsula, due to its complex orography, is recognized as a hotspot region for plant agrobiodiversity (Giupponi et al. 2021; Canella et al. 2022), with a particular richness in common bean landraces diversity. Common bean has always represented a valuable source of livelihood for the Italian society, mostly for rural economies, where this legume was labelled as "the poor man's meat" and served as an inexpensive meat alternative during the centuries (Scarano et al. 2014).

The long tradition of common bean cultivation in Italy and its strong link with rural agriculture has given rise to many landraces, with peculiar morphological, nutritional, and organoleptic traits and original local names, that are frequently associated to specific localities. Indeed, many Italian common bean landraces gained international and national quality marks linked to their geographical origins, such as "Indication of Protected Origin" (IGP), ''Traditional Agricultural food Products'' (PAT) and Slow Food Presidium (Venora et al. 2009; Scarano et al. 2014; Lo Bianco et al. 2015).

"Badda" (ball) bean from Siciliy, "Dente di morto" (dead man's tooth), "Solfariello" (sulphurous, yellowish) and "dell'occhio" (eye-shaped) beans from Campania region, "a pisello" (pea-shaped) and "del Purgatorio " (purgatory) from Lazio region, "a ughia'' (nail-shaped), ''Russa janca'' (red and white) and “Ciuncu” (truncated) beans from Calabria region, “Zolfino” and “Sorana” from Tuscany, “Sarconi”, “Bianco di Rotonda” (white from Rotonda) “Verdolino” (greenish), “Ciuoto” from Basilicata region, “Lamon” and “Spagnolet nano” from Veneto region and “Billò” from Piedimont, are only few of the many common bean landraces still present and cultivated in several restricted areas of Italy (Negri 2003; Lioi et al. 2005; Piergiovanni and Lioi 2010; Paniconi et al. 2010; Mercati et al. 2013; Scarano et al. 2014).

With the current focus on sustainable development set by Agenda 2030 of the United Nations (https://sdgs.un.org/goals) common bean offers a great opportunity as a strategic resource to meet many environmental and social challenges, such as climate change mitigation, global food security, sustainable agriculture and good health and well-being (Vidak et al. 2023).

Indeed, several studies reported the presence of a complex chemical composition in common bean genotypes, directly related to a plethora of structurally and functionally diverse metabolites, showing beneficial and effective roles in prevention and/or regulation of chronic conditions, such as obesity, diabetes, cancer and cardiovascular diseases (Wang et al. 2020; Ferreira et al. 2021).

Furthermore, common bean could serve as a source of metabolites involved in plant stress response, adaptation to environmental constraints and in addressing the present and the future climate change challenges (Bulut et al. 2023). Indeed, several common bean primary and secondary metabolites, with their functional diversity, play an important role in fine-tuning the environmental stress tolerance and allowing to obtain high yield performance and quality traits with low inputs conditions (Makhumbila et al. 2023; Nina et al. 2023). Moreover, the nitrogen-fixing properties of this legume species improve soil fertility and productivity, having a low impact on the carbon footprint and supporting the biodiversity of agroecosystems (Everwand et al. 2017).

Therefore, given the common bean potential of delivering multiple services in line with sustainability principles, it represents the future natural resource able to achieve the Sustainable Development Goals (SDGs) promoted by Agenda 2030 of United Nations, in terms of zero hunger/food security, good health and well-being, climate change mitigation, promotion of sustainable use of ecosystems and halt of biodiversity loss (Martinez-Juarez et al. 2015).

3.1.2 "Alto Medio Sannio" common bean landraces

Among different common bean (*Phaseolus vulgaris* L.) accessions collected throughout the "Alto Medio Sannio" inner area, the "Due Facce", "Ciliegino" and "Fagiolo D'Acqua" landraces showed the highest diversity in terms of distinct seed shape/colour features, special end-use qualities, and specific traditional knowledge as detailed below and in the next paragraphs.

3.1.2.1 "Ciliegino" common bean landrace

"Ciliegino" common bean landrace showed a really narrow geographic distribution in "Alto Medio Sannio" inner area. Indeed, this local bean variety has been cultivated only in the territory of Vastogirardi municipality, and, in recent time, its cultivation was extended in the neighbouring municipality of Carovilli.

"Ciliegino" bean takes the name from its seed shape, small and oval, and cherry red seed coat colour. It is a local variety cultivated in mountainous area of Vastogirardi village (1200 m a.s.l.), particularly appreciated by the local community for its sweet taste and often used for the preparation of a traditional handmade pasta meal, typical of this area, called in dialect "Cazzariegl' e fasciuol'" (Fig. 13).

Figure 13. Preparation of the traditional meal "Cazzariegl' e fasciuol'" with "Ciliegino" bean.

Referring to the historical memories of the elderly population of Vastogirardi, this bean landrace has been cultivated in the village since late 1800's or early's 1900, when it was probably brought to

Molise territory through Italian emigrants in the American continent returning to their native lands. Indeed, an extensive bibliographic research allowed to recognize the presence of several bean varieties, showing morphological features similar to “Ciliegino”, in South America, in particular in Ecuador and Colombia, where they are called “Bolon Rojo” and “Bola Roja” (https://mcp.bolsamercantil.com.co/ArchivosPublicados//PDF/PubId=85725_DCE%20FRIJOL%20BOLA%20ROJA.pdf) (Fig. 14a). In detail, this latter, is found to be particularly adapted to grow on high elevation (Botero Degiovanni et al. 2021), and this evidence is coherent with the high-altitude adaptation displayed by “Ciliegino” on highlands of Vastogirardi.
Recently, another heirloom bean, with morphological similarities of “Ciliegino” and named as “True Red Cranberry Bean”, was discovered by a seed collector in Northeastern America (Maine and Vermont), where it was used to be cultivated as a primarily source of livelihood by the ancient native American tribes “Abenaki” (https://www.fondazioneslowfood.com/en/ark-of-taste-slow-food/true-red-cranberry-bean/) (Fig. 14b).

Furthermore, the presence of two local bean landraces similar to “Ciliegino” was also found in Spain. This is the case of “Alubia Roja de Ibeas” (Fig. 14c) and “Alubia de Anguiano” (Fig. 14d) cultivated in two different villages in the North of Spain, Ibeas and Anguiano, respectively and, in particular, this latter is also awarded with geographical indication marker (https://alubiadeanguiano.com/; https://ibeasdejuarros.es/events/feria-de-la-alubia-ibeas/). These two traditional bean varieties are particularly appreciated by the local communities, which are actively involved in the organization of marketing activities and cultural and gastronomy events in order to incentivize their on-farm conservation.

Most of the Italian germplasm banks websites do not report a wide distribution of “Ciliegino” bean landrace along the national territory. Indeed, a similar bean variety resulted to be cultivated only in Basilicata region, in the hilly areas of Sarconi municipality (636 m a.s.l.), named by the local population as “San Michele Rosso”, for the red colour of its seed coat and for the time when it ripens, usually on the day dedicated to Saint Michele (Fig. 14e). It is renowned for its peculiar taste and short cooking time, and it is associated with the “Indication of Protected Origin” (IGP) quality marker (https://www.fagiolibelisario.it/).

On the whole, according to the information collected from the bibliographic research, all the American and European common bean varieties similar to “Ciliegino” are mainly cultivated in mountainous areas, suggesting their particular adaptation to high-altitude conditions.

Figure 14. American, European and Italian common bean varieties showing morphological similarities of "Ciliegino". Picture of Bola Roja as reported in the Instituto Nacional De Vigilancia De Medicamentos y Alimentos (INVIMA) of Colombia (a); True Red Cranberry Bean (https://beancyclopedia.com/index.php/True_Red_Cranberry) (b); Alubia Roja de Ibeas (c); Alubia de Anguiano (d); San Michele Rosso from Basilicata (e).

3.1.2.2 "Due Facce" common bean landrace

"Due Facce" is a white and black/purple/brown seed coat common bean landrace cultivated in several "Alto Medio Sannio" districts, where it owns different dialect names according to the local traditions of the cultivation site. Indeed, it is named "Suocera e Nuora" in Sant'Angelo del Pesco municipality, "Monachella" in Vastogirardi and "Pinto" in Agnone (Fig. 15).

According to the farmers' knowledge gathered, this bean variety is generally sowed after the 20th of May in the territories of "Alto Medio Sannio" inner area, in days without Bora wind, which could harden the seeds and negatively affect the cooking process. It is particularly appreciated by the local communities for its taste and, unlike most of the common bean landraces collected in "Alto Medio Sannio", it could be harvested as unripen pods and consumed as "green beans".

Literature review and Italian germplasm bank websites consultation revealed that "Due Facce" bean landrace is widely spread across the entire Apennine ridge: in Tuscany is cultivated in Piazza al Serchio (LU), where is called "Mascherino", in Abruzzo is named "Socere e Nore" and is cultivated in Abbateggio (PE), while in Basilicata is known as “Munachedda Rossa”, “Munachedda Nera” and “Tuvagliedda Rossa”, according to the dark part of seed coat colour, and is cultivated in the hilly lands of Sarconi (PZ) (Fig. 15) (http://germoplasma.regione.toscana.it/MESI_Menu/Elemento.php?ID=876; https://www.parks.it/parco.nazionale.majella/dettaglio_prodotto.php?id_prodotti=1762; https://www.fagiolibelisario.it/). Moreover, this bean landrace goes by the name of “Badda” in Sicily, for the round shape of its seeds. In detail, two different varieties are cultivated in the area of Polizzi Generosa (PA), the “Badda Nero” and “Badda Bianco”, with white and black and white and light brown seed coat, respectively (Paniconi et al. 2010) (Fig. 15).

Figure 15. Map of Italy with several «Due Facce» common bean landraces cultivated along the Apennine ridge. Tuscany (a); Abruzzo (b); Molise (c); Basilicata (d); Sicily (e).

3.1.2.3 “Fagiolo d’Acqua” common bean landrace

“Fagiolo d’Acqua” is a common bean landrace cultivated in two municipalities of “Alto Medio Sannio” inner area, Pietrabbondante and Carovilli. It has small, round and light brown coloured seed coat, with brown hilum (Fig. 16a). It is commonly used in soups because of its thin skin and it owns its name to the old farming location, near water sources, where it was used to grow in the past. No evidence regarding the cultivation of this landrace in the past times in the surrounding area of Pietrabbondante area was found in historical issues of the municipal archive, however, local elder people asserted the long-time presence of this landrace in the farmlands of the area.
The websites consultation showed the presence of a similar common bean landrace in Basilicata region (Sarconi, PZ), where is called “Verdolino” and awarded with the IGP (Indication of Protected Origin) marker (https://www.fagiolibelisario.it/) (Fig. 16b).

On the other hand, among all the legume landraces collected during the on-farm recovery activity throughout “Alto Medio Sannio”, “Fagiolo d’Acqua” displayed high morphological similarity with “Fagiolo della Levatrice” (midwife bean), cultivated in the village of Castel del Giudice (Fig. 16c). According to the historical memories of the farmer who actively manages the cultivation of this landrace, the first seeds of “Fagiolo della Levatrice” were brought to Castel del Giudice in 1938 from Bondeno (FE, Emilia Romagna region) by a lady, Elsa Cavallaro, who moved to the little village in order to provide assistance to pregnant women and newborns. Following the local custom, bean seeds were offered as dowry good for the young brides and this is probably the reason why “Fagiolo della Levatrice” beans were introduced in the territory of Castel del Giudice. Furthermore, always according to the farmer, “Ruviotto” bean, an ancient common bean landrace cultivated between Veneto and Emilia Romagna region, could be the ancestor of the actual “Fagiolo della Levatrice” (Fig. 16d). However, after 1950 information regarding the midwife Elsa Cavallaro got lost by the historical memories of older people of Castel del Giudice, and there are no further evidence about her moving in other (neighbouring) territories, therefore deeper analysis should be performed in order to verify the possible phylogenetic relationship between “Fagiolo d’Acqua” and “Fagiolo della Levatrice”.

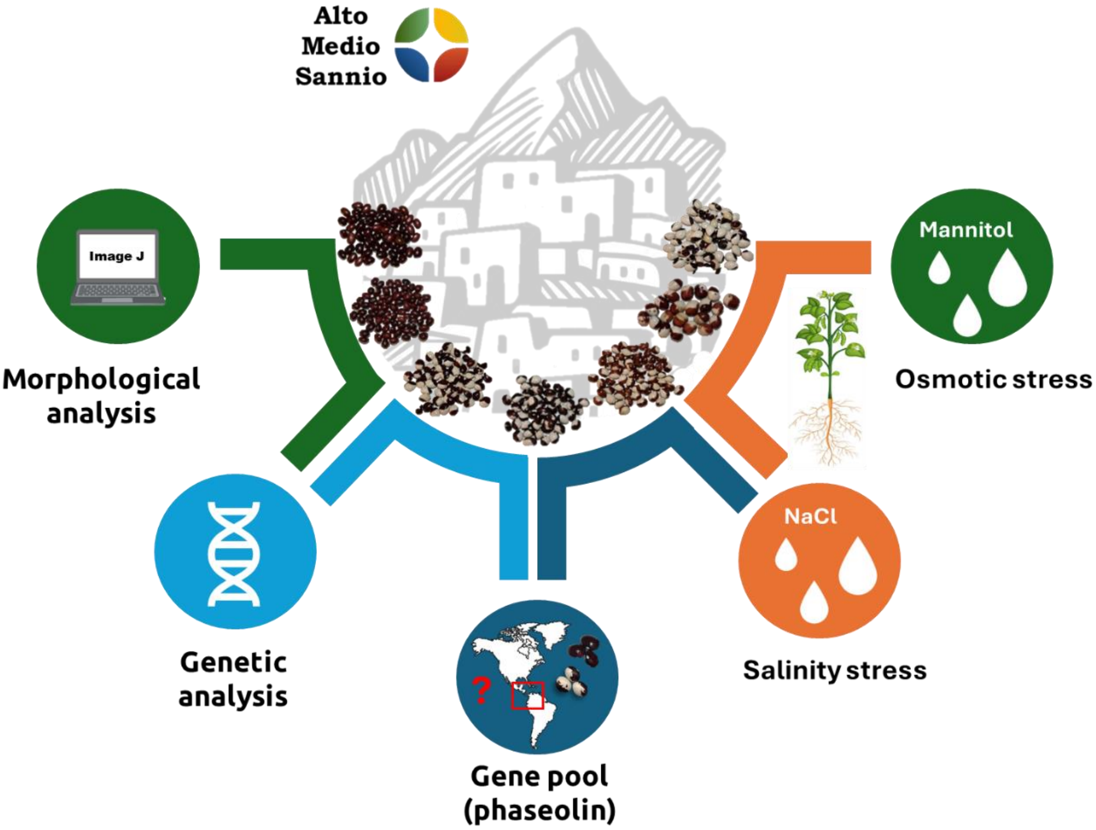
Figure 16. Common bean landraces displaying seed morphological similarities. “Fagiolo d’Acqua” – “Alto Medio Sannio” (a); “Verdolino” – Basilicata region (b); “Fagiolo della Levatrice” – “Alto Medio Sannio” (c); “Ruviotto” – Emilia Romagna and Veneto region (d).

Considering the peculiarity of the three above mentioned common bean landraces (*Phaseolus vulgaris* L.), “Due Facce”, “Ciliegino” and “Fagiolo D’Acqua”, they were selected for all subsequent multi-level characterization steps (Paper I, Paper II and Paper III-*in preparation*), in order to unveil their potential distinctiveness and enhance their conservation and valorisation.

3.2 A Multi-Level Approach as a Powerful Tool to Identify and Characterize Some Italian Autochthonous Common Bean (*Phaseolus vulgaris* L.) Landraces under a Changing Environment – Paper I

In the present study, published on *Plants*, 11: 2790 (*2022*; https://doi.org/10.3390/plants11202790), the morphological, genetic, and biochemical analyses were integrated with stress response-related studies to characterize the diversity of seven Italian autochthonous common bean landraces (Ciliegino, San Michele Rosso, Monachella, Mascherino, Pinto, Tuvagliedda Rossa and Suocera and Nuora). The results showed that the morphological descriptors and the neutral molecular markers represent powerful tools to identify and distinguish diversity among landrace populations, but they cannot correlate with the stress tolerance pattern of genetically similar populations. The study also supported the use of proline as a biochemical marker to screen the most salt-sensitive bean landraces. Thus, to fully elucidate the future dynamics of agrobiodiversity and to establish the basis for safeguarding them while promoting their utilization, a multi-level approach should always be included in any local and national program for the characterization/conservation/use of genetic resources. This study should represent the basis for further joint research that effectively contributes to set/achieve Italian priorities towards sustainability in the framework of emerging environmental, societal, and economic challenges.

Paper I


Alto
Medio
Sannio
Image J
Morphological analysis
Mannitol
Osmotic stress
Genetic analysis
NaCl
Salinity stress
?
Gene pool (phaseolin)

plants

MDPI

Article

A Multi-Level Approach as a Powerful Tool to Identify and Characterize Some Italian Autochthonous Common Bean (*Phaseolus vulgaris* L.) Landraces under a Changing Environment

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Abstract: A prime role in matters of agrobiodiversity is held by landraces, which serve as a repository gene pool able to meet sustainable development goals and to face the ongoing challenges of climate change. However, many landraces are currently endangered due to environmental and socio-economic changes. Thus, effective characterization activities and conservation strategies should be undertaken to prevent their genetic and cultural erosion. In the current study, the morphological, genetic, and biochemical analyses were integrated with stress response-related studies to characterize the diversity of seven Italian autochthonous common bean landraces. The results showed that the morphological descriptors and the neutral molecular markers represent powerful tools to identify and distinguish diversity among landrace populations, but they cannot correlate with the stress tolerance pattern of genetically similar populations. The study also supported the use of proline as a biochemical marker to screen the most salt-sensitive bean landraces. Thus, to fully elucidate the future dynamics of agrobiodiversity and to establish the basis for safeguarding them while promoting their utilization, a multi-level approach should always be included in any local and national program for the characterization/conservation/use of genetic resources. This study should represent the basis for further joint research that effectively contributes to set/achieve Italian priorities towards sustainability in the framework of emerging environmental, societal, and economic challenges.

Keywords: agrobiodiversity; phaseolin; ISSR marker; morphological descriptors; plant diversity; stress response

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1. Introduction

The safeguarding of agrobiodiversity, as an extension of the concept of biodiversity conservation, refers specifically to the preservation of all varieties/landraces of species of agricultural interest. Besides being an integral part of the local traditional knowledge and cultural heritage [1], landraces usually show the best adaptation to the pedoclimatic conditions of restricted geographical areas [2], and they are generally appreciated for their distinctive taste and high nutritional value [3]. Furthermore, they represent a source for lower-input agricultural systems and genetic crop improvement programs, guaranteeing food diversity for humans and other living beings [4,5]. Thus, their conservation and production is vital to enhance the sustainable development in challenging climatic conditions, optimizing the farming systems, and valorizing the marginal areas of Italy [6–8].

In this sense, agrobiodiversity represents a relevant challenge for rural marginal area (39% of the agricultural land in Italy) development [9], at both national and regional scales, and in particular, to promote the profitability of mountain agricultural activities—sustainable productions, as well as the diversification of products and services offered by farms—and, thus, the permanence of specific population segments in inland, peripheral rural, and mountainous districts. However, during the last decades, due to complex social, economic, and cultural changes [10] in Italy, as well as in other developed European countries, important locally-adapted sources have been subjected to genetic erosion, reaching a rate of 72.8% in the south of the Italian Peninsula [11]. This phenomenon has prompted governments to take immediate action, assigning economic incentives and subsidies to candidate rural districts (National Strategy for Inner Areas), adhering to international strategies such as the EU Biodiversity Strategy 2020 [12] and the 2030 Agenda for Sustainable Development [13], and drawing up national guidelines for the conservation and enhancement of agrobiodiversity. In this regard, Italy recently recognized the importance of establishing a National Agrobiodiversity Register [14] to collect information on landraces from all Italian regions. However, out of 20 regions, 12 were uncharacterized (having no landraces) [15], although many unknown landraces likely exist, but are isolated on farms. Recently, a more complete list of all Italian herbaceous landraces was produced [16]. Most of these resulted from the *Fabaceae*, *Poaceae*, and *Solanaceae* families and are concentrated in the marginal area (sub-mountain, hilly, and foothill areas; 150–800 m.a.s.l.). The Tuscany region represents the richest region in herbaceous landraces, with 197 varieties, while Molise is the region with the highest density of landraces (number weighted on the area), where there were 50 varieties of common bean (*Phaseolus vulgaris* L.) identified [16]. This richness, also observed in other Apennine areas, is probably determined by a variety of environmental and anthropic factors, such as different bioclimatic levels (e.g., the transition from sea level to mountain altitudes over a short distance), gastronomic heritage, family-based agrosystems, and related low-input practices [17].

In particular, the common bean is an ancient legume crop [18,19], and a typical element of rural economies, allowing for the evolution of many landraces adapted to restricted areas, especially in the southern Italian regions [20]. Although some of common bean landraces are collected and stored in regional seed banks and/or in universities/institutes and facilities for ex situ conservation [21,22], others continue to survive only in marginal areas of several Italian regions through on-farm conservation. Indeed, most of these landraces are severely outdated and endangered due to the advanced age of the farmers who use them, the spreading and wide availability of new commercial varieties, and the socio-cultural context in which they are cultivated. These accessions are often poorly known, and it is therefore of paramount importance to preserve them as part of our heritage (diversity) through a fully effective characterization of each landrace, which would further promote the efforts in planning adequate safeguarding actions.

It is well known that the analysis of seed morphological parameters represents a powerful tool to identify and characterize landraces and discriminate among *P. vulgaris* populations [23,24]. However, in the postgenomic era, molecular markers have emerged as powerful tools for the analysis of germplasm diversity, showing a high rate of reproducibility and efficiency, with no influence from environmental factors [25]. Numerous kinds of genetic markers have been employed for the evaluation of common bean genetic variation, such as inter-simple sequence repeats (ISSR), simple sequence repeats (SSR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and single nucleotide polymorphism (SNP) [2,26–30]. ISSR has proven to be one of the best-suited molecular markers for the accurate assessment of genetic relationships among bean genotypes, with a high efficiency regarding genetic diversity quantification [31–33].

The analysis of seed storage proteins represents a valuable contribution to the overall germplasm evaluation and assessment of genetic diversity [34]. Unlike other legumes, the storage proteins in the common bean are mainly constituted by phaseolin, which accounts for 50% of the total proteins in mature seeds. Phaseolin consists of a number of polypeptides (Mr 54–44 kDa), which vary according to their eco-geographical origin and related domestication events. This fact supports the hypothesis that there are two major gene pools within the common bean germplasm, the Mesoamerican and Andean, therefore phaseolin can be considered as a biochemical marker [35].

Furthermore, landraces show a spectrum of responses to different stressors, which are a combined result of complex interactions among different morphological, physiological, and biochemical features [36–38], and which also contribute to the description of their genetic diversity. Thus, a multidisciplinary characterization approach, combining morphological, genetic, biochemical, and stress response-related studies, has proven to be a more efficient method of exploring landrace diversity and identifying distinctive landrace traits [15,25,39,40]. This approach may provide a better understanding regarding the genetic resources best suited for use in managing the current climatic variability and adapting to progressive climate changes [41,42].

Based on these premises, in the present study, the diversity of some Italian common bean landraces was assessed and explored by using a multi-level approach able to integrate morphological, genetic, and phaseolin pattern characteristics, along with their ability to counteract two types of stress (salt and osmotic stress) that frequently occur in the Mediterranean Basin and continuously increase due to the changes in the climate and anthropogenic activities.

2. Results

2.1. Seed Morphological Parameters and Genetic Data

In our morphological analysis, the main quantitative and qualitative seed morphological descriptors (see Materials and Methods) of seven common bean populations were examined (Table S1; Supplementary Materials). In the principal component analysis (PCA) scatter plot (Figure 1a), Principal Component 1 (PC1) and Principal Component 2 (PC2) accounted for 84.98% and 7.80% of the total variance, respectively. In PC1, two groups could be observed: the first group was comprised of Suocera and Nuora (SA), Monachella (MO), Tuvagliedda Rossa (TR), Mascherino (MA), and Pinto (PI) landraces, while the second group was made up of Ciliegino (CV) and San Michele Rosso (SMR) (Figure 1a). In PC2, MA and PI were well separated from the other populations (Figure 1a). The noted differences were related to seed total area, dark and light-colored areas, weight, and volume.

The dendrogram, resulting from the unweighted pair group method with arithmetic mean (UPGMA) clustering, was in agreement with PCA results and revealed two main clusters (Figure 1b). The first cluster was divided into two subclusters: subcluster 1a contained MO and SA (Euclidean distance $\approx$ 20), and MA and TR (Euclidean distance $\approx$ 17). Subcluster 1b was characterized only by PI, which showed $\approx$ 37.5 Euclidean distance with respect to the other populations of the same cluster. The second cluster contained SMR and CV (Euclidean distance $\approx$ 25) (Figure 1b).

The genetic relations among the autochthonous common bean landraces were also successively evaluated by using inter-simple sequence repeat (ISSR) markers [33]. The PCA resulted in a scatter plot with PC1 and PC2 scores that accounted for 52.73% and 23.11% of the total variance, respectively. Regarding PC1, two groups were identified: the CV, MA, PI, and SMR populations formed one group, and the TR, SA, and MO (Figure 1c) comprised the other. In PC2, CV was well separated from all the other populations (Figure 1c).

The dendrogram constructed using the UPGMA clustering confirmed the PCA results (Figure 1d). Indeed, two main clusters were identified: TR, SA, and MO were grouped in cluster 1, with the highest genetic similarity between SA and MO (Jaccard similarity index $\approx$ 0.91); while cluster 2 was divided into two subclusters: MA, PI, and SMR were grouped in subcluster 2a (Jaccard similarity index $\approx$ 0.81); and CV was assigned to subcluster 2b (Jaccard similarity index $\approx$ 0.69) (Figure 1d). The ISSR primers generated a number of bands between 4 and 8 (Table S2; Supplementary Materials): LOL2, LOL9, and LOL12 produced the highest number of total bands (NTB: 8), whereas PHV06 and PHV07 exhibited the lowest number (NTB: 4). The PHV06 banding profile was totally characterized by monomorphic bands (NTB: 4 and NMB: 4), with no polymorphism (0.00 % P), while LOL12 (NTB: 8 and NMB: 0) showed the 100.00% P. The values of resolving power (RP) ranged from 0.29 (LOL8) to 5.60 (LOL12), while polymorphism information content (PIC) ranged from 0.03 (LOL8) to 0.44 (LOL12) (Table S3; Supplementary Materials).

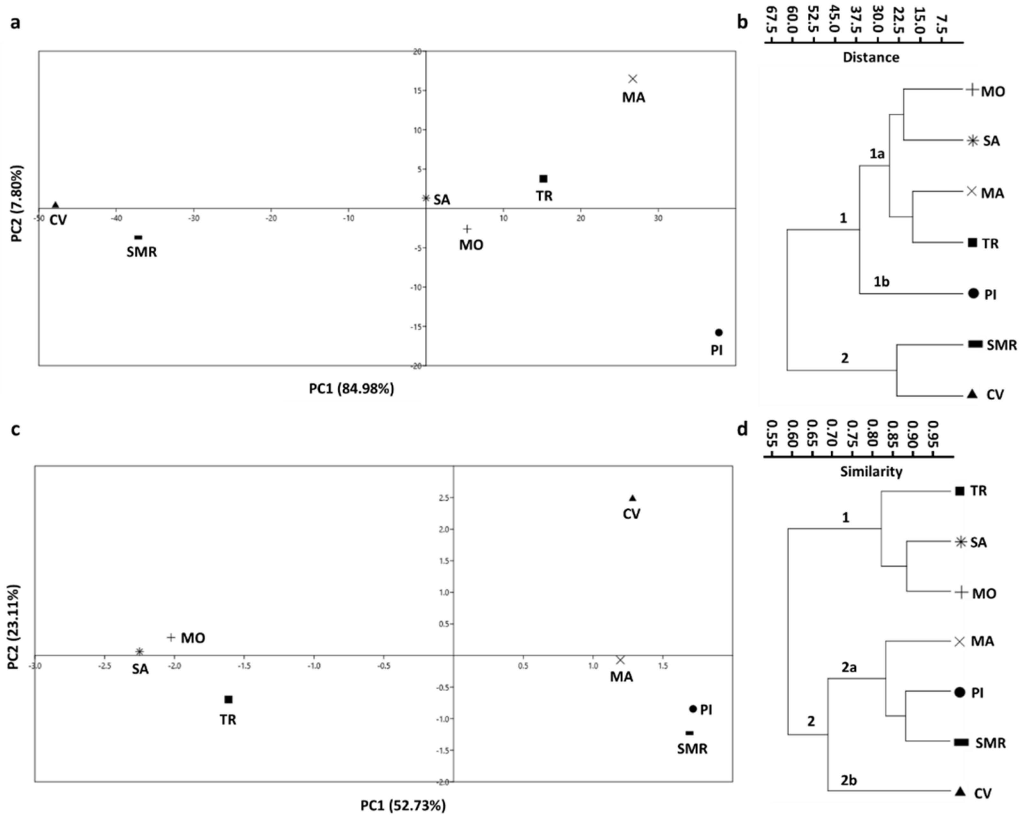

Figure 1. Principal component analysis (PCA) and UPGMA clustering of common bean morphological features and genetic data. Scatter plot of the PCA computed among seven populations of *Phaseolus vulgaris* L., using fifteen morphological features (**a**) and dendrogram resulting from a cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) with the Euclidean distance index (**b**). Scatter plot of the PCA computed on the seven bean populations, using eight inter-simple sequence repeat (ISSR) primers (**c**) and dendrogram resulting from UPGMA clustering (Jaccard similarity index) (**d**). CV: Ciliegino; SMR: San Michele Rosso; MO: Monachella; MA: Mascherino; PI: Pinto; TR: Tuvagliedda Rossa; SA: Suocera e Nuora.

2.2. Phaseolin Pattern Characterization

A detailed description and interpretation of the phaseolin diversity pattern (Mesoamerican and Andean gene pools) in cultivated common beans of different geographic origins was based on the high-quality 2-DE gel images and the PCR amplification of SCAR phaseolin (Phs), according to the methods of De la Fuente et al. [43].

The 2-DE maps revealed complex phaseolin patterns across the studied landraces (Figure S1; Supplementary Materials), which were characterized by a total of 26 spots that changed within each landrace (Figure 2). In particular, TR, MA, and PI showed a T-type (Tendergreen) phaseolin pattern (spots 3′/3, 5′/5, 6′/6, 7′/7, 9′/9, 10′/10, 12′/12, 15′/15, 18′/18, 19′/19, 22′/22, and 24); SMR and MO showed C-type (Contender) (spots 3′/3, 5′/5, 6′/6, 7′/7, 9′/9, 10′/10, 12′/12, 15′/15, 18′/18, 19′/19, 22′/22, and 25′/25) and A-type (Ayacucho) (spots 1′/1, 2′/2, 4′/4, 7′/7, 11′/11, 13′/13, 16′/16, 19′/19, and 22′/22) phaseolin patterns, respectively; while SA and CV were more similar to H-type (Huevo de Huanchaco) patterns (spots 3′/3, 4′/4, 5′/5, 7′/7, 9′/9, 11′/11, 12′/12, 15′/15, 18′/18, 19′/19, 21′/21, 23′/23, 25′/25 and 26′/26) (Figure 2).

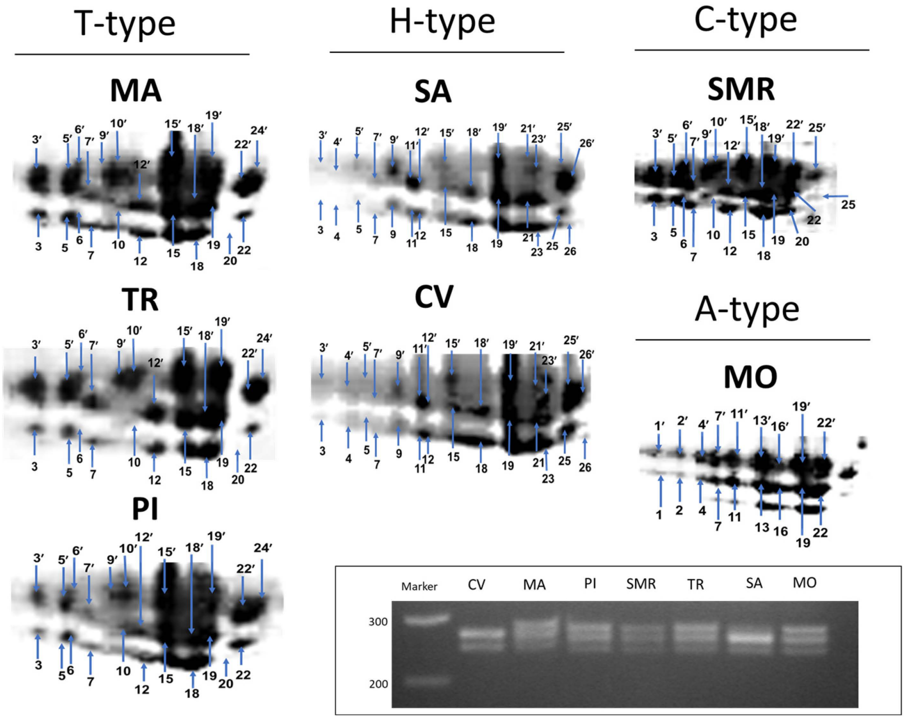

Figure 2. Phaseolin patterns. The 2-DE phaseolin spot patterns of seven common bean populations. Spots were numbered after comparison using the 2-DE maps reported in De la Fuente et al. [44]. Spots of different Mr and similar pI were denoted with and without apostrophes, respectively. The 2-DE imaging was performed using 50 µg of total protein loaded on Bio-Rad 7 cm long IPG strips containing pH 4–7 and 12% polyacrylamide gel. The amplification by PCR of the SCAR marker of the phaseolin in the seven common bean populations is shown in the rectangle. The ladder range is 200–300 base pairs (ApplyChem GmbH, Darmstadt, Germany). CV: Ciliegino; SMR: San Michele Rosso; MO: Monachella; MA: Mascherino; PI: Pinto; TR: Tuvagliedda Rossa; SA: Suocera e Nuora. C: Contender; T: Tendergreen; H: Huevo de Huanchaco; A: Ayacucho.

Overall, our 2-DE phaseolin profiles differed from those reported by De la Fuente et al. [43]. Indeed, the new spots that were identified for each population can be attributed to a variety of factors, including improvements in the protein extraction and resolution procedure. Additionally, CV showed some similarities with the Mesoamerican phaseolin pattern founded in the Sanilac (S-type) or Boyacá (B-type) accession used by De la Fuente et al. [43] (Figure 2). These results are fairly in agreement with those obtained by PCR amplification of the Phs SCAR that rendered two major profiles across landraces: two fragments of 249 and 275 bp were noted for CV and SA, typical of S-, B-, or H-type profiles, while three fragments of 249, 275, and 290 bp were obtained for MA, MO, SMR, TR, and PI, characteristic of T-, C-, or A-type profiles (Figure 2).

2.3. Morpho-Physiological Characteristics under Stress Conditions

In order to evaluate the effect of stresses on the different common bean landraces, the main plant morphological features, biomass distribution, and relative water content (RWC) were measured after 8 days of plant growth under control, osmotic-, and salt-stressed conditions.

The analysis of biomass allocation showed that the root (DW_{root}) and stem (DW_{stem}) dry biomass were decreased in SMR (64% DW_{root} and 51% DW_{stem}), PI (40% DW_{root} and 38% DW_{stem}), and SA (50% DW_{root} and 63% DW_{stem}) by the salt-stress treatment, and only in SA (43% DW_{root} and 67% DW_{stem}) by the osmotic-stress condition, when compared to the control plants (Figure 3a,b). In regards to the leaf dry biomass (DW_{leaf}), a decline under both types of stress was reported in SMR, MA, and SA (Figure 3c). In detail, in SMR, MA, and SA plants, DW_{leaf} was reduced by 52%, 38%, and 67% under salt stress, and by 43%, 51%, and 62% under osmotic stress, respectively (Figure 3c).

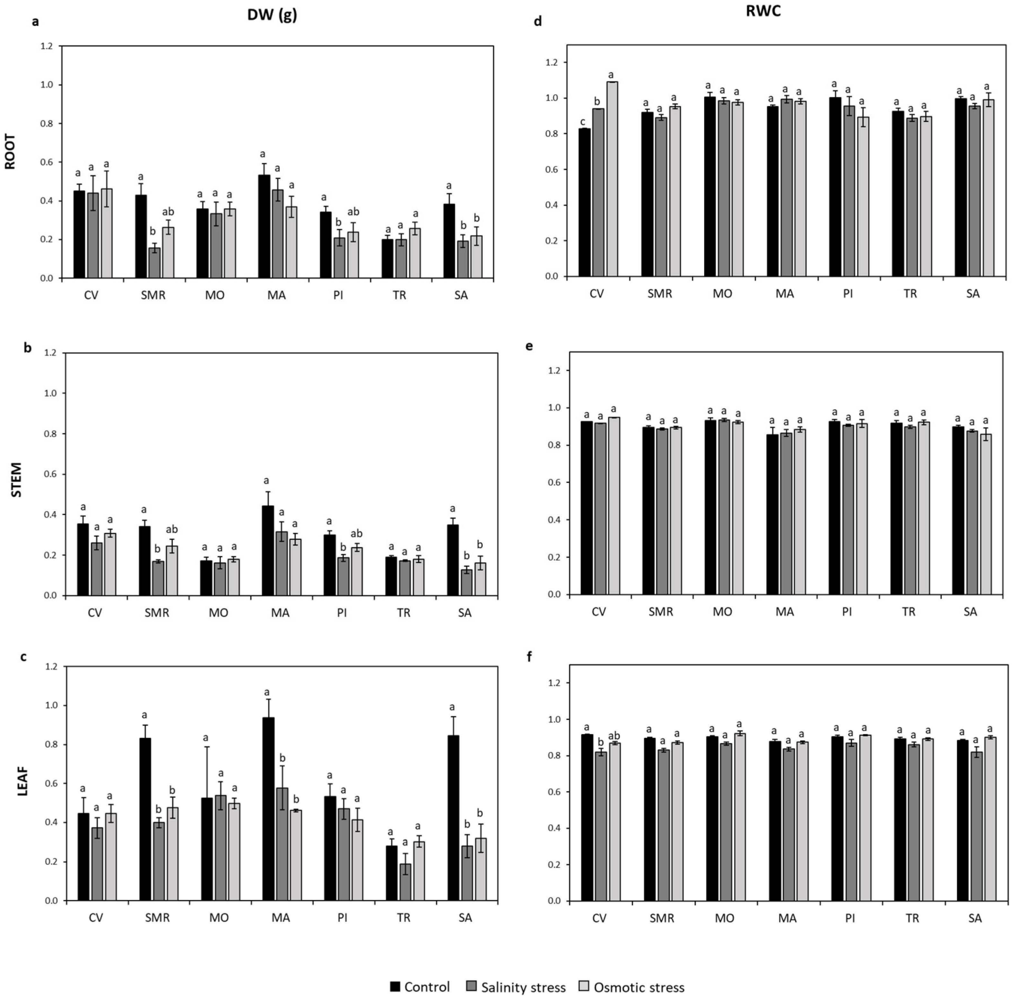

Figure 3. Dry biomass and relative water content. The dry biomass (DW; panel **a**–**c**) and relative water content (RWC; panel **d**–**f**) of roots, stems, and leaves of seven common bean populations, grown under controlled salt and osmotic stress conditions, were measured. Data represent the mean (n = 4) ± standard error. A Student's *t*-test was conducted to weigh the effects of different growth conditions ($p \leq 0.05$). Mean values marked with the same letter are not statistically different. CV: Ciliegino; SMR: San Michele Rosso; MO: Monachella; MA: Mascherino; PI: Pinto; TR: Tuvagliedda Rossa; SA: Suocera e Nuora.

Relative water content (RWC), measured in the three plant organs (roots, stems, and leaves), was proven to be differently affected by salt and osmotic stress. Indeed, the RWC_{root} in CV was increased by 13% and 32% under salt and osmotic stress, respectively. The RWC_{stem} results showed no change compared to the control, and in CV only, a reduction in the RWC_{leaf} (by 11%) was observed under salt stress (Figure 3d–f).

The analysis of the main morphological parameters showed that the stem height decreased by 23% in CV, 43% in MO, 29% in SMR, 14% in PI, and 38% in SA under the salinity stress condition with respect to the control; these reductions were observed under the osmotic stress in CV (20%), MO (22%), SMR (23%), MA (14%), and SA (18%) (Figure 4a). A decline in stem diameter was also reported in SMR, MA, and SA under the salt-stress conditions (with a reduction of 13%, 15%, and 31%, respectively) and osmotic-stress conditions (with a reduction of 17%, 23%, and 22%, respectively). The stem diameter also decreased in MO (14%) under osmotic-stress conditions (Figure 4b). The number of leaves was reduced in CV, MA, PI, TR, and SA under salt-stress conditions (decline of 28%, 29%, 24%, 22%, and 37%, respectively) and under osmotic stress (decline of 22%, 31%, 22%, 18%, and 45%, respectively) (Figure 4c).

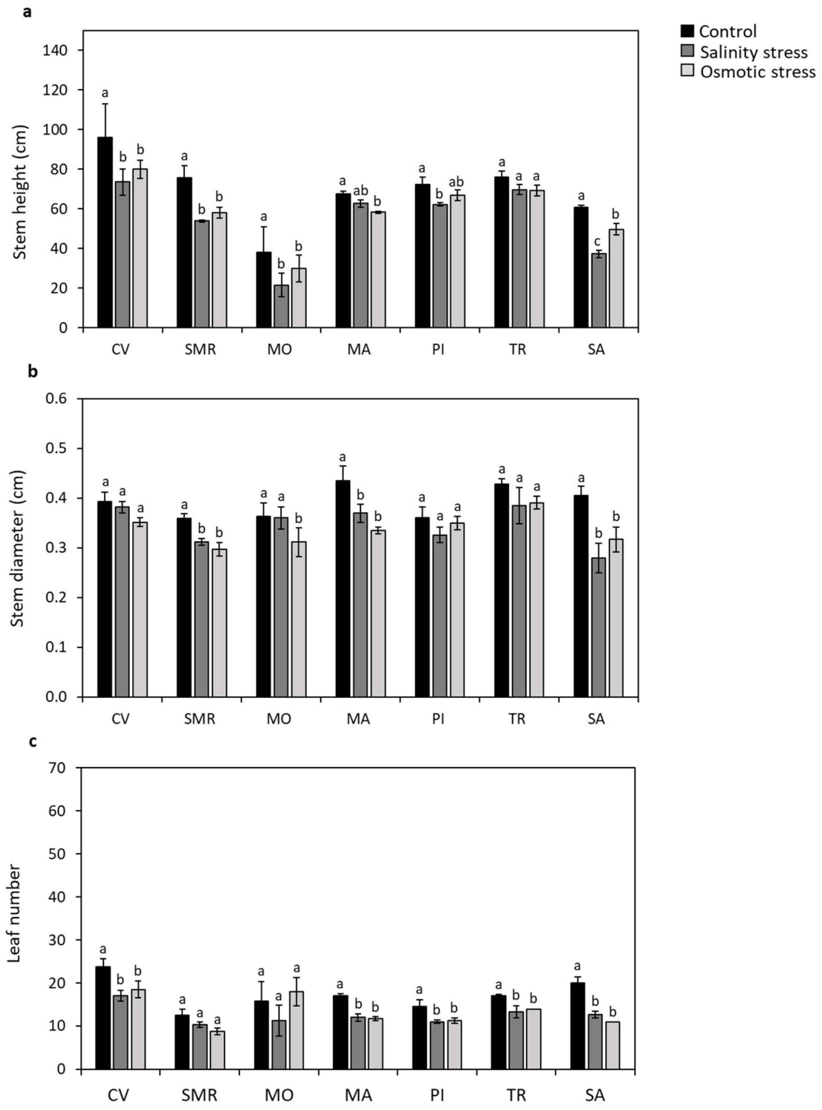

Figure 4. Plant morphological features. Stem height (**a**), diameter (**b**), and leaf number (**c**) of seven common bean populations grown under controlled, salt-, and osmotic-stress conditions. Data represent the mean (n = 4) ± standard error. A Student's t-test was conducted to weigh the effects of different treatments ($p \leq 0.05$). Mean values marked with the same letter are not statistically different. CV: Ciliegino; SMR: San Michele Rosso; MO: Monachella; MA: Mascherino; PI: Pinto; TR: Tuvagliedda Rossa; SA: Suocera e Nuora.

2.4. Biochemical Analysis

2.4.1. Proline and Malondialdehyde (MDA) Content

Proline was differently distributed in the above- and below-ground parts of common bean populations growing in controlled, salt-, and osmotic-stress conditions. In particular, the salt stress induced an increase in root proline content in SMR (105%), PI (193%), MO (50%), TR (62%), and SA (26%) (Figure 5a). The osmotic stress led to a 23% reduction in CV, while an increase was observed in MO (27%) and PI (116%), compared to the control condition (Figure 5a). The proline was also accumulated in leaves under the salt-stress condition. In detail, proline increased by 108% in SMR and by 68%, 57%, and 52% in MA, PI, and TR, respectively, over the control (Figure 5b). In contrast, the osmotic treatment decreased the leaf proline amount in CV and SA by 29% and 30%, respectively (Figure 5b).

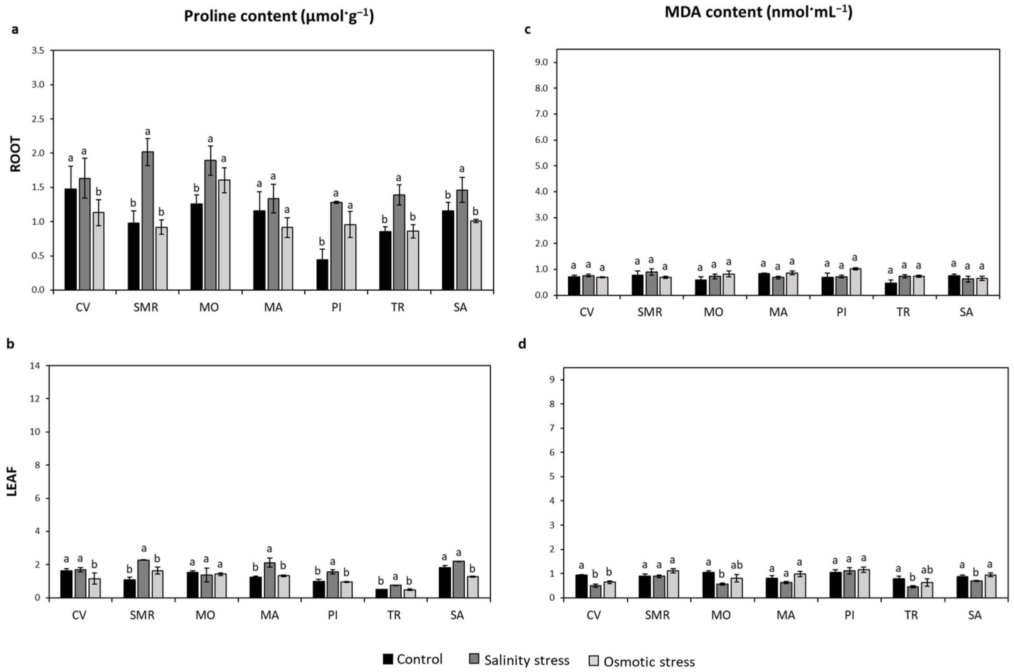

Figure 5. Proline and MDA content. The proline (µmol·g^{-1}; panel **a** and **b**) and malondialdehyde (MDA, nmol·mL^{-1}; panel **c** and **d**) contents were measured in the root and leaf of seven common bean populations grown under controlled, salt-, and osmotic-stress conditions. Data represent the mean (n = 4) ± standard error. A Student's *t*-test was conducted to weigh the effects of different treatments ($p \leq 0.05$). Mean values marked with the same letter are not statistically different. CV: Ciliegino; SMR: San Michele Rosso; MO: Monachella; MA: Mascherino; PI: Pinto; TR: Tuvagliedda Rossa; SA: Suocera e Nuora.

The analysis of MDA content, directly related to oxidative damage, reported no lipid peroxidation in the roots of any of the bean populations subjected to both stress conditions (Figure 5c). Conversely, at the leaf level, compared to the control, the MDA was reduced by 46%, 46%, 42%, and 22%, in CV, MO, TR, and SA, respectively, under salt stress, and by 31% in CV under osmotic stress (Figure 5d).

2.4.2. Photosynthetic Pigment Content

The analysis of the photosynthetic pigments revealed different accumulations of chlorophylls (total Chl A and Chl B) and carotenoids, depending on the bean population considered and the type of stress (Table 1). In detail, the salt stress reduced the total chlorophyll by 27% in CV, while increasing it by 25%, 16%, and 23% in MO, PI, and TR, respectively, compared to the control (Table 1). The Chl A/Chl B was reduced in SMR (8%) and increased in MO (13%), whereas the carotenoids increased by 45%, 23%, 27%, and 27%, in MO, PI, TR, and SA, respectively (Table 1). Under the osmotic-stress condition, the total chlorophyll content was affected only in CV and MO. In detail, it was 17% lower in CV and 20% higher in MO, with respect to the control (Table 1). Furthermore, reduced Chl A/Chl B values were observed in SMR (10%) and PI (14%), while increased Chl A/Chl B values were observed in MO (8%). Finally, the carotenoid content increased by 28% and 23% in MO and SA, respectively (Table 1).

Table 1. Leaf photosynthetic pigment content. Total chlorophyll (Chl) content ($\mu g \cdot mg^{-1}$), chlorophyll A and B ratio (Chl A/Chl B), and carotenoid content ($\mu g \cdot mg^{-1}$) of seven common bean populations grown under control, salt-, and osmotic-stress conditions. Data represent the mean ($n = 4$) ± standard error. A Student's *t*-test was conducted to weigh the effects of different treatments ($p \leq 0.05$). Mean values marked with the same letter are not statistically different. CV: Ciliegino; SMR: San Michele Rosso; MO: Monachella; MA: Mascherino; PI: Pinto; TR: Tuvagliedda Rossa; SA: Suocera e Nuora.

		Control	**Salinity Stress**	**Osmotic Stress**
Total Chl ($\mu g \cdot mg^{-1}$)	CV	1.34 ± 0.05 a	0.98 ± 0.04 b	1.11 ± 0.02 b
	SMR	1.12 ± 0.06 a	1.13 ± 0.14 a	1.08 ± 0.03 a
	MO	0.92 ± 0.02 b	1.14 ± 0.06 a	1.09 ± 0.03 a
	MA	1.61 ± 0.09 a	1.53 ± 0.06 a	1.72 ± 0.10 a
	PI	2.19 ± 0.08 b	2.56 ± 0.03 a	1.98 ± 0.17 b
	TR	1.41 ± 0.03 b	1.73 ± 0.03 a	1.15 ± 0.11 b
	SA	1.21 ± 0.06 a	1.41 ± 0.11 a	1.39 ± 0.09 a
Chl A/Chl B	CV	3.28 ± 0.05 a	3.22 ± 0.08 a	3.27 ± 0.01 a
	SMR	4.02 ± 0.05 a	3.71 ± 0.10 b	3.57 ± 0.07 b
	MO	3.19 ± 0.38 b	3.63 ± 0.26 a	3.44 ± 0.04 a
	MA	3.39 ± 0.04 a	3.22 ± 0.05 a	3.34 ± 0.04 a
	PI	3.54 ± 0.03 a	3.62 ± 0.27 a	3.02 ± 0.33 b
	TR	3.54 ± 0.02 a	3.33 ± 0.01 a	3.43 ± 0.04 a
	SA	3.23 ± 0.03 a	3.18 ± 0.03 a	3.28 ± 0.07 a
Carotenoids ($\mu g \cdot mg^{-1}$)	CV	0.19 ± 0.01 a	0.18 ± 0.01 a	0.19 ± 0.00 a
	SMR	0.16 ± 0.01 a	0.18 ± 0.02 a	0.15 ± 0.01 a
	MO	0.12 ± 0.00 b	0.18 ± 0.01 a	0.16 ± 0.01 a
	MA	0.26 ± 0.02 a	0.25 ± 0.01 a	0.26 ± 0.02 a
	PI	0.31 ± 0.01 b	0.39 ± 0.01 a	0.29 ± 0.03 b
	TR	0.23 ± 0.01 b	0.29 ± 0.00 a	0.18 ± 0.02 b
	SA	0.17 ± 0.01 b	0.21 ± 0.02 a	0.21 ± 0.01 a

3. Discussion

Over the last decades, agrobiodiversity has been jeopardized by several anthropologic pressures, including environmental degradation, rapid changes in land use, and the modernization of agriculture systems based on monoculture farming [44]. This has led to the abandonment of many traditional landraces, inducing a progressive loss of the genetic variability encompassed by the locally adapted germplasms [45], as well as the disappearance of the important traditional knowledge associated with their cultivation [15].

Appropriate characterization activities represent the main tools to assess plant diversity, which, in turn, could help to enhance conservation strategies and to ensure the sustainable use of these valuable plant genetic resources [46]. Furthermore, these activities may provide information about the genetic resources that are best suited for managing the current climatic variability and adapting to progressive climate change [42]. Indeed, landraces show a spectrum of responses to different stressors that are the combined result of the complex interactions among different morphological, physiological, and biochemical features [36–38], and which also contribute to the description of their diversity. A wide variety of methods have been used to investigate similarities and relationships among *Phaseolus vulgaris* landraces, and relevant differences were found in Italian common bean populations [47]. However, few studies have evaluated landrace diversity as related to environmental stresses.

Accordingly, a multi-level characterization approach—combining morphological, genetic, biochemical, and stress response-related studies—was used in this study to explore the diversity of the common bean landrace and to identify distinctive climate-smart traits [15,39]. In detail, an integrated approach using morphological, genetic, biochemical, and stress response analyses was used to characterize seven Italian *P. vulgaris* autochthonous landraces in order to (i) investigate the diversity and the relationships among the populations, and (ii) evaluate the stress response under salt- and osmotic-stress conditions, two of the main abiotic stresses occurring in the Mediterranean area.

Phenotypic characterization showed an appreciable morphological variation in seed descriptors (seed size, roundness, shape, color, and type of pattern) that separated the landrace populations into two main groups: one formed by CV and SMR, showing full red coat color, and the other composed of MO, SA, MA, and TR (with MO more similar to SA, and MA more similar to TR), with a bicolored (light and dark) seed coat pattern. The differences were mainly associated with seed coat color patterns and some morphometric descriptors related to seed size and shape.

Although the bicolor seed landrace is more widespread among the Apennine Italian regions, each population is named differently according to the place of cultivation or the morphological features of the seeds, resulting in slight differences. In detail, the SA, PI, and MO populations have all been cultivated by local communities in neighboring geographical areas of Alto Molise (SA: Sant'Angelo del Pesco—805 m.a.s.l.; PI: Agnone—830 m.a.s.l.; MO: Vastogirardi—1200 m.a.s.l.) for at least 50 years. The SA seeds are oval and characterized by white and purple coats, while MO and PI are white and burgundy, with a round and oval shape, respectively. The TR and MA populations, also characterized by seeds with bicolor coats, are cultivated in the other two Italian Apennine Regions. More specifically, TR is a white and mottled red bean landrace cultivated in the hilly areas of Basilicata (Sarconi—636 m.a.s.l.), and it is particularly appreciated for its taste and short cooking time; it is associated with the PGI (Protected Geographical Indication) quality marker. MA is a white and burgundy bean seed cultivated since the late 1800s in a hilly area of Tuscany (Piazza al Serchio—536 m.a.s.l.). In all bicolor landraces, the dark color was predominant, except for in PI, where a larger white area was found (see Table S1; Supplementary Materials).

The CV and SMR landrace populations took their name from the red color of their seed coat. In detail, the CV population is characterized by small, oval, red cherry-colored seeds, and it is typically cultivated only in Vastogirardi Village in the Molise region. SMR is a landrace cultivated in the hilly areas of Basilicata (Sarconi—636 m.a.s.l.), characterized by small, oval, ruby red beans, and which is also associated with the PGI quality marker. Furthermore, only CV exhibits small or medium-size seeds (about 32 g/100-seed weight), while all the other populations are characterized by a larger size (≥40 g/100-seed weight) (see Table S1; Supplementary Materials).

Analyzing the genetic pool of origin, the results confirm that all bean landrace populations derive from the Andean gene pool, as they exhibited the four typical Andean phaseolin characteristics (C-, H-, T-, and A-types). In detail, TR, MA, and PI showed the T-type phaseolin pattern, SA and CV exhibited the H-type pattern, SMR expressed the C-type pattern, and MO presented the A-type pattern. The PCR-based SCAR marker of phaseolin confirmed that all of the landraces belonged to the Andean genetic pool, revealing two bands typical of the H-type in the case of CV and SA and three bands for SMR, TR, MO, MA, and PI, common in the C-, T-, and A-type gene pools. This result supports the proven predominance of the Andean genotypes over the Mesoamerican examples in the Mediterranean Basin. Furthermore, a prevalence of C-type over T-type phaseolin has been reported in Italian and European common bean landraces [48,49], while the H-type phaseolin has proven to be more unusual among the common bean populations in Europe [22]. Moreover, it has been found that cultivars belonging to the Mesoamerican gene pool show small or medium-sized seeds (<25 g or 25–40 g/100-seed weight), while their Andean counterparts have larger seeds (>40 g/100-seed weight) [22]. This evidence is not completely supported by our data, in which the 100-seed weight of CV, SMR, and SA, associated with the Andean gene pool, was 32.2 g, 39.9 g, and 40.0 g, respectively (see Table S1; Supplementary Materials); however, similar exceptions were also found by Logozzo et al. [48] and Piegiovanni et al. [50].

Genetic data, obtained by using ISSR molecular markers, differentiate the previously morphological and phaseolin related landraces into two new main groups. Collectively, TR, SA, and MO were grouped together, with a higher genetic similarity observed between SA and MO, while MA, PI, and SMR were grouped together, and CV stood alone. Although ISSR molecular markers can give us a sense of the patterns and the extent of diversity found among landrace populations [10,51], the small amount of polymorphic loci data assayed in this study might only provide a rough indication of genetic relationships among landraces, which could be further analyzed on a smaller scale.

The frequent practice of seed exchange among the farmers [50], especially in central Italy, where the exchange of seed materials occurred frequently over the years, following transhumance pathways [51], could explain the higher genetic similarity within the *P. vulgaris* landraces originating from different Italian regions. This practice generally affects the genetic structure of the germplasm, resulting in a closer relatedness with populations cultivated in different Italian regions. In this regard, the Molise region, as a geographically central transhumance region, can act as a connecting element of the Italian Apennine regions [52].

Analyzing the bean landrace populations' ability to tolerate salt and osmotic stress, landraces displayed specific morpho-physiological and biochemical changes in the three plant organs (leaf, stem, and root), which were strictly related to the genotype and type of stress (salt or osmotic stress). In detail, salt stress adversely affected the overall growth of the SMR and SA landrace populations, reducing biomass accumulation in all three organs. Furthermore, it negatively impacted the root and stem biomass accumulation in PI, but only the leaf biomass in MA populations. The negative effect of salt stress on biomass accumulation was found to be strictly correlated with the high levels of proline in the different organs of the affected populations. On the contrary, osmotic stress only impacted the overall biomass accumulation of the SA landrace population and the leaf biomass of SMR and MA. No correlation with proline content was found. Depending on the landrace, a decrease in stem and leaf biomass was also associated with a decrease in stem diameter, branching, and/or height, as well with a decrease in leaf number and/or area. It is widely reported that salt stress has a higher impact on plant growth and development compared to osmotic stress [53], since it is responsible for both osmotic (cell dehydration) and toxic (ions accumulation) effects on plant cells, impairing several morpho-physiological parameters of plants [54]. Several studies reported a positive correlation between proline accumulation and stress tolerance in plants [55,56]. However, in some plant species, and in particular, in *P. vulgaris* plants, proline levels were higher in salt stress-sensitive cultivars compared to salt stress-tolerant examples [57,58]. These authors assumed that proline accumulation is a symptom of injury, rather than an indicator of salinity resistance, and that its biosynthesis presumably occurred as a consequence of disturbance in cell homeostasis, reflecting damage in response to salt stress.

However, the negative impact of salt or osmotic stress on plant growth was not associated with oxidative stress, as reported by the unchanged or even decreased MDA contents in the leaves and root of the populations with impaired growth.

It is reported that the accumulation of proline can alleviate membrane damage and reduce MDA levels in plants subjected to stress conditions [59]. However, proline is not only functional in preventing lipid membrane peroxidation, but it is also involved in other physiological functions when plants experience stress. As these functions include osmoprotection, free radical scavenger activity, macromolecule protection from denaturation, regulation of cytosolic acidity, and regulation of programmed cell death, proline homeostasis is essential for generating energy for metabolically demanding cells [60]. It is therefore likely that its importance is related to its ability to integrate growth/development according to environmental cues, and that the plant must develop specific strategies to effectively channel proline for optimizing the growth–defense tradeoffs. However, while there was an unequivocal association of higher proline contents with growth inhibition induced by salt stress, it was uncorrelated with osmotic stress. Indeed, in the SA landrace population, despite osmotic stress inducing a reduction in plant growth parameters (biomass and morphological traits), the proline content remained unchanged. However, plant stress responses may vary, considering the type, intensity, and duration of the stress. Moreover, even though stress tolerance mechanisms are based on specific stress responses, not all responses are relevant for tolerance. Proline clearly plays more than one role in the plant, and other aspects are important in the regulation of proline metabolism. For example, a partial catabolization of proline to pyrroline-5-carboxylate (P5C), which is toxic for certain tissues and leads to apoptosis, could be hypothesized to affect the overall plant growth [61].

Thus, proline accumulation could be considered as a general response to salt stress, rather than a tolerance mechanism. The unchanged proline content observed in the CV, MO, and TR populations, which are tolerant to both salt and osmotic stress, confirmed this hypothesis. Thus, other ion osmotic adjustments, including those related to K^+, Na^+, Cl^-, and organic osmolyte (sugar alcohols or ammonium compound), could also fulfill osmoprotective functions [62].

Furthermore, the involvement of an effective antioxidant defense system, composed of enzymatic and non-enzymatic components, could combat the harmful effects of oxidative stress on the cellular components [63]. Among different redox-balancing agents, it could be assumed that carotenoids, along with chlorophyll, could act as defense molecules against stress [64]. This evidence corresponds with the higher levels of chlorophylls and carotenoids found in MO and TR landraces. Indeed, carotenoids, besides representing essential pigments in photosynthesis, also play a major role in oxidative stress tolerance, since they protect the photosynthetic apparatus by quenching harmful free radicals, formed naturally during photosynthesis and under exposure to stress conditions [65]. Total chlorophyll content and/or Chl A/Chl B ratios were also altered under stress conditions, as a means of maximizing photosynthetic efficiency [66].

The CV bean populationwas not affected by salt or osmotic adversity in term of biomass accumulation but showed a decrease in RWC and proline amounts at root and leaf level, as well as a decrease in total chlorophyll. The RWC is an important indicator of water status in plants because it reflects the balance between water supply to the tissues and transpiration rate [67]. Reductions in RWC under salt and osmotic stress, caused by low osmotic potential, are commonly reported in the literature [68,69]. In detail, it has been argued that salt-tolerant plants decrease the hydraulic conductance of their roots, thereby reducing the delivery of (salty) water to the shoot and resulting in reduced water potential in their leaves [70]. The osmotic-induced low water potential could also accelerate the degradation of structural proteins [71], from which most of the osmolytes are synthesized. This could explain why a decrease in both RWC and proline amounts was observed in CV leaves, and a negative correlation was observed in CV roots (high RWC and low proline). However, other active (augmentation of other solutes within the cells, such as sugar or ammonium compounds) and/or passive (loss of cell water) osmotic adjustments could play a role in stress response—allowing plant tissues to retain water, even at low water potentials, maintaining turgor—and indirectly, in growth and productivity under water deficit conditions [72].

The lower amounts of chlorophyll could be interpreted as a tolerant trait and, in particular, an alternative route to produce H_2O_2 by the photocatalytic activity of chlorophyll itself, which acts as a secondary messenger in various stress-responsive signaling pathways, and which have been found essential for “salt stress preparedness” in tolerant plant species [73,74].

4. Materials and Methods

4.1. *Phaseolus vulgaris* Landraces

The seeds of seven common bean (*Phaseolus vulgaris* L.) landraces, cultivated in the Molise region (Italy), and in other two Italian Apennine regions (Tuscany and Basilicata) were analyzed (Figure S2; Supplementary Materials). Specifically, four landraces were collected from local growers in the geographical areas of Alto Molise, represented by the Vastogirardi (Ciliegino, CV, and Monachella, MO), Agnone (Pinto, PI), and Sant’Angelo del Pesco (Suocera e Nuora, SA) municipalities of the rural districts of the Alto Medio Sannio Inner Area [75]. All these municipalities are considered by the National Strategy for Inner Areas (SNAI), which aims to fight the depopulation and further marginalization of Italian inland areas by promoting territorial development and cohesion. One landrace, called Mascherino (MA), was collected from a single farm located in Piazza al Serchio (LU, Tuscany region, Italy) and, the two others, Tuvagliedda Rossa (TR) and San Michele Rosso (SMR), awarded in Italy with the PGI (Protected Geographical Indication) quality marker, were purchased from Belisario Farm (Sarconi, PZ, Basilicata region, Italy). All the seeds, collected from different landrace populations, were divided into two lots: one assigned to the Germplasm Bank of the University of Molise for long-term storage, and the other used for seed multiplication and for future reference.

4.2. Seed Morphological Traits

A total of fifteen morphological parameters were measured for each common bean seed population. In detail, quantitative morphological parameters, such as area (mm^2), perimeter (mm), major axis length (mm), minor axis length (mm), roundness, dark-colored seed area (mm^2), and light-colored seed area (mm^2), were measured from digital images using the software Image J (Version 1.51i Wayne Rasband-NIH; https://rsb.info.nih.gov/ij/ accessed on 20 January 2022), while 100-seed weight (g), 100-seed volume (mL), and density ($g \cdot mL^{-1}$) were measured with a precision balance and a graduated cylinder.

Qualitative seed descriptors were visually assessed using Image J software analyzing the following characteristics: seed coat pattern (absent, bicolor, spotted bicolor), number of seed coat colors (one, two, more than two), primary/main seed coat color (red, white, dark), predominant secondary seed coat color (none, white, dark), and seed shape (round, oval) [76,77].

Principal component analysis (PCA) was performed to define the role of each morphological characteristic in the grouping of accessions. Cluster analysis was also conducted by UPGMA (unweighted pair group methods using arithmetic averages) analysis to create a dendrogram with Past Version 4.03 software.

4.3. DNA Analysis

For each landrace population, seeds were germinated in a growth chamber under controlled conditions (25 °C), and total genomic DNA was extracted from five individuals (youngest leaf of 10-day-old seedlings); the samples were processed separately. The DNA extraction was performed using the Invisorb® Spin Plant Mini Kit (Stratec Molecular GmbH, Berlin, Germany). DNA was quantified by measuring the absorbance at 260 nm on a spectrophotometer, and individual stock concentrations were adjusted to 20 $ng \cdot \mu L^{-1}$ for PCR. A total of 8 inter-simple sequence repeat (ISSR) primers, reported in Marotti et al. [2] and Peña-Ortega et al. [28], were used for the genetic analysis (Table S3; Supplementary Materials). Amplification reactions were carried out in volumes of 25 µL, containing H_2O MilliQ, 20 $ng \cdot \mu L^{-1}$ template DNA, 1 unit of GOTaq DNA polymerase (Promega, Madison, WI, USA), 2.5 mM each of dNTP (Sigma-Aldrich, St. Louis, MO, USA), 25 mM MgCl2 (Promega, Madison, WI, USA), and 10 µM primer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in 5x Green color Go Taq Flexi buffer (Promega).

Polymerase chain reactions (PCRs) were run in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 3 min at 94 °C, for initial denaturation, 36 cycles of 45 s at 94 °C for denaturation, 1 min at annealing temperature, 1 min at 72 °C for extension, followed by 5 min at 72 °C for a final extension of the single strands. ISSR-amplified fragments were resolved on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light. Gels were scanned with ChemiDoc (Bio-Rad), and amplification profiles were analyzed with the Quantity-One Band Analysis software (Bio-Rad). Bands were scored according to presence or absence, and the raw data were processed with Past Version 4.03 software to obtain a standardized matrix and perform PCA and UPGMA clustering (Jaccard's similarity index). Finally, for each ISSR marker, the number of total bands (NTB), number of monomorphic bands (NMB), number of polymorphic bands (NPB), percentage of polymorphic bands (% P), resolving power (RP), and polymorphism information content (PIC) were calculated, according to the methods of Abdelaziz et al. [78].

4.4. Phaseolin Analysis

4.4.1. Protein Extraction and Two-Dimensional Electrophoresis (2-DE) Separation

The total proteins were extracted from three independent samples (1.0 g of a pool of 30 dry seeds), as described by Scippa et al. [79]. The protein quantity in the extracts was measured following the Bradford method, using bovine serum albumin as the standard.

For isoelectric focusing (IEF) analysis, immobilized pH gradient (IPG) strips (7 cm pH 4–7, Bio-Rad) were rehydrated overnight with 125 mL of rehydration buffer [6 M urea, 2% (*w*/*v*) CHAPS, 0.5% (*v*/*v*) Triton X-100, 20 mM dithiothreitol (DTT) and 1% (*w*/*v*) carrier ampholytes pH 3–10] containing 50 µg of total proteins to obtain the best resolution of the area of the 2-DE gels, wherein the phaseolin polypeptides are located [43].

Isoelectric focusing (IEF) was performed with the PROTEAN IEF Cell system (Bio-Rad) at 12 °C at the following voltages: 250 V (90 min), 500 V (90 min), 1000 V (180 min), and 8000 V, for a total of 55 kVh. Focused strips were incubated in the equilibration solution (50 mM Tris pH = 8.8, 6 M urea, 30% glycerol, and 2% SDS) with 2% dithiothreitol (DTT) for 20 min at room temperature and then with 2.5% iodoacetamide (IAA) for the same time and under the same conditions.

Two-dimensional electrophoresis was carried out using a Protean apparatus (Bio-Rad) and 12% polyacrylamide gels (30% Acrylamide mix, 1.5M Tris pH 8.8, 10% SDS, 10% Ammonium persulfate, Temed), with 120 V applied for 2–3 h. Each sample was run in triplicate. Standard proteins (Bio-Rad) were used to estimate the molecular weight of the protein spots.

Gels were fixed for 1 h with a solution of ethanol 40% and acetic acid 10%, stained for 16–18 h with Brilliant blue G-Colloidal Concentrate (Sigma Aldrich), rinsed for 4 days, and scanned using a Chemi Doc (Bio-Rad) device. Image analysis was performed using the PDQuest software, version 8.8 (Bio-Rad). Spot detection and matching between gels were performed automatically, followed by manual verification. After normalization of the spot densities against the whole-gel densities, the percentage volume of each spot was averaged for the three different replicates for each gel.

4.4.2. Amplification of the Phaseolin (SCAR) Marker by PCR

Amplification of the sequence-characterized amplified region (SCAR) marker of the phaseolin seed protein (Phs) locus was carried out by the PCR on three individuals in each population. Amplification reactions were carried out in volumes of 25 µL, containing H_2O MilliQ, 20 ng·µL^{-1} template DNA, 2 units of GOTaq DNA polymerase (Promega), 2.5 mM of dNTP (Sigma-Aldrich), 25 mM $MgCl_2$ (Promega), and 10 µM primer (Invitrogen, Thermo Fisher Scientific) in 5x Green color Go Taq Flexi buffer (Promega, WI, USA).

The sequence for the upstream Phs primer is 5′-AGCATATTCTAGAGGCCTCC-3′, and the sequence for the downstream Phs primer is 5′-GCTCAGTTCCTCAATCTGTTC-3′. The PCR primers were selected from regions of complete identity between the T and S phaseolin sequences [80] covering the region where the 15 bp repeat (present in the a-type genes) and a 21 bp direct repeat (third intron) are located.

PCR reactions were run in a T100 Thermal Cycler (Bio-Rad) under the following conditions: 3 m at 94 °C, for initial denaturation, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 5 min at 72 °C for a final extension of the single strands. Amplified fragments were resolved on a 2.5% agarose gel along with a 100–1000-bp ladder (ApplyChem GmbH, Darmstadt, Germany), stained with ethidium bromide, and visualized under UV light. The gels were scanned with a ChemiDoc (Bio-Rad) device.

4.5. Plant Growth Conditions and Stress Treatments

Seeds of each population were germinated in plateau pots on moistened vermiculite and placed in a growth chamber under controlled conditions (16 h light/8 h dark cycle under 25 °C temperature) for 20 days.

Four plants for each population were successively transplanted on a moistened mixture of soil and vermiculite (2:1) in 0.5 L pots (diameter = 11 cm) placed in plastic trays (12 pots per tray), and grown under different conditions: (i) control—plants were irrigated with 50 mL of distilled water; (ii) salt stress—plants were irrigated with 50 mL of 200 mM NaCl solution; and (iii) osmotic stress–plants were irrigated with 50 mL of 180 mM mannitol solution. The salt and osmotic concentrations were chosen to reproduce the osmotic potential of 0.8 MPa for both conditions [81]. The solutions were renewed every day for 8 days, and all plants were grown in an environmental chamber under the following controlled conditions: long-day photoperiod (16 h of light and 8 h of darkness), light intensity yield of approximately 250–300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (light meter sensor—HD2302.0—Delta Ohm; Caselle di Selvazzano, Italy) at pot height, a temperature of 23 °C, and a relative humidity range between 40–50%. Plant material (roots, stems, and leaves) was harvested after 8 days of growth and subjected to further analysis.

4.6. Plant Morpho-Physiological Analysis

Plant morphological analyses were performed by measuring the stem height and the leaf number, and by using ImageJ Version 1.51i software (Wayne Rasband-NIH https://rsb.info.nih.gov/ij/ accessed on 20 January 2022) to measure the stem diameter.

In addition, leaf, stem, and root biomass allocations were determined after two days of drying in an oven at 80 °C (dry weight, DW), and the relative water content (RWC) of the three organs (root, stem, and leaf) was also calculated using the formula reported in Smart and Bingham [82]: $[(FW—DW)/(TW—DW)] \cdot 100$.

All the measurements were performed on four plants and expressed as mean ± standard error. The statistical differences among treatments and populations were determined through a Student's *t*-test, and significance was accepted at $p \leq 0.05$.

4.7. Biochemical Analysis

4.7.1. Proline Content

Proline content was measured in the roots and leaves according to the method of Carillo and Gibon [83], with some modifications. Briefly, plant material (0.05 g), powdered with liquid nitrogen, was homogenized in 1 mL of 70% ethanol, incubated at 95 °C for 20 min, and cooled in an iced bath. The mixture was centrifuged (10 min, 14,000× *g*), and the supernatant was recovered and stored at −20 °C.

For proline determination, 500 µL of extract (supernatant) was added to 500 µL of reaction mix (50% of 2% ninhydrin and 50% of 60% glacial acetic acid); the solution was incubated at 95 °C for 20 min, cooled in an iced bath, centrifuged (10 min, 10,000 rpm), and allowed to stand for 24 h in the dark at 4 °C. Next, the samples were centrifuged (15 min, 10,000 rpm), and their absorbance was determined by a spectrophotometer (Bio-Rad) at 520 nm. The proline concentration was determined as mean ± standard error of triplicate measurements using a standard curve and expressed as $\mu mol \cdot g^{-1}$ using the equation reported by Carillo and Gibon [83].

The statistical differences among treatments and populations were determined through the Student's *t*-test, and significance was accepted at $p \leq 0.05$.

4.7.2. Lipid Peroxidation Assay

Lipid peroxidation in the roots and leaves was determined by using the thiobarbituric acid (TBA) reaction, followed by the measurement of malondialdehyde (MDA) content, as reported by Ben Abdallah et al. [84]. Plant material (0.05 g), powdered with liquid nitrogen, was extracted with 0.5 mL of 0.1% trichloroacetic acid (TCA). The samples were centrifuged at 15,000× *g* for 10 min, and 250 µL of supernatant was added to 1 mL of 0.5% TBA prepared in a 20% TCA solution. The solutions were incubated at 95 °C for 30 min, vortexed, cooled in an ice bath, and then centrifuged at 10,000× *g* for 15 min. The specific absorbance of the supernatant was measured at 532 nm, while the non-specific absorbance was measured at 600 nm. The MDA concentration was expressed as nmol·mL^{-1} (mean ± standard error of triplicate measurements) and calculated as reported by Hodges et al. [85].

The statistical differences among treatments and populations were determined through the Student's *t*-test, and significance was accepted at $p \leq 0.05$.

4.7.3. Photosynthetic Pigment Content

The total chlorophyll (Chl), the Chl A/Chl B ratio, and the carotenoid contents were determined spectrophotometrically in the leaf tissues, according to the method reported by Polzella et al. [86]. Briefly, fresh leaves (0.1 g), powdered with liquid nitrogen, were homogenized in 1.8 mL of N, *N*-dimethylformamide (DMF), and stored for 48 h at 4 °C. The samples were centrifuged at 14,000× *g* for 5 min and their absorbance was determined at 664 nm, 647 nm, and 480 nm. Photosynthetic pigment content was expressed as µg·mg^{-1} of fresh weight, and as the mean ± standard error of triplicate measurements.

The statistical differences among treatments and populations were determined through the Student's *t*-test, and significance was accepted at $p \leq 0.05$.

5. Conclusions

The present study demonstrates that a multi-level characterization approach, combining morphological, genetic, biochemical, and stress response-related studies, proves to be an efficient method to explore landrace diversity and identify climate-smart distinctive traits in these dynamic populations.

Indeed, relevant information was obtained on the diversity of some Apennine common bean landraces, as well as their ability to tolerate salt and osmotic stresses. The genetic distance among landrace populations did not correlate with their stress tolerance level. In particular, the CV and MO population, cultivated in the Molise region, and TR, cultivated in Basilicata region, were found to be salt-tolerant, whereas MA (Tuscany region), SMR (Basilicata region), and SA and PI (Molise region) were found to be the most sensitive to these stresses. The osmotic stress negatively impacted only the SA landrace population. The practice of frequent exchange among the farmers of central Italy could have affected the genetic structure of the germplasm and/or the plant plasticity, resulting in a closer relatedness with populations cultivated in different Italian regions. Furthermore, the results suggest that proline could be used as a large-scale biochemical screening marker for salt-sensitive bean landraces; however, it is not suitable for use in the case of osmotic stress. Other biochemical traits, such as the amount of sugar alcohols, ammonium compounds, or redox-balancing agents, could be used as general biomarkers of bean stress tolerance. The screening and identification of other common bean landrace populations susceptible or tolerant to salinity and osmotic stress are of great interest for identifying climate-smart landraces for increasing the productivity of staple food crops in stressful environments, which is of interest for plant adaptation to climate change and for the maintenance of yield stability under marginal conditions.

However, short-term and long-term field experiments should be conducted to monitor whether prolonged exposure to salt and osmotic stress may prevent plant growth and to determine whether plant priming could be a complementary and useful strategy to mitigate the injurious effects of salt and osmotic stress. More in-depth "omics" studies will be needed to shed light on the molecular mechanisms adopted by these locally adapted genetic plant resources to cope with salinity and osmotic stresses and to identify important traits useful for plant tolerance/adaptation to the ongoing climate change.

Further investigation should be also planned to explore the nutritional potential of different common bean landrace populations, characterizing the chemical diversity of their seeds and evaluating the possible presence of specific phytochemical markers of landraces and/or nutraceutical compounds important for human health. Starting from these suggestions, agrobiodiversity valorization could represent an effective tool for encouraging models for the sustainable production and use of food. The results of this study could also promote the cultural and socioeconomic value of the marginalized Apennine Italian areas in which these genetic resources are cultivated, transforming currently marginal areas into productive lands.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11202790/s1, Table S1: Morphological descriptors and analysis; Table S2: ISSR banding profile in common bean landraces; Table S3: Primers used for ISSR analysis and their respective markers' performance indexes; Figure S1: The 2-DE profiles of common bean landraces; Figure S2: Autochthonous Italian common bean seed accessions.

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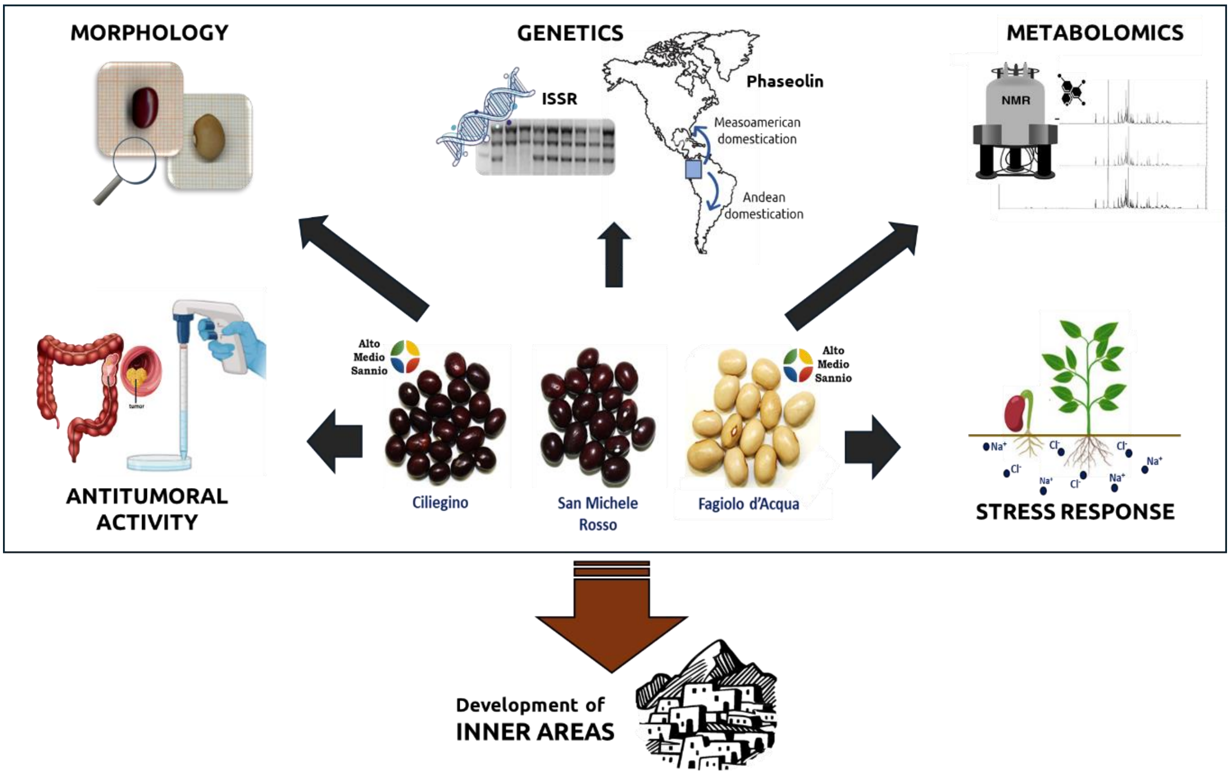
3.3 Building the Resilience of Marginal Rural Areas Using a Complementary Characterization Approach: Possible Beneficial Health Effects and Stress Tolerance of Italian Common Bean (*Phaseolus vulgaris* L.) Landraces – Paper II

In the present work, published on *Diversity*, 16(4): 240 (*2024*; https://doi.org/10.3390/d16040240), a comprehensive approach was used to characterize seed morpho-colorimetric traits, genetic diversity, and NMR metabolomic profiles of three Italian common bean landraces (Ciliegino, San Michele Rosso and Fagiolo d'Acqua). Specific physiological and biochemical features (antioxidant molecules, osmolytes, structural reorganization of photosynthetic pigment, etc.) together with the accumulation of distinctive metabolites contribute to the description of the observed diversity among landraces in terms of salt stress response and antiproliferative abilities on intestinal human cancer cells. This information could be useful to establish their value in terms of environment and/or human health "service", both essential to translating landraces into competitive products, a prerequisite for envisioning appropriate strategies for their conservation and driving force for revitalization of marginal rural areas.

Paper II


MORPHOLOGY
GENETICS
ISSR
Phaseolin
Measoamerican domestication
Andean domestication
METABOLOMICS
NMR
Alto Medio Sannio
Ciliegino
San Michele Rosso
Fagiolo d'Acqua
Alto Medio Sannio
tumor
ANTITUMORAL ACTIVITY
Na⁺
Cl⁻
STRESS RESPONSE
Development of
INNER AREAS

diversity

MDPI

Article

Building the Resilience of Marginal Rural Areas Using a Complementary Characterization Approach: Possible Beneficial Health Effects and Stress Tolerance of Italian Common Bean (*Phaseolus vulgaris* L.) Landraces

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Abstract: Common bean landraces, besides contributing to the preservation of the social and cultural identity of the local communities of the production area, typically display adaptability to adverse agro-climatic conditions. This adapted germplasm is a repository of the gene pool and also shows typical phytochemical profiles, representing an essential source of bioactive components. However, genetic erosion is progressively affecting this genetic material, creating serious threats to its cultivation in marginal rural areas and use as a source of biodiversity and bioproducts. In the present work, a comprehensive approach was used to characterize the seed morpho-colorimetric traits, genetic diversity, and NMR metabolomic profiles of three Italian common bean landraces. Specific physiological and biochemical features (antioxidant molecules, osmolytes, structural reorganization of photosynthetic pigment, etc.), together with the accumulation of distinctive metabolites, contribute to the description of the observed diversity among the landraces in terms of the salt stress response and antiproliferative abilities on intestinal human cancer cells. This information could be useful in establishing their value in terms of environmental and/or human health "service", both essential to translating landraces into competitive products, a prerequisite for envisioning appropriate strategies for their conservation and a driving force for the revitalization of marginal rural areas.

Keywords: antiproliferative activities; genetic diversity; inland areas; ISSR; NMR-based metabolomics; phaseolin; seed morpho-colorimetric descriptors; salt stress response

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1. Introduction

Over the last few decades, agricultural ecosystems have increasingly lost their biological diversity based on local landraces. This loss has been due to the use of modern intensive farming systems characterized by high-input and high-yielding crop varieties. Thus, there has been an abandonment of the use of traditional cropping practice, landrace cultivation and on-farm conservation [1].

Local landraces, besides contributing to the preservation of the social and cultural identity of the local communities in the production area, are repositories of gene pools that enrich agrobiodiversity, sustainably maintaining and stabilizing ecosystems to make them functional [2]. Indeed, this local germplasm shows adaptability to different agroclimatic conditions, maintaining considerable diversity among and within populations [3]. They are also characterized by typical phytochemical profiles, as compared to more common modern varieties, representing an essential source of important nutraceutical and bioactive components with multiple beneficial effects for human health [4].

The Mediterranean basin has a long and rich history of cultivation of several landraces [5,6]. Particularly, since ancient times, legume landraces have been the backbone of the Mediterranean agroecosystems, providing an excellent and inexpensive source of high-quality nutrients and representing a staple food for the human diet [7,8]. Among them, the common bean (*Phaseolus vulgaris* L.), an annual herbaceous plant with different growth habits, varying from determinate/indeterminate bush to climber/semi-climber vine, is a global resource for direct human consumption [9]. Independently domesticated in Andean South America and Mesoamerica some 7 million years ago, it achieved widespread distribution in Europe after several introductions from the New World, combined with direct trade among other Mediterranean countries over the centuries [10,11]. Thus, this legume species has adapted to grow in a wide range of environments, generating a huge diversification that serves as a source of plant stress response and adaptation to environmental constraints [12]. High yields and quality traits could be achieved with low inputs, thus addressing current and future climate change challenges [13]. Moreover, its nitrogen-fixing properties improve soil fertility and productivity, with a low impact on the carbon footprint and supporting agroecosystem biodiversity [14]. From the nutritional point of view, the common bean is regarded as "a grain of hope", particularly for poor communities, being rich in protein, complex carbohydrates, fiber, vitamins, unsaturated fatty acids, such as linoleic and oleic acids, and inorganic minerals (iron, zinc, copper, phosphorous, and aluminum) [15,16]. In addition to representing a valuable source of nutrients, lectins (phytohemagglutinins, PHAs; PHA-E and PHA-L), condensed tannins and phytic acids exert major antinutritional effects, reducing the bioavailability of important micronutrients for human dietary use, especially iron and zinc [17]. Furthermore, several studies reported the presence of a complex chemical composition in common bean genotypes, which is directly related to a plethora of structurally and functionally diverse metabolites. Many of these metabolites show beneficial and effective roles in the prevention and/or regulation of chronic conditions, such as obesity, diabetes, cancer, and cardiovascular diseases [18,19].

In recent years, common beans have drawn attention for their unique nutraceutical profiles and their role in agriculture sustainability, climate change mitigation and global food security. FAO report [20] stated that "the answer to mitigate, adapt and reduce the effects of climate change come in the form of a single seed: the pulse".

Given these considerations, common beans could be considered as the potential future natural resources able to achieve the Sustainable Development Goals (SDGs) promoted by the United Nations Agenda 2030: zero hunger/food security, good health and well-being, climate change mitigation, promotion of the sustainable use of ecosystems, and halting biodiversity loss [21,22].

However, like all major crops, the common bean is sensitive to salinity stress [15], one of the most alarming abiotic stresses caused by climate change in the Mediterranean region [23], inducing multiple alterations in plant morpho-physiology and biochemical processes. The negative effects of salt on plants are related to a reduction in the osmotic potential, water deficit, nutrient imbalance, and ion toxicity [24]. Furthermore, a high level of salt hampers the photosynthetic process in all its stages, limiting the CO_2 supply to leaves and causing ultrastructural changes in chloroplasts, alterations of the polypeptide composition of the photosystems, reduced electron flow from PSII to PSI, impaired chlorophyll biosynthesis or accelerated degradation, and accumulation of reactive oxygen species (ROS) [25].

However, plant salt tolerance is dependent on the growth development stage [26]; the germination and early seedling stages are known to be the most sensitive to stress, as also reported in common bean varieties [27,28]. Although severe impairments are commonly described in *P. vulgaris* plants under salinity conditions [29,30], several cultivars respond better to salt stress [31–33], also demonstrating high potential for salinity tolerance during early vegetative growth. Thus, the observed substantial diversity in salinity tolerance among landraces could represent an important prerequisite for identifying stress-tolerant traits/markers able to address the threats posed by climate change.

However, globalization and technological modernization of agricultural practices in the last few decades have drastically reduced and genetically eroded traditional and local bean agro-ecotypes, restricting them to isolated local markets or causing them to completely disappear [34]. The survival of common bean landraces therefore occurs in limited mountainous and hilly areas along the Apennine ridge of the central and southern regions, prevailing in internal and marginal territories where traditional forms of agriculture are practiced by elderly farmers, mostly for family subsistence [35]. Although these areas suffer from demographic decline and disadvantaged conditions, they still have remarkable environmental resources (high-quality agricultural products and natural landscapes) and cultural assets (archaeological and historic settlements) capable of creating new income opportunities and promoting territorial competitiveness [36]. From this perspective, as detailed in the Italian public policy National Strategy for Inland Areas (Italian SNAI) plan, "place-based" strategies must be implemented to valorize the local resources (particularly agricultural products) and, thus, promote territorial development and the economic and social regeneration of these fragile Italian territories [37]. In this context, the assessment of the existing morphological and genetic diversity of germplasm represents the first step in a characterization process focused on the preservation and possible exploitation of endangered local resources [38].

Among them, the characterization of phaseolin isoforms allows the identification of the Andean or Mesoamerican gene pool of the populations, helping to assess the genetic structure of beans from different geographical locations [39,40].

During the last years, nuclear magnetic resonance (NMR) spectroscopy-based metabolomics has turned into a fast and reliable analytical tool to characterize the metabolite diversity and estimate the nutritional quality of several crops [12,41,42]. However, despite this notion, to the best of our knowledge, only a few published articles have reported the use of NMR metabolomics to characterize the diversity of common beans [43,44]. It could be fundamental to establish landraces' value and peculiarities in terms of environment and human health "services", both essential to translating them into competitive products and a prerequisite for envisioning appropriate strategies for their on-farm conservation.

Based on these premises, in the present study, a comprehensive characterization of three Italian autochthonous *P. vulgaris* L. landraces was performed at the morphological, genetic and metabolomic levels, also examining the presence of promising bioactive compounds involved in plant stress tolerance or with some antiproliferative capability in intestinal human cancer cells. Successively, to tackle the climate change challenge, the ability of these landraces to counteract salinity stress was evaluated by monitoring the main plant morpho-physiological and biochemical parameters after 15 days of salt stress exposure.

2. Materials and Methods

2.1. Plant Material Collection

Seeds of three common bean (*Phaseolus vulgaris* L.) landrace populations were collected from local farmers in Italian hilly and mountainous areas and successively stored ex situ in the Molise Germplasm Bank. Ciliegino (CV) and Fagiolo d'Acqua (FDA) are cultivated with low input and traditional farming systems in two different marginalized areas of the Molise region located, respectively, in Vastogirardi [41°46′9.651″ (N) 14°15′28.894″ (E)] and Pietrabbondante [41°43′9.829″ (N) 14°22′47.892″ (E)] municipalities, both belonging to the Alto Medio Sannio Inner Area [45]. In detail, CV takes its name from the cherry red seed coat color, also small and oval, and is particularly appreciated for its sweet taste. FDA is an oval bean with a light brown seed coat, commonly used in soups because of its thin skin and whose name refers to the old farming location, near water sources, where it was grown in the past.

San Michele Rosso (SMR) is cultivated by Belisario Farm [Sarconi, PZ; 40°14′38.04″ (N) 15°51′55.439″ (E)], in the hilly areas of the Basilicata region, and is associated with the PGI (Protected Geographical Indication) quality marker. It owes its name to the red color of its seed coat and to the time when it ripens, usually on the day dedicated to Saint Michele, and it is particularly appreciated for its taste and short cooking time.

All the populations are characterized by round-shaped seeds and white hilum but different seed coat colors: CV and SMR show a full shiny red coat color, while FDA presents a uniform light brown coat color (Supplementary Figure S1).

2.2. Landrace Diversity Assessment

2.2.1. Seed Morphological Analysis

A total of 14 seed morpho-colorimetric parameters were measured on a digital image of 10 seeds for each common bean population by using software Image J (Version 1.51iWayne Rasband-NIH; https://rsb.info.nih.gov/ij/, accessed on 20 April 2023). Only the seed weight (g), volume (mL), and density (g·mL^{-1}) were measured on 100 seeds. The morpho-colorimetric features were selected by referring to Loddo et al. [46], and the measurements were conducted in triplicate and expressed as the mean ± standard error.

2.2.2. DNA Analysis: ISSR and SCAR Amplification

The total genomic DNA was used to investigate the genetic relationships among the common bean populations by using a total of 8 inter-simple sequence repeat (ISSR) primers and the sequence-characterized amplified region (SCAR) marker of the phaseolin (Phs) locus (Supplementary Table S1), following the same procedures reported in Falcione et al. [47].

2.2.3. NMR Metabolomic Profiling

The extraction of all the metabolites was performed according to the procedure applied by Samukha et al. [48]. Briefly, powdered seed (1.5 g) was incubated for 1 h at room temperature with CH_2Cl_2/MeOH/H_2O (2:1:1) The resulting lipophilic and hydroalcoholic phases were accurately separated and vacuum dried in a Rotavapor (R-114, Büchi, Switzerland), keeping the temperature at 30 °C to inhibit the decomposition of the thermolabile compounds. Next, the samples were placed in a SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA) and successively transferred to the freeze dryer (CHRIST, Hosterode am Harz, Germany) for two days until complete solvent evaporation. All the experiments were performed in quadruplicate to ensure their reproducibility.

For the NMR experiments, the aqueous extract (7 mg) was resuspended in 500 µL of deuterated water (D2O, 99.95% D, Sigma-Aldrich, Milan, Italy) containing 0.1 mM sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TMSP 0.75 wt% sodium salt, 99.9% D, Sigma-Aldrich, Milan, Italy), for 1 H spectral reference, and phosphate-buffered saline (PBS) at pH 6. The lipophilic extract (7 mg) was solubilized in 500 µL of deuterated chloroform ($CDCl_3$, 99.8%, Sigma-Aldrich, Milan, Italy) adding Tetramethylsilane $Si(CH_3)_4$ (TMS, 0.03% v/v, 99.8% D, Sigma-Aldrich, Milan, Italy) as an internal standard. Finally, the samples were transferred into NMR tubes and analyzed via ^1H and ^{13}C NMR spectroscopy, homonuclear correlation spectroscopy (2D $^1H-^1H$ COSY), heteronuclear single quantum correlations (2D $^1H-^{13}C$ HSQC) and heteronuclear multiple bond correlation (2D ^1H–^{13}C HMBC).

One-dimensional (1D) and two-dimensional (2D) (COSY, HSQC and HMBC) spectra were acquired on a Bruker DRX-600 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), equipped with 5 mm TXI 1H/D-13C/15N-GRD Z816801/0193 probes, operating at 298 K. The acquisition parameters for the ^1H–NMR (1D) were: FID size = 64 k, dummy scans = 4, spectral width = 11, number of scans = 512, acquisition time = 2.18 s, relaxation delay = 5 s, receiver gain = 90.5 and FID resolution= 0.45 Hz. The acquisition parameters for the ^1H–NMR (2D) were: COSY dummy scans = 16, number of scans = 32, relaxation delay = 1.5 s, receiver gain= 228; HSQC dummy scans = 16, number of scans = 64, relaxation delay = 1 s, receiver gain = 2050; HMBC dummy scans= 16, number of scans = 96, relaxation delay = 1 s, receiver gain = 2050. Data acquisition was achieved by using TOPSPIN 3.5 software (Bruker BioSpin GmbH, Rheinstetten, Germany).

Phase and baseline corrections were performed for the NMR data processing. For the data calibration, the TMSP shift signal was adjusted to 0.0 ppm using TOPSPIN 3.5 software (Bruker BioSpin GmbH, Rheinstetten, Germany).

Metabolite profiling was assigned by comparison with the Human Metabolome Database (HMDB; http://www.hmdb.ca/, accessed on 20 May 2023) and the signal assignments were confirmed by recording and analyzing the 1D and 2D NMR experiments. These were ^{13}C spectroscopy (1D), homonuclear correlation spectroscopy (2D, ^{1}H–^{1}H COSY), heteronuclear single quantum correlations (2D, ^{1}H–^{13}C HSQC) and heteronuclear multiple bond correlation (2D, ^{1}H–^{13}C HMBC). To differentiate the samples through the NMR spectra, we carried out a multivariate statistical analysis of the NMR qualitative data using projection methods.

For the quantification of the metabolites, considering that the signal intensity in the ^{1}H NMR spectrum is proportional to the molar concentration of the metabolites [49], the most abundant compounds were quantified by an integration of the ^{1}H NMR signals, using TSP as the internal standard, and considering several parameters, such as the mass of the target compound [μg] in the solution used for the ^{1}H NMR measurement, the molecular weight of the target compound [$g \cdot mol^{-1}$], the relative integral value of the ^{1}H NMR signal of the target compound, the relative integral value of the ^{1}H NMR signal of the standard compound, the number of protons belonging to the ^{1}H NMR signal of the standard compound, the number of protons belonging to the ^{1}H NMR signal of the target compound, the concentration of the internal standard (TSP) in the solution used for the ^{1}H NMR measurement [$mmol \cdot L^{-1}$], and the volume of the solution used for ^{1}H NMR measurement [mL]. The quantification of the metabolites was expressed in mg/g of dry weight of bean seeds.

2.3. Anti-Proliferative Activity of Seed Aqueous Extracts on Caco-2 Cell Line

Cancer coli-2 (Caco-2) cells were cultured at 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Merck Life Science, Milan, Italy), containing 10% (*v*/*v*) fetal bovine serum (FBS) (Merck Life Science, Milan, Italy), L-glutamine (2 mM), and penicillin/streptomycin solution. The growth curve experiments were assessed by seeding Caco-2 cells into 12-well plates (50,000 cells for each well). Then, 5 h and 48 h after seeding, the cells were treated with the bean extracts (CV, FDA, SMR) at different concentrations: 0.1 $\mu g \cdot mL^{-1}$, 1 $\mu g \cdot mL^{-1}$, 100 $\mu g \cdot mL^{-1}$, while the control cells received DMSO (dilution 1:1000 in cell culture medium) as the vehicle. Cell counts were performed after 96 h, and the results were expressed as the means ± SD (standard deviation).

2.4. Seed Germination Behavior under Salt Stress

To germinate, sterilized seeds from each population were placed in 9 cm diameter Petri dishes on Whatman filter papers moistened with 2 mL of distilled water (control; 10 seeds) or with 200 mM NaCl solution to reproduce the osmotic potential of −0.8 MPa [50] (salt stress; 10 seeds). All the Petri dishes were subsequently kept in a growth chamber under controlled environmental conditions (25 °C with a photocycle of 16 h of light and 8 h of dark), and the solutions were renewed every 2 days.

The number of seeds germinated was recorded for 8 days (Supplementary Figure S2) and the data were expressed as the germination capacity/germination percentage and as the germination speed (mean ± standard error of three biological replicate). The germination capacity was obtained by dividing the number of germinated seeds in each Petri dish (n) by the total number of seeds tested (N), multiplied by 100 ($G = n/N \cdot 100$). The germination speed (T_{50}), expressed as the number of days required to reach 50% of the final germination capacity, was calculated using the formula reported in Kouam et al. [51].

2.5. Plant Performance under Salt Stress

Eight *Phaseolus vulgaris* plants for each population were grown in a growth chamber (16 h light/8 h dark cycle under 25 °C temperature) in the control and salt stressed conditions for 15 days, as reported in Falcione et al. [47] (Supplementary Figure S2). At the end of the experiment, the soil electrical conductivity (EC) was measured (control soil EC: 1.35 $dS \cdot m^{-1}$; salt-stressed soil: 9.57 $dS \cdot m^{-1}$, data not showed) and plant material (roots and leaves) was harvested and subjected to further analyses.

The plant morphological changes were analyzed after the 15 days of treatment by measuring the fresh (FW) and dry biomass (DW) of different organs, together with the relative water content (RWC) and the photosynthetic pigments. In detail, the leaf and root FW was immediately measured after harvesting. Then, each organ was incubated in the dark in deionized water for 4 h and weighed to determine the turgid weight (TW). These samples were, successively, dried at 80 °C for 48 h and the DW was recorded. The RWC was calculated using the formula reported in Smart [52]: $[(FW - DW)/(TW - DW)] \cdot 100$. All the morpho-physiological measurements were performed on four plants and expressed as mean ± standard error.

The total chlorophyll (Chl), Chl A/Chl B ratio, and carotenoid contents were assessed following the spectrophotometric procedure described in Polzella et al. [53] and expressed as $\mu g \cdot mg^{-1}$ (mean ± standard error of triplicate measurements).

The plant stress response was evaluated in the roots and leaves of different landraces by analyzing the proline and sugar content and estimating the lipid peroxidation. In detail, the proline content and lipid peroxidation were evaluated as reported by Falcione et al. [47].

Briefly, the proline concentration was spectrophotometrically determined at 520 nm as the mean ± standard error of triplicate measurements using a standard curve and expressed as $\mu mol \cdot g^{-1}$ using the equation reported by Carillo and Gibon [54]. The lipid peroxidation, expressed as the malondialdehyde (MDA) content, was measured by using the thiobarbituric acid (TBA) method according to Abdallah et al. [55] as the mean ± standard error of triplicate measurements and was expressed as $nmol \cdot mL^{-1}$ (molar extinction coefficient reported by Hodges et al. [56]).

The total carbohydrate content was measured by using the Total Carbohydrate Assay Kit (MAK104, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions, with some modifications. In detail, plant material (50 mg) was homogenized in 200 µL of ice-cold Assay Buffer and the samples were centrifuged at 13,000× *g* for 5 min. Next, 150 µL of sulfuric acid (H_2SO_4) was added to 30 µL of supernatant and the mixture was heated at 90 °C for 15 min in the dark. Then, 30 µL of Developer were added to each sample and mixed well using a horizontal shaker for 5 min. Successively, H_2O MilliQ was added to each sample and the absorbance was read at 490 nm with a spectrophotometer (Bio-Rad, South Granville, NSW, Australia). The amount of total carbohydrates present in the samples was determined from a standard curve made by dilutions of 2 $mg \cdot mL^{-1}$ D-Glucose standard and expressed as $mg \cdot g^{-1}$ of fresh weight and as the mean ± standard error of triplicate measurements.

2.6. Statistical Analysis

The statistical analysis of the genetic and seed morpho-colorimetric data was conducted using Past Version 4.03 software (https://past.en.lo4d.com/windows, accessed on 1 October 2023). In detail, Principal Component Analysis (PCA) was carried out to define the role of each morphological trait in the grouping of accessions and cluster analysis was performed by unweighted pair group methods using arithmetic averages (UPGMA) with the Euclidean distance index to create a dendrogram. Principal Coordinate Analysis (PCoA) was carried out to estimate the overall genetic relationship among populations and UPGMA clustering was used with Jaccard's similarity index to create a dendrogram.

The ^1H NMR qualitative data were imported into MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/, accessed on 15 October 2023) and PCA and Partial Least Squares Discriminant Analysis (PLS-DA) were performed. The quality of the PLS-DA model was evaluated using the correlation coefficient R_2 and a cross-validation correlation coefficient Q_2. Furthermore, the Variables Importance in Projection (VIPs) score (value > 1) for the

PLS-DA was selected. Student's *t*-test was performed to determine significant quantitative differences in the metabolite levels among populations ($p \leq 0.05$).

Analysis of the anti-proliferative activity of the seed aqueous extracts on the Caco-2 cell line was performed by using two-way ANOVA followed by a Bonferroni post-test ($p \leq 0.05$) on GraphPad Prism 5 (GraphPad, La Jolla, CA, USA) for Windows.

Differences between the control and treated samples in terms of the germination behavior and plant morpho-physiological (biomass, RWC and photosynthetic pigment content) and biochemical (proline, lipid peroxidation and total carbohydrate contents) parameters were determined through a Student's *t*-test ($p \leq 0.05$).

3. Results

3.1. Seed Morphological Traits and Genetic Data

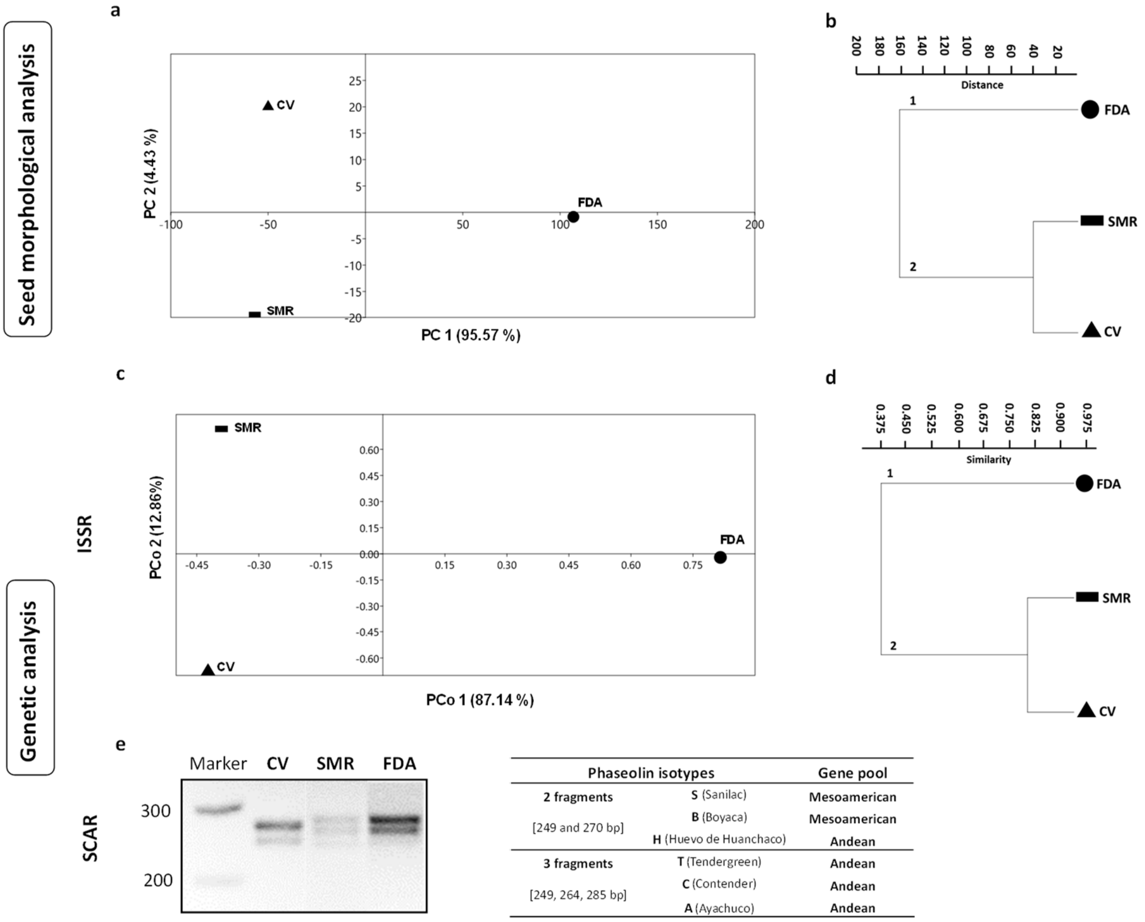
The results of the seed morpho-colorimetric analysis are shown in Supplementary Table S2. The Principal Component Analysis (PCA) of the 14 seed morpho-colorimetric descriptors noted differences among the populations. In detail, in the PCA scatter plot (Figure 1a), along Principal Component 1 (PC 1), CV and SMR both appeared in negative coordinates values, while FDA projected in the positive coordinates. On the other hand, on the Principal Component 2 (PC 2), CV showed positive values, while FDA and SMR were placed along negative values. These differences were mainly related to the seed volume, seed weight, and seed coat colorimetric parameters (max gray value, modal gray value, and median values of the pixels in the selected image), accounting for 95.57% and 4.43% of the total variance, on the PC1 and PC 2, respectively (Supplementary Figure S3). The dendrogram, resulting from the unweighted pair group method with arithmetic mean (UPGMA) clustering, revealed two main clusters: cluster 1 was characterized by only FDA (Euclidean distance $\approx$ 160), and cluster 2 contained both CV and SMR (Euclidean distance $\approx$ 40) (Figure 1b).

The genetic relationships among the autochthonous common bean populations were analyzed by using eight inter-simple sequence repeat (ISSR) markers. The PCoA analysis of the amplified products resulted in a scatter plot with PCo 1 and PCo 2 scores that accounted for 87.14% and 12.86% of the total variance, respectively (Figure 1c). Regarding PCo 1, on the one hand, CV and SMR both showed negative coordinates values; on the other hand, FDA was distributed in positive coordinates (Figure 1c). Along PCo 2, only SMR was placed in positive values, while FDA and CV displayed negative values (Figure 1c). In the corresponding dendrogram, constructed using the UPGMA clustering, two main clusters were identified: FDA was assigned to cluster 1 (Jaccard similarity index $\approx$ 0.38); CV and SMR were grouped in cluster 2 (Jaccard similarity index $\approx$ 0.84) (Figure 1d).

The phaseolin pattern analysis [PCR amplification of sequence-characterized amplified region (SCAR) marker of the phaseolin (*Phs*) locus] allowed the assignment of the three common bean populations to the Andean gene pool. However, as also shown in Falcione et al. [47], the CV profile consisted of two fragments of 249 and 275 bp, typical of S-, B-, or H-type phaseolins, while both SMR and FDA contained three fragments of 249, 275 and 290 bp, characteristic of T-, C-, or A-type phaseolin profiles (Figure 1e).

3.2. NMR Metabolomic Profiling Data

The ^{1}H NMR spectra of the common bean seed aqueous and lipophilic extracts showed a similar chemical profile among the populations. In detail, the ^{1}H NMR spectrum of the aqueous extract revealed a total of 25 metabolites, which ranged from 0.90 to 10 ppm: 3 sugars, 13 amino acids, 5 organic acids, and 4 other miscellaneous compounds (Figure 2). The ^{1}H and ^{13}C NMR chemical shifts (Figure 2a) were assigned based on the 2D-NMR experiments (COSY, HSQC, and HMBC) and comparison with published data in the HMDB (Human Metabolome Database) (https://hmdb.ca/, accessed on 20 May 2023).



Phaseolin isotypes		Gene pool
2 fragments [249 and 270 bp]	S (Sanilac)	Mesoamerican
	B (Boyaca)	Mesoamerican
	H (Huevo de Huanchaco)	Andean
3 fragments [249, 264, 285 bp]	T (Tendergreen)	Andean
	C (Contender)	Andean
	A (Ayachuco)	Andean

Figure 1. Analysis of common bean morpho-colorimetric and genetic diversity. Scatter plot of the PCA computed among three populations of common bean (*Phaseolus vulgaris* L.) using fourteen morpho-colorimetric features (**a**) and dendrogram resulting from a cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) with the Euclidean distance index (**b**). Scatter plot of the PCoA computed on the three bean populations, using eight inter-simple sequence repeat (ISSR) primers (**c**) and dendrogram resulting from UPGMA clustering (Jaccard similarity index) (**d**). Phaseolin (Phs) SCAR marker banding patterns. The ladder range is 200–300 base pairs (ApplyChem GmbH, Darmstadt, Germany) (**e**). CV: Ciliegino; SMR: San Michele Rosso; FDA: Fagiolo d'Acqua.

In detail, in the first region of the ^{1}H NMR spectra (from 0.9 to 3.0 ppm), the signals of several amino acids (leucine, isoleucine, valine, threonine, alanine, proline, methionine, GABA, aspartate and asparagine) and organic acids (lactic acid, pipecolic acid, malic acid and citric acid) were detected (Figure 2b). The second region (from 3.0 to 4.5 ppm) was characterized by the presence of organic acids (pipecolic acid and malic acid), sugars (sucrose, raffinose, fructose, galactose, stachyose), and other miscellaneous compounds (choline and trigonelline) (Figure 2c). In the third region (from 4.5 to 5.9 ppm), several various sugars, such as stachyose, raffinose and sucrose, were observed (Figure 2d). The fourth region (from 5.9 to 7.9 ppm) was characterized by the signals of aromatic groups from amino acids (tyrosine, phenylalanine and tryptophan) and other compounds, such as uridine (Figure 2e). Finally, in the fifth region (from 7.9 to 9.2 ppm), evident signals of trigonelline and formic acid were found (Figure 2f).

The analysis of the ^{1}H NMR spectrum of the seed lipophilic extracts, obtained from the three common bean populations, showed the presence of different metabolite classes: fatty acids in the first region, which ranged from 0.9 to 3.0 ppm, and glycerol and polyunsaturated fatty acids (PUFA), in the second one, which ranged from 3.0 to 6.0 ppm

(Supplementary Figure S4). The assignments of the ^{1}H NMR chemical shifts of the compounds were performed as described for the aqueous extract.

a

		Metabolite	Assignment	δ ^{1}H	δ ^{13}C
Amino acids	**1**	**Alanine (Ala)**	β-CH_3	1.48 (d, 7)	16
	2	**Asparagine (Asn)**	β,β′-CH_2	2.86 (dd, 7.3, 16.8) 2.95	33.7
	3	**Aspartate (Asp)**	β,β′-CH_2	2.84 (dd, 4.8, 17.3) 2.7	
	4	**γ-aminobutyrate (GABA)**	α-CH_2	2.31 (t,7.3)	34.4
			γ-CH_2	3.00 (t,7.3)	35.8
	5	**Isoleucine (Ile)**	γ-CH_3	1.01 (d, 7)	15.2
	6	**Leucine (Leu)**	δ-CH_3	0.93 (d, 7)	22.3
			δ′-CH_3	0.90 (d,7)	20.6
	7	**Methionine (Met)**	γ-CH_2	2.17	26.6
			-S- CH_3	2.13 s	14.6
	8	**Phenylalanine (Phe)**	2,6-CH	7.58 m	-
			3,5-CH	7.33 m	-
	9	**Proline (Pro)**	γ-CH_2	2.00 m	-
	10	**Threonine (Thr)**	γ-CH_3	1.33 (d, 6.7)	19.5
	11	**Tyrosine (Tyr)**	2,6-CH	6.9	-
			3,5-CH	6.57	-
	12	**Tryptophan (Trp)**	8-CH	7.54 (d, 8.0)	
			9-CH	7.74 (d, 8.0)	
	13	**Valine (Val)**	γ-CH_3	0.99 (d,7)	16.3
			γ′-CH_3	1.05 (d, 7)	17.8
Sugars	**14**	**Sucrose**	CH-1 (Glc)	5.42 (d, 4)	92.7
			CH-4 (Glc)	3.47 (t, 10)	69
			CH-3′ (Fru)	4.23 (d, 9)	76.3
			CH-4′ (Fru)	4.05 (t)	74
			CH_2-1′(Fru)	3.68	61.6
	15	**Raffinose**	CH-1 (Glc)	5.42 (d, 4)	92.7
			CH-1 (Gal)	5.00 (d, 4)	98.2
			CH-3 (Fru)	4.22 (d, 9)	74.1
			CH-3 (Glc)	3.77 (t)	72.6
	16	**Stachyose**	CH-1 (Glc)	5.44 (d, 4)	92
			CH-1 (Gal-T and Gal-I)	5.00 m	98.3
			CH-3 (Fru)		
			CH_2-1 (Fru)	4.21 (d, 9)	76.3
				3.68	61.6
Organic acids	**17**	**Citric acid**	α,γ-CH	2.59 (d, 17.5)	44.4
			α′,γ′-CH	2.71 (d, 17.5)	
	18	**Formic acid**	-CH	8.44 s	-
	19	**Lactic acid**	CH_3	1.25 (d, 7)	22.8
	20	**Malic acid**	α-CH	4.30 (dd)	70.3
			β-CH	2.35 (dd)	33.8
	21	**Pipecolic acid**	CH-3,4,5	2.22-1.67 m	
			CH-3′,4′,5′	1.73-1.87 m	
			CH-6	3.00 (td, 12.5,3.2)	43.5
			CH-6′	3.41 dd	
			CH-2	3.59 (dd)	58.9
			N-H	2.17 m	
Other compounds	**22**	**Choline**	-$N(CH_3)_3^+$	3.20 s	53.6
	23	**Guanosine**	CH-8	5.92	
	24	**Uridine**	CH-6	5.91 d 7.87 d	
	25	**Trigonelline**	H-2	9.13 s	145.7
			H-4,6	8.83 m	145.5
			H-5	8.09 m	127.7
			-N-CH_3	4.44 s	48.2

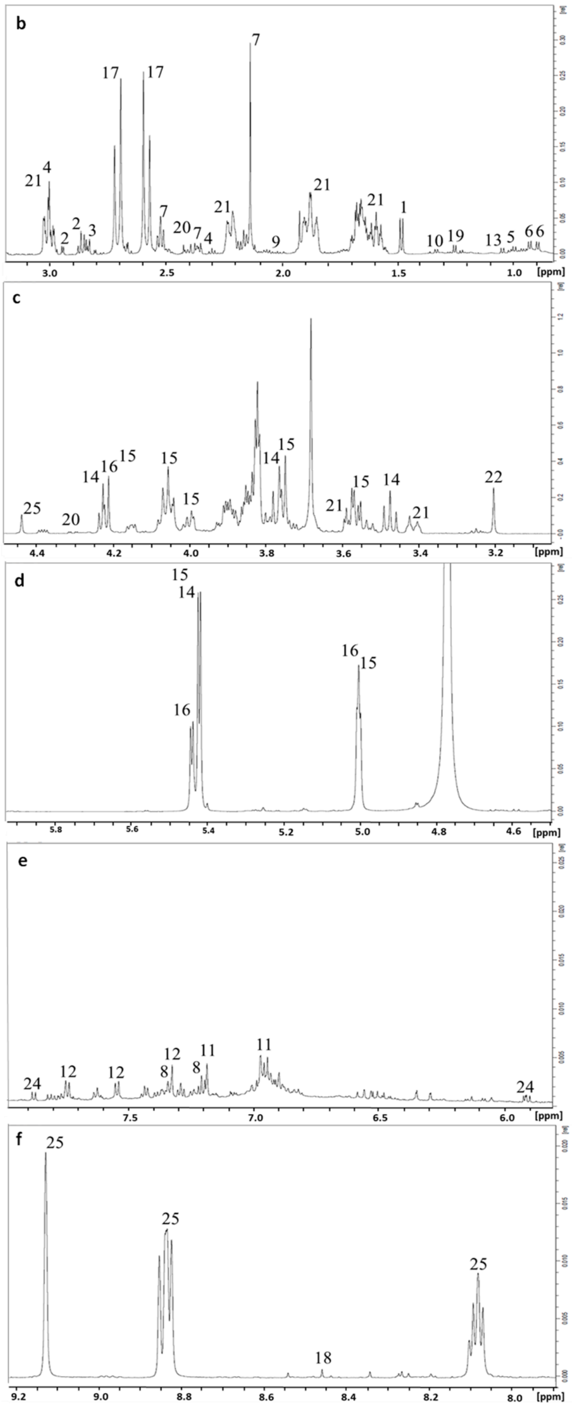


Figure 2. ^{1}H NMR metabolomic profile of common bean seed aqueous extracts. ^{1}H and ^{13}C chemical shift assignment (δ, ppm) of the 25 metabolites detected in the seed aqueous extract of the common bean population (**a**). Expanded ^{1}H NMR spectrum of the San Michele Rosso common bean landrace from: 0.9 to 3.0 ppm (**b**); 3.0 to 4.5 ppm (**c**); 4.5 to 5.9 ppm (**d**); 5.9 to 7.9 ppm (**e**); 7.9 to 9.2 ppm (**f**). d: doublet, dd: double doublet; m: multiplet; s: singlet, t: triplet; tp: triple doublet.

The data of the ^{1}H NMR spectra of the aqueous seed extracts were imported for the construction of the PCA and Partial Least Squares Discriminant Analysis (PLS-DA) (Figure 3).

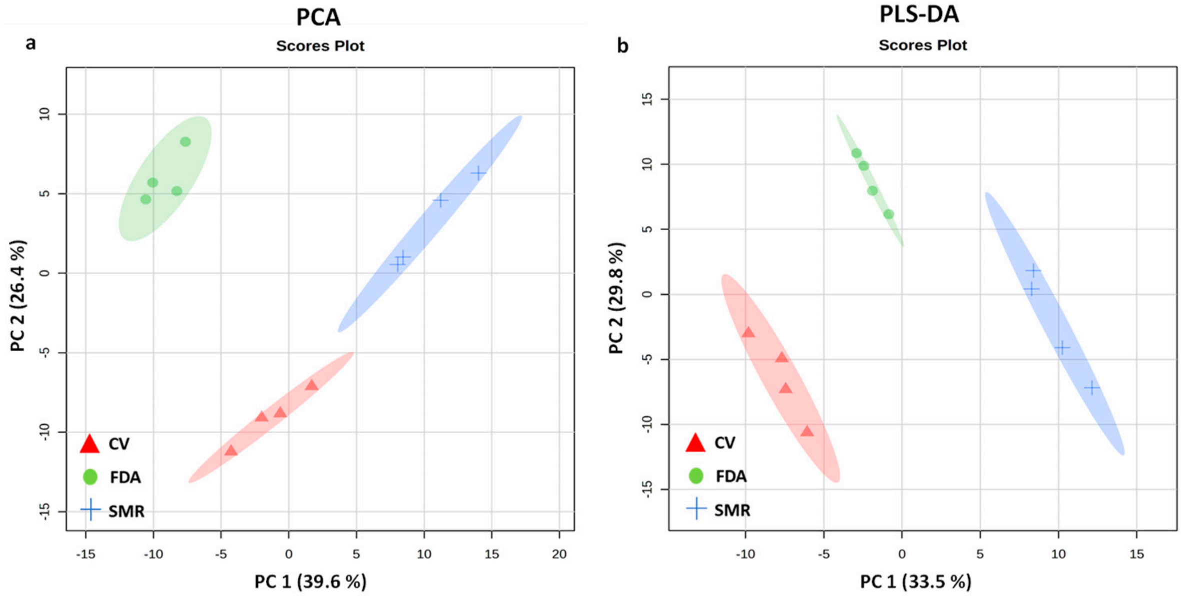


Figure 3. Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) computed on NMR qualitative data of common bean aqueous seed extracts. Score plot of the PCA (**a**) and score plot of the PLS-DA (**b**). CV: Ciliegino; SMR: San Michele Rosso; FDA: Fagiolo d'Acqua.

In the PCA score plot, PC1 and PC2 accounted for 39.6% and 26.4% of the total variance, respectively. Three separate groups, related to CV, SMR and FDA populations, can be observed. In detail, in PC1, FDA and CV were displayed along negative coordinates, with an outlier point of CV placed in positive values, while SMR was positioned in positive coordinates. The three populations can also be discriminated by PC2: FDA and SMR were placed in positive values, while CV was displayed along negative PC2 values (Figure 3a). The corresponding PCA loadings plot is reported in Supplementary Figure S5a.

PLS-DA was then applied to improve the group separation and to deeply investigate the sample classification according to the metabolic profiles expressed by the three different populations. The PLS-DA regression ($R_2 = 0.98$; $Q_2 = 0.95$) showed a good group separation along all the components in the score plot (PC1 = 33.5%; PC2 = 20.7%). In particular, CV and FDA were projected in negative PC1 coordinates, while SMR displayed along positive PC1 values. Regarding PC2, FDA and CV were placed in positive and negative values, respectively; conversely, SMR appeared in both positive and negative values (from 5 to -10) (Figure 3b). From the corresponding loadings plot (Supplementary Figure S5b), the metabolites responsible for data clustering in the PLS-DA, with a Variables Importance in Projection (VIPs) score > 1, were selected. The 10 most discriminant metabolites are illustrated in the VIPs plot in Supplementary Figure S6.

3.3. *Quantification of Metabolites in Aqueous Common Bean Seed Extracts*

Among all the metabolic profiles, the most intense and distinct signals of the ^{1}H NMR spectra have been quantified. The dominant compounds, detected in the three bean populations, turned out to be sugars (sucrose and stachyose) and organic acids (citric and pipecolic acid) (Figure 4).

Sucrose and stachyose showed high contents in all three bean populations (sucrose: 0.0016–0.0021 mg·g^{-1} DW; stachyose: 0.0015–0.0019 mg·g^{-1} DW), with no significant variation in the sucrose content among the populations. In contrast, for stachyose, on the one hand, the amount present in CV showed no significant differences compared with SMR and FDA; on the other hand, the sugar concentration in SMR was significantly higher than that in FDA (Figure 4).

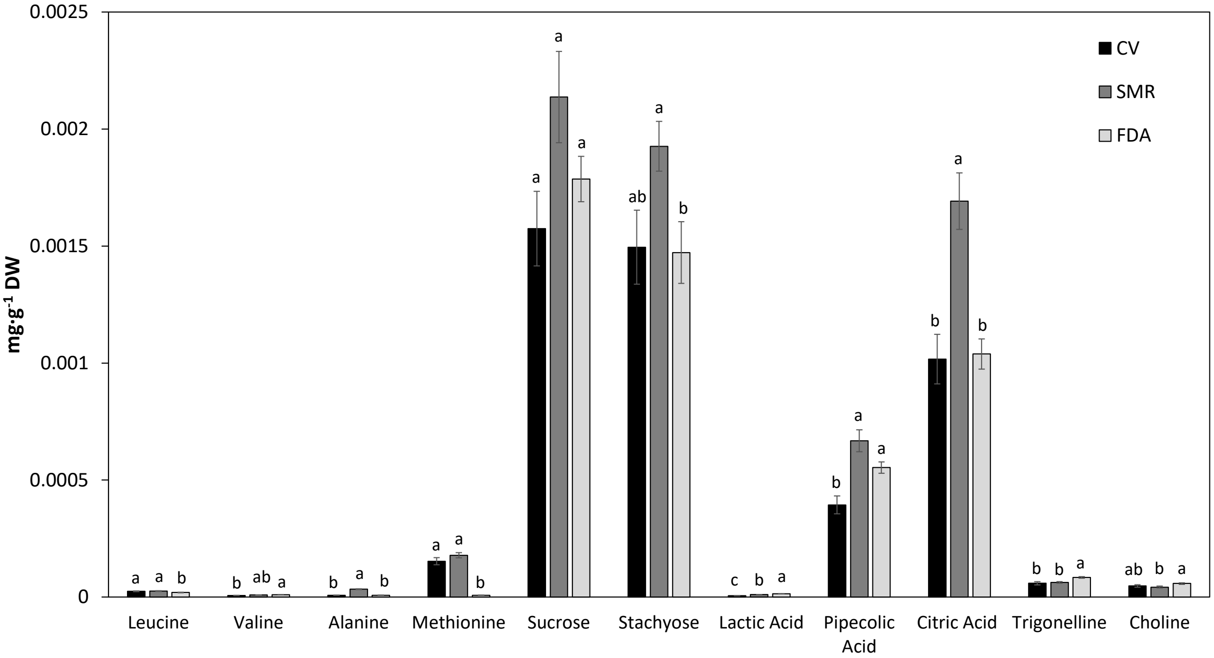


Figure 4. Quantification (mg·g^{-1}DW) of metabolites in aqueous seed extracts of three common bean landraces. The most intense and distinct metabolite signals of the ^{1}H NMR spectra have been quantified. Data are expressed as the mean ± standard error and calculated using four replicates. Student's *t*-test was performed to determine significant differences ($p \leq 0.05$) in the metabolite levels in the common bean populations. Mean values marked with the same letter are not statistically different. CV: Ciliegino; SMR: San Michele Rosso; FDA: Fagiolo d'Acqua.

The three bean populations also contained substantial levels of organic acids (lactic acid: 5.40×10^{-6}–$1.35 \cdot 10^{-5}$ mg × g^{-1} DW; pipecolic acid: 0.0004–0.0007 mg·g^{-1} DW; citric acid: 0.0010–0.0017 mg·g^{-1} DW), with the highest content detected for citric acid, followed by pipecolic and lactic acid (Figure 4). Regarding citric acid, SMR had the highest amount compared to CV and FDA; the latter showed no significant differences between them. In addition, SMR and FDA showed no significant differences in the pipecolic acid content, but these concentrations were higher than those observed in CV. Lastly, the highest lactic acid content was found in FDA, followed by SMR and CV (Figure 4).

Regarding amino acids, leucine, valine and alanine showed lower concentrations (leucine: 1.91×10^{-5}–2.51×10^{-5} mg·g^{-1} DW; alanine: 7.15×10^{-6}–3.35×10^{-5} mg·g^{-1} DW; valine: 6.42×10^{-6}–9.05×10^{-6} mg·g^{-1} DW) than methionine (7.33×10^{-6}–1.78×10^{-4} mg·g^{-1} DW). The methionine concentrations in CV and SMR were not significantly different but were higher than the content in FDA. For leucine, equivalent contents were found in CV and SMR, which were found to be higher than in FDA. SMR showed no differences in the valine concentrations compared with CV and FDA; however, the valine content in FDA was higher than in CV. Finally, SMR reported the highest alanine amount compared with CV and FDA (Figure 4).

Other compounds, such as trigonelline and choline, showed quite low content (trigonelline: 5.83×10^{-5}–8.37×10^{-5} mg·g^{-1} DW; choline: 4.22×10^{-5}–5.75×10^{-5} mg·g^{-1} DW). In detail, an increased trigonelline content was observed in FDA compared to CV and SMR. CV showed no differences with respect to SMR and FDA for choline, but FDA had a higher choline amount than SMR (Figure 4).

3.4. Anti-Proliferative Activity of Bean Aqueous Extracts on Caco-2 Cell Line

The aqueous bean extracts had different effects on the cell growth of the Caco-2 line (Figure 5). Specifically, after 96 h, the Caco-2 cell proliferation was significantly reduced following the administration of all three concentrations of CV extracts (Figure 5a). On the other hand, the increasing doses of SMR extracts induced no change in cell growth compared with the control (Figure 5b). Conversely, interestingly, all three concentrations of FDA extracts increased the number of Caco-2 cells compared to the vehicle-treated cells (Figure 5c).

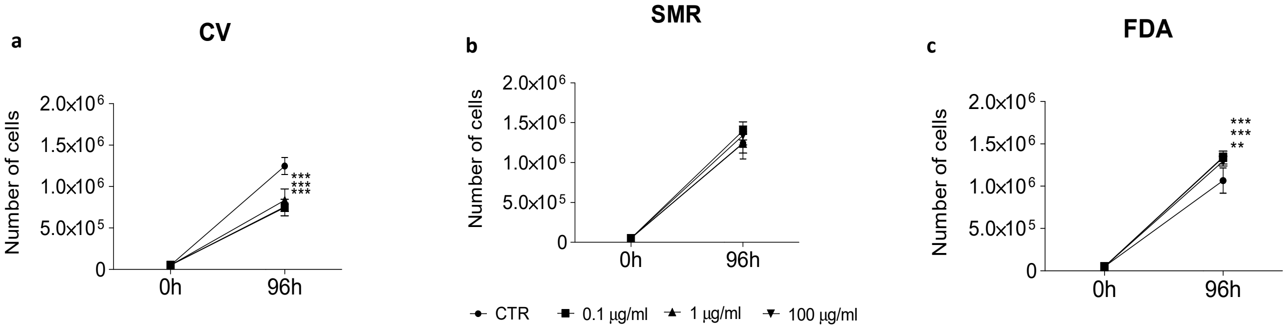


Figure 5. Effects of aqueous bean extract administration on cell growth in the Caco-2 cell line. Cell proliferation was assessed in Caco-2 cells treated with vehicle (DMSO, CTR) and CV (**a**), SMR (**b**) and FDA (**c**) aqueous extracts at the doses of 0.1 $\mu g \cdot mL^{-1}$, 1 $\mu g \cdot mL^{-1}$, 100 $\mu g \cdot mL^{-1}$. n = 3 independent experiments. Data represent the means ± standard deviation. Statistical analysis was carried out by using two-way ANOVA, followed by a Bonferroni post hoc test. ** $p < 0.01$; *** $p < 0.001$.

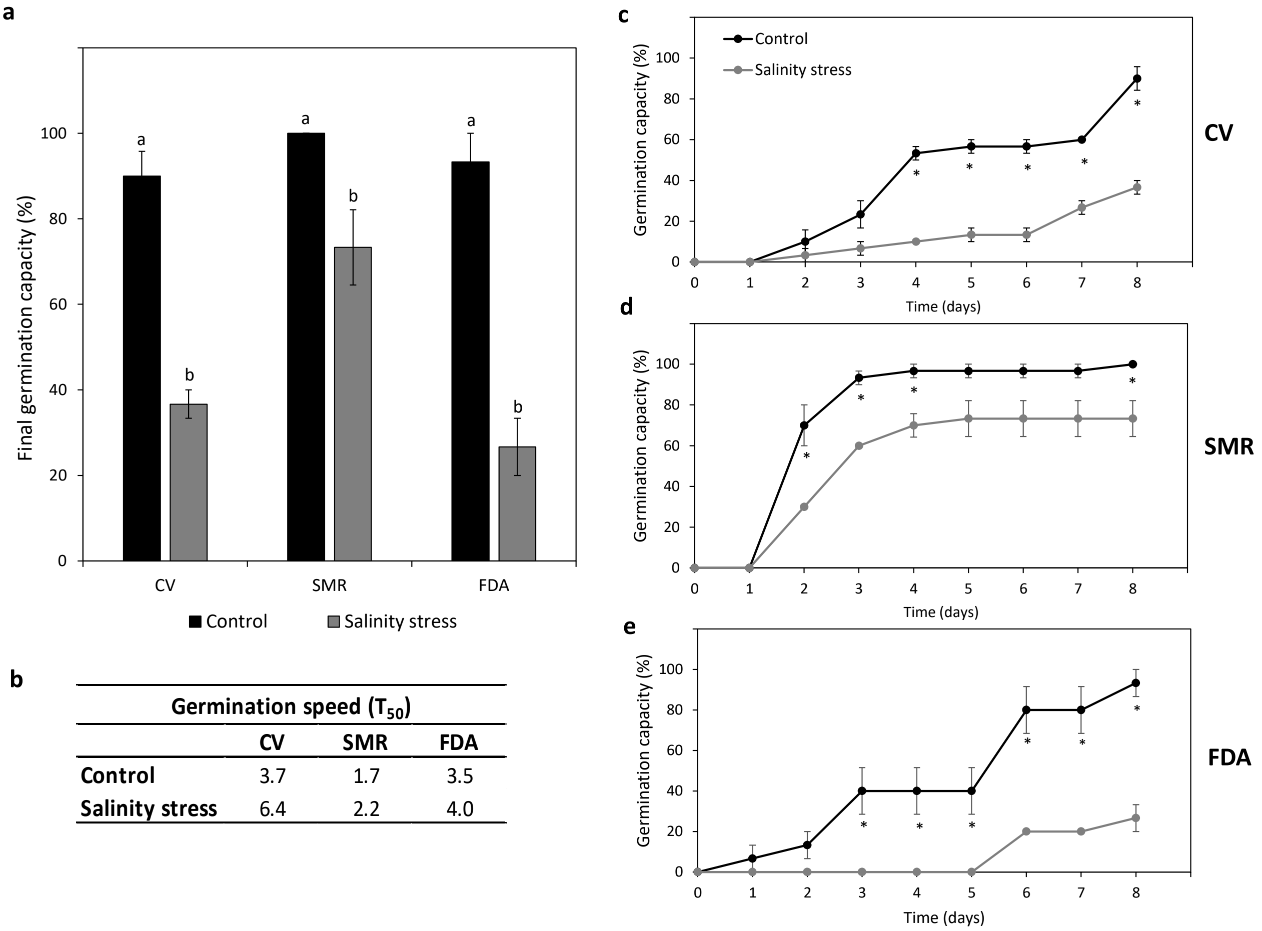
3.5. Effects of Salt Stress on Germination and Plant Morpho-Physiological and Biochemical Responses

3.5.1. Germination Behavior

Seeds of the CV bean population that received no stress treatment had a final germination capacity of 90%. In comparison, a 37% final germination capacity was observed under salt stress treatment after 8 days (Figure 6a). Furthermore, the germination speed was also negatively affected by salinity stress, as indicated by the higher T_{50} value under salinity stress (T_{50} = 6.4) compared to control (T_{50} = 3.7) (Figure 6b). No difference was observed between the control and the stress condition during the first three days, while from the fourth day (4d) to the eighth day (8d), salinity stress induced a reduction in the germination rate. In detail, the salt stress condition decreased the germination capacity by five times at the fourth day with respect to the control (53%) until two times at the eighth day compared to the control condition (90%) (Figure 6c).

The SMR bean population reached 100% final germination capacity under controlled conditions, while a final germination percentage of 73% was observed under salinity stress (Figure 6a). A slight delay in the germination speed was reported under salinity stress (T_{50} = 2.2) with respect to the controlled conditions (T_{50} = 1.7) (Figure 6b). Salt treatment had little impact on the germination rate and only at the 2 d, 3 d, 4 d, and 8 d. More specifically, under salt stress conditions, the germination capacity was 2-fold lower (2 d) and 1-fold lower (3 d, 4 d and 8 d) than the control (2 d: 70%, 3 d: 93%, 4 d: 97%, 8 d: 100%) (Figure 6d).

The FDA untreated seeds reached the final germination of 93% at the 8 d, while the salt-treated showed a final germination capacity of 27% (Figure 6a). A slight decrease in the germination speed was also reported under salinity stress (T_{50} = 4.0) compared to the untreated seeds (T_{50} = 3.5) (Figure 6b). In detail, no significant differences were reported between the germination rates under controlled and salinity stress conditions during the first 2 days, whilst from the 3rd d to the end of germination test (8 d), the germination percentage was 0% (3 d, 4 d and 5 d), 20% (6 d and 7 d) and 27% (8 d) under salt stress, compared to control (3 d–5 d = 40%, 6 d–7 d = 80%, 8 d = 93%) (Figure 6e).



Germination speed (T_{50})

	CV	SMR	FDA
Control	3.7	1.7	3.5
Salinity stress	6.4	2.2	4.0

Figure 6. Seed germination under control and salinity stress conditions. Final germination capacity reached by seeds of common bean populations on the last day (8th) of the germination test (**a**). Germination speed, expressed as T50, of each common bean population under control and salinity stress conditions (**b**). Germination capacity of Ciliegino (**c**), San Michele Rosso (**d**) and Fagiolo d'Acqua (**e**) under both experimental conditions. Data represent the mean (n = 10) ± standard error. Mean values, marked with the asterisk, are statistically different. Student's t-test was applied to weigh the effects of different treatments ($p \leq 0.05$). Mean values marked with the same letter are not statistically different. CV: Ciliegino; SMR: San Michele Rosso; FDA: Fagiolo d'Acqua.

3.5.2. Plant Morpho-Physiological Data

The analysis of the biomass allocation showed that the fresh (FW_{root}) and dry (DW_{root}) root biomass did not change significantly in the SMR plants, while they were negatively affected by salinity stress in CV and FDA (Figure 7a,d). More specifically, salinity stress reduced the FW_{root} by 44% in CV and 56% in FDA, and it decreased the DW_{root} by 69% in CV and by 58% in FDA, compared with the control plants (Figure 7a,d). A decline in both the fresh and dry weight was observed in CV and FDA leaves under salt stress treatment: the leaf fresh weight (FW_{leaf}) was reduced by 89% in CV and by 90% in FDA, while the leaf dry weight (DW_{leaf}) declined by 44% and by 56% in CV and FDA, respectively. Conversely, salinity stress led to a reduction in the SMR FW_{leaf} by 86%, while the SMR DW_{leaf} was not affected by salt stress compared to the control (Figure 7b,e).

The root relative water content (RWC_{root}) was not influenced by the salt treatment. However, a decline in the leaf relative water content (RWC_{leaf}) was shown in all three bean populations under salinity stress compared to the control. More specifically, the CV, SMR and FDA RWC_{leaf} were reduced by 63%, 76%, and 78%, respectively (Figure 7g,h).

The analysis of the photosynthetic pigments revealed that salt stress induced an increase in the total chlorophylls in only CV (220%) and FDA (28%), whilst no significant change was found in SMR, compared to the control (Figure 7c). On the other hand, the Chl A/B ratio was decreased by 31% in CV, by 72% in SMR and by 45% in FDA under salinity stress (Figure 7f). Finally, salt stress caused an increase in the carotenoid content in all three populations compared to untreated plants: by 234% in CV, 22% in SMR, and 145% in FDA (Figure 7i).

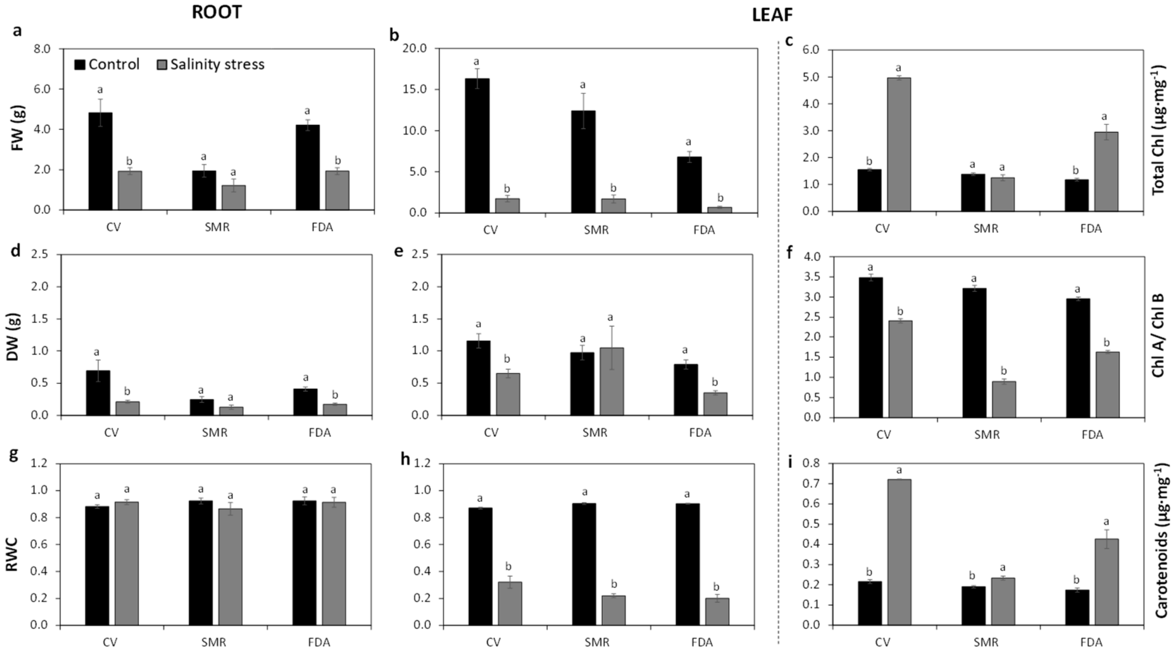


Figure 7. Morpho-physiological parameters analyzed in the three common bean landrace populations. Fresh biomass (FW; (**a**,**b**)), dry biomass (DW; (**d**,**e**)), relative water content (RWC; (**g**,**h**)) of roots and leaves and total chlorophyll content (Chl; (**c**)), chlorophyll A and B ratio (Chl A/Chl B; (**f**)) and carotenoid content (**i**) of leaves of the three common populations, grown under controlled and salt stress conditions, were analyzed. Data represent the mean (FW, DW and RWC n = 4; total Chl, Chl A/Chl B, carotenoids n = 3) ± standard error. A Student's *t*-test was conducted to weigh the effects of the different growth conditions ($p \leq 0.05$). Mean values marked with the same letter are not statistically different. CV: Ciliegino; SMR: San Michele Rosso; FDA: Fagiolo d'Acqua.

3.5.3. Plant Biochemical Data

Salt stress led to increased proline in the roots and leaves of the bean populations (Figure 8a,d). In detail, proline increased in the roots of CV (116%) and SMR (38%), while no change was found in the FDA roots, compared to the control plants (Figure 8a). The leaf proline level was found to be sharply increased by salinity stress in all three bean populations: CV (1091%), SMR (823%), and FDA (1197%) (Figure 8d).

Salt stress differently affected the total carbohydrate content in the common bean roots and leaves (Figure 8b,e). Specifically, salt stress led to a decline in the root total carbohydrates in CV, SMR and FDA by 36%, 45% and 40%, respectively (Figure 8b). On the contrary, a significant total carbohydrate content increase was observed in the leaves of the three bean populations: CV (116%), SMR (123%) and FDA (1791%) (Figure 8e).

The analysis of the MDA content, directly related to oxidative damage, indicated a decrease in the lipid peroxidation levels in the SMR (by 44%) and FDA (by 34%) roots under salt stress treatment, whilst CV showed unchanged root MDA levels, compared with the control (Figure 8c). In the leaves, salt stress led to an increase in the MDA levels in all the bean populations: the MDA level in CV was increased by 1359%, in SMR by 871%, and in FDA by 462% (Figure 8f).

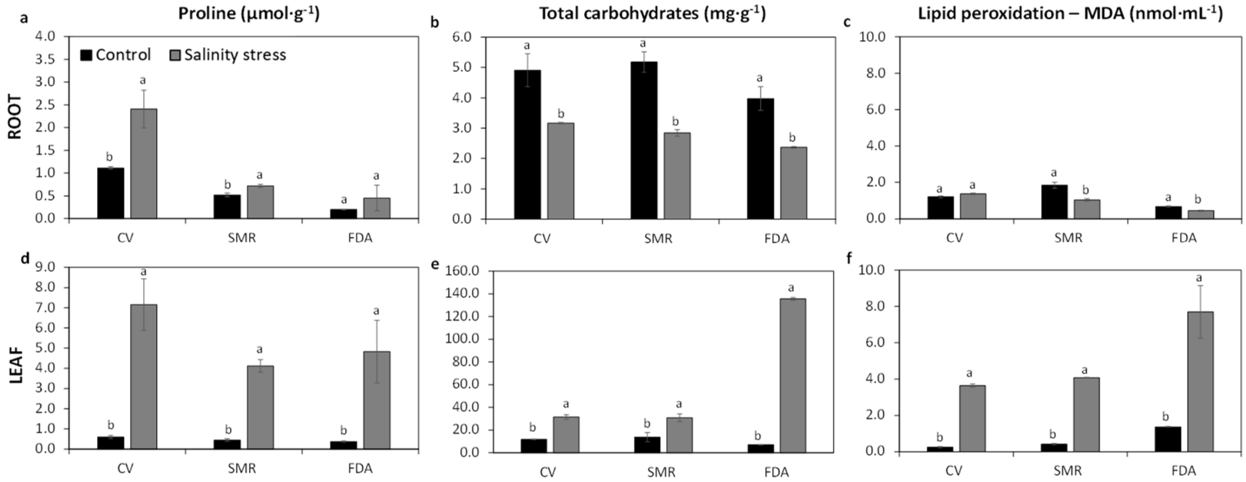


Figure 8. Biochemical parameters analyzed in the three common bean landrace populations. Proline (**a**,**d**), total carbohydrates (**b**,**e**) and malondialdehyde (MDA; (**c**,**f**)) contents were measured in the roots and leaves of the three common populations, grown under controlled and salt stress conditions. Data represent the mean (n = 3) ± standard error. A Student's *t*-test was conducted to weigh the effects of the different growth conditions ($p \leq 0.05$). Mean values marked with the same letter are not statistically different. CV: Ciliegino; SMR: San Michele Rosso; FDA: Fagiolo d'Acqua.

4. Discussion

In the present work, for the first time, the combined use of morphological, biochemical, molecular, and metabolomic data allowed for the differentiation of three Apennines landraces of *Phaseolus vulgaris* called "Ciliegino", "Fagiolo D'Acqua", and "San Michele Rosso". In detail, despite CV and SMR sharing similar seed colorimetric features, CV seeds are bigger and more rounded compared to SMR. Indeed, besides these red-colored seed populations (CV and SMR) being grouped together and well separated from FDA in the genetic and morphological dendrograms, they were well differentiated along the PCA and PCoA. However, the diversity assessment could not be unequivocally performed when studies deal with few accessions [57], considering that a higher genetic distance between CV and SMR populations was observed in our previous work [47].

Their diversity also emerged by analyzing the phaseolin type: despite all the three common bean populations belonging to the Andean gene pool, SMR and FDA showed three typical bands of phaseolin, while the CV profile was characterized by two bands, as also reported in Falcione et al. [47]. This result matches with other studies reporting a higher frequency of the Andean gene pool among common bean germplasm dispersed in Italy, the Iberian Peninsula, and the Balkan area [10,35]. This suggests that most of the common bean landraces were brought to Italy more likely through Spain rather than directly from America—between the 16th and 17th centuries many Italian regions, including Molise, were under Spanish control. However, a certain extent of admixture in the phaseolin diversity also indicated the occurrence of past (or recurring) hybridization events among gene pools [58], in combination with adaptation to different environments [59]. Thus, the CV population, subjected to different domestication flux, could be as putative hybrids between the two Mesoamerican and Andean gene pools. Our research, carried out by collecting the historical memories of the elderly inhabitants of Vastogirardi village, suggested that CV seeds could have arrived in Molise territory between the late 1800s and the early 1900s through Italian emigrants to the United States and their return to Italy. The CV could originate from an old local bean landrace, recently discovered by some farmers and seed collectors, showing the same small, round and red coat seeds; it is named "True Red Cranberry Bean", which used to be cultivated by the ancient native American tribe "Abenaki" in the northeastern areas of the United States, primarily in the region of Maine [60,61].

Alongside some similarities in the metabolomic profile, mainly due to amino acids, sugars, organic acids, nucleosides, and other miscellaneous compounds [62,63], specific metabolites also varied quantitatively among the landraces.

In detail, lactic acid, trigonelline and choline, as well as other molecules and micronutrients, highly enriched in the FDA seed aqueous extract, might play a role in FDA's ability to stimulate the cell proliferation of colon cancer cells [64,65] as evaluated in this work. Excess lactic acid has been shown to promote tumor growth: it acts as an energy source, signaling molecule and key tumor immunosuppressive factor [66]. Trigonelline is an alkaloid formed from nicotinic acid that, even at very low concentrations, may act as a phytoestrogen able to elicit cell growth of estrogen receptor (ER)-expressing cells [67]. Since Caco-2 cells express ERβ and are responsive to the proliferative effects induced by estrogens [68], it is possible to speculate that trigonelline promotes Caco-2 cell growth by acting as an endocrine disruptor. Furthermore, it has recently been shown that, in vitro, choline not only promotes cell proliferation but is also linked to malignant transformation and cancer progression [69].

In contrast to what was observed in FDA, the low amounts of lactic acid and pipecolic acid found in the CV seed extracts may have played a suppressive role in cancer cell proliferation, causing the decrease in the number of Caco-2 cells at all the doses here tested. A recent study showed that silencing pyrroline-5-carboxylate reductase 1 (PYCR1), a biosynthetic enzyme that converts Δ1-piperidine-6-carboxylate to pipecolic acid, induced apoptosis and cell cycle arrest in prostate and hepatocellular carcinoma [70]. A similar effect could be hypothesized in colon cancer. However, some other classes of metabolites, such as phenolic compounds, generally contained in red-skinned beans may be responsible for the observed antiproliferative effects on colon cancer cells rather than lactic and pipecolic acid alone [71,72].

Other metabolites could be actively involved in the different abilities of the three common bean landraces to counteract salinity stress. Several studies reported that salinity hampers seed germination, inhibiting many seed enzymatic activities and cellular processes, directly inactivating proteins, producing reactive oxygen species (ROS) or interfering with mineral nutrition [73]. Furthermore, seed hydration, one of the crucial steps in the germination process, is inhibited by salinity-induced osmotic stress; this prevents or delays the germination process [74].

Compared to CV and FDA, effective SMR seed adaptive mechanisms seem to be able to cope with the adverse effects of salinity stress. The high amounts of citric acid and some amino acids, such as alanine, leucine and methionine, detected by the ^{1}H–NMR spectra analysis, could play a protective role against salinity stress. Citric acid, acting as an antioxidative defense molecule, was probably involved in the recovery from ROS generated during stress conditions [75]. Free amino acids, such as alanine and leucine, in addition to serving as substrates for protein synthesis, also act as osmoprotectants under several abiotic stress conditions [76]. Methionine plays a key role in the synthesis of brassinosteroids, cytokinins and auxins to regulate/enhance plant growth, also functioning as a cofactor of antioxidants, vitamins, and polyamines [77]. Pipecolic acid acts as a critical regulator of plant immunity induced in response to biotic and abiotic stresses [78]. Thus, all these compounds might have played a role as compatible osmolytes, providing for: (i) osmotic adjustments; (ii) maintenance of cellular turgor; and/or (iii) alleviation of salt stress injury.

The SMR landrace also showed better performance than CV and FDA at the plant stage. Indeed, salt stress applied for 15 days had a negligible impact on the biomass accumulation in the roots and leaves of SMR plants, despite leading to a reduction in the leaf relative water content, an important indicator of plant water status [79]. It is argued that, under salinity stress conditions, plants decrease the hydraulic conductance of their roots to retain water and prevent its loss to salty soil. This mechanism could reduce the supply of (saline) water to the shoots, leading to reduced water potential in the SMR leaves [80].

As reported in several stress-sensitive grain legumes, including *P. vulgaris* [79,81,82], the growth retardation observed in CV and FDA could be attributed to the adverse effects of salinity on cell division and elongation: the excessive accumulation of salts around the root zone affects the water and nutrient uptake by the bean plantlets.

The combined results of the complex interactions among different molecular and biochemical features, such as the photosynthetic pigment content, lipid peroxidation and osmolyte accumulation (sugars and proline), generally contribute to the description of the observed diversity among landraces [83,84], but not in this case. Indeed, despite the low accumulation of sugars (and high proline) in all three landraces, the roots of SMR and FDA seemed to respond better than CV and the leaf counterpart, as demonstrated by the decrease in the amount of malondialdehyde (MDA) in this lower organ.

MDA, one of the end products of polyunsaturated fatty acid peroxidation in cells, is often used as a marker of oxidative damage [81]; on the other hand, it is known that proline, sugars, and carotenoids are all compounds that may alleviate oxidative stress damage [85].

Several studies have reported that the carbon translocation patterns from source (leaves) to sink (roots, stems, fruits, and seeds) are altered by salinity stress, leading to a consequent accumulation of sugars in the mature leaves to the detriment of the roots [86,87]. Similar results were also described in the studies conducted by Moles et al. [88], where soluble sugars were concentrated in tomato leaves to a greater extent than in the roots under salinity stress conditions.

In the leaves of all three common bean populations, salt stress induced a reduction in the ChlA/ChlB ratio and an increase in the amount of carotenoids. However, the total chlorophyll only increased in the CV and FDA leaves, while no change occurred in SMR due to the strong reduction in the ChlA content being counterbalanced by the increase in ChlB. Moreover, the proline, total sugars, and MDA content increased in the leaves of all three populations subjected to salt stress. Thus, besides the general role of the above-mentioned antioxidant molecules in alleviating oxidative stress and in osmoprotection [85], the structural reorganization in the main pigment–protein complexes may have contributed to maximizing the light harvesting and increasing the photosynthesis efficiency in SMR plants under stress conditions [89].

In some plant species, including *P. vulgaris*, the proline levels were higher in salt stress-sensitive cultivars compared to salt stress-tolerant examples as a consequence of disturbance in cell homeostasis, reflecting damage in the response to salt stress, thus a symptom of injury rather than an indicator of salinity resistance [47]. Furthermore, it is reported that prolonged salinity stress induces substantial proline accumulation to minimize salinity stress-related detrimental effects on plants [90]. This finding could explain the observed proline increase in all the populations, both the decreasing (CV and FDA) and unchanging (SMR) biomass accumulation, and the different ability of the CV and SMR populations to counteract mid- and long-term stress imposition, showing opposite behavior after 8 and 15 days of salt stress [47].

Taken together, the collected results led us to hypothesize the involvement/modulation of other enzymatic and non-enzymatic ROS detoxification systems that act differently in roots and leaves [91]. Although there are universal antioxidant defense mechanisms for all tissues, each organ uses the mechanisms that are most efficient for it and in harmony with its other metabolic networks, at a particular point of its age, to cope with salinity and the resulting oxidative stress [92].

Moreover, as recently demonstrated by Hernandez-Guerrero et al. [43], considering that common bean cultivars show analogous metabolomic profiles in the seeds and leaves, we can speculate an equivalent role of some detected compounds in the two organs. The high amount of citric acid, together with some free amino acids, such as alanine, leucine and methionine, could be able to counteract the salinity stress in the leaves of SMR when compared to CV and FDA and could represent climate-smart distinctive features of these dynamic landraces.

5. Conclusions

The complementary characterization approach performed in this study allows us to recognize three Apennines common bean landraces as a unique source of morphological/genetic diversity and bioactive chemical features. Although the use of neutral molecular markers, together with the analysis of genetic pool and morphological descriptors, can provide insights into the patterns and amounts of diversity found among landrace populations, they cannot be essential to translating landraces into competitive products. Conversely, NMR-based metabolomics results in a fast and reliable analytical tool to estimate similarities and differences in valuable bioactive compounds as potential resources to meet some contemporary societal challenges—plant biodiversity preservation/sustainable development under climate change and human well-being. The collected data also highlighted that plants undergo specific spatial and temporal physiological and biochemical reprogramming to adapt/balance growth and respond to stressful conditions. A mass spectrometry-based metabolomics approach should be used to decipher all the actors involved in the response of both above and below ground plant organs.

Further studies should be devoted to the investigation of the anti- and pro-tumoral mechanisms of common bean extracts in colon cancer cells, performing different typologies of assays aimed at evaluating the effects of total extracts or purified metabolites on apoptosis, senescence and autophagy processes.

Overall, this information, besides supporting the conservation strategies for this endangered locally adapted germplasm, could also act as a driving force for the development and promotion of the inland marginal areas, where these resources are generally confined.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d16040240/s1, Table S1: Primers used for ISSR and SCAR analysis; Table S2: Seed morpho-colorimetric descriptors and analysis; Figure S1: Plant material collection; Figure S2: Plants and seeds subjected to salt treatment; Figure S3: Most discriminant variables in the seed morpho-colorimetric analysis; Figure S4: ^{1}H NMR spectra of common bean seed lipophilic extracts; Figure S5: Loadings plot of the corresponding PCA and PLS-DA computed on the NMR qualitative data; Figure S6: Variables Importance in Projection (VIPs) plot of the PLS-DA.

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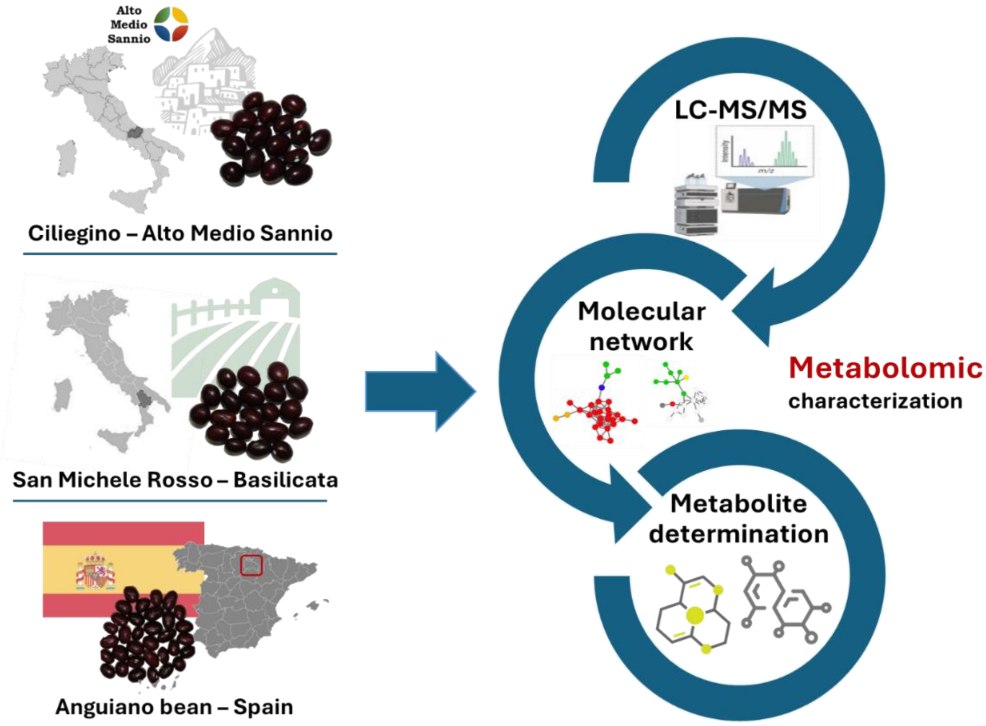
3.4 Metabolite Profiles of Common Bean (*Phaseolus vulgaris* L.) Landraces under the Spotlight of Mass Spectrometry-based Metabolomics – Paper III (*in preparation*)

Based on the promising results obtained in our previous study for “Ciliegino” bean, due to its proven antitumoral activity on colon cancer cells, and for “San Michele Rosso”, for its marked salinity stress tolerance, we undertook a further characterization activity on these two populations by using an untargeted mass spectrometry-based metabolomics approach. Furthermore, considering the presence of similar common bean varieties in other countries of the world and the past Spanish dominance in the Italian peninsula during the XVI and XVII centuries, which could have led to the diffusion of Spanish common bean germplasm into the Italian country, we decide to include also the “Alubia Roja de Anguiano” (Spain) in the characterization program, in order to compare the chemical profiles among these landrace populations, unveil their metabolomic fingerprint and evaluate their interconnections.

Paper III

in preparation



Introduction

Ushering into the metabolomics era, mass spectrometry (MS)-based metabolite characterization has evolved as an adept methodology to discern chemodiversity in metabolic pathways and phytochemicals, complementing the omics-cascade alongside genomics and proteomics. Metabolomics enables unbiased, high-throughput screening and characterization of the metabolite gamut in plant extracts through chromatographic separation, high resolution MS, and enhanced detection sensitivity (Chen et al. 2013). Indeed, LC-MS represents one of the major analytical techniques to determine global metabolite profiles and to dissect the immense plant chemodiversity, due to its capability of analysing a wide range of metabolites including secondary/specialized metabolites (e.g. alkaloids, benzoids, flavonoids, terpenes, isoprenes, glucosinolates and phenylpropanoids) and highly polar and/or higher molecular weight molecules (oligosaccharides and lipids) (Hill and Roessner 2015).

Recently, untargeted LC-MS-based metabolomic approach has been successfully applied in many studies aimed at exploring the chemical diversity of several plant species, including rice landraces, sweet potato cultivars, *Amaranthus* spp., *Butia* spp. and legume species (Hoffmann et al. 2017; Bennett et al. 2021; Bulut et al. 2023; Udhaya Nandhini et al. 2023; Abdel-Moez et al. 2023), providing a useful tool to profile the metabolome differences and identify distinctive chemical traits characterizing the plant samples.

Moreover, this powerful approach was also used for discriminating among rice samples products produced using different agricultural practices (Xiao et al. 2018), evaluate the variance in metabolites content of common bean (*P. vulgaris* L.) accessions under challenging environmental conditions (Mecha et al. 2022) and to trace the geographic origin of 151 grain maize (*Zea mays* L.) samples from several countries (Schütz et al. 2021) and *Cannabis sativa* L. genotypes of different regions of China (Li et al. 2022), enabling to discover differential metabolites with potential ability of chemotypic or geographical markers.

Compared with NMR spectroscopy, MS-based metabolomics provides an excellent approach that can offer a combined sensitivity and selectivity platform for metabolomics research. Indeed, MS is superior in allowing analysis of secondary metabolites at very low concentrations (picomole to femtomole) and the combination of diversified array of operational principles, such as ionization techniques and mass analyser technology, enables to increase the number of metabolites that can potentially be detected (Emwas 2015).

Several studies have reported the use of LC-MS metabolomics to characterize the diversity of common bean allowing to detect and quantify their major nutritional and anti-nutritional compounds (Llorach et al. 2019; Bulut et al. 2023). Moreover, this technique was used to dissect the chemical composition at a tissue-specific level in several accessions of common bean belonging to different gene pools, enabling to assign the chemical classes and/or the putative structure for approximately 39% of all measured metabolites (De Souza 2013).
However, considering the wide diversity characterizing the common bean landraces, only fragmented information are available regarding the differences in the metabolomic profiles of this legume species.

Furthermore, LC-MS could also shed light on seed specialized metabolites modifications and/or decorations, allowing their detection and characterization via the construction of molecular network (Wang et al. 2019). Indeed, the majority of the structural diversity in the plant specialized metabolome arises from "decorative" biosynthetic steps, such as hydroxylations, methylations, glycosylations, acylations and prenylations, which contribute to the huge variability of activities and functions of seed and plant specialized metabolites (Barreda et al. 2024). The decorations are also subjected to dynamic change during growth and development of the plants or in response to environmental stimuli, representing a key element in the adaptive capacity of plants to changes in their environment, performing metabolic regulatory functions, detoxifying activities as well as increasing the solubility of specialized metabolites (Desmet et al. 2021).

Based on these premises, in the present study untargeted metabolomics analysis, based on liquid chromatography coupled with mass spectrometry (LC-MS/MS), was performed with the goal of exploring the seed chemical variability of three common bean landrace populations coming from different locations and evaluating the possible presence of distinctive metabolic signatures characterizing each common bean population. This information may provide additional insights into the compositional, nutraceutical, and functional properties of common bean landraces.

Material and methods

Plant material

Seeds of three common bean landrace populations (Fig. 17) were collected from traditional farmers and local farms in Molise and Basilicata region (Italy) and in North of Spain. In particular, "Ciliegino" (CI) was collected by local farmers in Vastogirardi district (1200 m a.s.l.), belonging to "Alto Medio Sannio" inner area, while "San Michele Rosso" (SMR) was purchased from Belisario farm in Sarconi

(PZ), Basilicata region, and awarded with IGP (Indication of Protected Origin) quality marker. On the other hand, "Alubia Roja de Anguiano" (CI SP) was obtained from traditional farmers' association (https://alubiadeanguiano.com/), actively involved in the on-farm maintenance of this landrace in Anguiano, a little village in the region of La Rioja, Northern Spain, and assigned with DOP (Denomination of Protected Origin) attestation. All the common bean landrace populations are cultivated in mountainous or hilly areas (Vastogirardi 1200 m a.s.l.; Sarconi 636 m a.s.l.; Anguiano 663 m a.s.l.) and are characterized by small, oval, and shiny red coat-coloured seeds, with white hilum. Seeds of the three common bean populations are stored ex situ in Molise Germplasm Bank.

Figure 17. Plant material collection. Seeds of the three common bean landrace populations analysed in the present study. Ciliegino from Vastogirardi, "Alto Medio Sannio" inner area (a); San Michele Rosso from Sarconi, Basilicata region, awarded with IGP quality marker (b); Anguiano bean from Spain, assigned with DOP quality marker (c).

Metabolite determination

The extraction of polar and non-polar metabolites was performed according to the procedure applied by Boutet et al. (2022) with some modifications. Briefly, 10 mg of dry seeds of each common bean populations were powdered with liquid nitrogen and homogenized in 1 mL of MeOH/acetone/H_2O/Trifluoroacetic acid (TFA) (30:42:28:0.1) extraction buffer and 500 ng of Apigenin (Extrasynthese) (used as internal standard). The mixtures were placed in an ultrasonication bath for 1-2 min and shacked for 30 min at 4 °C in a Thermomixer (Eppendorf). Next, the samples were centrifuged for 10 min at 4 °C at 10 000 rpm and supernatants were collected in glass tubes. The remaining pellet was extracted once again, and the second supernatants were collected and pooled with the one obtained from the first extraction step. Successively, 1.8 mL of the pooled extracts were transferred in a SpeedVac vacuum concentrator and dried at room temperature until complete solvent evaporation. The extraction procedure was performed in triplicate to ensure the reproducibility. The dried extracts were dissolved in 150-200 µL H_2O containing 10% acetonitrile and transferred in HPLC vials.

Untargeted metabolomic analysis was performed by using a UHPLC system (Ultimate 3000 Thermo) coupled with a quadrupole time-of-flight mass spectrometer (Q-Tof Impact II Bruker Daltonics, Bremen, Germany). Nucleoshell RP 18 and a reversed-phase column were used for chromatographic separation. Samples were injected in positive and negative ionization modes (ESI+ and ESI-) and ESI+ and ESI– were analysed using MZmine 2.52 software (http:// mzmine.github.io/ accessed on 1 August).

Metabolite annotation of untargeted metabolomic data was performed following several consecutive steps. First, LC-MS/MS data were compared using a homemade library (IJPB metabolomic platform) containing more than 150 standards or common experimental features. Second, LC-MS/MS data were searched against available MS^2 spectral libraries (Massbank NA, GNPS Public Spectral Library, NIST14 Tandem, NIH Natural Product, and MS-Dial). Third, not-annotated metabolites, belonging to molecular network clusters with annotated metabolites, were assigned to the same chemical family. In detail, this approach is based on the reconstruction of the potential biochemical conversions occur ring in the set of detected features along with the MSMS spectral similarity analysis. The result is represented by a network in which nodes correspond to metabolites and edges correspond to putative biological relationship. Metabolites accumulation was normalized according to the internal standard (Apigenin, 500 ng/sample) and weight of seeds used for the extraction.

Statistical analysis

The online MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca/ accessed on 15 October 2023) was used to perform the statistical analysis of the data. For univariate and multivariate analyses, the median data were used to normalize the relative amounts of data for identified metabolites. A one-way ANOVA was performed on the dataset, followed by post-hoc Tukey's honestly significant difference (HSD) test with an adjusted p value ≤ 0.05. The unsupervised approach of pattern identification, known as Principal Component Analysis (PCA), was first used to investigate the data matrix's inherent variation. Following that, Partial Least Squares Discriminant Analysis (PLS-DA), a supervised classification method, was performed. The quality of PLS-DA model was evaluated using the correlation coefficient R_2 and a cross-validate correlation coefficient Q_2. Furthermore, Variables Importance in Projection (VIPs) score (value>1) of PLS-DA were selected. A hierarchical clustering analysis (HCA) dendrogram with heatmap representation was performed with the Pheatmap R package in order to evaluate the differential metabolite accumulation among the common bean populations. The dissimilarity was measured using the Euclidean distance, and the clusters were described using Ward's approach.

Enrichment analysis was performed with R software package and by using a hypergeometric test, and the FDR was controlled across all the terms at level 0.05. The annotated and unknown metabolic categories of differentially accumulated metabolic features (DAMfs) were used as metabolite reference set.

Preliminary results

Principal Component Analysis (PCA) and Partial Least Squares-Discriminant analysis (PLS-DA)

Positive and negative electrospray ionization of LC-MS/MS metabolomic data revealed the presence of 583 peaks of known and unknown metabolites, called as metabolic features (Mfs).

Total metabolites detected by LC-MS/MS were imported for the construction of Principal Component Analysis (PCA) and Partial-Least-Squares Discriminant-Analysis (PLS-DA).

In the score plot resulted from PCA (Fig. 16a), Principal Component 1 (PC1) and Principal Component 2 (PC2) accounted for 58% and 17.6% of the total variance, respectively.

Three distinct groups, related to the three different common bean populations, could be observed, revealing significant variations in their chemical compositions. In detail, along PC 1, CI and CI SP were placed in negative values, while SMR appeared in positive coordinates. On the other hand, regarding PC 2, CI SP and SMR were projected along positive coordinates, while CI displayed negative values (Fig. 18a). The PCA corresponding loadings plot was reported in Fig. 18b.

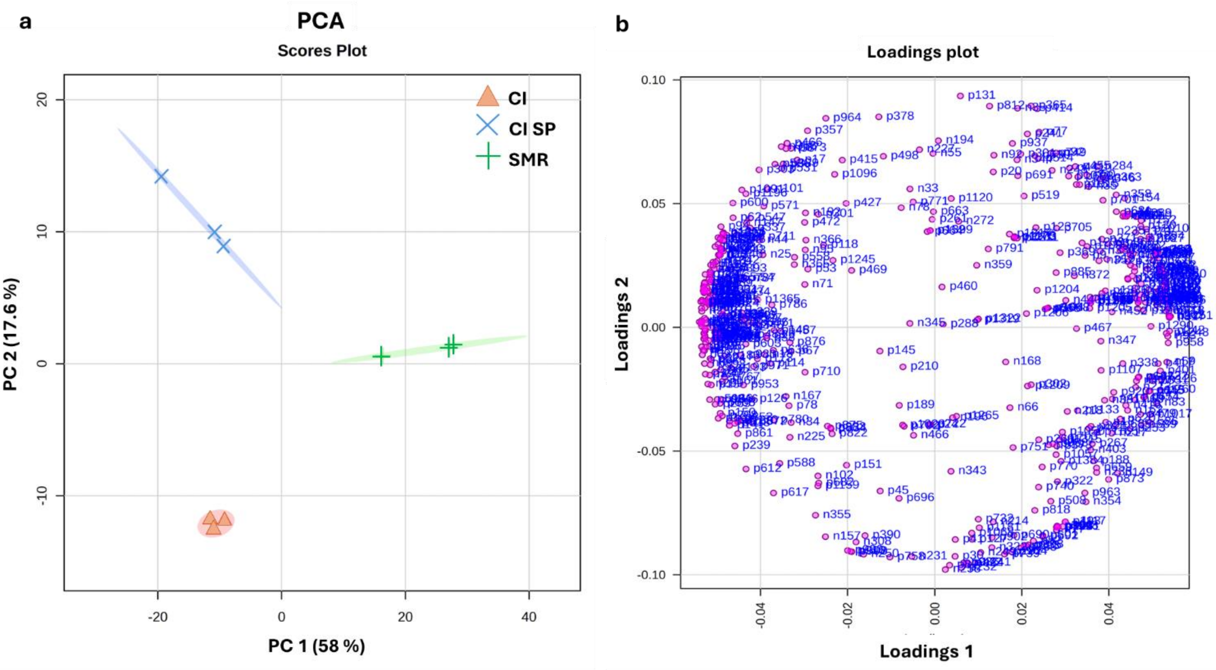


Figure 18. Principal Component Analysis (PCA) and the corresponding loadings plot computed on total metabolites detected by LC-MS/MS. Scores plot of the PCA (a); loadings plot of the PCA. The numbers refer to the Mfs detected by LC-MS/MS (b). CI: Ciliegino; CI SP: Anguiano bean; SMR: San Michele Rosso.

A clear distinction among the three bean populations emerged also from the PLS-DA. In the resulted score plots, PC 1 explained 19.3% of the variance, followed by the PC 2 with 56.1%. The PLS-DA regression (R_2=0.98; Q_2=0.78) showed a good group separation along the components. In detail, in PC 1, CI and SMR were placed along negative values, while CI SP projected along positive coordinates. Conversely, along PC 2, CI and CI SP showed negative values, while SMR was positioned along positive coordinates (Fig.19a).

The corresponding loadings plot (Fig. 19b) was inspected for metabolites responsible for PLS-DA data clustering. The 10 most discriminant metabolites, with Variables Importance in Projection (VIPs) score>1, are illustrated in the VIPs plot and are mainly represented by flavonoids (Fig. 20).

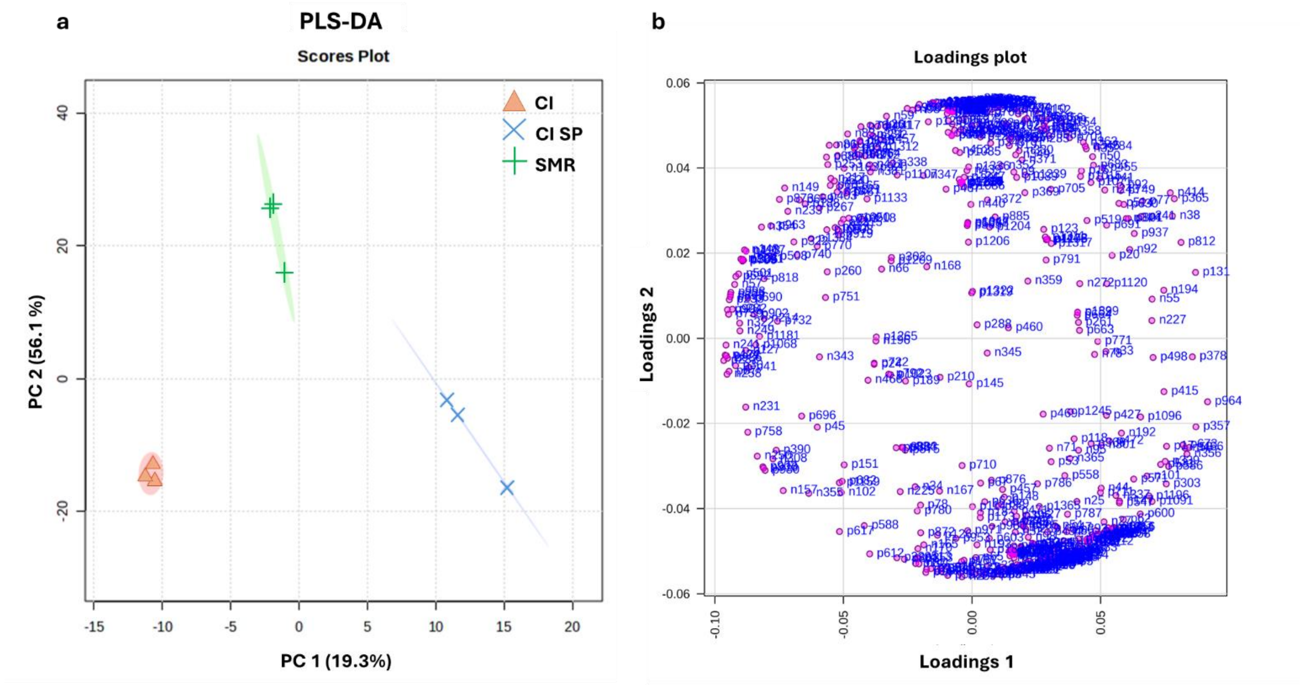


Figure 19. Partial-Least-Squares Discriminant-Analysis (PLS-DA) and the corresponding loadings plot computed on the total metabolites detected by LC-MS/MS. Scores plot of the PCA (a); loadings plot of the PLS-DA. The number refers to the Mfs detected by the LC-MS/MS (b). CI: Ciliegino; CI SP: Anguiano bean; SMR: San Michele Rosso.

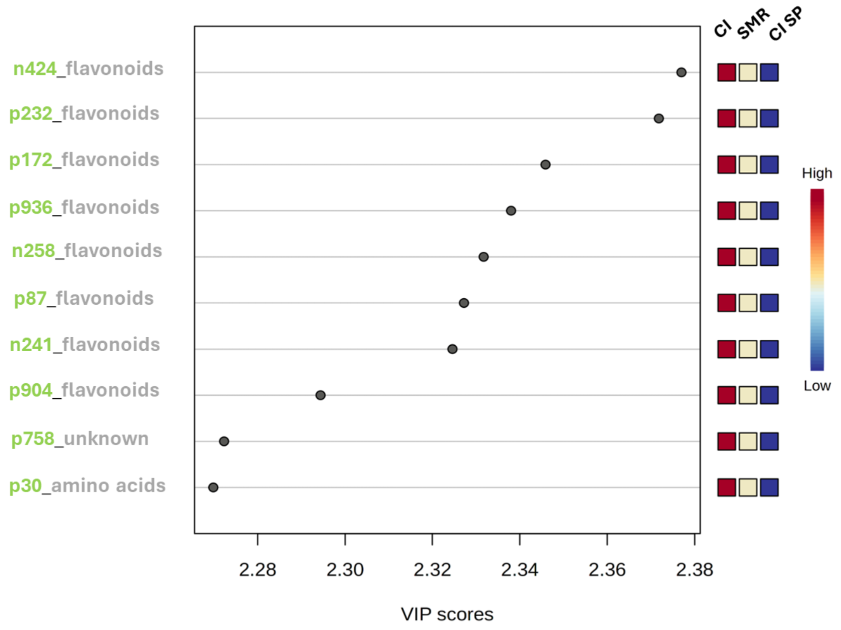


Figure 20. Variables Importance in Projection (VIPs) plot of PLS-DA.VIPs plot representing the 10 most discriminant metabolites in common bean populations. The x-axis reports VIPs scores, the y-axis shows the annotated metabolites. The coloured boxes on the right indicate the relative concentrations (red, high; yellow, intermediate; blue, low) of the corresponding metabolite in each bean population under study. CI: Ciliegino; CI SP: Anguiano bean; SMR: San Michele Rosso.

Metabolic profile differences among the three bean populations

The number of differentially accumulated metabolic features, also known as DAMfs, was identified by the one-way analysis of variance (ANOVA) statistical test. The ANOVA plot revealed that out of 583 total metabolites detected by LC-MS/MS, 344 were found to be differentially expressed with statistical significance among the populations. In detail, the red coloured dots represent the metabolites differentially expressed ($p \leq 0.05$) among the populations, while the green dots indicate the metabolite of without statistical significance (Fig. 21).

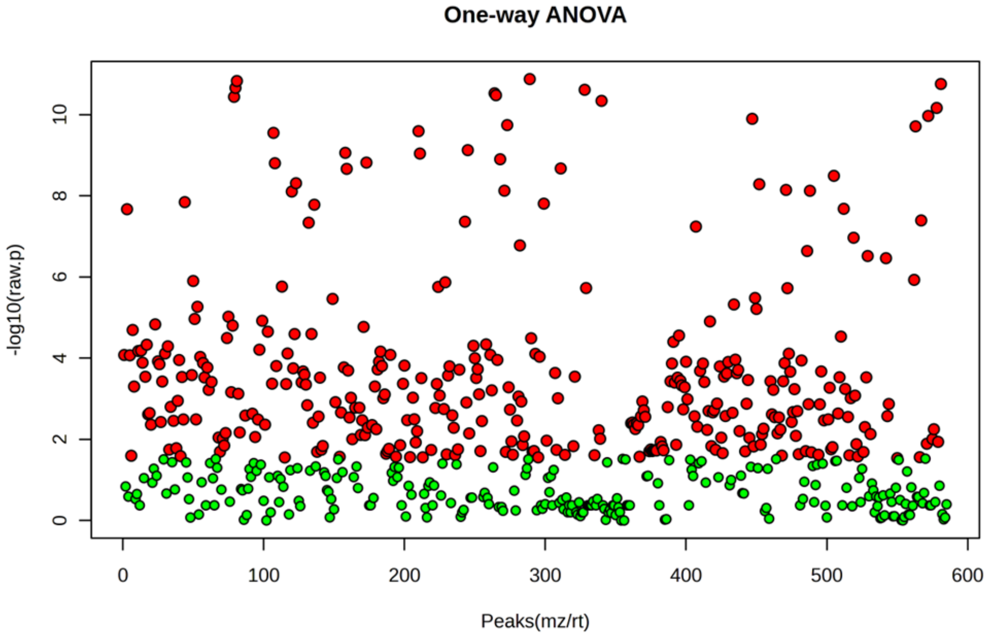


Figure 21. Analysis of variance (ANOVA) plot showing significantly ($p \leq 0.05$) detected metabolites. Each red dot represents one metabolite with $p \leq 0.05$, while the green dot represents metabolite without statistical significance.

Metabolite annotation

The annotation process was conducted on the significantly ($p \leq 0.05$) differentially accumulated metabolic features (DAMfs) identified by ANOVA test, by using a public database (GNPS Public Spectral Library) and internal standard, and by generating a metabolite molecular network with MetGem software (Fig. 22a). In particular, molecular networking allows to cluster compounds with a high degree of structural similarity according to their comparable MS^2 fragmentation pathways, and therefore enables to identify clusters of unknown metabolites using the MS/MS information for annotated compounds.

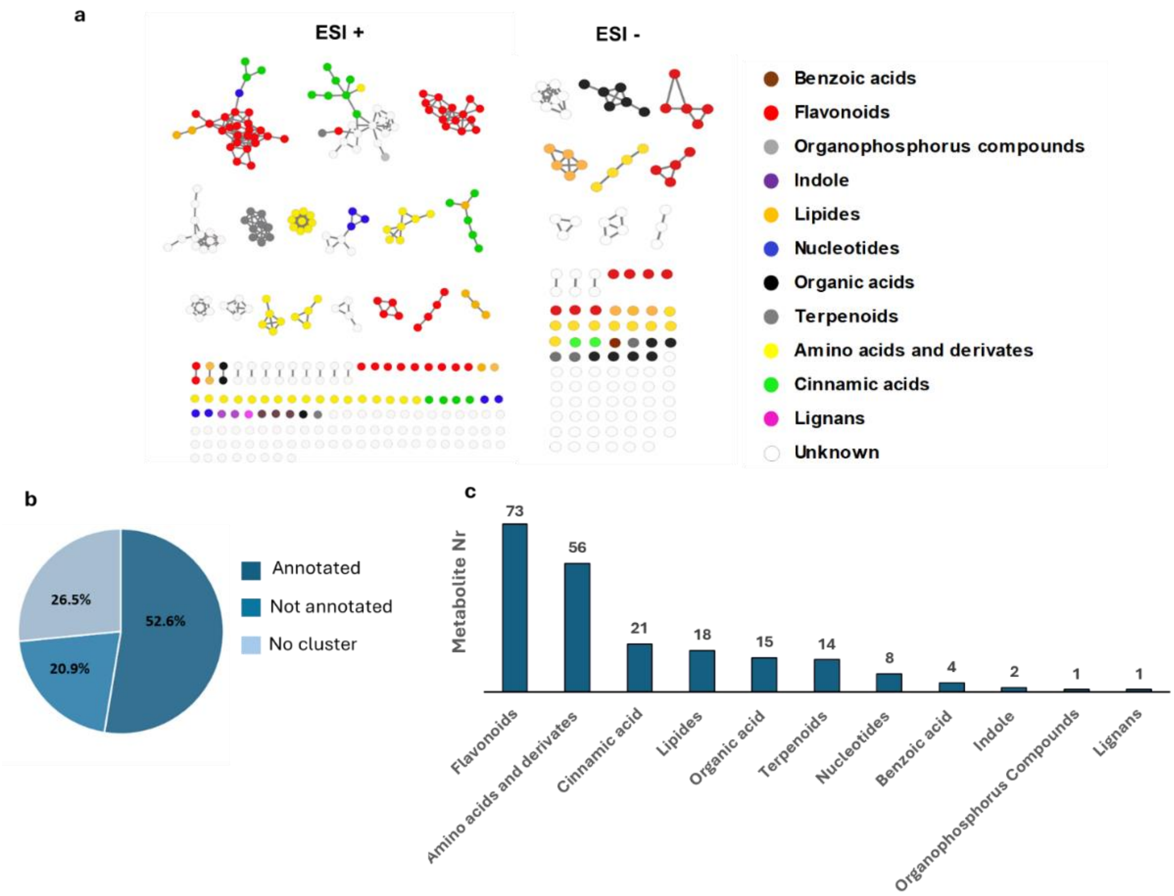


Figure 22. Annotation process of the significantly (p≤0.05) differentially accumulated metabolic features (DAMfs) among common bean populations. Percentage of metabolite annotated, not annotated and metabolites not included in a cluster and not annotated (a); Molecular networks for metabolite analyses carried out in positive (ESI+) and negative (ESI-) ionization modes. Each metabolic class is characterized by a different colour (b). The 11 chemical classes identified through the annotation process. The number of metabolites of each chemical category is shown (c).

In detail, the preliminary annotation process performed with the above-mentioned methods allowed to identify and categorize into chemical classes 52.6% of the DAMFs.
On the other hand, 20.9% of the DAMfs metabolites were encompassed in clusters that could not be associated to a metabolic class and 26.5% of the DAMfs metabolites were not included in a cluster and not annotated (Fig. 22b).

The annotated metabolites were categorized into 11 different putative chemical classes: flavonoids resulted to be the most represented metabolic category (73), followed by amino acids and derivates (56), cinnamic acid (21), lipids (18), organic acids (15), terpenoids (14), nucleotides (8), benzoic acid (4), indole (2), organophosphorus compounds (1) and lignans (1) (Fig. 22c).

Hierarchical clustering analysis (HCA) and enrichment analysis

Hierarchical cluster analysis (HCA) of the statistically significant ($p \leq 0.05$) representative metabolites was performed to further investigate the variation of metabolites among the bean populations. The metabolite patterns distributions among samples are visualized as a heatmap (Fig. 23).

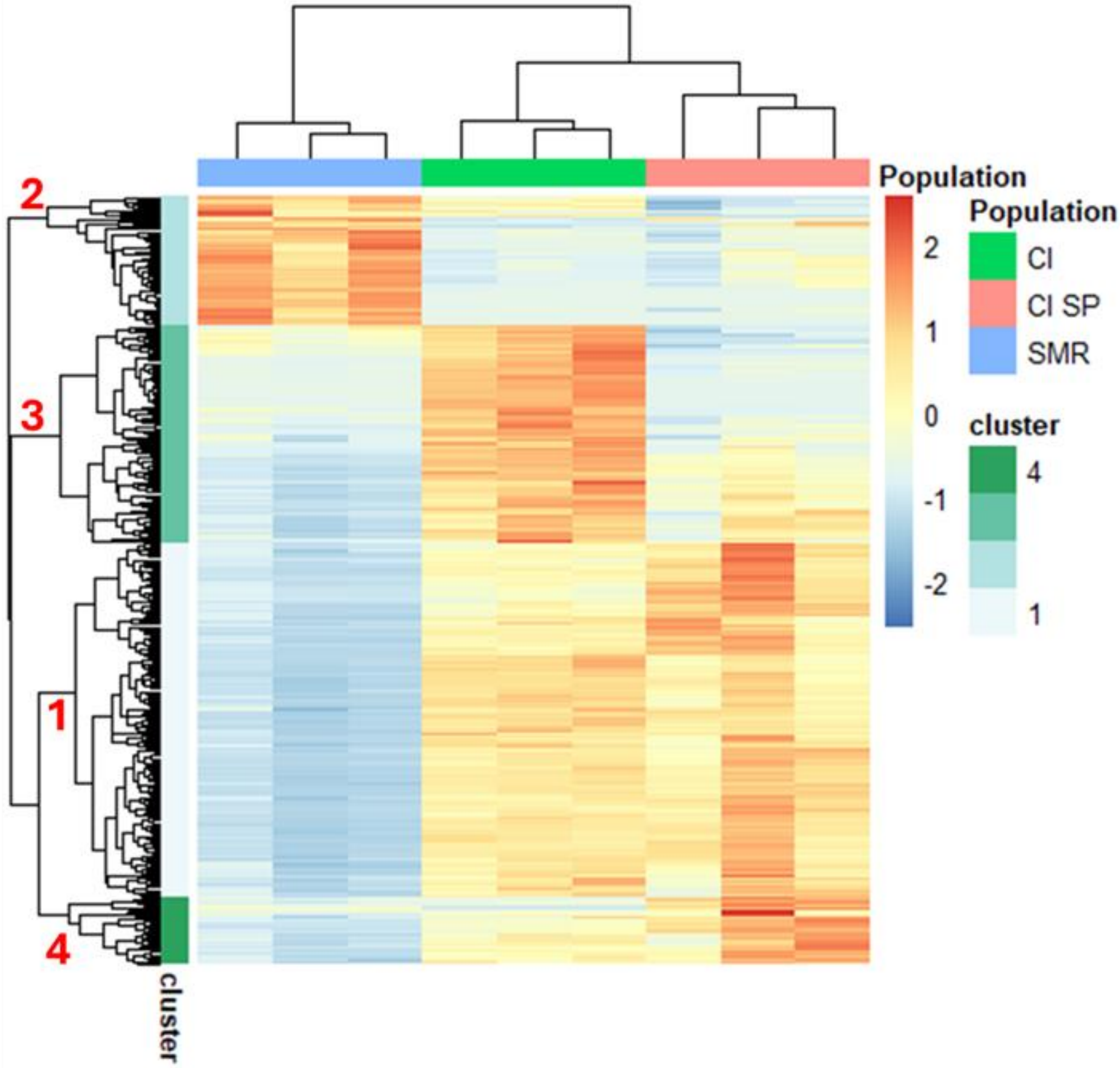


Figure 23. Heatmap representing the variations in metabolite accumulation among the three common bean populations. Each line refers to a single metabolic feature (Mf), while columns refer to common bean populations. On the right the colour key scale indicates the trend of metabolite accumulation (red, high; yellow, intermediate; blue, low).

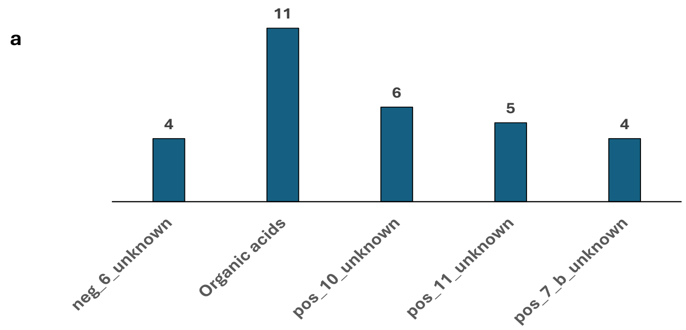
The HCA heatmap results grouped CI and CI SP together, indicating that these two populations have more similar metabolite profiling with respect to SMR, which was clustered alone. Analysing the differential metabolite distribution among the three bean populations, metabolites of cluster 1, showed a sharp decrease in SMR, compared to CI and CI SP, where higher levels are reported.

Metabolites of cluster 2 significantly accumulated in SMR with respect to CI and CI SP, while as regards metabolites of cluster 3, higher levels were reported in CI, compared to SMR and CI SP. In the case of cluster 4, increased metabolite amounts were displayed in CI SP, whereas moderate and low levels were reported in CI and SMR, respectively (Fig. 23).

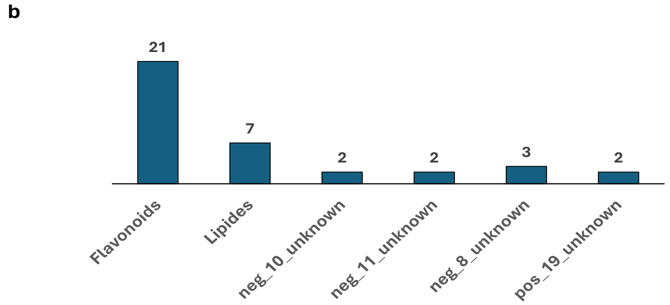
The enrichment analysis of metabolic clusters was performed in order to identify population-specific metabolic features. Among the DAMfs of cluster 1, organic acid (11 DAMf) and four unknown categories, named neg_6 (4 DAMf), pos_10 (6 DAMf), pos_11 (5 DAMf) and pos_7 (4 DAMf), were found to be enriched compared with the metabolite reference set library, suggesting a putatively distinctive accumulation of these metabolic features in CI and CI SP populations.
Similarly, among the DAMfs of cluster 2, flavonoids (21 DAMf), lipids (7 DAMf), and four unknown categories - neg_10 (2 DAMf), neg_11 (2 DAMf), neg_8 (3 DAMf), pos_19 (2 DAMf) - were found to be enriched and putatively specific of SMR. In the case of cluster 3, a distinct metabolic category characterized by not-annotated metabolites, also known as no_category (23 DAMf), was found to be enriched and over-accumulated in CI. Instead, among the DAMfs of cluster 4, no metabolic category was found to be enriched, probably due to the limited metabolite number of cluster 4 (Fig. 24).
Further details regarding the enriched metabolic categories are shown in the tables of Fig. 24.

Organic acids and flavonoids, which are found to be increased in CI and CI SP and SMR, respectively, belong to the promising groups of bioactive compounds with strong antioxidant and anti-inflammatory properties.

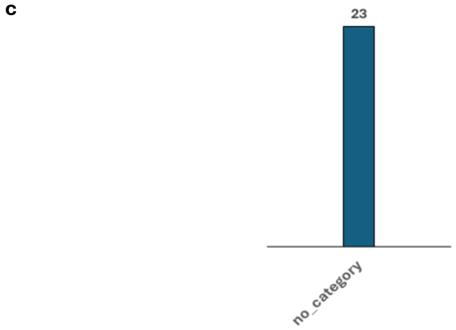
In detail, organic acids are central to cellular metabolism, representing intermediates of carbon metabolism in plant cells and being involved in various biochemical pathways, such as glycolysis, tricarboxylic acid cycle, photorespiration, glyoxylate cycle, and photosynthetic C_4 cycle (Drincovich et al. 2016). Besides their proven role in assisting plant growth under harsh environmental conditions and in alleviating the adverse effects of abiotic stresses (succinic, malic and galacturonic acids) (Khan et al. 2020), several studies reported their anticarcinogenic activity (ascorbic and citric acids), serving as potent antioxidants and redox homeostasis regulators in tumor microenvironments (Ayat 2019; Maekawa et al. 2022). Furthermore, various experimental research described their critical role as peeling agents to treat skin disorders (salycilic acid), as neuroprotectors in several brain diseases (malic acid) and as anti-inflammatory compounds in the treatment of antigen-induced arthritis (*cis*-aconitic acid) (Arif 2015; Niaz et al. 2022; Oliveira et al. 2022)



Term	Urn success	Urn failures	Trial success	Trial effective	Urn percentage success	Trial percentage success	P value over
neg_6_unknown	4	340	4	159	1.16	2.52	0.044714
Organic acids	15	329	11	159	4.36	6.92	0.028868
pos_10_unknown	6	338	6	159	1.74	3.77	0.00926
pos_11_unknown	5	339	5	159	1.45	3.14	0.020384
pos_7_b_unknown	4	340	4	159	1.16	2.52	0.044714



Term	Urn success	Urn failures	Trial success	Trial effective	Urn percentage success	Trial percentage success	P value over
Flavonoids	60	284	21	58	17.4	36.2	0.000116
Lipids	11	333	7	58	3.202	12.07	0.017384
neg_10_unknown	2	342	2	58	0.581	3.45	0.028019
neg_11_unknown	2	342	2	58	0.581	3.45	0.028019
neg_8_unknown	3	341	3	58	0.872	5.17	0.004588
pos_19_unknown	2	342	2	58	0.581	3.45	0.028019



Term	Urn success	Urn failures	Trial success	Trial effective	Urn percentage success	Trial percentage success	P value over
no_category	58	286	23	97	16.9	23.7	0.026691

Figure 24. Enriched metabolic categories. Cluster 1 (a); cluster 2 (b); cluster 3 (c). Histograms show the annotated and not-annotated enriched metabolic categories of each metabolic cluster using differentially accumulated metabolic features (DAMfs). Further details of the enriched metabolic categories are displayed in the tables. Term: metabolic feature (Mf); Urn success: number of the specific metabolic feature out of the metabolite reference set detected; Urn_failures: number of the metabolite reference set detected except the specific metabolic feature number; Trial_success: number of the specific metabolic feature in the selected cluster; Trial_effective: total number of metabolic features in the selected cluster; Urn_percentage_success: % of the specific metabolic feature out of the metabolite reference set detected; Trial_percentage_success: % of the specific metabolic feature in the selected cluster; p value≤0.05.

Likewise, flavonoids possess multiple health benefits, displaying antioxidant and anti-inflammatory effects in several metabolic disorders such as cardiovascular disease, cancer, obesity, and diabetes. Their chemical structure indicates that they act as radical scavengers, oxygen quenchers, and hydrogen-donating antioxidants, playing a main role in boosting endogenous antioxidants and in preventing the formation of ROS and their subsequent cell damage (Mahmoud et al. 2019). Moreover, flavonoids have been reported to exert varying degree of antibacterial activity, outperforming several common antibacterial agents, such as tetracycline, erythromycin, norfloxacin, ciprofloxacin, and oxacillin in the treatment of various multidrug-resistant bacterial strains (Shamsudin et al. 2022).

Concluding, the obtained results allowed to evaluate the natural variance in metabolites content of common bean landraces and to perform a preliminary identification of hyper-accumulated/over-represented metabolic categories in each population. In the future, the completion of the annotation process could be used as a baseline for the full elucidation of further population-specific metabolic features. These differentially accumulated metabolites could act as potential chemotypic markers of the common bean populations evaluated, highlighting distinctive chemical traits of these genotypes.

4 General conclusions and future perspectives

Local landraces are recognized as important natural resources which could serve as a basis to reach sustainable development and to cope with future climatic scenarios. However, their fast disappearance due to the substitution with improved modern varieties has required the impellent necessity to take up characterization activities for an efficient conservation and use of these endangered genetic material. These initiatives could be essential to establish the value and the potential distinctiveness of these local resources, which, in turn, may play a pivotal role in enhancing the resilience and economy of the marginal rural territories where these resources are traditionally maintained "since time immemorial".

With this idea, the objective of this doctoral thesis, was to recover, characterize and conserve the plant agrobiodiversity of the "Alto Medio Sannio" inner area. Therefore, after conducting a collecting mission throughout this inland area to recover local legume landraces and the closely linked cultural information, an extensive experimental activity has been undertaken - paper I, II and III - in order to perform a comprehensive characterization of several common bean (*P. vulgaris* L.) landraces. In detail, these studies identified some common bean landraces as a source of morphological, genetic and phytochemical diversity, enabling us to identify also "climate-smart" bean populations tolerant to short-term salt and osmotic stress (paper I). The presence of valuable bioactive compounds, revealed by nuclear magnetic resonance-based metabolomics, resulted responsible of the antitumoral activity and the long-term salinity stress tolerance observed in some populations (paper II). Finally, the deep mass spectrometry-based metabolomics approach defined metabolomic fingerprint of three common bean landraces, allowing the preliminary identification of differentially accumulated metabolites among the populations (paper III, *in preparation*). Further analysis of these metabolites, in term of the corresponding biological significance and the possible involvement in the environmental stress tolerance, will enable to determine the local resources value in regard to nutritional, therapeutic and/or the abiotic/biotic stress resilience potential.

Taken together, the results collected in the above-mentioned studies, besides providing strong basis to foster the conservation strategies towards this endangered locally-adapted germplasm, also serve as an incentive for the development and the promotion of the Italian marginal inland territories, where these resources are generally confined. Indeed, this local genetic material represents a bridge between the past, in terms of popular culture and traditional knowledge, and the future, given their possible use under changing environments and in sustainable agriculture. Therefore, the preservation and the valorisation of local germplasm, along with the intangible cultural heritage associated, could represent a valid means of propulsion for activating an economic regeneration in marginalized areas.

In a prospective view of this doctoral research, further studies should be dedicated to evaluating the effects of common bean seed extracts on different cancer cell culture models, such as human hormone-dependent mammary (MCF7), liver (HepG2) and prostate (PC3) cancer cell lines, in which evident *in vitro* cytotoxic effects have been widely reported. Moreover, further efforts may be proposed to elucidate the role of specific purified common bean metabolites in cancer progression pathways, in antimicrobial activity and/or in several biological process, e.g. lipid metabolism, in which common bean bioactive compounds, such as protein hydrolysates, flavonoids and saponins, have been reported to exert potentially meaningful beneficial effects.

More in general, it could be essential to foster local citizens engagement and participation in landraces cultivation initiatives, contributing to the successful conservation of the local resources and to the resilience and well-being of both local communities and the broader agroecosystems. In this context, the implementation of dissemination activities across the community, aiming at communicating local people their role in the conservation and in the sustainable use of biodiversity, could play a strategic role in strengthening the value of landraces and in guaranteeing the maintenance of the agrobiodiversity and, at the same time, the permanence of traditional social heritage in rural territories.

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Annex I

Report of the research activity performed during the study period abroad (3 months 19/09/2022−20/12/2022) at Seed Bank Laboratory of the Department of Biology, Faculty of Science, University of Malta, Msida

Background

Agrobiodiversity is the basis of multiple ecosystem services essential to sustain human well-being and future food security demands (Mercer *et al*., 2010). The conservation and the management of this wide and vital sub-set of biodiversity is a key issue in the current climate change scenario and, despite the ongoing conservations efforts, agrobiodiversity is under severe threat in many regions of the world because of several factors, including land use intensification, structural transformations in the agricultural sector, climate change and urbanization (Pautasso *et al*., 2012; Kumar *et al*., 2020). One example of this decline in agrobiodiversity is the widespread disappearance of landraces, locally adapted plant genetic resources with valuable cultural significance, as well as high genetic diversity, able to easily adapt to local and unfavourable agro-environmental conditions and face the current climate change challenges (Casanas *et al*., 2017).

In particular, common bean (*Phaseolus vulgaris* L.) landraces are important grain legume which are undergoing a gradual process of genetic erosion due to intensification of food production which caused a displacing of this traditional germplasm by the improved and high yielding varieties. However, these threatened and locally adapted resources are characterized by a huge diversity in terms of plant physiology, seed characteristics (e.g., size, shape and colour), reproductive cycle and many other qualitative and quantitative traits, representing a source of valuable alleles still unexplored and which could serve as adaptive attributes under a changing environment (Caproni *et al*., 2019; Kyratzis *et al*., 2019). Therefore, it is of paramount importance to identify and characterize common bean landraces in order to investigate their diversity and unravel their unique and specific traits, with the prospect of enhancing future conservation strategies to safeguard these plant genetic resources.

In the last two decades, a significant increase in the application of software-assisted image analysis has been highlighted in the plant biology research field (Smykalova *et al*., 2011). In particular, several studies proved the importance of using image analysis for the characterization of cultivated species, such as *Cucumis melo* L., *Olea europaea* L., *Malus domestica* (Suckow) Borkh. (Sabato *et al*., 2015; Piras *et al*., 2016; Sau *et al*., 2018) and for the identification of unknown plant seed material in archeobotany (Orrù *et al*., 2013; Ucchesu *et al*., 2017). Moreover, the validity of the image analysis technique has successfully been applied to identify and discriminate among legume landraces (lentil, bean, pea), evaluating several morphometric and colorimetric features of seeds (Venora *et al*., 2007; Venora *et al*., 2009; Smykalova *et al*., 2011). Seed size, shape, colour and texture can be quickly obtained and processed by this automatised system, showing the potential to replace human visual assessments and providing accurate, repeatable and objective measurements (Lo Bianco *et al*., 2015).

Therefore, at the current status, it is possible to assert that seed image analysis represents a reliable, cost-effective and non-destructive method to investigate the diversity among plant species, which could be used as a complementary tool to the widely used molecular methods for the characterization of the landraces (Orrù *et al*., 2012).

Furthermore, the characterization of the full set of chromosomes of a species, as known as karyotype, could provide key cytogenetic information regarding plant systematics, evolution and phylogenetic relationships and for assisting plant breeding programs (Baltisberger *et al*., 2016; He *et al*., 2022; Yu *et al*., 2022).
Karyotype analysis could be performed with several approaches, including software assisted-image analysis of metaphase chromosomes, fluorescence in situ hybridization (FISH) and flow cytometry and it is able to define the number, size and morphology of chromosomes (Rois *et al*., 2018; Lima *et al*., 2019; Jiang, 2019).
Variation in chromosomes number, size, morphology and staining properties could occur within a taxon (e.g. family, genus or species) and even an intra-specific variation in genome size was detected in a common bean collection (Baltisberger *et al*., 2016; Tuna *et al*., 2020).
Furthermore, information on plant genome size, collected through karyotype analysis, could give important insights also on plant stress tolerance, since a higher fitness under environmentally stressful conditions have been detected in plants with smaller genomes (Meyerson *et al*., 2020).

Therefore, based on these premises, karyotype investigation could provide relevant information on genetic aspects and phylogenetic relationships of common bean landraces, playing a main role in the characterization of these plant genetics resources.

During these three months of research activity at the University of Malta, the characterization and the assessment of phylogenetic relationships among seven autochthonous and endangered common bean populations have been performed by using software assisted-seed image analysis and karyotyping of root tip cells of germinating seeds.

Plant material

Seeds of seven autochthonous common bean (*Phaseolus vulgaris* L.) populations, cultivated in the Molise region (Italy) and in other two Italian Apennine regions (Tuscany and Basilicata) were analyzed (Fig.1). Specifically, four landraces were collected in Alto Molise area, represented by Ciliegino (CV), Monachella (MO), Pinto (PI) and Suocera e Nuora (SA), two from Basilicata, called San Michele Rosso (SMR) and Tuvagliedda Rossa (TR), and one from Tuscany, named as Mascherino (MA).
Ciliegino and San Michele Rosso show full red seed coat colour, while Monachella, Mascherino, Pinto, Tuvagliedda Rossa and Suocera e Nuora are characterized by a bicoloured (light and dark) seed coat pattern (Fig. 1).

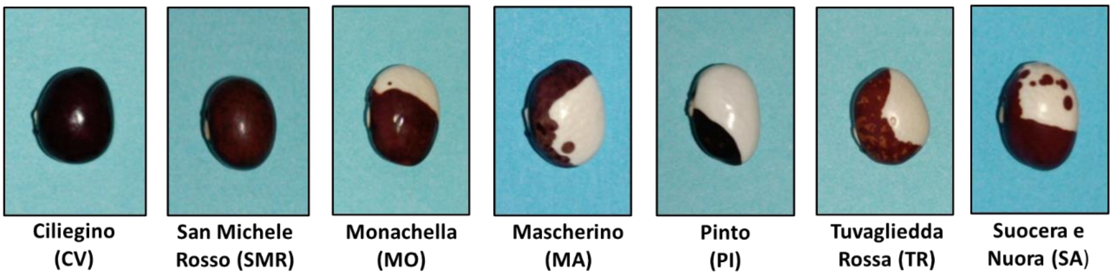


Figure 1. Seven Italian autochthonous common bean populations analyzed during the research activity.

Karytoype analysis

In order to set the protocol, karyotyping analysis was first performed on *Allium cepa* L. root tips, according to the method reported by Guetat *et al*. 2015, with some modifications.

Briefly, *Allium cepa* bulbs were put to germinate for 2-3 days and, after 1 h at 4 °C, 1.5-2 cm root were cut and pretreated with 0.002 M 8-hydroxyquinoline (Sigma-Aldrich) for 2 h at 20-25 °C and another 2 h at 4 °C. After that, root tips were fixed in 3:1 ethanol and acetic acid and stored at –20 °C overnight. The meristems were washed in 10 mM citrate buffer for three minutes and then macerated in a mixture of 2% cellulase (G-Biosciences) in citrate buffer and 20% pectinase (from *Aspergillus niger*) (Sigma-Aldrich) in 40% glycerol in 10 mM citrate buffer for 1 h at 37 °C. Subsequently, root tips were washed in 10 mM citrate buffer for three minutes, hydrolyzed for 10-15 min in 1 N HCl at 60 °C and stained in Schiff's reagent solution (Carl Roth, Germany) overnight in the dark at 4 °C. Stained meristems were squashed on a slide in a drop of 45% acetic acid for three minutes and a drop of aceto-carmine (4%) (Merck) for five minutes. Finally, the cover slip was mounted with D.P.X. mountant (Sigma-Aldrich) and the slides were observed using Nikon Eclipse Ci microscope. Photomicrographs of well-spread metaphases were taken and processed with Swift Imaging 3.0, while chromosome count and measurements were made from digital images using IdeoKar 1.3 (http://agri.uok.ac.ir/ideokar/index.html accessed 15 November 2022).

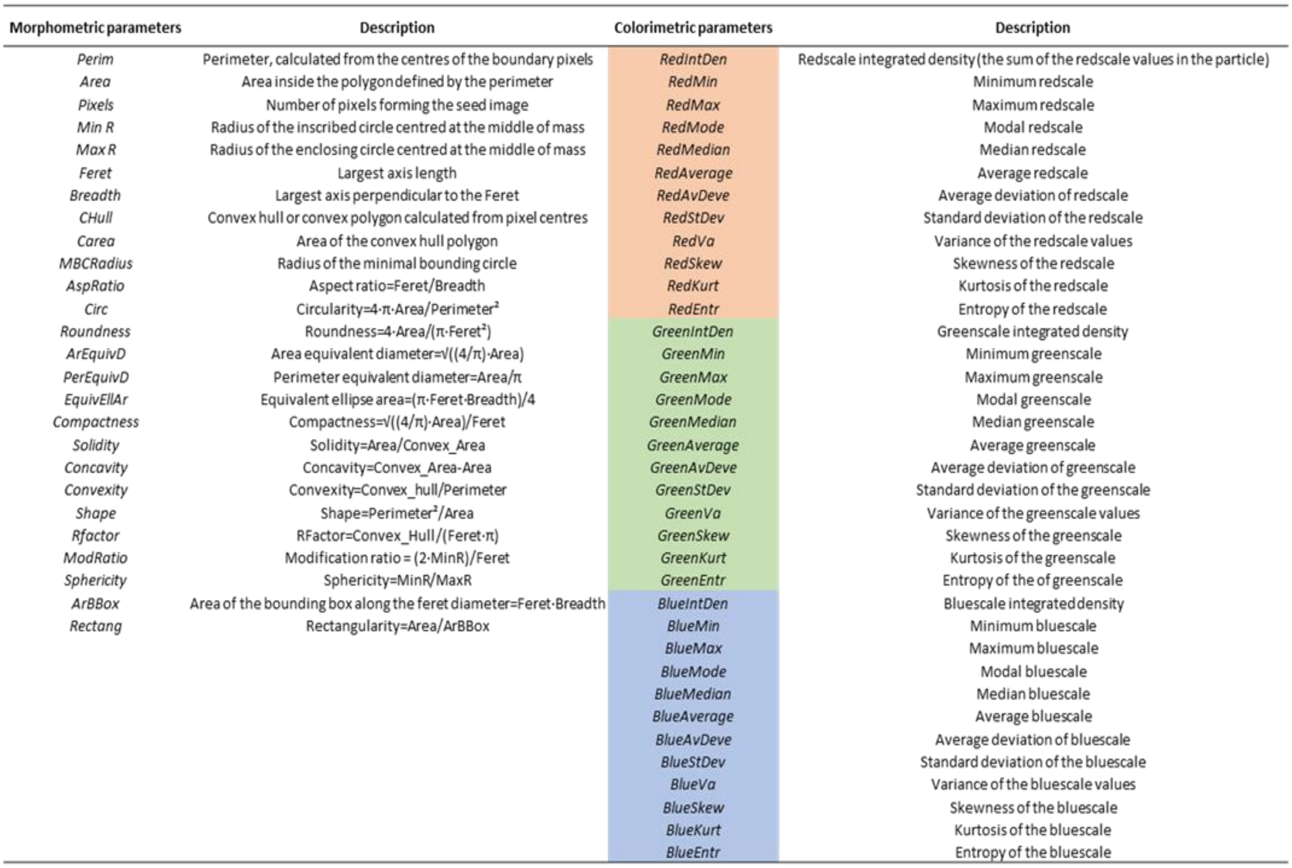
However, a more specific protocol for karyotype analysis of root tip cells of common bean was performed according to the method reported by Fonseca *et al*. 2013, with some modifications.

In detail, common bean seeds were grown on moist filter papers in Petri dishes for 4-5 days and put at 4 °C for 1 h before cutting root 1,5-2 cm long. Then, the root tips were pretreated with 0.002 M 8-hydroxyquinoline (Sigma-Aldrich) at 10 °C for 18 h, fixed in ethanol-glacial acetic acid (3:1) for 2 h at room temperature and stored overnight at –20 °C. The meristems were washed in 10 mM citrate buffer for three minutes and then digested in a solution containing 2% cellulase (G-Biosciences) and 20% pectinase (from *Aspergillus niger*) (Sigma-Aldrich) in 40% glycerol in 10 mM citrate buffer for 1h 30 min at 37 °C. Subsequently, the roots were washed in 10 mM citrate buffer for three minutes and stained in Schiff's reagent solution (Carl Roth, Germany) overnight in the dark at 4 °C. The roots were dissected in 45% acetic acid for three minutes, squashed under a cover slip, subsequently removed by freezing in at –80 °C for two hours, air-dried for 30 min and stained with one drop of aceto-carmine (4%) (Merk) for five minutes. Finally, the cover slip was mounted with D.P.X.

mountant (Sigma-Aldrich) and the slides were observed using Nikon Eclipse Ci microscope. Photomicrographs of well-spread metaphases were taken and processed with Swift Imaging 3.0.

Seed image analysis

A total of 150 seeds for each population were scanned by using the smartphone camera and the android application *HP Smart* (https://www.hpsmart.com/it/it, accessed on 30 September 2022), placing 50 seeds at a time on a light-blue background to obtain a proper image detail resolution. The seeds were arranged separately from each other in order to avoid overlap and digital images were analyzed using the free software package ImageJ (Version 1.51i Wayne Rasband-NIH; https://rsb.info.nih.gov/ij/ accessed on 30 September 2022).
In detail, the plugin Particles8 (https://blog.bham.ac.uk/intellimic/g-landini-software/ accessed 30 September 2022) was used to measure 61 morpho-colorimetric features (Table 1) (25 morphometric and 36 colorimetric) for each common bean populations.

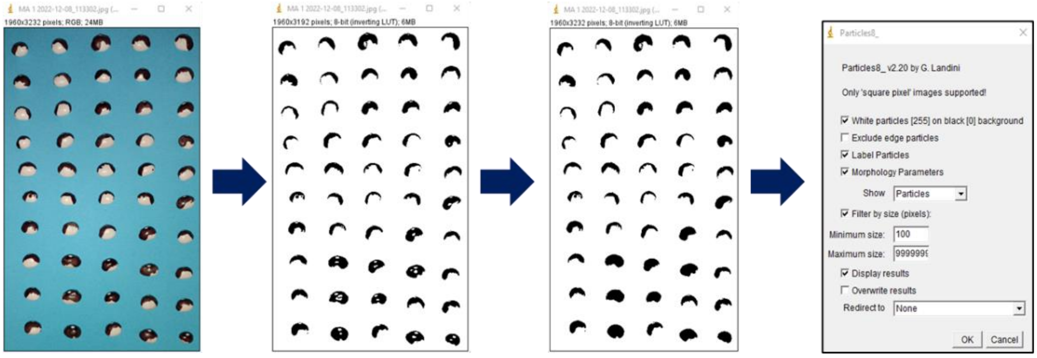


Morphometric parameters	Description	Colorimetric parameters	Description
Perim	Perimeter, calculated from the centres of the boundary pixels	*RedIntDen*	Redscale integrated density (the sum of the redscale values in the particle)
Area	Area inside the polygon defined by the perimeter	*RedMin*	Minimum redscale
Pixels	Number of pixels forming the seed image	*RedMax*	Maximum redscale
Min R	Radius of the inscribed circle centred at the middle of mass	*RedMode*	Modal redscale
Max R	Radius of the enclosing circle centred at the middle of mass	*RedMedian*	Median redscale
Feret	Largest axis length	*RedAverage*	Average redscale
Breadth	Largest axis perpendicular to the Feret	*RedAvDeve*	Average deviation of redscale
CHull	Convex hull or convex polygon calculated from pixel centres	*RedStDev*	Standard deviation of the redscale
Carea	Area of the convex hull polygon	*RedVa*	Variance of the redscale values
MBCRadius	Radius of the minimal bounding circle	*RedSkew*	Skewness of the redscale
AspRatio	Aspect ratio=Feret/Breadth	*RedKurt*	Kurtosis of the redscale
Circ	Circularity=4·π·Area/Perimeter^2	*RedEntr*	Entropy of the redscale
Roundness	Roundness=4·Area/(π·Feret^2)	*GreenIntDen*	Greenscale integrated density
ArEquivD	Area equivalent diameter=√((4/π)·Area)	*GreenMin*	Minimum greenscale
PerEquivD	Perimeter equivalent diameter=Area/π	*GreenMax*	Maximum greenscale
EquivEllAr	Equivalent ellipse area=(π·Feret·Breadth)/4	*GreenMode*	Modal greenscale
Compactness	Compactness=√((4/π)·Area)/Feret	*GreenMedian*	Median greenscale
Solidity	Solidity=Area/Convex_Area	*GreenAverage*	Average greenscale
Concavity	Concavity=Convex_Area-Area	*GreenAvDeve*	Average deviation of greenscale
Convexity	Convexity=Convex_hull/Perimeter	*GreenStDev*	Standard deviation of the greenscale
Shape	Shape=Perimeter^2/Area	*GreenVa*	Variance of the greenscale values
Rfactor	RFactor=Convex_Hull/(Feret·π)	*GreenSkew*	Skewness of the greenscale
ModRatio	Modification ratio = (2·MinR)/Feret	*GreenKurt*	Kurtosis of the greenscale
Sphericity	Sphericity=MinR/MaxR	*GreenEntr*	Entropy of the of greenscale
ArBBox	Area of the bounding box along the feret diameter=Feret·Breadth	*BlueIntDen*	Bluescale integrated density
Rectang	Rectangularity=Area/ArBBox	*BlueMin*	Minimum bluescale
		BlueMax	Maximum bluescale
		BlueMode	Modal bluescale
		BlueMedian	Median bluescale
		BlueAverage	Average bluescale
		BlueAvDeve	Average deviation of bluescale
		BlueStDev	Standard deviation of the bluescale
		BlueVa	Variance of the bluescale values
		BlueSkew	Skewness of the bluescale
		BlueKurt	Kurtosis of the bluescale
		BlueEntr	Entropy of the bluescale

Table 1. List of the 61 morpho-colorimetric parameters measured on each common bean populations by Particles8 plugin from ImageJ v. 1.51.

The image acquisition step involves creating the input images to be processed by Particles8 plugin order to obtain accurate measurements of the morpho-colorimetric features of the samples. These image adjustments include reducing the analyzing area (*Crop*), duplicating the image (*Duplicate*) in order to perform the subsequent colorimetric analysis, transforming the image in the only format accepted by Particles8 plugin (8-bit) (*Make binary*), eliminate potential holes in the image (*Fill holes*) due to light artefacts and setting the minimum filter size of the particles to be analyzed (Fig. 2).

Principal component analysis (PCA) and cluster analysis, using the unweighted pair group method with arithmetic mean (UPGMA) and the Euclidean distance index, were performed on the results obtained by using *Past* software (version 4.03 https://www.nhm.uio.no/english/research/resources/past/ accessed 20 January 2022).



Particles8_ v2.20 by G. Landini

Only 'square pixel' images supported!

☑ White particles [255] on black [0] background
☐ Exclude edge particles
☑ Label Particles
☑ Morphology Parameters
Show: Particles
☑ Filter by size (pixels):
Minimum size: 100
Maximum size: 999999
☑ Display results
☐ Overwrite results
Redirect to: None

Figure 2. Image processing steps to create the input images for Particles8 plugin analysis.

Results

Karyotype analysis

Several well-spread metaphases could be observed in *Allium cepa* root tip cells from photomicrographs (Fig. 3), although the images lack the appropriate quality and details in order to accomplish a complete karyotype analysis with IdeoKar 1.3 software.

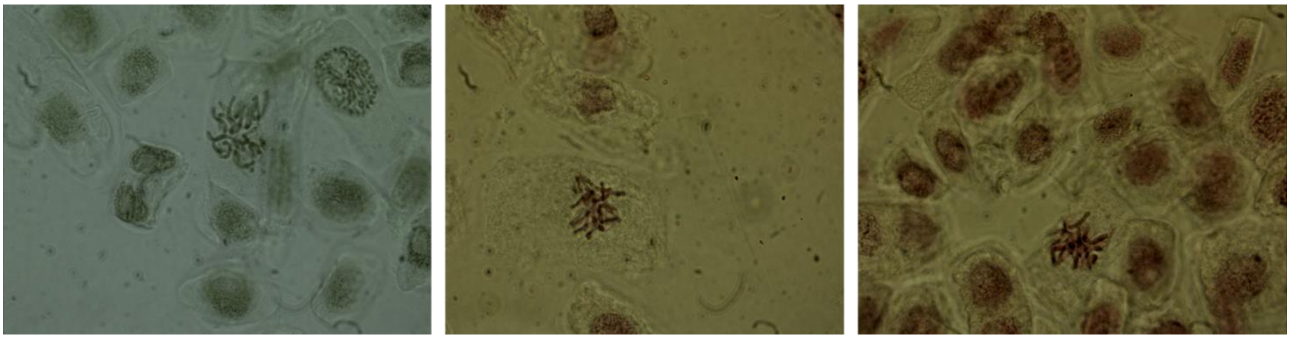


Figure 3. Metaphase plates in root tip cells of Allium cepa observed with 100x microscope oil immersion objective.

On the other hand, it was not possible to perform karyotype analysis on common bean root tips cells; as showed in Fig. 4, after conducting Guetat *et al.* modified protocol, common bean cells showed thick cell walls which did not allow to carry out chromosomes observation and analysis.

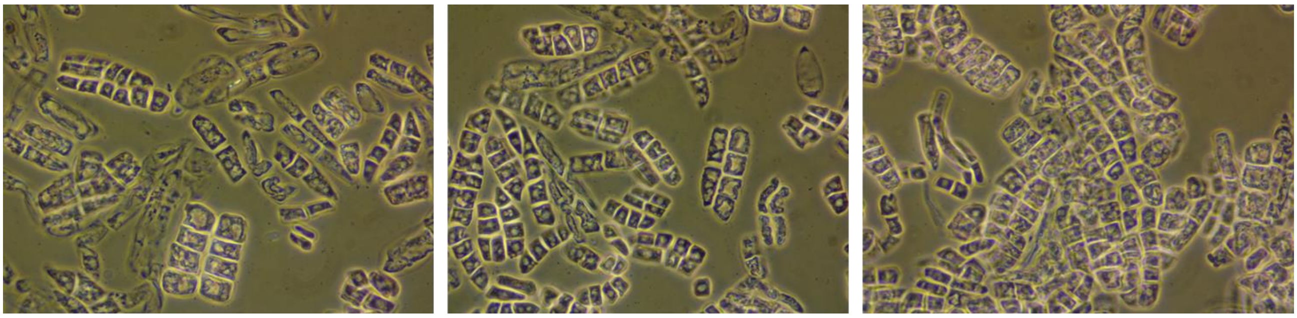


Figure 4. Common bean root tip cells observed with 40x microscope objective after conducting Guetat et al. modified protocol.

Similar results have been found also after performing Fonseca *et al*. modified protocol (Fig. 5), where cells lack definition and chromosomes observation could not be conducted.

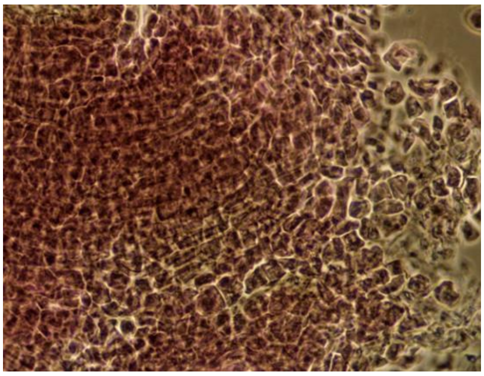


Figure 5. Common bean root tip cells observed with 40x microscope objective after conducting Fonseca et al. modified protocol.

Seed image analysis

A seed image analysis on the seven autochthonous common bean populations have been already carried out in Falcione *et al.* 2022. In detail, a total of 15 seed descriptors (area, perimeter, major axis length, minor axis length, roundness, dark-coloured area, light-coloured area, 100-seed weight, 100-seed volume, 100-seed density, seed coat pattern, number of seed coat colours, main seed coat colour, predominant secondary seed coat colour and seed shape) were evaluated on 10 seeds for each population by using ImageJ software.

The results obtained displayed a higher similarity between CV and SMR, showing full red coat colour, and among MO, MA, PI, TR and SA, with a bicolored (light and dark) seed coat pattern, with MO more similar to SA, and MA more similar to TR.

Based on these results, a more in-depth characterization of these common bean populations has been carried out by using Particles8 plugin of ImageJ, which provided a more comprehensive analysis of the relationships existing among the populations, showing different results compared to those obtained in the aforementioned study.

In the principal component analysis (PCA) scatter plot (Fig. 6a), Principal Component 1 (PC1) and Principal Component 2 (PC2) accounted for 69.67% and 25.79% of the total variance, respectively.
In PC1, four groups could be observed: the first group was composed of Monachella (MO), Mascherino (MA), Suocera and Nuora (SA) and San Michele Rosso (SMR) landraces, while Pinto (PI), Ciliegino (CV) and Tuvagliedda Rossa (TR) formed three separated groups (Figure 6a).
In PC2, CV was well separated from all the other populations (Figure 1a). The noted differences were mostly due to colorimetric features, such as *Minimum Greenscale* (GreenMin), *Bluescale Integrated Density* (BlueIntDen), *Greenscale Integrated Density* (GreenIntDen) and *Redscale Integrated Density* (RedIntDen).
The dendrogram, resulting from the unweighted pair group method with arithmetic mean (UPGMA) clustering, was in agreement with PCA results and revealed two main clusters (Figure 6b). Cluster 1 is divided in several subclusters: subcluster 1a and 1c are formed only by CV and TR, respectively, while subcluster 1b grouped together SMR, MA, SA and MO, with a higher similarity between SMR and MA and SA and MO. On the other hand, cluster 2 was characterized only by PI.

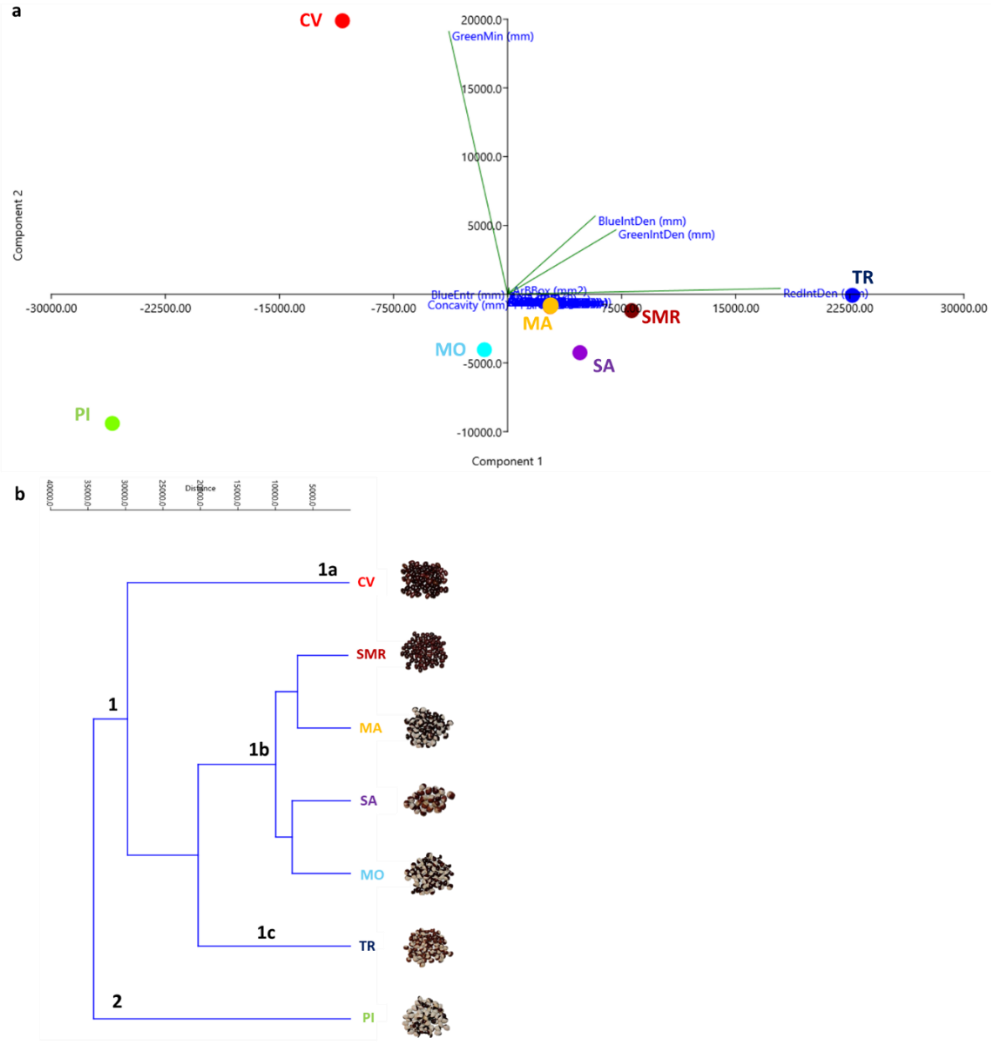


Figure 6. Principal Component Analysis (PCA) (a) and Cluster Analysis (b) of the morpho-colorimetric data obtained by Particles8 plugin from ImageJ.

Future perspectives

As a future direction, thanks to the promising results obtained by the proposed image analysis tool in the discrimination of common bean landraces, we plan to perform a seed morpho-colorimetric analysis by using another brand-new plugin from ImageJ called SeedsAnalyzer (Loddo *et al.*, 2021). The resulting dataset will be compared with Particles8 plugin results and an evaluation of the correctness and validity of the extracted seed features between the two plugins will be carried out.

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Conference participation

- UNIMOL PhD EXPO 2024 (Università degli Studi del Molise, Campobasso, 24.01.2024) – poster presentation: “Falcione M., Renella A., Samukha V., Colardo M., Simiele M., Scippa G.S., Segatto M., and Trupiano D. Complementary characterization of Italian common bean (*Phaseolus vulgaris* L.) landraces to unravel differences in their beneficial health effect and stress tolerance ability”.

- Departmental Research Day 2023 (Università degli Studi del Molise, Isernia, 18.12.2023) – poster presentation: “Renella A., Falcione M., Corso M., Scippa G.S and Trupiano D. Characterization and valorization of autochthonous legume ecotypes of Molise region”.

- Progetto Demetra. Buone pratiche per un’agricoltura sostenibile e multifunzionale (Università degli Studi del Molise, Termoli-CB-, 10.07.2023) – **oral presentation**: “Linea 2. Recupero di cultivar vegetali tradizionali ad elevato contenuto proteico”.

- CYBO–Conference of Young Botanists (Università Libera di Bolzano, Bolzano, 09-10.02.2023) – **oral presentation**: “Falcione M., Simiele M., Renella A., Scippa G.S., Di Martino P. and Trupiano D. A multi-level analysis to identify and characterize some Italian autochthonous common bean (*Phaseolus vulgaris* L.) landraces under a changing environment”.

- Departmental Research Day 2022 (Università degli Studi del Molise, Termoli-CB-, 20.12.2022) – conference abstract: “Falcione M., Simiele M., Renella A., Scippa G.S., Di Martino P. and Trupiano D. A multi-level approach as a powerful tool to identify and characterize some Italian autochthonous common bean (*Phaseolus vulgaris* L.) landraces under a changing environment”.

- UNIMOL PhD EXPO 2022 (Università degli Studi del Molise, Campobasso, 19.12.2022) – conference abstract: “Falcione M., Simiele M., Renella A., Scippa G.S., Di Martino P. and Trupiano D. An integrated approach to characterize some Italian common bean (*Phaseolus vulgaris* L.) landraces under a changing environment”.

- Riunione annuale dei gruppi di lavoro “Biologia Molecolare e Cellulare” e “Biotecnologie e Differenziamento” SBI (Società Botanica Italiana) (Università La Sapienza, Roma, 16-17.06.2022) – participant.

- Riunione annuale dei gruppi di lavoro “Biologia Molecolare e Cellulare” e “Biotecnologie e Differenziamento” SBI (Società Botanica Italiana), (Dipartimento di Scienze ed Innovazione Tecnologica, Università degli Studi del Piemonte Orientale, Alessandria, 16-18.06.2021) – **oral presentation**: “Falcione M., Scippa G.S., Renella A., Di Martino P. and Trupiano D. Characterization of salinity and osmotic stress response of some autochthonous common bean (*Phaseolus vulgaris* L.) landraces of Molise region (South-Central Italy)”.

- Third Mediterranean Plant Conservation Week (Mediterranean Agronomic Institute, Chania, 27.09.2021-01.10-2021) – poster presentation: “Falcione M., Scippa G.S., Renella A., Di Martino P. and Trupiano D., Recovery, characterization and conservation of some autochthonous common bean (*Phaseolus vulgaris* L.) landraces of Molise (South-Central Italy)”.

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