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A Bioprospecting Multidisciplinary Approach to Valorise Biodiversity: The Case of Bowman-Birk Protease Inhibitors in *Vigna unguiculata* (L.) Walp.

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1. Abstract

Natural biodiversity has always been an important source for humans since ancient times. Despite its relevance, biodiversity is experiencing a dramatic decline and many species are at risk of extinction. Climate change and human activity are the main drivers of non-sustainable landscape conservation and management. Extensive conservation efforts and thorough research are needed in order to mitigate the biodiversity loss, along with their valuable genetic, biochemical and metabolic features. Conventional agriculture by exploiting domesticated, but homogeneous, cultivar crops is constantly at risk due to the temperature increase caused by climate change. The opportunity to save or dampen climate change effects is provided by indigenous species that are domesticated in little local contexts, but actually are little investigated.

In this framework, this PhD thesis has the general scope of developing a strategy to valorise natural biodiversity, including minor cultivars and endangered species, taking into account different integrative scientific aspects. The first objective of this thesis is the rediscovery of a traditional African legume, *Vigna unguiculata* (L.) Walp., and assess its adaptability to stressful conditions typically caused by climate change or undemanding agricultural practices. Moreover, the research for bioactive compounds is a mighty added value to give impulse and consciousness of the species potential. The identification of complexes with peculiar health-promoting properties is fundamental to develop new healthier cultivar crops. For this purpose, exploration of natural genetic diversity of known legume bioactive compounds, the Bowman-Birk protease inhibitors (BBIs), and appraisal of their nutraceutical properties are the second objective of the project. This work follows a bioprospecting approach, from the investigation in natural contexts of suitable plant species and their phytocomplexes to the laboratory evaluation of their healthy activities for human applications.

To reach these goals, a multidisciplinary experimental overview has been applied by integrating different approaches to create a fluid and coherent

workflow. The demonstration of *Vigna unguiculata* as suitable species for climate change was carried out with a field experiment and subsequent laboratory analyses to evaluate its production parameters (biomass and grain yield) and metabolic features (i.e., starch, protein content and characterisation of free amino acids). The genetic diversity of this species was explored through molecular biology techniques and *in silico* computational and phylogenetic analyses. The nutraceutical features were established by biochemical procedures and cellular biology *in vitro* and *in vivo* tests on different ageing and cancer models.

The experimental workflow implemented in this PhD study aims at considering biodiversity as an element of value not only in terms of conservation, but also of exploitation and use of biologic diversity resources to improve environmental sustainability and human well-being. Our work delivers new perspectives to encourage the employment of *Vigna unguiculata* in wider agricultural systems, commonly found in other parts of the world.

This thesis work is divided into three main parts. The first issue addressed is the suitability of *Vigna unguiculata* for sustainable agricultural systems. From the point of view of cultivation needs, it is possible to consider *V. unguiculata* as undemanding both in terms of water demand and for the agronomic practices such as ploughing and fertilisers. This makes this legume particularly suitable for conservation agriculture practices both in developing countries and well as in countries where climate change is having a dramatic impact on indigenous crops such as the Mediterranean basin.

This legume is also an important resource of essential macronutrients and micronutrients such as amino acids. To further enhance this species and create a positive lever to promote its cultivation globally, we also wanted to focus on the presence of bioactive molecules with direct action on humans. The genetic exploration was one of biggest biodiversity overviews to the best of our knowledge and took into account almost 200 accessions, comprehending both wild and domesticated entries and covering the great majority of *Vigna unguiculata* subspecies. Through the

genetic investigation, I have identified 13 isoforms of BBI in different wild and cultivated accessions, distributed in the African continent and in other areas of the world. Furthermore, we managed to develop an extraction purification procedure to isolate single isoforms and characterise them.

Our data suggest that BBIs of *V. unguiculata* are molecules with a great natural genetic and biochemical diversity. Moreover, the demonstration of BBI-related bioactivities on different models make them very promising as a high-value natural compound for human wellbeing. The direct action on different tumour cell lines has suggested a possible therapeutic application also in synergy with some drugs already on the market (i.e. Cetuximab). This opens up opportunities for future research both on similar phylogenetically related species and genera, and on the analysis and structure of the BBI to evaluate the most effective forms also in *in vivo* systems.

In conclusion, this PhD project demonstrates that i) bioprospection of local species and indigenous cultivars directed to the search for bioactive molecules useful for human well-being also represents an important lever for safeguarding; ii) the knowledge of the evolution and diversification of the plants of interest is a fundamental tool for improving bioprospecting actions and for identifying molecular variants of bioactive compounds; iii) the analysis of the functional efficacy of bio-active compounds in *in vitro* and *in vivo* systems is the fundamental step to be able to bring scientific research dedicated to the enhancement of biodiversity in an operational context. This is an essential phase to stimulate private investors and businesses to bring economic and social value and biodiversity conservation.

2. The bioprospecting as a driver towards a more sustainable and respecting world

Biological diversity, better known as biodiversity, is defined as 'the variety of living species on Earth' and comprehends not only the diversity of species, but also the diversity among species and ecosystems (Pascual et al., 2021). Even though it comprises all species widespread all over the world, only at the end of the last century biodiversity has become a global concern due to the increasing number of endangered species: around 1 million species are threatened with extinction at the moment (Diaz et al., 2019). Human activity and climate change are the main drivers of habitat degradation and these changes are acting too fast to comply with the natural rhythms of species adaptation and evolution. For these reasons the United Nations Convention on Biological Diversity defined concrete objectives published in 'zero draft' text which was presented to the 15th Conference of the Parties in October 2020 in Kunming, China. In the text, a concrete 10-year strategy to halt and reverse species decline has been described. Two main points deserve particular attention: the protection of at least 30% of the planet and the restoring at least 15% of ecosystems in priority areas by 2030. In response to these challenges, the EU has recently adopted the European Green Deal, which aims at achieving a sustainable and carbon-neutral economy by 2050 (EC, 2019). In line with the UN Decade of Restoration (UN, 2019), the European Green Deal also represents a strong commitment by the European Commission to legally binding restoration of degraded habitats, the services they provide and the biodiversity they hold, and provides financial support over the next decade for restoring ecosystems (EC, 2019). Unfortunately the times are very short and the risks for biodiversity erosion are high. In 2015 all United Nation members signed and adopted the 2030 Agenda for Sustainable Development in order to face the biggest global issues - social, economic and environmental issues- in the least time possible. In particular, 17 Sustainable Development Goals (UN, 2015) and 169 targets

were set as objectives to direct national and international efforts to reach people wellness, economic prosperity and planet preservation.

The World Wide Fund for Nature (WWF, 2020) estimates that humans are using 25% more resources than the planet can have at best and this is reflected in the consequent loss of biodiversity. These data are tightly linked because every extra resource used by human activity is a resource that is subtracted from nature. A solid review by Cardinale et al. (2012) summarises that the loss of biodiversity reduces consequently resource collection, processing and recycling of the main nutrients. All of this goes ultimately to negatively impact the entire ecosystem structure and efficiency and to a collapse of the system itself. But this is only a face of the medal: a great biodiversity is able to better prevent and/or constrain the outbreak of novel pathogens. The last years were shocked by the COVID-19 pandemy, but in the last decades it was not the only one (e.g., SARS, Ebola, HIV and severe flu forms). As stated by Schmeller et al. (2020), a little change in the balance in one or more ecosystem interactors (Fig. 1) has a tremendous impact on the insurgence and severity of diseases. In particular, one of the interactors that is changing more is the natural environment. The average global temperature has been increasing almost linearly since the first years of the XXI century and predictions estimate that around the 2050 temperature will rise above 3°C degrees creating serious problems for flora and fauna. In particular, many current staple crops will reduce dramatically their production due to the consequent shortage of available water and incremented heat stress (Wheeler and Von Braum, 2013, Abraham et al., 2014)

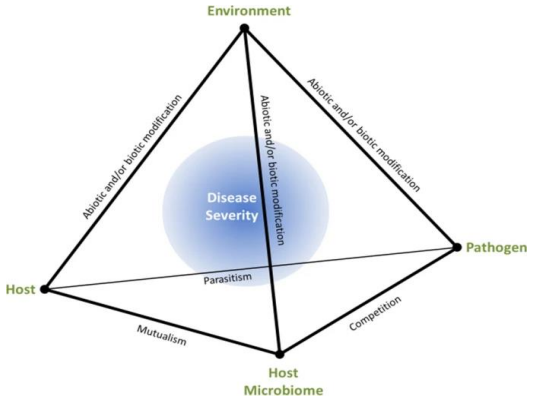

Fig. 1: Disease pyramid of interactions between actors. (Schmeller et al., 2020)

Although the erosion of biodiversity is an issue of global concern, it is difficult to develop efficient conservation strategies, especially in developing countries where the costs of managing reserves and biodiversity are often unsustainable. For this reason, it is necessary to find effective levers capable of enhancing biodiversity, drawing profits from reinvesting in the protection and safeguarding of different species. One interesting strategy is to link biodiversity to human well-being through bioprospecting. Bioprospecting, also known as biodiversity prospecting, is a dynamic and methodical search for natural ingredients and genes in wildlife that have the potential to be turned into industrial products by biological, genetic, and chemical manipulation and without harming nature (Pushpangandan et al., 2018). As shown in Fig. 2, published papers with “Plant Bioprospecting” as keywords of the work are increasing exponentially (PubMed database). The rising interest in the biodiversity issue led many scientists to implement this approach and integrate it with modern techniques. New methods involve high-throughput screening and other automated programs to identify, screen, and isolate novel wild bioactive compounds from different organisms and biomasses.

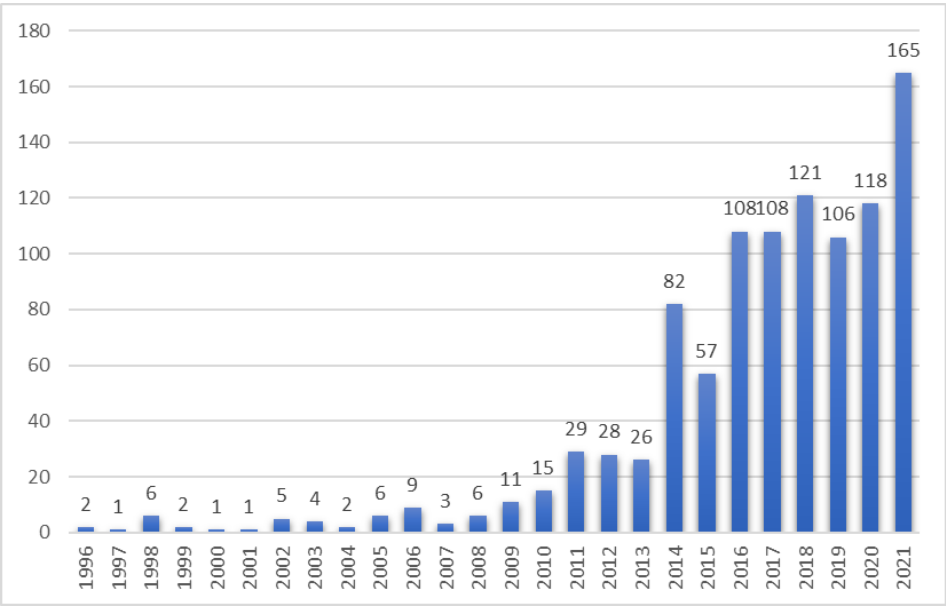

Fig. 2: published papers trend with keywords “plant bioprospecting”, from 1996 to 2021 (PubMed Database)

There is an ocean of untapped potential in unknown bioresources that can provide industries with novel developments. Among these, the cosmetics and medical industries are investing more and more energy and research in this direction. There is a tremendous number of examples of natural molecules derived from every natural taxon. Just to give a number, Sorokina et al., (2020 and 2021) created a database (Coconut Database) to list all existing natural products (NPs): at the moment there are 407270 known NPs, but this list comprises only small molecules and no other types of molecules, such as proteins.

Organism	Bioactive agent	Effects for human health	References
Penicillium genus	Penicillin	Antibiotic	Bennett and Chung, 2001
Taxus genus	Taxanes	Antimitotic, for tumour therapy	Crown and O'Leary, 2000
Salix genus	Acetylsalicylic acid	Non-steroidal anti-inflammatory (Main effect)	Schror, 2016
Limulus polyphemus	Amebocytes Lysate	Bacterial LPS detection	Novitsky, 1994
Elephants	Multiple tumour-suppressor genes	Tumours avoidance	Greaves and Ermini, 2015
Naked-Mole Rats	Multiple factors		Shepard and Kissil, 2020
Bats	Peculiar Antiviral Response	Viruses tolerance	Irving et al., 2021

Tab. 1: Examples of emblematic organisms sources of bioactive(s) molecules for human well-being

In Tab. 1 is presented a brief and non-exhaustive list of bioactive compounds retrieved by nature, as can be seen there are organisms very distant in the evolutionary process but every one of them had or has nowadays a game-changing role for research and medical applications. In this optic, the research is making great efforts and investments to understand and provide information on biodiversity.

Thanks to biodiversity, humanity had the possibility to discover compounds (bioprospecting) and implement therapies to fight and overcome light and serious diseases, from headache to cancer. The biodiversity valorisation through the discovery and use of genes, molecules or metabolic pathways opens the question related to the

access and benefit-sharing of biodiversity resources to prevent biopiratery and overexploitation actions. For these reasons, the Convention on Biological Diversity developed the Nagoya Protocol dedicated to set up a guideline on providing fair access to users of biodiversity sources. The protocol aims to fairly distribute benefits between the providers of genetic resources (such as biodiversity-rich countries) and users of genetic resources (such as biotechnology or pharmaceutical companies, universities, collections such as botanical gardens or genebanks) deriving from scientific research and development on genetic resources. This aspect is very important both because many developing countries are rich in biodiversity that could be plundered without rules, but also because, thanks to the revenues obtained from the exploitation of resources, it will be possible to support conservation and restoration programs in disadvantaged areas of the planet.

3. Domestication and effects of the Green Revolution

One of the main drivers that led to species and intra-species biodiversity loss is domestication. Domestication is a human-driven process that has the objective to artificially select plants that naturally exhibit characteristics considered useful by farmers. If the Neolithic has been the period of the birth of agriculture in numerous areas of the world, the period between the 1960s and early 2000 was a period when domestication reached probably one of its highest peaks in history. The domestication process requires the presence of cofactors such as the occurrence of plants, of appropriate environmental conditions as well as the cultural variations of local populations (from hunters to farmers). Many areas of the world have not seen the birth of local domestic varieties and have had to wait many years before obtaining very productive cultivars. In some cases, only wild or proto-domestic species were propagated, which did not guarantee high yields. This happened for example in many African countries where high productive crops arrived later (about 1990-2000) due to political and social controversies. Although this has produced serious economic, food and social difficulties, these countries retain a great wealth of local varieties with great potential for development (Altieri and Koohafkan, 2008).

Another essential step of agricultural history was the Green Revolution of the 1960s, which made further changes to domestic species and cultivation practices that increased yields. This was mainly concentrated in very fertile areas, therefore many developing countries have not been subject to agricultural improvements.

Being promoted during the Green Revolution, several new cultivars were selected and in combination with the mechanisation, the use of fertilisers and the use of agrochemistry, a general increase in terms of yield of many crops occurred (Power and Follett, 1987, Evenson and Gollin, 2003, Pingali, 2017). At first, these improvements were positively evaluated, but

after a few decades they raised a lot of issues and concerns, mainly in terms of reckless land exploitation and misuse of the chemical compounds that contributed heavily to pollution and finally in climate change. The strong selection of improved varieties has further reduced the cultivated species to the global level and has concentrated the research on only a few monocultures.

Fifty years of indiscriminate selection of a few crops caused a coverage of agricultural lands by really few species: over 50% of consumed proteins are from wheat, maize, rice, barley, sorghum, oats, rye and millet (Sands et al., 2009). Cereal proteins are considered of a lesser quality than other crops, such as legumes (Simopoulos, 1999). In Fig. 3 is reported how the traditional domestication and green revolution processes impacts on natural diversity (Zhang et al., 2017). From naturally growing vegetable species, some plants show elitary traits that are useful and make early farmers work easier. These plants, then, were subject to a first skimming to select only the ones that responded better to human manipulation. Finally, the last step is the employment of very few plants, but this implies the creation of a diversity bottleneck, exacerbated by breeding with identical plants making the population very homogeneous.

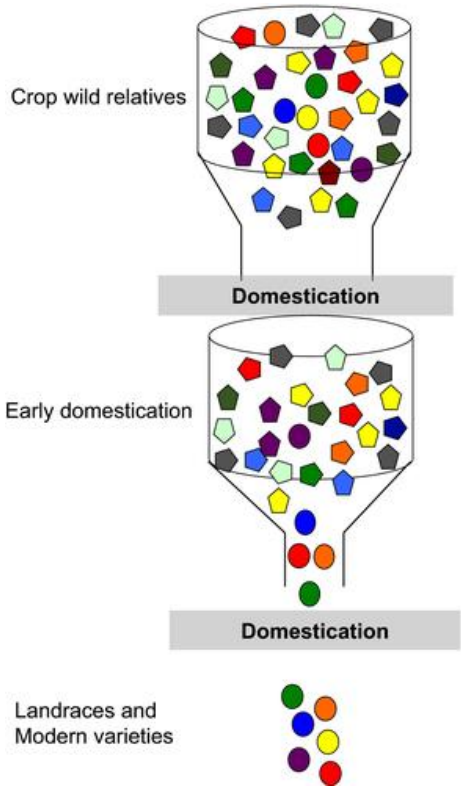

Fig. 3: graphic representation of domestication process, from wild landraces to current cultivated varieties. (Zhang et al., 2017)

The need of super-productive cultivars led humans to select and breed plants that are very productive, but very fragile and prone to diseases or pests without the use of strict and massive use of agrochemistry (Signore et al., 2018). One emblematic case occurred in 1970 and 1971 with an epidemic disease brought by a fungus, *Bipolaris maydis*. The main outcome was a tremendous loss of corn crops (~15%) and, obviously, a loss of money (~1 billion dollars) (Bruns et al., 2017). Lack of genetic diversity was the cause for the disease spread all over the country, damaging cultivations and, consequently, the economy. The episode of maize and *Bipolaris maydis* was not the only one in history, many of them occurred recently and are still being fought (e.g., *Xylella fastidiosa*), but the common points are the genetic homogeneity and the rapid spread of diseases.

A smart approach to sustainable agriculture is to reinvent and innovate traditional agricultural practices as well as the ancient varieties and proto domestic accessions in order to identify strategies and opportunities to reduce the risks related to the use of agrochemicals and the consume natural resources (i.e., water, soil) improving the health of ecosystems (Hermoso et al., 2022).

4. The “wild” relatives in natural biodiversity: de-novo domestication?

The genetic diversity of domesticated plants is significantly lower than wild relatives (Zhang et al., 2017) and this is a strong risk factor for the onset of diseases and their rapid diffusion. As a matter of fact, when a population is genetically homogeneous and geographically diffused, pathogens will acquire the ability to spread much faster and cover greater spaces in smaller times (Heal et al., 2004). Therefore, wild species or wild plant relatives are the real pool of genes and genetic fluxes, both in the single individual and in the entire population (Ramanatha Rao and Hodgkin, 2002). Worldwide, current crops are basically monocultures and, genetically speaking, very, if not totally, homogeneous. Conversely, indigenous varieties, often cultivated in small farms have less genetic homogeneity and, in some cases, retain good levels of heterozygosity as they have not been subjected to targeted improvement, as happened for staple crops (Newton et al., 2011, Wang et al., 2014). For example, diversity found in a single cultivated field of Cassava in Brazil resulted to be as diverse as the entire collection of accessions in the “Centro Internacional de Agricoltura Tropical'' (CIAT) (Colombo, 1996). However, the greatest genetic diversity can be only found in “wild” relatives. This term refers to those plants that are genetically related to cultivated crops but have not been exploited or disturbed by human activity (WRs, CIAT) WRs are distributed all over the world, but the more interesting data is the estimated number. Fifty-sixty thousand WR are naturally present and more than 10000 could have a direct outcome for food security (Maxted and Kell, 2009, Majeed et al., 2021). Considering the deleterious effects of monocultures and the need of new impulse to lower environmental impact, scientists, stakeholders but also policymakers are starting to take into account WR as possible and ready solutions in crop improvement. In the previous paragraph the SCLB was presented as one of the crucial examples of how monocultures can be defective and easily infested. The

gene resistance transfer from a WR into cultivated corn made them tolerant to the pest (Prischmann et al., 2009). This is only a case of WR recovery of resistance genes, but there could be many different possibilities, such as tolerance to abiotic stresses, improvement of nutritional profile or expression of peculiar bioactive compounds.

As demonstrated, WRs are extremely important to improve already existing cultivars, basically of every crop species. Breeding though is a very complex methodology that requires various attempts to obtain the desired result and this is time and resource consuming. In the last five years, a new approach has been investigated to solve or at least improve the agricultural and biodiversity situation: the *de-novo* domestication. On the whole, the domestication process was very simple and led by selecting characteristics that were convenient or that made life easier, such as lack of pod-dehiscence and pods that do not open when seeds are mature to prevent food losses (Takahashi et al., 2020). However, the new domestication processes also aim to obtain species suitable for modern diets, i.e., rich in macronutrients and micronutrients necessary to prevent hidden hunger. Furthermore, the new agricultural varieties will have less impact on natural resources and spontaneous biodiversity. Therefore, the diet of the future must in fact be healthy and sustainable (Willett et al., 2019).

Fernie and Yan (2019) summarised the domestication phases that humans passed during history and described the actual state of the art. In particular, the *de-novo* domestication is a recent approach that promises to optimise production but also to valorise the wild landraces by re-domesticating them or exploiting the “wild” knowledge to upgrade current cultivars. This kind of process is no more phenotype-driven or need-driven but aims at creating a cultivar/crop that has the best characteristics from already domesticated plants and WR.

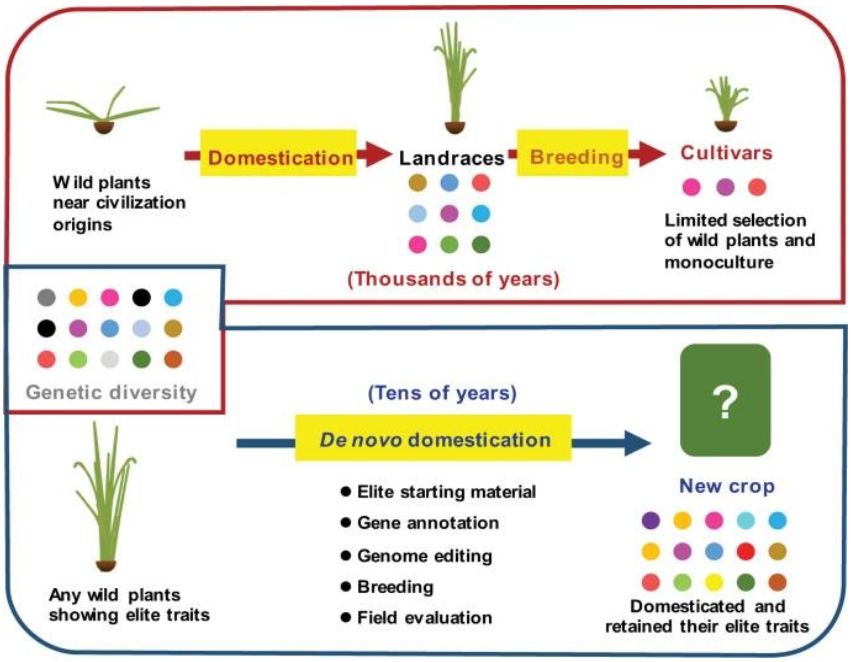

Fig. 4: schematic representation of conventional and *de-novo* domestication processes (Yu and Li, 2022).

To do so, it is necessary to implement a workflow that permits to exploit all the available or gatherable data from literature, laboratory analyses and bioinformatics and then apply them using modern technologies. *De-novo* domestication can potentially mimic the domestication process, but in much less time (Fig. 4) and much more respectful in terms of natural biodiversity. The strategy to reach the objective can be divided into 4 steps (Gasparini et al., 2021, Yu and Li, 2022):

-1 Selection of appropriate plant material

-2 Establishment of a transformation procedure and comparison with annotated genomes

-3 Editing step, domestication-related genes and breeding into a field verification

-4 Cultivar approval and registration

Probably, the most difficult step is the third one, because in many cases there is a lack of basic data information, such as annotated reference genomes. Furthermore, this step requires advanced editing techniques

and sometimes some plant species result to be difficult to edit (Atkins et al., 2020). However, all these advances are very promising and show a huge potential for the purpose.

An alternative and synergic opportunity derives from areas that were partially or not interested by Green Revolution changes. An emblematic example is represented by the African continent, in particular the Sub-Saharan area. Crops of the Green Revolution did not catch on and were not considered due to unfavourable social, climatic and soil conditions (Ejeta, 2010). African farmers tend to grow and feed on local landraces that are completely unexplored and represent the means to improve agricultural controversies.

Therefore, there are many autochthonous edible plants that could be seen as unexplored items in the context of agrobiodiversity and that could contribute to ameliorate agricultural issues worldwide. Among these plants, some legumes show interesting perspectives. For example, *Vigna unguiculata* L. presents a great nutritional value with great richness in terms of amino acids and essential micronutrients, such as folic acid (Olaleke et al., 2006). These species, known as Neglected and Underutilised Species (NUS) or African Indigenous Vegetables (AIVs), can offer both biodiversity immediately exploitable to 'tame' local species, and to find genes and metabolic pathways to be transferred to cultivated species (Gregory et al., 2019, Popoola et al., 2019).

5. Fabaceae and the *Vigna* genus

Although plant biodiversity offers numerous opportunities for human nutrition, one of the most current issues is that of obtaining both micronutrients, amino acids, peptides and proteins from plants. Fabaceae is a very numerous and heterogeneous family of the eudicotyledons taxon. One of the unique features of this family is the ability to establish and maintain a strong symbiosis with N-fixing bacteria, where these gain protection by the plant while the plant obtains useful N compounds. In agricultural terms, these plants do not need the addition of N chemical compounds and are also used in renewal of N poor soils (Raza et al., 2020).

Evolutionary speaking, Fabaceae family has not a clear time origin point, but a combination of fossils data and genetic markers hypothesises the occurrence of Fabaceae about 50-60 Mya (Lavin et al., 2005). In just a few million years the Fabaceae family developed a great biodiversity of genera and species, reaching great numbers of more than 800 genera and 20000 species, 22939 to be precise (Lewis et al., 2005, Kew Garden, 2022). However, only 13 genera (*Pisum*, *Vicia*, *Lens*, *Lathyrus*, *Trifolium*, *Medicago*, *Cicer*, *Phaseolus*, *Glycine*, *Vigna*, *Cajanus*, *Arachis* and *Lupinus*) are considered the major legume crops and, among them, there are less than 60 species that are currently cultivated, even though these genera comprehend about 1350-1400 different species (Smykal et al., 2014). In other words, agriculture exploits less than 5% of the potential that nature has developed during the evolution process and the risk is to underutilise or totally neglect the rest of species, impoverishing and losing the extreme Fabaceae biodiversity.

Among all Fabaceae genera, the *Vigna* genus stands out for its peculiar characteristics and its great pool of wild species and subspecies. Among legumes, only a few species are currently employed even if the Fabaceae family is extremely biodiverse, comprising over 20000 species. Members of the *Vigna* genus are gaining more and more social and economic importance, especially in the African continent where animal protein

intake is sometimes very limited if not impossible (Marconi et al., 1997, Duranti, 2006, Gogoi et al., 2018). The *Vigna* genus comprises more than one hundred species, 13 of them are currently domesticated and only 3 of them are of African origin.

Considering i) the diffusion of the *V. unguiculata* species in Africa and at the global scale, ii) the amount of scientific and technical projects dedicated to the collection and conservation of the seeds of this plant and iii) the nutritional value of the seeds of this plant, this PhD project is mainly dedicated to investigate the phylogenetic relationship among cultivars and wild varieties of this species as well as to study the biological properties (bioprospecting) of *V. unguiculata* varieties to better identify the most interesting and active fraction and molecules. Nowadays, several manuscripts reported either the nutritional properties of both seeds and leaves of this plant, and the presence of bioactive compounds starting from secondary metabolites up to small peptides and proteins (Jayathilake et al., 2018, da Silva et al., 2018, da Silva et al., 2021). The presence of an annotated reference genome provides a huge quantity of high-quality data (Lonardi et al., 2019). The sequencing itself provides only sequence information without the link to the functional role of the single genes or genome areas. Annotation brings all necessary information to foresee and evaluate in a logical way structural features and functional roles (Salzberg, 2019). This creates the possibility to compare or scavenge information from public resources, using genomes as a reference.

Briefly, *Vigna unguiculata* is a domesticated African legume but has spread globally and is better known as cowpea (Herniter et al., 2020). It originated with much probability in South-East Africa and therefore it diffused in all Africa (Fig. 5). This hypothesis is supported by the exclusive presence of wild subspecies in Africa and the greatest variability is found in the South-Eastern portion of the continent (Richard, 1848; Piper, 1913; Faris, 1965; Maréchal, 1978, Padulosi and NG 1997).

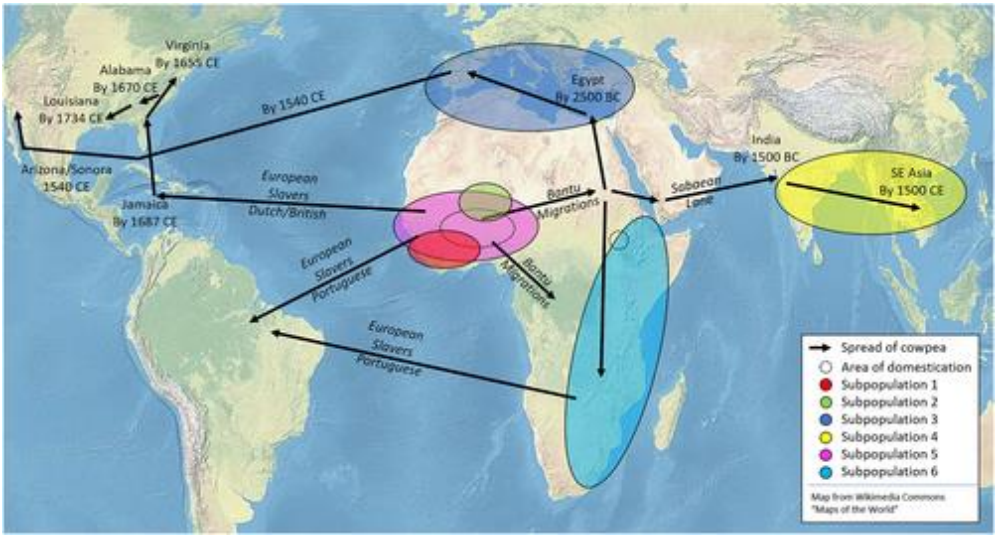

Fig. 5: diffusion hypothesis of the domesticated cowpea (Herniter et al., 2020)

One of the most appreciated agricultural qualities is the ability to grow in stressful conditions without any specific treatment or care. One of the greatest challenges for cowpea is its valorisation among farmers, in fact, *V. unguiculata*, is listed among African Indigenous Vegetables (AIVs) that are "plant species consumed in specific locations as part of traditional diets and have the potential to diversify cropping systems, increase farm income, and add a range of vital micronutrients to diets" (World Vegetable Centre). For this reason, studies on cowpea are not only important for the research of balanced food and promising bioactive compounds, but also for society and sustainability in developed countries. Although this is suitable for growing globally, in Europe and the United States it is scarcely present on the market because consumers prefer legumes rich in carbohydrates and specific class of proteins such as the common bean (*Phaseolus vulgaris* L.) the broad bean (*Vicia faba* L.), pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.). These staple crops are also more suitable for intensive cultivation and are compatible with the qualitative and quantitative standards imposed by the industrial supply chains. Only in recent years has arisen the need to consider legumes both as an alternative (or supplementary) source of animal proteins and as a source of bioactive molecules to promote human health. This opens up

the opportunities to introduce new species of legumes (minor varieties, local cultivars, species coming from marginal areas) also in international markets even in the face of the fact that wild and proto domestic legumes are very resistant plants able to grow with little or no fertilisers, stressful conditions and in intercropping with other crop species, usually maize or wheat (Ngwira et al., 2012). However, their healthy nutritional profile makes them exceptional candidates to be elected as future food. Legumes are commonly known as the most important sources of proteins in the vegetable world. In fact, legumes can be represented by a great percentage of proteins comprehending almost all essential amino acids (20-40%, Duranti, 2006). Given the great protein content, legumes have been defined as “poor man’s meat” and could the best representants as a pivotal crop for European sustainable agri-food transition (Ferreira et al., 2021): as a matter of fact, legume cultivation is more sustainable than animal protein production since it produces less gas emissions and needs less water (Semba et al., 2021). However, dietary nutritional profile and cultivation suitability are not the only positive aspects among legume proteins. Glycosides, tannins, saponins, alkaloids, and many other bioactive substances in grain legume seeds increased the importance of legumes for human consumption.

In recent years, scientific research has also focused on the identification of legume small peptides and proteins with actions on cellular and physiological well-being (Muzquiz et al., 2012, Jayathilake et al., 2018). Some studies showed diverse biological effects of these peptides as antioxidants, antihypertensives, anti-inflammatory, antimicrobial, antithrombotic, antidiabetic, hypocholesterolemic, and even immunomodulators. These beneficial effects aid in preventing and treating chronic illnesses, particularly inflammatory disorders, obesity, and cardiovascular diseases (Juarez-Chairez et al., 2022).

Small proteins are the object of this research project due to their key role in the plant such as defensive compounds against biotic and abiotic agents, but, especially, for their wide range of promising bioactivities on human cells. Among these, the Bowman-Birk protease inhibitors

(hereafter BBIs) stand out for their peculiar tertiary structure and the ability to interact with two targets at the same time (Birk, 1985, Clemente and Domoney, 2006) Although this small protein has been described in *V. unguiculata*, its structural and functional diversity has been little investigated. Considering its key role as an element of defence against insect attack, it is plausible to think that this small protein has been positively selected at an evolutionary level and that different varieties, distributed in different geographical areas and subjected to different environmental conditions, may have selected more or more variants. less active than BBI. Another interesting aspect is understanding the bioavailability of these peptides and the ability to reach cellular targets.

6. The Bowman-Birk protease inhibitor family

Bowman-Birk protease inhibitors are small proteins of about 70-80 amino acids and a molecular weight of about 6-9 kDa that act as Serine-Protease inhibitors (Clemente and Domoney, 2006). Nonetheless, its structure is what really defines these proteins. In fact, they possess 14 conserved cysteines that realise 7 disulphide bonds, making BBIs extremely resistant to many physico-chemical stresses (Fig. 6, Odani and Ikenaka 1972, Ramasarma et al., 1995, Safavi and Rostavi, 2013).

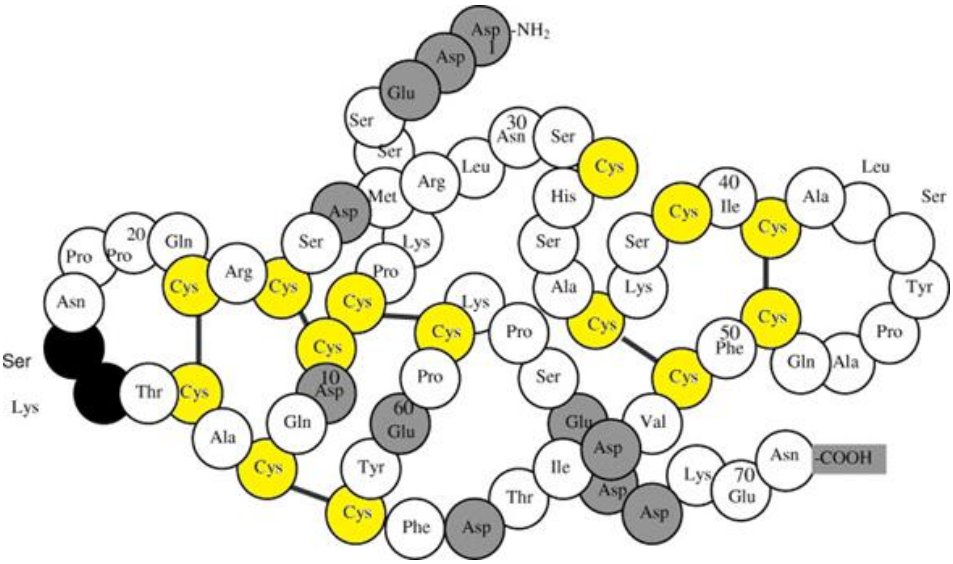

Fig. 6: Primary structure of a soybean trypsin-chymotrypsin Bowman-Birk inhibitor. In yellow are evidenced the 14 cysteines involved in structural conformation.

Their physiological role is to defend developing plants from insects attack by inhibiting digesting enzymes, trypsin and chymotrypsin (Birk, 1985). BBIs are also called “double-headed” because of the presence of two distinct domains that interact independently, but not covalently, with their targets that are trypsin and/or chymotrypsin. Domains are nonapeptides flanked by two cysteines that fold them properly to obtain

the inhibitory loops (Bode and Huber, 1993). The affinity with trypsin or chymotrypsin defines the isoform and is determined by a single amino acid (in position P1): the first domain is very conserved and P1 amino acid is Arginine or Lysine, the second domain is more variable and can exhibit at P1 Arginine, and consequent trypsin affinity, or Threonine, Phenylalanine or Leucine, and so affinity for chymotrypsin. Depending on the combination of domain targets, two major isoinhibitors can be identified: the trypsin-trypsin BBI (TT-BBI) and the trypsin-chymotrypsin BBI (TC-BBI) (Laskowski Jr. and Sealock, 1980, Clemente and Domoney, 2006). BBIs though are not represented only by these two isoforms but are encoded by a multi-genic family and every isoform can be processed naturally in a post-translational phase (Domoney et al., 1995). This is due to the co-evolution with insects and increases the number of possible existing isoforms and some processes could change their affinity capacity (Domoney et al., 1995, Harsulkar et al., 1999).

Bowman-Birk protease inhibitors (BBIs) are famous amidst legume bioactive compounds, however, there is not a thorough state of the art of their evolution. BBIs are commonly present in the Fabaceae family and some Poaceae species (Cereals), but there is evidence for an ancient origin of this protein group (Odani et al., 1986, James et al., 2017). As a matter of fact, there are BBI domain-like proteins that were found in very basal plants, such as Lycopodiophyta (*Selaginella moellendorffii*) and archaic Angiosperms (*Isoetes drummondii* and *Amborella trichopoda*) (James et al., 2017). So, the evolutionary divergence of BBI or BBI-like ancestors happened way before the occurrence of Fabaceae, about 370 million years ago against the 50-60 Mya for the Fabaceae. Curiously, events of evolutionary convergence of similar domains were detected in distant species (*Helianthus annuus*, Luckett et al., 1999, Elliott et al., 2014) and even in animals (*Odorrana grahami*, Li et al., 2007).

However, BBIs are not found in every plant lineage due to the development of other protease inhibitors with the same function (Huma and Khalid, 2007, James, 2017).

BBIs are becoming more and more relevant for the bioactivities they exert as nutraceutical compounds. Their capability to basically interact with SER-protease proteins or domains makes them very suitable for novel applications other than for defending the plant. The better acknowledged bioactivity is the ability to act as chemopreventive agents or to interfere with tumour progression. Different cancer models have been studied with pure BBIs or BBIs enriched fractions and results are very promising, in particular for the selectivity shown by the treatment (Bosland et al., 2002, Clemente et al., 2005, Clemente et al., 2010, Riley et al., 2016, Olias et al., 2019). Furthermore, some works tried to hypothesise the mechanism of action of BBI in tumour cells. In two works (Joanitti et al., 2010, Mehdad et al., 2016) on breast cancer *in vitro* models, researchers investigated and evaluated apoptosis, lysosomal permeabilization, mitochondrial impairment and interactions with proteasome 20S generated by BBIs. Mehdad and co-workers (2016) hypothesised that BBIs by interacting with Proteasome 20S interfere with tumour protein turnover and thus leading to cell death.

BBIs have other nutraceutical properties worth to be mentioned: anti-inflammatory capacity (Utrilla et al., 2015, Jin et al., 2016, Akbari et al., 2019), antibacterial effect (Martins et al., 2018) and also anti-viral ability (Ma et al., 2016). The most relevant points that still remain unclear concern the bioactivities associated with the different isoforms, the possible synergistic effect of BBI with other molecules present in the metabolome of *V. unguiculata* and the selective action on tumour cells compared to healthy ones. At the production level, however, it remains to be clarified whether there is a stable expression of this protein, such as organ or plant tissue and what are the possible elements capable of promoting gene expression for translation.

7. Aim of the PhD Project and experimental design.

The general objective of my thesis work concerns the protection of plant biodiversity through targeted bioprospecting actions aimed at identifying bioactive molecules with high added value for the human health sector. Specifically, my thesis work has been developed to explore and valorise natural biodiversity of *V. unguiculata* L. focusing on its Bowman-Birk protease inhibitor family. The experimental design is summarised in Fig. 7.

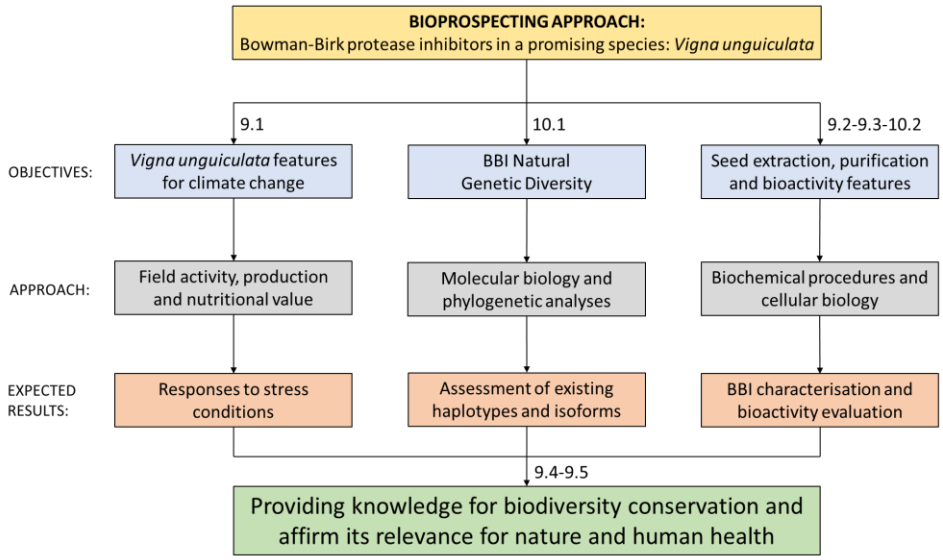

Fig. 7: experimental design of my PhD work. Numbers refer to the chapters where the objective is addressed in depth.

More in depth, here is explained every aim and which methodologies have been applied to reach the different goals.

1. The first aim for this study is to assess *V. unguiculata* L. feasibility and usefulness in future agricultural fields to face climate change (Chapter

9.1). In particular, the work involves a field experiment and evaluation of growth parameters and nutrients production performances. The Italian field experiment mimicked the cultivation conditions in the African continent and stresses that could occur due to climate change. Biomass and grain yield are evaluated as production performance in addition to measurements of soil properties and morphophysiological analyses. To assess the suitability of *Vigna unguiculata* L. for diet, chemical characterisation is performed with the evaluation of macronutrients: starch, protein and free amino acid profile.

2. The second aim of this project is to explore and evaluate natural diversity of Bowman-Birk protease inhibitors in *Vigna unguiculata* accessions (both wild and cultivated varieties) (Chapter 10.1). This molecule is correlated with numerous bioactivities and properties, also investigated in Chapters 9.2 and 9.3. Understanding the existing diversity of genes encoding BBIs is crucial to find isoforms that interact better with their molecular targets and could be potentially more effective not only for the plant but also for exerted bioactivities. DNA screening and molecular biology techniques are applied to extract, purify and sequence amplified gene targets through PCR. Subsequently, phylogenetic analyses and different selection models are performed to evaluate episodic or diffused positive or negative selection.

3. The third aim of this study is to determine which and how potent bioactivities (Chapters 9.2 and 9.3) *Vigna unguiculata* extracts can exert. To estimate these properties, extraction and purification procedures are set up. At first, aqueous extractions are performed to obtain starting material to be characterised and tested on *in vitro* and *in vivo* models. The extracts are purified with biochemical procedures and then, proteomic analyses are performed to identify the compounds enclosed in the extracts or fractions. Then, extracts are administered to different cellular and *in vivo* models. Additionally, optimised extraction and purification techniques are applied to reach single BBI isoform purity. Subsequently

quantitative assays on trypsin and chymotrypsin are performed to assess the inhibition potency of the albumin fraction and purified isoforms. Purified BBI are further identified with peptide fingerprinting and precise mass analyses (Chapter 10.2).

4. The fourth aim is to create the state of art of the knowledge learnt during the whole project by revising two aspects of the work. The first part reviews the existing literature regarding the *Vigna* genus, analysing and valorising African species considering the perspective of human activity and what effects it exerts (Chapter 9.4). The second part summarises the nutritional properties and bioactive compounds of legumes, keeping an eye on elderly diet and effects on health (Chapter 9.5).

8. Methods

The experimental design described in the previous paragraph is addressed with different techniques and methodologies.

8.1 Sample collection

The thesis project involves extensive research work on African accessions of *V. unguiculata* and of some related species (*Vigna davyi*, *Vigna membranacea*, *Vigna vexillata*, *Vigna radiata*, *Vigna subterranea*, *Vigna frutescens*, *Vigna lobatifolia*, *Vigna reflexo-pilosa*). About 200 accessions of seeds are collected from 3 germplasm banks (International Institute of Tropical Agriculture - Nigeria, Meise Botanic Garden - Belgium, Centro Nacional de Recursos Fitogeneticos - Spain). The collected material is stored in cool and dry places. Preliminary germination and growth tests allowed us to identify the most suitable varieties for open field experiments and those more recalcitrant to germination, therefore suitable only for biomolecular analysis and bioprospecting on seeds.

8.2 Field experiment

The field experiment is conducted to assess the suitability of *Vigna unguiculata* L. in typical climate change stress conditions. This experiment is carried out at Cerzoo experimental fields in San Bonico (Piacenza, Italy). Plants of *V. unguiculata* are grown under different agronomic conditions: conventional agriculture (tillage and no cover crops) and conservation management (no-tillage and presence of cover crops) during a whole cultivation season (from May to August). Eight plots measuring 2x2 metres are divided into four plots under conventional agriculture and the other four with conservation management. Every plot is then sub-divided into 2 parts: one is watered as in conventional practices, the other is subjected to water scarcity regime (additional details on Material and Methods of Chapter 9.1). The experiment also evaluates production and morphophysiological parameters (biomass, grain yield, photosynthetic

efficiency and soil properties) to determine the ability of *Vigna unguiculata* to cope with climate-related stresses.

8.3 Laboratory experiments

8.3.1 Bioactive molecules extraction and purification procedures

Extraction is a fundamental step to recover compounds of interest in the phytoextract of *V. unguiculata* seeds, leaves and other tissues. In our work we set up two different types of extraction procedures on dry seeds. The first one is described in chapters 9.1, 9.2 and 9.3. It is an aqueous extraction to extract all hydrophilic molecules, especially proteins and free amino acids. In Chapter 9.2, this extraction procedure is then refined by the addition of purification steps, in order to separate groups of macromolecules basing on their physicochemical characteristics and individuate the ones exerting the anticancer bioactivity. The first purification step is obtained with a 3 kDa cutoff filter used to separate proteins and polysaccharides from small molecules. Then, a separation by charge is set up to divide positively from negatively charged proteins. The last step is a size exclusion chromatography to define the molecular weight of the bioactive fraction. In Chapter 10.2, instead, a different extraction method is employed. This procedure was optimised by Rubio et al., (2014) for protein extraction from great amounts of starting material. Briefly, a borate buffer is the initial extracting solution. Then changes of pH and precipitation with Ammonium Sulphate let proteins to be extracted. This method already divides proteins into three macro categories: Legumins, Vicilins and Albumins. Albumin fraction is the one containing BBIs. From albumins, single BBI isoforms can be retrieved by two in tandem chromatographies: a size exclusion followed by a cation exchange chromatography. These extracts, fractions or purified proteins are ready to be used in subsequent assays.

8.3.2 Chemical characterisation of cowpea extracts

Extract characterisation is important to understand the molecular profile and identify the agents of bioactivity. Aqueous extracts (Chapters 9.1, 9.2 and 9.3) are evaluated in terms of starch (enzymatic assay) and protein (Bradford reagent) contents with spectrophotometric techniques. Free amino acid profile is obtained by High Performance Liquid Chromatography that permits to show every amino acid and how their profile changed under different cultivation conditions. In Chapters 9.2 and 9.3 proteomic analyses are performed to identify protein and peptides. In addition, in Chapter 9.2, information of the macro molecular components are provided by H-NMR analyses.

8.3.3 Bioactivity assessments

To evaluate bioactivity features and properties of *Vigna unguiculata* L. extracts, assays on *in vitro* and *in vivo* models are performed. In Chapter 9.2 extracts are tested on colorectal cancer cell lines. Most of the studies focus on this type of tumour cells because it can be the first target of bioactive compounds in food. In particular, to assess potential intracellular targets, a panel of four tumour cell lines presenting mutations on growth and survival pathways is utilised. The same treatments are performed on a healthy mucosa cell line, to observe eventual toxicities on healthy cells. Furthermore, an experiment of extract-drug (Cetuximab, election drug for colorectal cancer treatment) complementation is performed to assess the potency of extract in combination with a commercial drug but also to evaluate eventual synergic behaviours. Expression patterns of molecular targets involved in cellular growth and survivability are explored to find affected primary or secondary cellular targets. In Chapter 9.3, we want to explore different potential bioactivities. The focus is settled on ageing and age-related disease. Furthermore, we choose very evolutionary distant models to understand if the responses are coherent between them. *Saccharomyces cerevisiae* and Drosophila melanogaster are involved in senescence

experiments evaluation with quantification of precise molecular marker in the senescence pathway (Snf1/AMPK, FOXO, SIRT1, NOTCH, Heme oxygenase). Caenorhabditis elegans and the human neuroblastoma cell line SH-SY5Y are used to assess the extracts potential in interfering with α-synuclein self-assembly, which causes Parkinson's disease.

8.3.4 Evaluation of genetic diversity of BBI gene

The genetic diversity of BBI DNA sequences is investigated to understand how a gene could vary across the natural biodiversity of *V. unguiculata* accessions distributed in African countries. We want to appraise how many haplotypes of the BBI genes can be found in a representative sample of *Vigna unguiculata* accessions distributed in different African countries (Chapter 10.1). To reach this scope, DNA is extracted from seedlings, amplified with specific primers, purified and sequenced.

8.4 Computational techniques

8.4.1 Phylogenetic analyses

Sequences derived from DNA screening (chapter 7.3.4) are aligned and heterozygotes are processed with the PHASE algorithm to figure out the most probable haplotype. Subsequently the sequence alignment is analysed with phylogenetic and network techniques to discern the relationship between haplotypes and isoforms. Phylogenetic trees are made with a maximum likelihood approach and to infer a strong tree, sequences from other *Vigna* species are added. Finally, analyses of selection are performed with different techniques to identify eventual episodic or diffused selection processes.

8.4.2 Computational analyses

Isoforms found by the genetic screening of the BBI gene, are then exploited to perform *in silico* interaction energy analyses. This step is designed to evaluate computationally isoforms found in natural diversity

and estimate which one could be further studied. Isoforms that interact better with their physiological target could, with much probability, exert a stronger or more effective bioactivity in a health perspective.

8.4.3 Statistical analyses

In Chapters 9.1 and 9.2 statistical models are applied to support our data properly. In Chapter 9.1, data derived from field experiments were analysed with R, applying Linear Mixed Effects Models (LME) or Generalised Linear Mixed Effects Models (GLMM). However, time dependent data show a non-linearity in the residual patterns, therefore, a Generalised Additive Mixed Model is applied. Concerning the plant performances and chemical characterisation, a GLMM analysis is performed setting the plot as random effect.
In Chapter 9.2, similarly, statistical analysis is implemented. In particular, GLMM analyses are applied to cell viability assay, proliferation assays and protein dependency to assess responses differences between the healthy cell line and the cancerous ones. One-way ANOVA and Dunnett tests are carried out to demonstrate combinatorial effects of extracts and the cetuximab. Finally, a linear model is used to determine differences between expression levels of pEGFR, AKT and ERK.

9. Publications produced during the thesis work

9.1 Sustainability Perspectives of *Vigna unguiculata* (L.) Walp. Cultivation under No Tillage and Water Stress Conditions

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Abstract

Nowadays, agriculture is facing the great challenge of climate change which puts the productivity of the crops in peril due to unpredictable rain patterns and water shortages, especially in the developing world. Besides productivity, nutritional values of the yields of these crops may also be affected, especially under low mechanization and the low water availability conditions of the developing world. Conservation agriculture (CA) is a topic of emerging interest due to the provision of adequate yields and reduced environmental impact, such as greenhouse gas emissions, by being based on three main principles: minimum soil disturbance (reduced or no tillage), cover crop maintenance, and crop rotation. The aim of this study was to assess the impact of CA management on the growth performance and the nutritional profile of cowpea (*Vigna unguiculata* L. Walp), a pulse of African origin, commonly known as black eye bean under field conditions. A field experiment was designed to assess the effect of conventional tillage (CT) and no-tillage (NT) combined with the usage of a set of cover crops, coupled to normal and deficient water regimes. Cowpea was revealed to be able to grow and yield comparably at each level of the treatment tested, with a better ability to face water exhaustion under CA management. After a faster initial growth phase in CT plots, the level of adaptability of this legume to NT was such that growth performances improved significantly with respect to CT plots. The flowering rate was higher and earlier in CT conditions, while in NT it was slower but longer-lasting. The leafy photosynthetic rate and the nutritional profile of beans were slightly influenced by tillage management: only total starch content was negatively affected in NT and watered plots while proteins and aminoacids did not show any significant variation. Furthermore, significantly higher carbon and nitrogen concentration occurred in NT soils especially at the topmost (0–5 cm) soil horizon. These findings confirm the capability of CA to enrich soil superficial horizons and highlight that cowpea is a suitable crop to be

grown under sustainable CA management. This practice could be pivotal to preserve soils and to save agronomical costs without losing a panel of nutrients that are important to the human diet. Due to its great protein and aminoacidic composition, *V. unguiculata* is a good candidate for further cultivation in regions of the word facing deficiencies in the intake of such nutrients, such as the Mediterranean basins and Sub-Saharan countries.

Keywords: **conservation agriculture**; **no-tillage**; **climate change**; **drought stress**

1. Introduction

The Green Revolution (1960–1980s) was aimed at improving the agronomic productivity and nutritional features of the major staple crops worldwide (e.g., maize, rice, and wheat) [**1**]. Most of the crop varieties were selected to deal with emerging environmental and biotic stresses (i.e., desertification, nutrient-poor soils, and extreme temperatures) and were expected to produce yields several times higher than minor crops and the local varieties. Unfortunately, overcoming some of these obstacles was not always possible in a sustainable way and during the past three decades, cultivation practices have been demanding a higher and higher use of water and agrochemicals (e.g., fertilizers, pesticides, and herbicides), to enhance (or maintain) maximum crop yields [**2**,**3**]. Environmental hazards, the poor maintenance of long-term plant and soil productivity and the higher costs in terms of agrochemicals and energy consumption produced the modern crisis of agriculture. To address this crisis and environmental concerns of the consumers, in recent years, the principles of Sustainable Agriculture were continuously promoted worldwide [**4**,**5**]. Therefore, for the green revolution of the 21st century, the practices of Organic farming (OF) and Conservation Agriculture (CA) are deemed environmentally friendly approaches to agriculture. Traditionally, OF is based on the creation of the correct ecosystems for the crop productivity with a holistic approach that considers maintenance and health of the soil, plants, and livestock, with strictly regulated use of

external inputs while focusing on farm production and recycling of needed products (e.g., composting wastes and green mulching) and the adoption of integrated strategies against plant pests. On the other hand, CA represents a set of three crop management principles: (i) direct planting of crops with minimum soil disturbance, (ii) permanent soil cover by crop residues and cover crops, (iii) crop rotation [**6**]. Through these strategies, CA guarantees an optimum environment for the rhizosphere to capture nutrients and water [**5**]. The adoption of no-till (NT) and the maintenance of a crop residue mulch on the surface have assumed an important role, especially in the geographical areas characterized by consistent rainfall and the consequent risk of soil leaching [**7**]. Although most of the production zones in the Mediterranean region are characterized by hot summers and rainy winters, global warming has been increasing the risk of (i) soil degradation due to soil losses in response to the greater drought and torrential rainfall; (ii) soil salinization due the increase of droughts, irrigation, and sea level; and (iii) soil carbon stock depletion because of the increase of temperature and drought [**8**]. Therefore, the application of CA principles has the potential in the Mediterranean regions to preserve soil structure and fertility, as well as to improve productivity and quality of crops [**9**,**10**,**11**].

Promotion and research on CA in many instances have focused on the first two principles, which are minimum soil disturbance/no-tillage and surface crop residue. Species belonging to Fabaceae could also enhance soil fertility thanks to the nitrogen-fixing symbionts. Moreover, there are several legume crops that are also able to grow under stress conditions [**12**], such as water/salt stress, and could be adapted to no-tilled soils. Sub-Saharan Africa is an important source of stress-resistant legume grain cultivars, such as species belonging to the genera *Vigna* and *Lablab* [**13**,**14**,**15**], and some of these could also be adapted to grow under the Mediterranean climatic conditions.

In this study, we selected *Vigna unguiculata* L. Walp (also known as cowpea) to investigate the ability of this species to grow in the Mediterranean region under CA conditions. The species was adequate for such study due to the fact that its beans are rich in proteins and carbohydrates and have relatively low-fat content [**16**]. Moreover, *Vigna unguiculata* beans show an aminoacidic pattern that is complementary to that of many foods consumed in the Mediterranean area, such as cereal grains. These aspects make *V. unguiculata* a 'strategic' crop for the Mediterranean diet. Furthermore, *V. unguiculata* is attracting the attention of consumers and researchers due to its beneficial health properties, including anti-diabetic, anti-cancer, anti-hyperlipidemic, anti-inflammatory, and anti-hypertensive properties [**17**]. Specifically, in this work, the response of *V. unguiculata* to NT soil management both with and without irrigation was investigated. Plant growth features and plant productivity, in terms of straw biomass and grain yield, were evaluated. At the same time, the response of the soil carbon (C) and nitrogen (N) stock to NT was verified. Emissions of greenhouse gases CO2 and N2O from NT soils are highly variable and depend on complex interactions among soil properties (i.e., soil water content, soil C and N), microbes, and the cultivated plant. Usually, the increased soil organic C (SOC) in surface layers of no-till soils is widely found but may not be associated with increased C sequestration throughout the soil profile [**18**]. Therefore, the evaluation of the relative carbon balance under NT vs. CT is essential to better estimate the potential of NT to sequester additional C into the soil. Furthermore, there is no accordance in the scientific literature about the effect of NT in N sequestration and dynamics [**18**]. Moreover, the metabolic features of the seeds, in terms of nutritional components after boiling (to imitate the conditions of consumption and the effective intake for humans), were assessed.

2. Material and Methods

2.1. Experimental Design and Treatments

A one-year field experiment was carried out on a long-term field study (initiated in 2010) at the CERZOO experimental research station in Piacenza (45°00’18.0” N, 9°42’12.7” E; 68 m above sea level), Po valley, Northern Italy. The soil is a fine, mixed, mesic, Udertic Haplustalf (Soil Survey Staff 2014), with a silty clay loam texture (sand 122, silt 462, and clay 416 g kg−1) in the upper layer (0–30 cm). The main physical-chemical properties of the soil are reported in Fiorini et al. [**19**]. The climate is temperate, and the mean annual temperature and precipitation are 12.2 °C and 890 mm, respectively. Climatic data were collected from an automated meteorological station positioned in the experimental field (**Figure S1 in Supplementary Materials**). The experimental design was a randomized complete block (RCB) with four repetitions and two tillage treatments: conventional tillage (CT) and no-tillage (NT). In detail, (i) CT included an autumn plowing (35 cm) and two passages of rotating harrow in spring (15–20 cm) to prepare the seedbed, and (ii) NT consisted of direct sowing on a soil untilled for 7 years using a double-disk opener planter for seed deposition. Between 2011 and 2017, the crop sequence was a three-year crop rotation, with soybean (*Glycine max* L. Merr.), durum winter wheat (*Triticum turgidum* L. var. durum), and maize (*Zea mays* L.). During winter off-seasons, a mixture of winter cover crops was sown in NT plots, right after harvesting the previous main crop. The species composing the cover crops mixture were rye (*Secale cereale* L.), hairy vetch (*Vicia villosa* L.), crimson clover (*Trifolium incarnatum* L.), Italian rye-grass (*Lolium multiflorum* Lam.), and radish (*Raphanus sativus* L.). In 2018, 15 m2 (5 m × 3 m) within each plot (1430 m2; 65 m × 22 m) were cropped with *V. unguiculata*, both under CT and NT. The experiment was established to compare responses of cowpea cultivation to

contrasting tillage systems. NT and CT planters were calibrated in order to obtain the same sowing depth in both treatments. The distance between planting rows was 50 cm, and the distance between seeds on the same row was 10 cm. Sowing of cowpea was carried out on May 18th (sowing depth: 3–4 cm) and the harvest took place on August 9th. No fertilizers were applied during the growing season, and weeds were suppressed weekly by hands. On July 20th, when cowpea plants were at the beginning of the blooming, each plot was divided into two subplots. The first one was sprinkler irrigated to prevent water stress (20 mm per time, for a total of three irrigation events), whereas, in the second sub-plot, any kind of natural or artificial water input was prevented by temporarily covering those sub-plots through greenhouses to induce and to simulate the dry season.

2.2. Biomass and Morphophysiological Sampling and Analysis

During the whole growing seasons, plants (N = 320) were measured weekly for a total of 7 surveys to detect the following parameters: the dimension of the canopy (cm), the total number of leaves, and number of flowers. Plants were labelled univocally in order to follow the growing performance of each individual over time. After the greenhouses settlement, to evaluate the role of water exhaustion on plants, in each subplot three plants were randomly chosen to be undergone to Pocket PEA Chlorophyll Fluorimeter (Hansatech Instruments, Pocket PEA, 2013) measurements, providing an estimate of the Fv/Fm ratio. Fv is the fluorescence variable, calculated as Fm − Fo, where Fo is the fluorescence origin and Fm is the fluorescence maximum. Measurements took place from the 5th to the 7th survey on the same labelled plants in a time range between 10 a.m. and 1 p.m. After the 7th survey, grain yield and above-ground biomass weight were measured by harvesting three randomly selected 2.0 × 1.0 m squares from each subplot. Above-ground biomass was manually cut at the soil level and weighed. Grain and straw were also separated. The dry weight biomass of cowpea (grain and straw) was

gravimetrically determined by drying biomass at 70 °C until constant weight. Fruits derived from the remaining plants were harvested and stored at −20 °C before phytochemical analyses.

2.3. Measurement of Soil Physical Properties

To determine soil C and N stock, soil bulk density (BD, 0–30 cm), SOC, and N concentration in the 0–20 cm layer (0–5 and 5–15 cm) were measured right after harvesting cowpea. Four randomly selected undisturbed soil core samples were collected on August 20th, 2018 from each subplot, using a steel auger of 5 cm diameter. Soil BD was determined according to the cylinder method [**20**], while samples to determine SOC and N concentration were air-dried, ground with a rubber pestle, and sieved to 2 mm. About 1 g of dry soil per each sample was weighed and used to determine C and N concentration by Dumas combustion method with an elemental analyzer varioMax C:N (VarioMax C:NS, Elementar, Germany). Soil carbonate removal was not necessary due to the low carbonate content in the soil. To estimate the effect of watering on soil, the gravimetric water content was measured on a weekly basis, both in watered (W) a non-watered (NW) plots (**Figure 1**). From May 18th to July 20th precipitation events occurred for a total of 121 mm. After July 20th, in W sub-plots, precipitation and irrigation events consisted of 38 and 60 mm (three irrigations of 20 mm each), respectively.

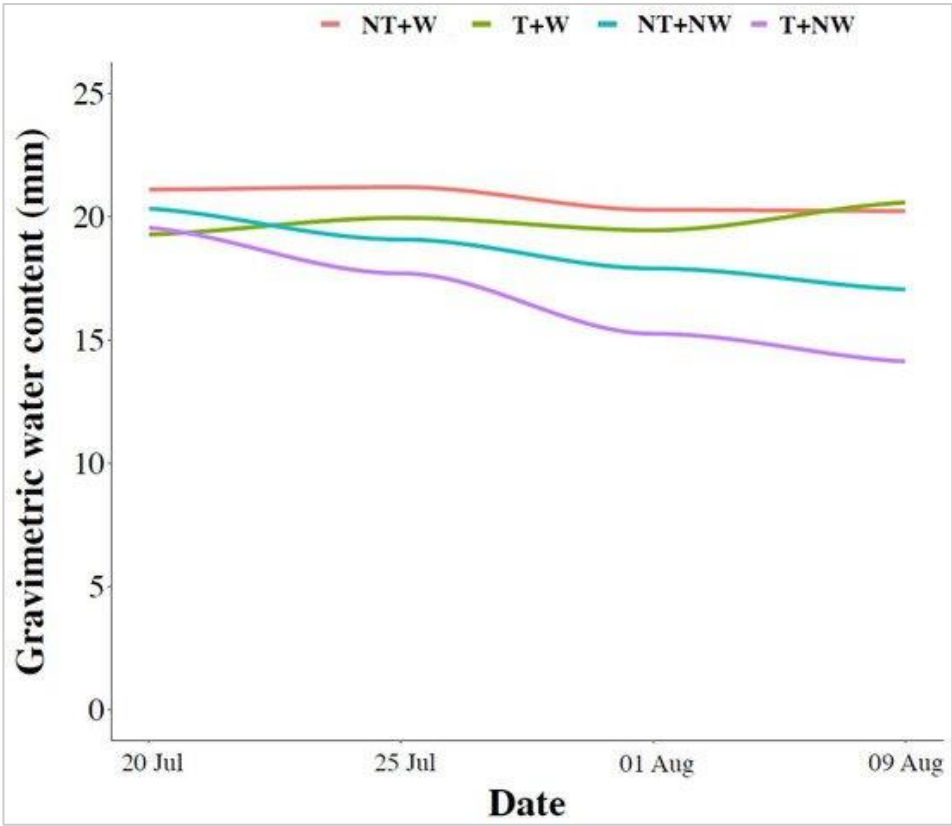

Figure 1. Trend of the gravimetric water content in the four different treatments. CT = conventional tillage, NT = no tillage, W = watered, NW = not watered.

2.4. Chemical Characterization of V. unguiculata Seeds

Phytochemical analysis was carried out on the cowpea seeds that were boiled in water for one hour and then were left to cool down for another hour, as suggested by Olaleke et al. [**21**], in order to mimic the conditions of cooking and consumption. Subsequently, seeds were dried at 50 °C overnight and then ground to a fine powder.

2.4.1. Evaluation of Total Starch Content (TSC)

The TSC content was indirectly evaluated by measuring the amount of NADPH in samples after an enzymatic treatment by using the Kit Megazyme® Total Starch AOAC Method 996.1 1 and AACC Method 76.13. Briefly, 50 mg of dry powder was added to 200 µL of ethanol 80% *v/v* and 1 mL of KOH 2 M and left stirring for 20 min at 4 °C. Then, 4 mL of a sodium acetate buffer 1.2 M Ph = 3.8 were added followed by the addition of 50 µL of α-amylase (8300 U/mL) and then 50 µL of amyloglucosidase (AMG, 3300 U/mL). Samples were incubated for half an hour at 50 °C and then centrifuged at 3000 rpm for 10 min to recover the supernatant. For each sample, the reaction mixture was prepared as follows in a quartz cell: 1 mL H2O, 25 µL of the supernatant, 50 µL of a buffer solution pH = 7.6, 50 µL NADP+/ATP. The solution was incubated for 3 min at room temperature and then the absorbance was read at 340 nm against the blank containing water instead of sample. Then, 10 µL of a solution containing hexokinase (HK) and glucose-6-phosphate-dehydrogenase (G6PDH) was added. After an incubation of 5 min at room temperature, the absorbance was read against the blank again at 340 nm. TSC is expressed as g of total starch per 100 g of dry powder.

2.4.2. Total Protein Content (TPC)

The extraction of amino acids and proteins from dry seeds was performed as in Olaleke et al. [**21**] with minor modifications. Briefly, 2 g of dry powder were extracted in 50 mL of an aqueous solution of theanine at a concentration of 10 µg/mL. Theanine was chosen as internal standard as it is not biosynthesized in beans. The solution was stirred at 500 rpm for 5 min. Then samples were centrifuged at 5000 rpm for 30 min and the supernatant was recovered and freeze-dried. Yields of extraction were recorded by weighing freeze-dried extracts.

The Total Protein Content (TPC) was evaluated by using the Bradford assay as follows: 1 mL of 50% Coomassie-Brilliant Blue Bradford reagent (ThermoFisher) was incubated at room temperature with 2 µL of extract of known concentration for a minute. Absorbance was read against blank at 595 nm and fitted on a calibration curve made up with BSA (Bovine Serum Albumin) in a range between 0 and 6 mg/mL. TPC was expressed as g total proteins per g of extract and was then multiplied per the yield of extraction to be expressed on g of dry powder.

2.4.3. Amino Acidic Content and Characterization

The evaluation of the amino acid content was performed by High Performance Liquid Chromatography coupled to a Diode Array Detector (HPLC-DAD), 1260 Infinity II LC System (Agilent, 2018). A calibration curve was made up by using an amino acid mixed solution (Merck, analytical standard, 17 amino acids plus tryptophan) in a range between 0.078 mM and 1.25 mM. The column used for this analysis is an Agilent Poroshell HPH C18 (100 × 4.6 mm, 2.7 µm) with a guard column (AdvanceBio Oligo 4.6 × 5 mm, 2.7 µm) and it was kept at 40 °C. Mobile phases were phosphate buffer (10 mM Na2HPO4 pH = 8.2) and Acetonitrile:Methanol:Water (45:45:10). The elution program is (%B): 0–0.35 min 2%, 13.4 min 57%, 13.5 min 100%, 15.7 min 100%, 15.8 min 2%, 18 min end. Flow rate was constant at 1.5 mL/min. Solvents were HPLC grade, whereas the buffer, solutions and samples were pre-filtered with a 0.22 µm filter. As derivatizing agent, OPA (o-Phthaldialdehyde reagent, Merck) was chosen for its capacity to bind amino groups and act as fluorophore. The injection volume was 10 µL. The signal used to visualize the fluorescence was set at 338 nm bandwidth 10 nm with a reference wavelength of 390 nm bandwidth 20 nm. Spectra were collected during analysis in a range between 200 nm and 500 nm with a step of 2 nm, to have a side control and make identifications easier. All data were displayed and analysed on Agilent ChemStation software.

2.5. Plant Morphometry, Photosynthetic Efficiency, and Phytochemistry

Data deriving from the field activity were analyzed through the software R(Version 3.3.3 © 2019–2016) and particularly by the lme4 and glmmTMB, mgcv and gamm4 package. Linear Mixed Effect Models (LME) or Generalized Linear Mixed Effect Models (GLMM) were initially applied. However, when considering trends in time of morphological parameters, model validation confirmed non-linear pattern in the residuals, therefore, the application of GAMMs (Generalized Additive Mixed Models) was required after confirming through the AIC evaluation. Specifically, canopy was assumed to be Gamma distributed (data were considered Gamma-distributed because of the occurrence of negative fitted values), number of leaves and flowers was considered Poisson distributed but they were then switched to a negative binomial distribution to deal with overdispersion. Concerning the evaluation of Fv/Fm ratio from PEA measurements, only data from the 5th to 7th survey were provided, so the (b) model was directly run. This ratio is an index, therefore it was assumed to be beta-distributed. Models were run providing for a double random effect, which is the individual nested within the plot. Model validation was performed by plotting residuals from each model against fitted values as well as each covariate and random component. Because of the high tendency of violation of independence as a consequence of temporal correlations (data were collected week by week), a corARMA1 correction was provided for each model [**22**]. The selection of the best model was provided by following the Aikaike Criterion (AIC) through the anova function. Concerning plant chemical parameters, TPC, TSC, and total amino acidic content were analyzed through a GLMM as above, considering the plot as random factor (R, Version 3.3.3 © 2019–2016 and particularly by the lme4 and glmmTMB package). Data were assumed to be binomially distributed but necessitated a switch to a beta-binomial distribution due to over-dispersion. The fixed effect was time in interaction with the management condition (tillage, irrigation) in order to

evaluate their effect on response variables in time by exploiting 95% confidence bands.

2.6. Soil Properties

The soil C and N stock (Mg ha−1) at 0–30 cm depth was calculated as follows: profile soil stock (Mg ha−1) = (soil C/100) × BD (Mg m−3) × depth (m) × 10,000 (m2 ha−1). Likewise, soil N stock on the soil was determined. Data on soil C and N (both concentration and stock), as well as on grain yield and straw biomass of cowpea, were subjected to analysis of variance (ANOVA) with a split plot design following procedures outlined by Gomez and Gomez [**23**] and using the "agricolae" package of RStudio 3.3.3. The main-plot factor was the tillage system (NT vs. CT), while the subplot factor was water management (W vs. NW plots). When the Shapiro–Wilk test and the Levene's test did not confirm the assumptions of ANOVA, data were log-transformed before analysis. Tukey's honestly significant difference (HSD) as a post hoc was used to test for significant differences in variables among treatments with a *p*-value of 0.05 as the threshold for statistical significance.

3. Results

3.1. Biomass and Grain Yield

No significant differences were detected between grain yield on plant grown under NT and CT conditions (**Figure 2**) and with and without irrigation. Concerning the straw biomass, significant higher production in the W plots than in the NW ones (6.78 vs. 5.18 Mg ha−1; +31%) was observed. Conversely, no difference was found between CT and NT plots (**Figure 2**).

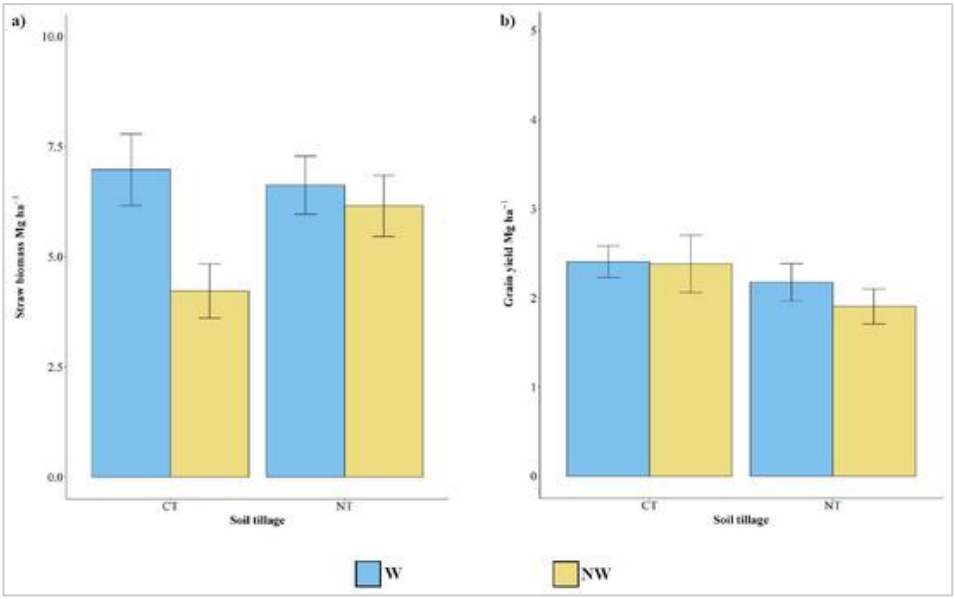

Figure 2. (**a**) Cowpea biomass and (**b**) grain yields (Mg ha−1). Values are mean ± SEM. CT = conventional tillage, NT = no tillage, W = watered, NW = not watered.

The interaction between the tillage system and water supply showed significant differences in cowpea straw biomass while not in the grain yield production (**Figure 2**). In detail, CT-W and NT-W had the highest straw biomass with 6.94 and 6.63 Mg ha−1, respectively. CT-NW had the lowest straw biomass (4.22 Mg ha−1), while NT-NW did not show a significant difference compared to the other conditions (**Figure 2**).

3.2. Morphometrics and Growth Parameters

Planting density was measured in all plots and subplots at the late flowering stage (R4-R5) and was as follows: under CT, the W (CT-W) and the NW (CT-W) subplots had an average of 16.8 and 15.3 plants m−2, respectively; under NT, the W (NT-W) and the NW (NT-NW) subplots had on average value of 18.5 and 17.5 plants m−2, respectively. **Figure 3** shows the results related to the morphometric parameters detected: number of flowers (intended as a reproductive parameter), dimension of the canopy, and total number of leaves (intended as growth parameters). The flowering period took place starting from the 4th week. As **Figure 3**a shows, CT plots revealed a sudden blooming followed by a likewise sudden interruption, while NT plots showed a more contained but constant blooming. Therefore, blooming was significantly higher between the 4th and the 5th week in CT plots and then significantly higher in NT plots. No effects were due to no irrigation (see 95% confidence bands). Concerning vegetative parameters, the obtained data (**Figure 3**b,c) suggested that a significant difference resides in the decrease of the total number of leaves caused by the absence of irrigation at the 6th week, followed by recovery during the last survey. As far as tillage is concerned, it was found that at the beginning of the growth season (from the 1st to the 3rd week) both plants canopy expansion and total number of leaves were significantly higher in CT, then during the 4th survey, no differences were detected between the two groups, while from the 5th week to the end NT plots showed a significantly higher performance for both the considered parameters.

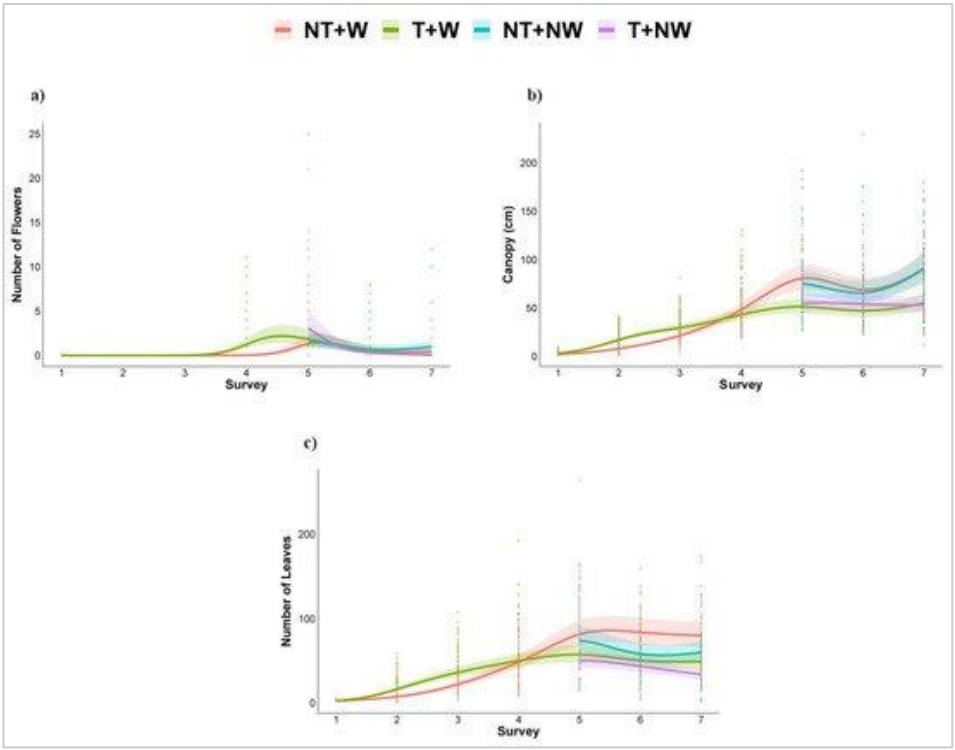

Figure 3. Models showing the trend of (**a**) number of flowers, (**b**) canopy length, and (**c**) number of leaves during the experimental period in the four treatments. CT=conventional tillage, NT=no tillage, W = watered, NW = not watered.

3.3. Efficiency of Photosynthesis and Metabolic Profile

Figure 4 shows the results relative to photosynthetic efficiency, TSC, TPC, and amino acid content. Results suggested that metabolic features were lowly affected by tillage management. Photosynthetic efficiency (**Figure 4**) was comparable between the two treatments ($\chi 2 = 5.03$, $p = 0.17$). Concerning proteins (**Figure 4**b), TPC was not significantly influenced by treatments ($\chi 2 = 6.14$, $p = 0.11$).

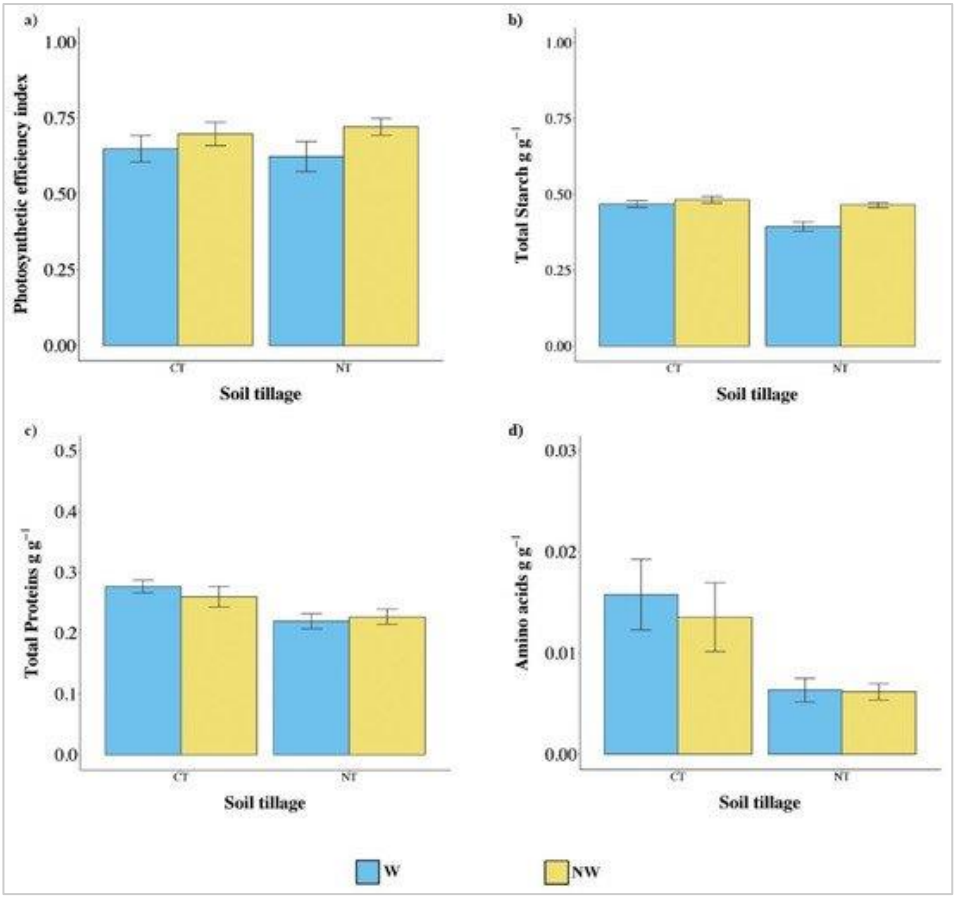

Figure 4. (**a**) Leafy photosynthetic efficiency, (**b**) TSC, (**c**) TPC, and (**d**) amino acids. Values are mean ± SEM. CT = conventional tillage, NT = no tillage, W = watered, NW = not watered.

Also, the total amino acidic content (**Figure 4**d) did not show any significant difference between treatments ($\chi 2 = 4.15$, $p = 0.25$), with an average amount ranging between 0.5% and 2% of the dry matrix. Finally, TSC was clearly influenced by treatments ($\chi 2 = 29.63$, $p < 0.001$). In particular, both CT ($\beta = 0.3 \pm 0.1$, $p = 0.004$;) and NW ($\beta = 0.29 \pm 0.05$, $p <$

0.001) caused an increase in the TSC of about 4.5% and 3.3%, respectively. The interaction resulted to be significant as NT coupled to W caused the most dramatic decrease in TSC (β = −0.23 ± 0.06, $p < 0.001$).

3.4. Soil Organic Carbon and Total Nitrogen

Concerning soil chemical characteristics, a significant difference in C and N concentration occurred between NT and CT, especially at the topmost (0–5 cm) soil horizon. In detail, C and N concentrations in this soil layer were 59% and 27% higher under NT than under CT. No significant difference in soil C and N concentration was found in the 5–30 cm soil layer. (**Table 1** and **Table 2**). Overall, soil C stock in the 0–30 cm soil layer was statistically affected by the tillage system and was 2.58 Mg ha−1 higher in NT than in CT soil (+5%). Conversely, soil N stock in the same soil layer did not statistically differ between NT and CT, even though NT tended to increase soil N stock value by 4% (**Table 1** and **Table 2**).

Table 1. Soil organic carbon. Values are mean ± SEM. Significance levels: * < 0.05, ** < 0.01, *** < 0.001.

Condition	Code	C Concentration 0–5 cm (g C kg^{-1} soil)	C Concentration 5–30 cm (g C kg^{-1} soil)	C Stock (Mg ha^{-1})
Tillage	CT	12.49 ± 1.48	12.39 ± 0.88	48.56 ± 3.37
	NT	19.92 ± 0.73	12.5 ± 0.76	51.15 ± 2.24
Signif.		***	n.s.	*
Water	W	16.17 ± 4.06	12.49 ± 0.76	49.98 ± 2.81
	NW	16.24 ± 4.22	12.4 ± 0.88	49.73 ± 3.51
Signif.		n.s.	n.s.	n.s.
Interaction	CT-W	12.46 ± 0.92	12.43 ± 1.03	48.69 ± 3.53
	CT-NW	12.51 ± 2.07	12.34 ± 0.87	48.43 ± 3.74
	NT-NW	19.88 ± 0.97	12.54 ± 0.54	51.27 ± 1.24
	NT-NW	19.96 ± 0.55	12.45 ± 1.02	51.03 ± 3.19
Signif.		n.s.	n.s.	n.s.

Table 2. Nitrogen content in the soil. Values are mean ± SEM. Significance levels: * < 0.05, ** < 0.01, *** < 0.001.

Condition	Code	N Concentration 0–5 cm (g N kg^{-1} soil)	N Concentration 5–30 cm (g N kg^{-1} soil)	N Stock (Mg ha^{-1})
Tillage	CT	1.49 ± 0.21	1.49 ± 0.1	5.84 ± 0.46
	NT	1.9 ± 0.21	1.57 ± 0.1	6.1 ± 0.37
Signif.		**	n.s.	n.s.
Water	W	1.72 ± 0.37	1.55 ± 0.11	6.04 ± 0.39
	NW	1.67 ± 0.22	1.52 ± 0.12	5.91 ± 0.48
Signif.		n.s.	n.s.	n.s.
Interaction	CT-W	1.41 ± 0.09	1.48 ± 0.1	5.76 ± 0.37
	CT-NW	1.57 ± 0.28	1.5 ± 0.12	5.93 ± 0.58
	NT-NW	2.03 ± 0.22	1.62 ± 0.06	6.32 ± 0.08
	NT-NW	1.76 ± 0.08	1.53 ± 0.13	5.88 ± 0.43
Signif.		n.s.	n.s.	n.s.

4. Discussion

4.1. Suitability of V. unguiculata for Mediterranean CA

Our findings, overall, suggest that tilling is not fundamental to guarantee cowpea growth and yield. Therefore, cowpea is a crop suitable for CA practices and could be cultivated in harsh conditions such as arid and semiarid regions, where not all the crops perform well. For instance, grain yield in common bean *Phaseolus vulgaris* L. is highly affected (−70%) under drought conditions [**24**]. Usually, tillage alters the physical-chemical properties of soil, and NT vs. CT may greatly impact plant growth and yield [**25**]. Unfavorable effects of NT on crop yield have been widely reported immediately after the conversion from CT [**26**]. This is because NT may increase soil strength and BD in the initial years due to transient

compaction of soil [**27**], thus reducing the root growth of plants [**28**]. However, it has also been shown that negative effect usually expires from three to five years after the conversion from CT to NT [**19**]. Our experimental activities with seven-year NT corroborated this improvement of yield potential under NT in the medium-long term. No-till is usually indicated to enhance the water content of soil [**29**], which is of primary importance to sustain crop yield, especially under arid and semi-arid climates [**30**]. Our data show that NT tended to mitigate soil water losses compared with CT and cowpea tolerated NW conditions well under NT. These results are in line with earlier studies reporting the high potential of NT to enhance crop yield in non-irrigated field management [**31**]. The efficacy of cowpea to grow under water deficient conditions and with reduced or no tillage was also documented by Moroke et al. [**32**] in the experimental area of Texas, and by Ahamefule and Peter [**33**] in Nigeria. Moroke and co-workers [**32**] suggested that the cultivation of this species enhances the residual soil water and the presence of surface residue management due to cover crops and NT systems usually increases the whole stored soil water. In the Mediterranean basin, mean temperatures are constantly increasing and precipitation pattern is changing towards hot and dry summer seasons as a consequence of climate change. In this context, European production of pulses has been constantly declining (from 5.8 to 1.8 million ha from 1961 to 2013), mainly due to the introduction of more specialized and intensive crops such as wheat, rice, and corn [**34**,**35**,**36**], but also as a consequence of productive instability of highly water-demanding species [**37**]. However, the European Commission [**38**,**39**] welcomes initiatives to increase the EU's plant protein production in a sustainable and agro-ecological way. Since cowpea can be considered as a leguminous species with reduced water demand and high drought tolerance [**40**], it may be considered to support the cultivation of legumes in Europe replacing currently cultivated species that have higher water demands [**37**]. In addition, we indicate that cowpea is also a reliable alternative to the common Mediterranean bean (*P. vulgaris*) in North-African countries, especially because of high

drought tolerance during the reproductive phase [**41**]. Therefore, combining NT through the cultivation of species and cultivars of pulses highly resistant to water stress (such as the cowpea) could support the resumption of legume cultivation in Europe and in the Mediterranean basin to deal with the ongoing claims about pulses and climate change [**42**]. Concerning the growth parameters, our experiment shows that plants initially grow better on CT soils, but after having joined a critical dimension the NT treatment helps to maintain and stimulate a more pronounced growth in a significant manner. This is particularly true if soil treatment is associated with NW. Generally, the two treatments on the global pattern are not significantly different, but this is due to a balance on the whole values associated with pretty large variations during the growing season. Also, leafy photosynthetic efficiency did not differ between treatments. Therefore, we highlight a great capability of the cowpea to grow under reduced tillage and low water regime. Many studies focused on the impact of CA on crop yields [**6**], while no attention was given to morphological adaptations and metabolic profiling. The latter is an important aspect, impacting on nutritional importance and sustainability of this crop. Generally, the main constituents of the seeds were not strongly affected by treatments (NT and NW) with the exception of TSC. TSC is normally related to a plant's ability to photosynthesize, therefore, some studies conducted on *V. unguiculata* seeds showed a reduction in TSC under NW, also in order to increase the number of free analytes able to gather water through osmosis [**43**]. Here, we found an increase in TSC in NW conditions. Maybe this could be related to the maintenance of a high photosynthesis rate, also without irrigation. As a matter of fact, NW is not always related to starch degradation in plant tissues [**44**]. These data further confirm a good resistance of this species against drought, a condition highly dangerous for many crop species not equally able to adapt to climate changes [**45**]. Finally, in terms of protein and amino acids, our analyses confirm that cowpea is an important source of these nutritional components and the growing condition did not affect their amount. Considering that in the Mediterranean area, especially in

the Eastern and African sides, diets are deficient in terms of protein and amino acid intake [**16**,**46**] and despite the change in food regimes that lead to a decrease of the intake of animal proteins, we estimate that cowpea could be a great support or even a crucial aliment to compensate these lacks. Moreover, the cowpea amino acidic content is two times higher than the widespread common bean *P. vulgaris* [**16**], therefore this species could be a good substitute for the traditional legume crops.

4.2. Conservative Cultivation of V. unguiculata Enhances Soil Fertility

As well documented, Fabaceae are able to accumulate organic nitrogen thanks to symbiotic interactions at the rhizosphere level. This phenomenon has positive effects also on soil fertility; however, soil management could also affect organic and inorganic components. For instance, CT is considered a major cause of soil C and N depletion as a consequence of soil organic matter mineralization [**47**]. NT has been widely indicated as a key strategy to increase C storage in arable soils. Our results are in line with this consideration as they show that converting CT to NT increased C stock by 2.58 Mg ha−1 on a silty clayey soil (**Table 1**), which means that NT increased the potential of soil to sequester C by 0.32 Mg ha−1 yr−1 under our experimental conditions. This is consistent with the previous findings of a review study by West and Post [**48**]. These authors reported that NT may increase C sequestration in soil by 0.20–0.57 Mg ha−1 yr−1, according to the complexity of crop rotation. Also Aguilera et al. [**49**], in a recent meta-analysis reported a 0.44 Mg ha−1 yr−1 higher soil C sequestration under NT in the surface 34 cm of soil under a temperate climate. Our findings also suggested that considering soil C expressed as concentration (g kg−1) instead of as mass (Mg ha−1) may lead to overestimating the role of NT for soil C sequestration. However, we underline that we have data from the last eight years about the used NT soil patches, and our surveys showed an increase in soil C concentration in the 0–30 cm soil layer of, on average, 11% compared with CT. Conversely, soil C stock, which takes into account soil BD, was

only 5% higher under NT than under CT. This is because lower BD of soil in the surface layers under NT (especially 0–5 cm) reduces the actual impact on soil C accumulation [**18**]. Nevertheless, NT did not reduce soil C stock in the subsurface soil layers (5–30 cm), and the net impact of NT on soil C stock in the 0–30 cm soil was positive. Concerning nitrogen, the soil tillage could increase soil oxygen exposure and this promotes soil organic matter mineralization and soil N depletion [**50**]. Therefore, increasing soil organic matter in soils is a key way not only to increase soil C stock and mitigate climate change but also to enhance soil fertility and thus sustain food production [**51**]. As expected, our results showed that the evolution in soil N concentration and stock followed a similar pattern to that of soil C levels. Converting CT to NT led to a significant increase of soil N concentration in the 0–5 cm soil layer (**Table 2**). These results confirmed that variation of soil C and N levels as induced by NT differed considerably depending on the surface (0–5 cm) or subsurface (5–30 cm) soil layers [**52**]. Mazzoncini et al. [**53**] also found that soil C and N accumulated under NT may be mainly attributed to soil C and N variation in the topmost (0–10 cm) soil layer, which is in substantial agreement with our results (**Table 1** and **Table 2**). This is mainly due to the fact that NT limits the direct input of fresh organic matter to the subsoil, thus reducing the downward movement of soil organic matter, which is usually increased only in the surface 10 cm of soil. In addition, reducing soil disturbance decreases N mineralization and losses especially in the topmost soil layers [**53**], due to a lower temperature [**54**] and aggregate turnover [**55**] in non-tilled soils than in tilled ones.

5. Conclusions

In conclusion, CA can offer many advantages in the Mediterranean context. First of these, is the saving of energy and costs. Studies performed on cowpea in a semiarid area of India showed that zero tillage practices provide a considerable energy saving (−17.1 GJ ha−1) due to the

lower input compared to CA [**56**]. The energy efficiency should be about 13 times higher in zero tillage systems than in conventional ones. However, Dixit et al. [**56**] showed that cowpea yield (intercropped with sorghum) was significantly higher in the context of conventional agriculture compared with NT. Another key point of CA is the ability to reduce the impact on the greenhouse effect by anthropogenic gases emission. As suggested by Powlson [**18**] the total carbon sequestration in NT condition could reach about 0.3 Mg ha−1 yr−1; our data confirm that the total CO2 sequestration per year was estimated to be 0.32 Mg yr−1. This is due also to the usage of a cover crop mixture during the winter period which provided for better capture of the CO2, while in Powlson [**18**] the meta-analysis took into account predominantly field not managed with cover crops. This underlines the importance of the integration of cover crops in zero tillage management. Finally, concerning the ability of the soil to save water, our data suggested an increase of SOC in NT area against CT. Previous studies suggested that in the context of the Mediterranean geographical area, an increase of about 0.4% SOC may lead to an increase of up to 34% of water saving [**57**]. Regarding all these elements and knowing that cowpea is a very interesting stress-tolerant minor crop with a short time maturity (about 60 days), cowpea could be introduced not only to relieve and reduce agricultural impact and climatic changes but also to supply a lot of vegetable-derived nutrients, like proteins and amino acids.

Supplementary Materials

The following are available online at **https://www.mdpi.com/2223-7747/9/1/48/s1**, Figure S1: Evolution of daily precipitation (bars) and average daily temperature (line) of the field site during the course of the experiment.

Author Contributions

L.G., A.F., D.P., N.T. and A.G. designed and performed the field experiment, L.G., A.F., D.P., F.G., analysed data, E.T. and C.M. contributed during field activity, E.P., V.T., A.G. and M.L. supervised and contributed to the paper writing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors have no conflict of interest to declare.

Abbreviations

CA: conservation agriculture; CT: conventional tillage; NT: no-tillage; W: watered; NW: not watered.

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Supplementary Material

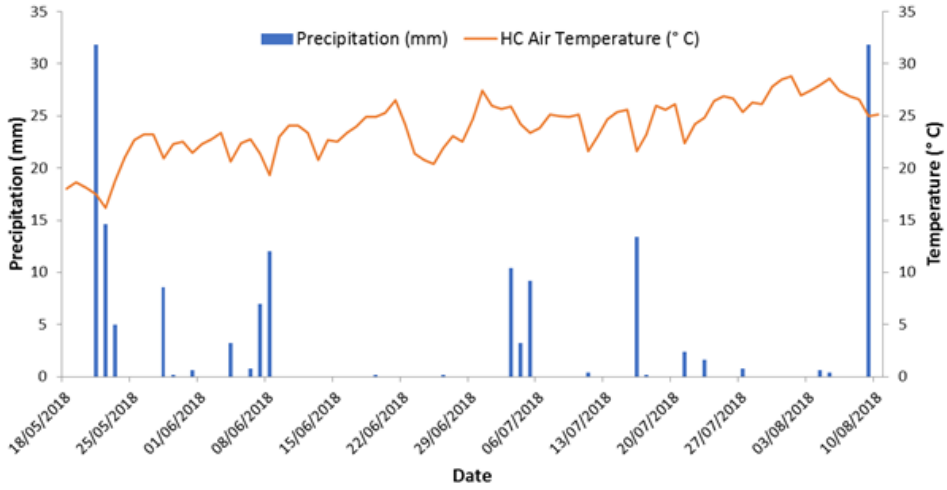

Figure S1: Evolution of daily precipitation (bars) and average daily temperature (line) of the field site during the course of the experiment.

9.2 Effectiveness of *Vigna unguiculata* seed extracts in preventing colorectal cancer

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Abstract

Colorectal cancer (CRC) is one of the most common types of cancer, especially in Western countries, and its incidence rate is increasing every year. In this study, for the first time *Vigna unguiculata* L. Walp. (cowpea) water boiled seed extracts were found to reduce the viability of different colorectal cancer (CRC) cell lines, such as E705, DiFi and SW480 and the proliferation of Caco-2 line too, without affecting CCD841 healthy cell line. Furthermore, the extracts showed the ability to reduce the level of Epidermal Growth Factor Receptor (EGFR) phosphorylation in E705, DiFi and SW480 cell lines and to lower the EC50 of a CRC common drug, cetuximab, on E705 and DiFi lines from 161.7 ng mL−1 to 0.06 ng mL−1 and from 49.5 ng mL−1 to 0.2 ng mL−1 respectively. The extract was

characterized in its protein and metabolite profiles by tandem mass spectrometry and 1H-NMR analyses. A Bowman-Birk protease inhibitor was identified within the protein fraction and was supposed to be the main active component. These findings confirm the importance of a legume-based diet to prevent the outbreak of many CRC and to reduce the amount of drug administered during a therapeutic cycle.

Introduction

Colorectal cancer (CRC) is one of the most frequent malignancy in the world. The outbreak of the majority of CRC forms is related to environmental factors, such as lifestyle and diet, while only a 5–10% of hereditariety is described.1 During carcinogenesis and cancer progression, the up-regulation of survival signals is mainly responsible for the abnormal proliferation of CRC cells,2 in which the Epidermal Growth Factor Receptor (EGFR) signaling pathway is thought to play a crucial role. EGFR is a transmembrane tyrosine kinase receptor triggering two signaling pathways: the RAS-RAF-MAPK axis involved in cell proliferation and the PI3K-PTEN-AKT pathway involved in cell survival and escaping from apoptosis.3 In the last decades, many drugs have been developed for the treatment of CRC. Among these, the monoclonal antibodies (MoAbs) cetuximab and panitumumab bind to the extracellular domain of EGFR when it is in the inactive configuration, compete for receptor binding by occluding the ligand-binding region, and thereby block ligand-induced EGFR activation, inducing its internalization and degradation.4 However, these drugs are expensive and still characterized by some side effects such as severe skin toxicity, occurring in approximately 80% of patients,5 corneal erosion,6 headache, pulmonary damages, general weakness and diarrhea.7 Moreover, they show efficacy in no more than 30% of patients.8 Indeed, it has been demonstrated that hyperactivating mutations occurring in downstream effectors (such as KRAS, NRAS and BRAF) represent the main mechanism of primary resistance. However, even in patients without any downstream mutations, the percentage of

efficacy of anti-EGFR MoAbs is less than 50%.9–18 All these concerns underline the need to identify new approaches, such as the application of nutraceuticals able both to elicit and expand the range of chemo-preventive actions, while reducing the amount of administered drugs during a therapeutic cycle.19 Fruit, vegetables and other edible plant parts are the primary sources for human nutrition and medicine.20 The increasing knowledge on plant biodiversity and biotechnology has dramatically changed the role of plant food on human health and wellbeing. Nutritional therapy and phytotherapy have emerged as new concepts and healing systems have quickly and widely spread in recent years. Considering that plant foods easily reach the stomach and the gut, these organs represent the most suitable targets to estimate the biological activities of food phytocomplexes.19 Pulses have received increasing attention in the last decades due to their nutraceutical properties, such as antioxidant, anti-inflammatory, hypoglycemic and other activities.21,22 Moreover, several studies have shown pulses anticancer properties related to the presence of specific classes of phytochemicals, such as resistant starch fermenting in the gut and being converted into SCFAs, proteins, like amylase and protease inhibitors, globulins and polyphenols.23 In Europe, the production of pulses is lower than in other continents and mainly limited to peas, chickpeas and faba beans, while in poor and developing countries other species and local cultivars are preferred due to higher accessibility.24 Among these minor crops, *Vigna unguiculata* L. Walp., also known as cowpea, stands out due to its adaptability to harsh environmental conditions, such as drought and minimum field tillage.25 Its seeds provide high amount of proteins, peptides, amino acids and other micronutrients such as folates and minerals (calcium, zinc and iron);23 leaves are also sometimes consumed fresh or boiled increasing the uptake of polyphenols and fibers in diet.26,27 Although the nutritional traits of this species are well documented, relatively little is known about the anticancer properties of this species, mainly due to bioactive peptides and polyphenols, but results are controversial and require further investigations.28,29 Therefore, in

this study *V. unguiculata* beans phytoextract was considered to evaluate potential anticancer activities. Specifically, phytochemical analyses were combined with bioactivity investigations to clarify the role of specific bean components as a possible supplement in EGFR-targeted therapies for CRC.

Experimental

Plant material and phytoextraction

V. unguiculata seeds from three batches were collected (Colfiorito, Italy, batches 17117 and 18039, and Castellani, Italy, batch 011018). Seeds were water boiled for one hour and left resting one more hour in water at room temperature (RT). This treatment aimed at emulating the typical boiling process of conventional food recipes. Seeds were then incubated overnight at 50 °C to dry completely and then they were grinded into a powder. Two grams of dry powder were extracted in 50 mL of milliQ water at RT with a magnetic stirrer for 5 minutes and then centrifuged at 5000*g* for 30 minutes at RT. Supernatants were then recovered and freeze-dried.

Separation of phytoextract components

Molecular weight-based separation. Ultra-2 mL Amicon filters (Merck-Millipore, Germany) with a cut-off of 3 kDa were used to begin the separation process of extracts. Freeze-dried extracts were resuspended in water at a concentration of 40 mg mL−1. After conditioning tubes with 2 mL of water, extracts were loaded and centrifuged till complete separation at 7197*g* at 4 °C. The upper and the lower fractions were collected, freeze-dried and stored at −20 °C. Protein content was determined using Coomassie Brilliant Blue G-250 method (Thermo Scientific Rockford, IL, USA) and using bovine serum albumin (BSA) as standard protein for calibration curve.

DEAE chromatography. DEAE chromatography was performed to further purify bioactive components. The upper fraction was resuspended in 5 mM Tris HCl pH 8 to reach a concentration of 40 mg mL−1. 1 mL of DEAE resin (DEAE Sephacel, GE-Healthcare, USA) was centrifuged at 5000*g* for 10 minutes at 4 °C to separate the resin from ethanol. Ethanol was removed, then 10 mL of water were added, centrifuged and supernatant was removed. The resin was further resuspended in 10 mL of 5 mM Tris HCl pH 8 for conditioning and centrifuged, then about 400 mg of sample were loaded and incubated on rotating wheel for 60 minutes at 4 °C. The mixture was subsequently loaded onto a column at a flow rate of 1 mL min−1 and eluted with 10 mL of 5 mM Tris HCl pH 8, 0.5 M NaCl at the same flow rate. The flowthrough and the eluted fractions were recovered and stored. Eluted fractions were dialyzed against PBS buffer (25 mM phosphate buffer pH 7.2, 0.15 M NaCl) with a 13 000 Da cut-off membrane overnight at 4 °C.

Size exclusion chromatography. A further purification was carried out using the AKTA Purifier Instrument (GE-Healthcare) equipped with a Superose 12 10/300 GL gel filtration column (GE Healthcare, Life Sciences, Little Chalfont, England), pre-equilibrated with PBS buffer. Elution was performed at a flow rate of 0.5 mL min−1 in the same buffer. A calibration curve was obtained by plotting elution volume parameters of a set of standard proteins against the logarithm of their molecular weights. Standards employed at 1 mg mL−1 were immunoglobulin G (150 kDa), bovine serum albumin (67 kDa), bovine β-lactoglobulin (35 kDa) and bovine cytochrome C (12.7 kDa) (Sigma Aldrich, St Louis, MO, USA).

Removal of the hydrophobic components. A further step of purification was carried out on the fractions isolated through the size exclusion chromatography by using SPE C-18 Bond Elute cartridges (Agilent Technologies, USA) in order to remove the hydrophobic components. Samples were passed through the cartridge and the unbound fraction was recovered.

Proteomic analysis

HPLC fractions were reduced, derivatized and digested with trypsin (protein: protease ratio 20 : 1) as described in30 before MS/MS analysis. Peptides separation was achieved on a Thermo Easy-nLC 1000, and MS data were acquired on a Thermo Q-Exactive–HF, with a data-dependent top 15 method, the survey full scan MS spectra (300–1650 *m*/*z*) were acquired in the Orbitrap with 60 000 resolution, AGC target 3e6, IT 20 ms. For HCD spectra resolution was set to 15 000, AGC target 1e5, IT 80 ms; normalized collision energy 28 and isolation width of 1.2 *m*/*z*. Raw label-free MS/MS files from Thermo Xcalibur software (version 4.1) were analyzed using Proteome Discoverer software (version 2.2, Thermo Fisher Scientific) and searched with Sequest algorithm against the proteome of NCBI Phaseoleae (release 05th August 2019) with minimum peptide length 6 amino acids, carbamidomethylation as fixed modification, Met oxidation and Arg/Gln deamidation as variable modifications.31 The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD017846.

1H-NMR metabolic profile

The total cowpea water boiled seed extract was suspended in H2O : D2O (9 : 1) at a final concentration of 10 mg mL−1. 3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TSP, final concentration 0.5 mM) was used as external reference and alanine doublet at 1.48 ppm as internal reference for chemical shift. The pH of the sample was verified with a microelectrode (Mettler Toledo, Columbus, OH, USA) and adjusted to 7.4 with NaOD and DCl. The acquisition temperature was 25 °C. All spectra were acquired on an Avance III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a QCI (1H, 13C, 15N/31P and 2H) cryogenic probe. 1H NMR spectra were recorded with *noesygppr1d* pulse sequences (Bruker library) and 256 scans, spectral width 20 ppm, relaxation delay 5 s. They were processed with 0.3 Hz line broadening,

automatically phased and baseline corrected. The 1H,1H-TOCSY (Total Correlation SpectroscopY) spectra were acquired with 24 scans and 512 increments, a mixing time of 80 ms and relaxation delay of 2 s. 1H,13C-HSQC (Heteronuclear Single Quantum Coherence) spectra were acquired with 48 scans and 256 increments, relaxation delay 2 s. The NMR data were processed using MestreNova 14.1.0 software (Mestrelab Research, Santiago de Compostela, Spain). Compound identification and assignments were done with the support of 2D NMR experiments and comparison with reported assignments.

Bioactivity assessment

Cell cultures. CCD841 (ATCC® CRL-1790™) human healthy mucosa cell line and Caco-2 (ATCC® HTB-37™) human colorectal cancer cell line were grown in EMEM medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 100 U mL−1 penicillin, 100 µg mL−1 streptomycin. E705 (kindly provided by Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy) and SW480 (ATCC® CCL-228™) human colorectal cancer cell lines were grown in RPMI 1640 medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 100 U mL−1 penicillin, 100 µg mL−1 streptomycin. DiFi human colorectal cancer cell line, kindly provided by Dr Josep Tabemero (Vall d'Hebron Institute of Oncology, Barcelona, Spain), was grown in Ham's F12 medium supplemented with heat-inactivated 5% FBS, 2 mM L-glutamine, 100 U mL−1 penicillin, 100 µg mL−1 streptomycin. All cell lines were maintained at 37 °C in a humidified 5% CO2 incubator. ATCC cell lines were validated by short tandem repeat profiles that are generated by simultaneous amplification of multiple short tandem repeat loci and amelogenin (for gender identification). All the reagents for cell cultures were supplied by Lonza (Lonza Group, Basel, Switzerland).

Viability assay. Cell viability was investigated using MTT-based *in vitro* toxicology assay kit (Sigma, St Louis, MO, USA), according to manufacturer's protocols. In detail, the different cell lines were seeded in

96-well microtiter plates at a density of 1 × 104 cells per well, cultured in complete medium and treated after 24 hours with increasing concentrations of total extract (0–4000 µg mL−1). In order to evaluate the combined effect of cowpea extract and cetuximab, 24 hours after the seeding the cells were treated with different concentrations of cetuximab (0–100 µg mL−1) and at fixed concentrations of cowpea total extract (200 and 1000 µg mL−1). After 48 hours at 37 °C, the medium was replaced with a complete medium without phenol red containing 10 µL of 5 mg mL−1 MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide). After 4 hours incubation more for CCD841 and 2 hours for CRC cells lines, formazan crystals were solubilized with 10% Triton X-100, 0.1 N HCl in isopropanol and absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage against untreated cell lines used as controls. Before each experiment the extract or the related fractions were filtered through a nitrocellulose 0.22 µm filter and the protein concentration was evaluated by using Coomassie Brilliant Blue G-250 (Thermo Scientific Rockford, IL, USA) and BSA as a standard protein. **Proliferation assay.** In order to evaluate the effect of cowpea total extract on the cellular proliferation of the CRC cell lines, the cells were counted at consecutive time points. The cells were seeded in 35 mm dish at a density of 1–2 × 105, treated with the extract (200 and 2000 µg mL−1) 24 hours after seeding and harvested by trypsinization at 24, 48 and 72 hours after treatment. Aliquots of the cell suspension were counted in Burker's chamber. All counts were expressed as total number of cells. **SDS–PAGE and western blot.** To examine the effect of extract on the EGFR phosphorylation, the CRC cell lines and the healthy cell line were seeded at 75 × 104 cells per 60 mm dish and treated for 48 hours with the total extract at 200 and 2000 µg mL−1. The cells were rinsed with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), containing protease and phosphatase inhibitors and 1 mM PMSF. Homogenates were obtained by passing 5 times through a blunt 20-gauge needle fitted to a syringe and then centrifuged at 15 000*g* for 30 minutes. Supernatants were analyzed

for protein content by the BCA protein assay.32 SDS-PAGE and western blot were carried out by standard procedures.33 Twenty or sixty micrograms of proteins were separated on 10% acrylamide/bis-acrylamide SDS-PAGE, transferred into a nitrocellulose membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% (w/v) dried milk in PBS for 30 minutes at RT and then probed overnight at 4 °C with the appropriate antibodies in 5% (w/v) BSA in PBS. After three 10 minutes washes with PBS containing 0.1% (v/v) Tween 20 (PBS-T), membranes were treated for 1 hour at RT with an HRP-conjugated secondary antibody diluted in 5% (w/v) dried milk in PBS. After three washes in PBS-T, detection was performed using an ECL plus detection system (Millipore, Billerica, MA, USA). Protein levels were quantified by densitometry of immunoblots using Scion Image software (Scion Corp., Frederick, MD, USA). The following primary antibodies (all purchased from Cell Signaling Technology, Danvers, MA, USA) were used: anti EGFR (dilution 1 : 1000), phospho-EGFR (Tyr1068; dilution 1 : 1000), p44/42 MAPK (ERK 1/2; dilution 1 : 1000), phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204; dilution 1 : 1000), Akt (dilution 1 : 1000), phospho-Akt (Ser473; dilution 1 : 1000), GAPDH (dilution 1 : 10 000) and vinculin (dilution 1 : 10 000). IgG HRP-conjugated secondary antibodies (purchased by Cell Signaling Technology, Danvers, MA, USA) were diluted 1 : 10 000.

Statistical analyses

Statistical analyses were performed by using software R, version 3.3.3. Packages used for the analyses and the graphs were lme, lme4, nlme, glmmTMB and ggplot2. The threshold of statistical significance was set at 0.05.

MTT assay. To analyse the impact of the amount of extracts on the viability of the different cell lines a Generalized Linear Mixed Effects Model (GLMM) was used. The response variable (% cells survival) was assumed to be binomial or beta-binomial distributed in case of

overdispersion.34 Fixed effect analysed was the concentration of extract in interaction with the cell line. Since three different batches were tested, they were treated as a random effect.

Proliferation assay. Data from proliferation assays were analysed by a GLMM. Response variable was the number of cells and it was assumed to be negative-binomially distributed. Fixed effect was growth time (up to 72 hours after the treatment) in interaction with the amount of extracts used for the treatment itself. For each cell line, 3 replicates were performed and therefore treated as random effect.

Combination experiment between cetuximab and total extract. One Way ANOVA was used to evaluate the effect of the supplementation of two different concentrations of extract on the EC50 of cetuximab. In order to compare the effect of the supplementation of extract at different concentrations (200 μg mL−1 and 1000 μg mL−1) against the control (cells treated only with cetuximab) a Dunnett test was performed.

Densitometric analysis. A Linear Model (LM) was performed to evaluate statistical differences regarding EGFR phosphorylation among the different extract concentrations in the 5 cell lines. The response variable was the level of EGFR phosphorylation while the categorical variable was the interaction between the cell line and the concentration of extract. The same analysis was performed on ERK and AKT phosphorylation levels.

Evaluation of protein-dependence. To verify the existence of a relationship between CCD841 and E705 cell viability and the amount of proteins in the samples, a GLMM was carried out considering a binomial distribution of the response variable and proteins amount in interaction with the cell line as fixed effect. Both total and purified extracts were taken into account, so that the different extract fractions (see paragraph 2.2) were considered the random component within the model.

Results & discussion

Effect of *V. unguiculata* extract on the viability of healthy and colorectal cancer cell lines

Starting from 2 g of dried seeds from 3 different batches the following extract yields were obtained: 102.4 ± 11 mg from the first batch, 203 ± 9 mg from the second and 81.8 ± 3.78 mg from the third. The protein content was equal to 4.168 ± 0.379 mg for the extraction of the first batch, 3.179 ± 0.203 mg for the second batch and 8.142 ± 0.407 mg for the third. Extracts of *V. unguiculata* from the three different batches were tested on healthy mucosa and CRC cell lines with different molecular profiles: Caco-2, E705 and DiFi cell lines, all wild type for EGFR, KRAS, NRAS, and BRAF genes, as well as SW480 cell line, carrying the KRAS G12 V mutation and wild-type for the other aforementioned genes. The DiFi cell line is characterized by a strong EGFR gene amplification profile. Fig. 1 reports the results of MTT assay on each cell line at different phytoextract concentrations. Data suggest a dose-dependent effect in E705 ($p < 0.001$), DiFi ($p < 0.001$) and SW480 ($p < 0.001$) cell lines, with a percentage of viability at 2000 µg mL−1 of 50%, 23% and 45%, respectively. Neither Caco-2 cancer cell line ($p = 0.672$) nor the healthy one CCD841 ($p = 0.301$) were affected by the treatment.

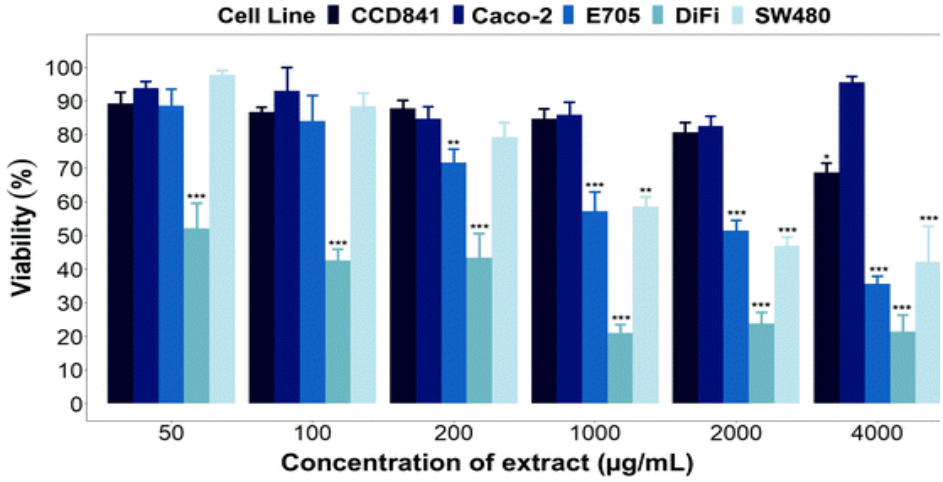

Fig. 1 Effects of *V. unguiculata* extract after 48 hours treatment on the viability of colon cell lines. Data are expressed as the mean percentage of viability of the three batches tested compared to the untreated control (*p < 0.05, **p < 0.01, ***p < 0.001, with Bonferroni's correction). The experiment was performed with increasing concentrations of phytoextract (from 50 µg mL−1 to 4000 µg mL−1).

Proliferation assays on healthy and CRC cell lines after extract treatment

To evaluate the cytostatic effect of the phytoextract, proliferation assays were performed on healthy and CRC cell lines treated with 200 and 2000 µg mL−1 extract at different times. Results showed a general cell growth decrease of the CRC lines (Fig. 2). In particular, the effect on Caco-2 was detected only at the highest dose after 60 hours treatment, whereas E705 proliferation showed a significant effect already after 30 hours at both concentrations. Noteworthy, at a concentration of 2000 µg mL−1, DiFi cells did not show any growth. Concerning SW480, the reduction in the growth rate at 2000 µg mL−1 extract was highly significant compared to the untreated control approximately after 60 hours treatment (confidence bands do not overlap). The healthy line CCD841 was found not to be affected by the treatment with the extract at any concentration tested (Fig. 1, ESI†). Therefore, our data indicate that components of *V. unguiculata* seed extract may play a role in cancer prevention, especially by slowing down cancer cells proliferation.

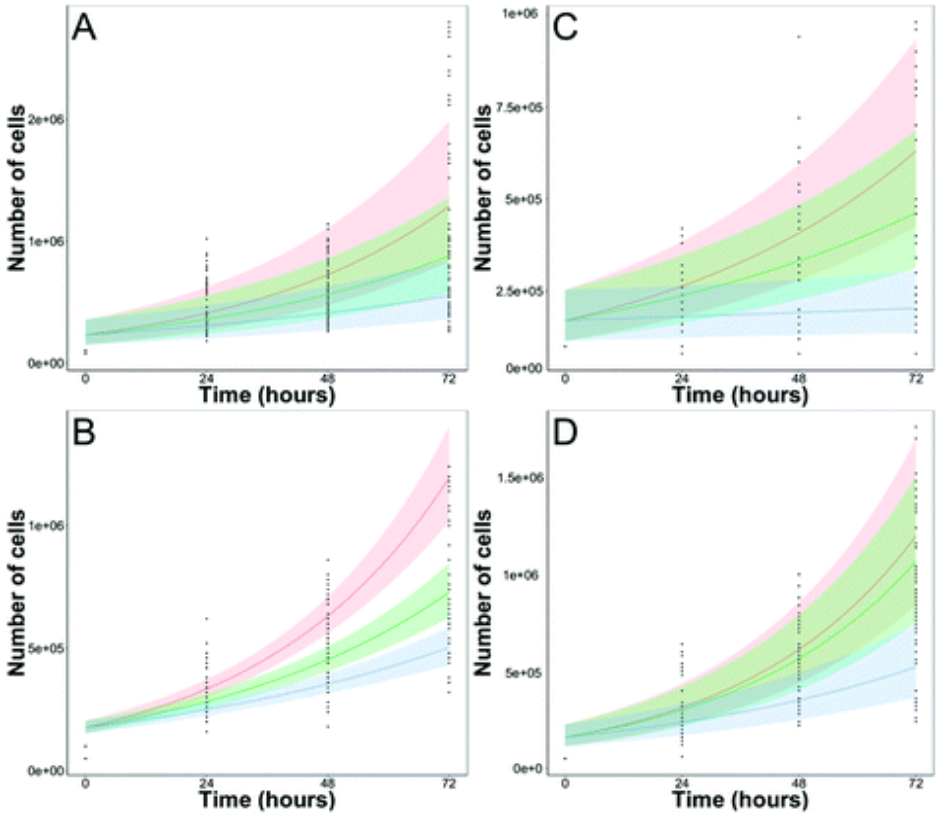

Fig. 2 Cell proliferation. CRC cell lines treated after 24 hours after seeding, harvested and counted at 24, 48 and 72 hours after treatment. A: Caco-2, B: E705; C: DiFi; D: SW480. Coloured lines represent the growth function in time of cells treated with different concentration of extract (red: control, green: 200 µg mL−1, blue: 2000 µg mL−1). Bands show the 95% confidence intervals. When bands overlap, there is no significant difference among treatments.

Analysis of EGFR phosphorylation and related downstream pathways in response to phytoextract

As EGFR signaling is one of the pathways mainly involved in CRC pathogenesis, the activation of EGFR and of the main downstream effectors (ERK for the MAP kinase pathway, Akt for the PI3k-Akt-mTOR

axis) were evaluated through western blot and densitometric analysis after treatment with 200 μg mL−1 and 2000 μg mL−1 extract (Fig. 3). Results showed that in E705 cells the level of phospho-EGFR significantly decreased at 200 μg mL−1 and even more at 2000 μg mL−1 extract ($p < 0.01$). DiFi and SW480 showed a decrease in phospho-EGFR levels only at 2000 μg mL−1 ($p < 0.01$). Conversely, no effects were shown on Caco-2, neither at 200 μg mL−1 nor at 2000 μg mL−1. As expected, no EGFR phosphorylation was found in CCD841. All CRC cell lines displayed high phospho-EGFR levels compared to the healthy control.35

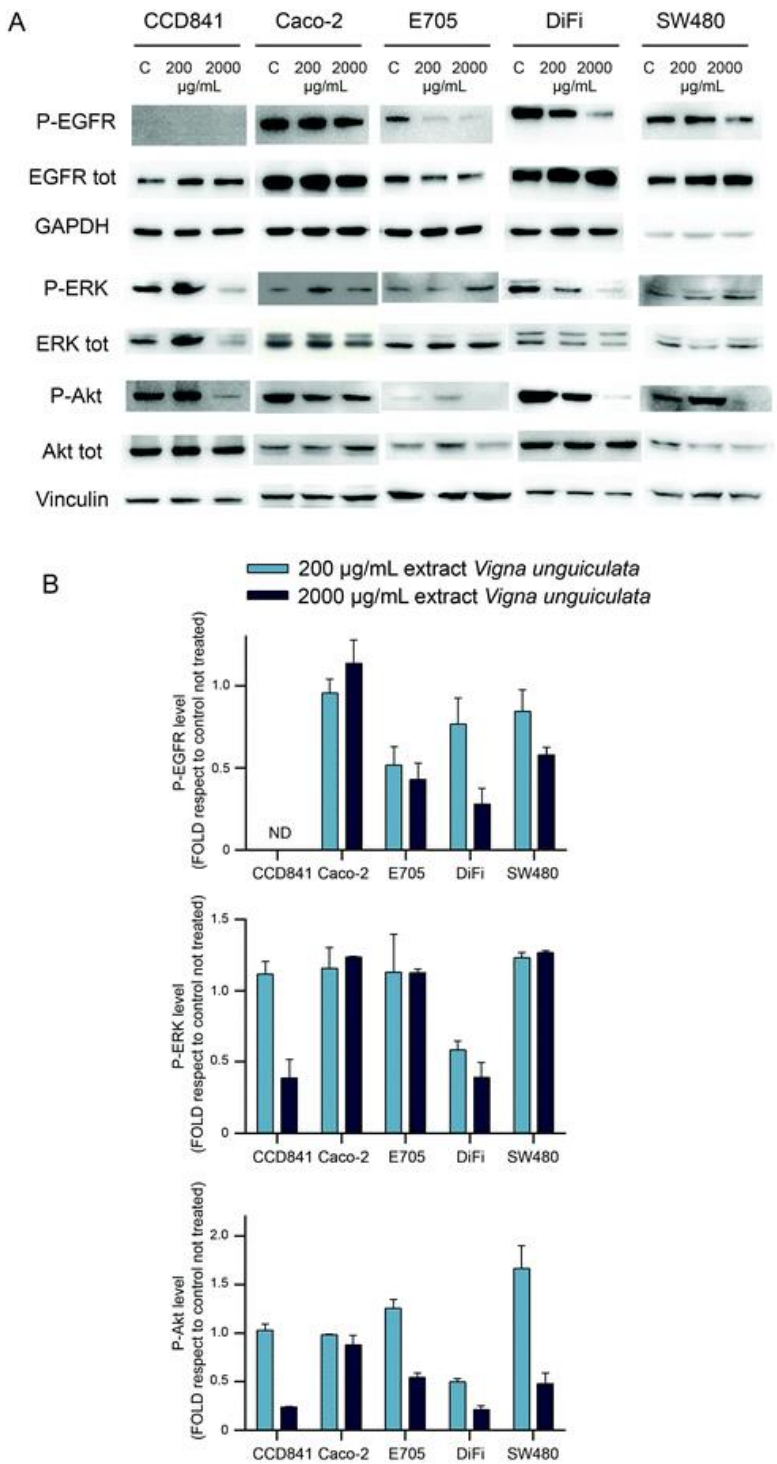
A
CCD841
Caco-2
E705
DiFi
SW480
C 200 2000 µg/mL
P-EGFR
EGFR tot
GAPDH
P-ERK
ERK tot
P-Akt
Akt tot
Vinculin
B
200 µg/mL extract Vigna unguiculata
2000 µg/mL extract Vigna unguiculata
P-EGFR level (FOLD respect to control not treated)
ND
P-ERK level (FOLD respect to control not treated)
P-Akt level (FOLD respect to control not treated)
CCD841 Caco-2 E705 DiFi SW480

Fig. 3 Western blotting analysis of EGFR, ERK and Akt phosphorylation in CRC and normal mucosa cell lines. (A) Representative western blotting performed on protein extracts (20 μg for DiFi cell line and 60 μg for the other cell lines in EGFR analysis and 60 μg for all cell lines in ERK and Akt analysis), using anti-P-EGFR, anti-EGFR, anti-P-ERK, anti-ERK, anti-P-Akt and anti-Akt antibodies. GAPDH and vinculin was used as loading control. (B) Determination of phosphorylation rate by densitometric analysis was performed with Scion Image Software. Data are expressed as the phospho/total ratio and each ratio is normalized on phospho/total ratio of not treated cell line.

The precise interplay of the two EGFR downstream pathways is still unknown, but most data suggest that the MAP kinase pathway is the most relevant one.8,36,37 As a matter of fact, the presence of mutations able to hyperactivate this pathway (such as those occurring in KRAS, NRAS and BRAF genes) is the main factor for ruling out EGFR-targeted therapies, that have been developed in the last 10 years.9 However, concerning the two EGFR downstream pathways, our data reveal that the situation is more complex. Indeed, ERK activation was downregulated only in the DiFi cell line in a dose-dependent manner ($p < 0.001$), while no variations in Caco-2, E705 and SW480 cells were detected. Diversely, a significant decrease in Akt activation was observed in DiFi cell line already at 200 μg mL−1 extract, while in E705 and SW480 cell lines only at the highest extract dose. In general, the responses of these cell lines were found to be concentration-dependent (E705 $p = 0.028$, DiFi $p < 0.001$, SW480 $p = 0.009$). A decrease in ERK ($p < 0.001$) and Akt ($p = 0.001$) activation was also observed in the CCD841 normal mucosa cell line, but only at the highest phytoextract concentration and with limited evidences at the viability and proliferation level (Fig. 1 and Fig. 1, ESI†). Overall, the alterations of the activation levels of EGFR and partly of its downstream effectors are in line with the viability and proliferation assays. In E705 and DiFi cell lines, both sensitive to anti-EGFR therapies, the decrease of EGFR

phosphorylation and the decrease of ERK and/or Akt activation led us to hypothesize a putative supplementary effect between *Vigna unguiculata* extracts and EGFR-targeted therapies. The SW480 cell line is characterized by the KRAS G12 V mutation and presents a constitutively activated MAP-kinase pathway, so that the significant decrease in EGFR phosphorylation can lead to the reduction of the PI3K-mTOR pathway with a decrease of Akt phosphorylation, leading cells to apoptosis. We cannot exclude that cell proliferation may be mediated by alternative, MAP-kinase independent pathways. The Caco-2 cell line shows a decrease in cell proliferation only at the highest dose but no alterations in the activation of EGFR and its downstream effectors are observed: for this cell line, that is characterized by a complete RAS-BRAF wild-type status, we can hypothesize that the driver is outside the EGFR pathway but acts on it through a putative dimerization of EGFR with other members of the EGFR family.38 Therefore the phytoextract plays a minor role, in accordance with the smaller decrease observed in cell proliferation. As for the normal mucosa cell line, the decrease of downstream pathways activation at the highest dose does not lead to any alteration in the viability and proliferation levels. It is conceivable that the administration of *Vigna unguiculata* extract treatment may have no effect on normal mucosa cells. This is an extremely important factor to avoid the tedious side effects of the majority of chemotherapies. Overall, our western blotting results clearly indicate that not only the MAP kinase axis, but both EGFR downstream pathways are relevant. At clinical level, as mentioned before, the presence of mutations able to hyperactivate this pathway, such as those occurring in KRAS, NRAS and BRAF genes, is the main factor for ruling out EGFR-targeted therapies.9 On the contrary, little is known about the P3K-Akt-mTOR pathway, if we exclude few, sporadic studies that investigated PIK3CA mutations and PTEN protein deregulations, but without an extensive confirmation by the wide scientific community.39–41 Having in mind that only up to 30% of RAS-BRAF wild-type cases may profit from the administration of EGFR-

targeted therapies, our data suggest a deeper investigation of the P3K-Akt-mTOR axis.

Extract ability to supplement cetuximab treatment

The significant reduction in EGFR activation may propose these extracts as potential co-drugs to be administered in combination with EGFR-targeted therapies, that have some adverse side effects and are effective in only up to 30% of treated patients. The ability of the extract to complement the cetuximab EGFR-targeted therapy was evaluated. CRC cell lines were treated with cetuximab (0–100 μg mL−1) and 200 μg mL−1 and 1000 μg mL−1 extract (Fig. 2, ESI†). These concentrations were selected on the basis of extract effect on the two drug-sensitive cell lines, *i.e.*, E705 and DiFi. As far as E705 line is concerned, results show that the EC50 of the drug decreased from 161.7 ± 18.3 ng mL−1 to 18.4 ± 9.8 ng mL−1 when E705 were treated with 200 μg mL−1 and to 0.06 ± 0.01 ng mL−1 in combination with 1000 μg mL−1 of extract. Regarding DiFi cell line, a similar pattern to that of E705 was observed. Specifically, the EC50 of the drug decreased from 49.5 ± 1.0 ng mL−1 to 12.4 ± 5.4 ng mL−1 in combination with 200 μg mL−1 of extract and to 0.2 ± 0.1 ng mL−1 with a treatment of 1000 μg mL−1 of extract. In both cell lines, differences between treatments and control were statistically relevant ($p < 0.05$). We assume the possible use of lower doses of drug in combination with cowpea extracts in the treatment of patients. A diet regime including pulses' consumption, such as cowpea, could be a turning point in targeted therapies: there are, in facts, evidences showing that consumption of pulses is linked to a lower CRC incidence rate.[42–46] Furthermore, some studies showed that it would be enough to include in the diet the consumption of approximately 100 g of legumes per week to prevent many forms of cancer, including CRC.[47]

Chemical characterization of the total extract (1H-NMR)

The metabolic profile of the total extract was characterized by NMR spectroscopy data exploited for primary and secondary metabolites identification following the approach developed for the analysis of complex plant extracts.48–53 The identification of metabolites was based on the analysis of mono and bidimensional NMR spectra and is in agreement with data from previous literature.54 Overall, 1H-NMR profile (Fig. 4) revealed the presence of amino acids (alanine, valine, serine, threonine, methionine, cysteine, arginine, aspartate, glutamate, proline, glycine, tryptophan, tyrosine, phenylalanine), organic acids (acetate, lactate, citrate, succinate, GABA, nicotinic acid), sugars (glucose, sucrose, raffinose among the most abundant), choline and uracil. In addition to previously reported results,54 we also observed trigonelline and a significant amount of broad resonances (indicated by red arrows in Fig. 4), corresponding to proteins, as confirmed also by typical TOCSY correlations in the amide NH region (Fig. 3, ESI†).

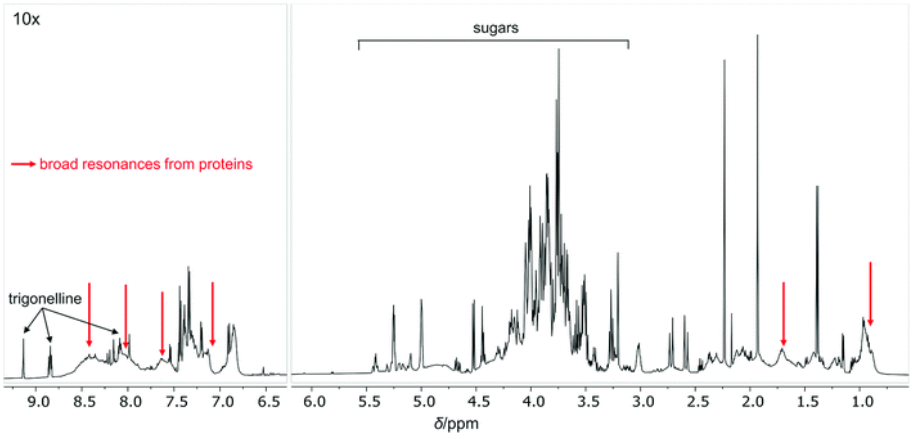

Fig. 4 1H-NMR profile of a total cowpea water boiled seed extract sample dissolved in H2O : D2O 9 : 1 at a final concentration of 10 mg mL−1, 25 °C, 600 MHz.

Fractionations of the bioactive components

To identify the molecules responsible for the selective cytotoxicity against CRC cells, the extract was progressively fractionated and tested to follow the bioactive component. The effects of the different fractions are expressed as the viability ratio between E705 cancer cells and CCD841 healthy cell line (Fig. 5A). Only fractions showing a viability ratio below 1 were considered active and further purified. At first, the separation through Centricon suggested that the bioactive component is a compound with a molecular weight higher than 3 kDa. DEAE chromatography and size exclusion chromatography (Fig. 4, ESI†) showed that it is a macromolecule with an overall negative charge at physiological pH, with an apparent molecular weight ranging from 8 kDa to 20 kDa. The P1, P2 and P3 fractions obtained by SEC were further loaded onto SPE C-18 Bond Elute cartridge to remove hydrophobic compounds. The active fraction (P1 plus P2) was effective also on the other sensitive CRC lines (Fig. 5B).

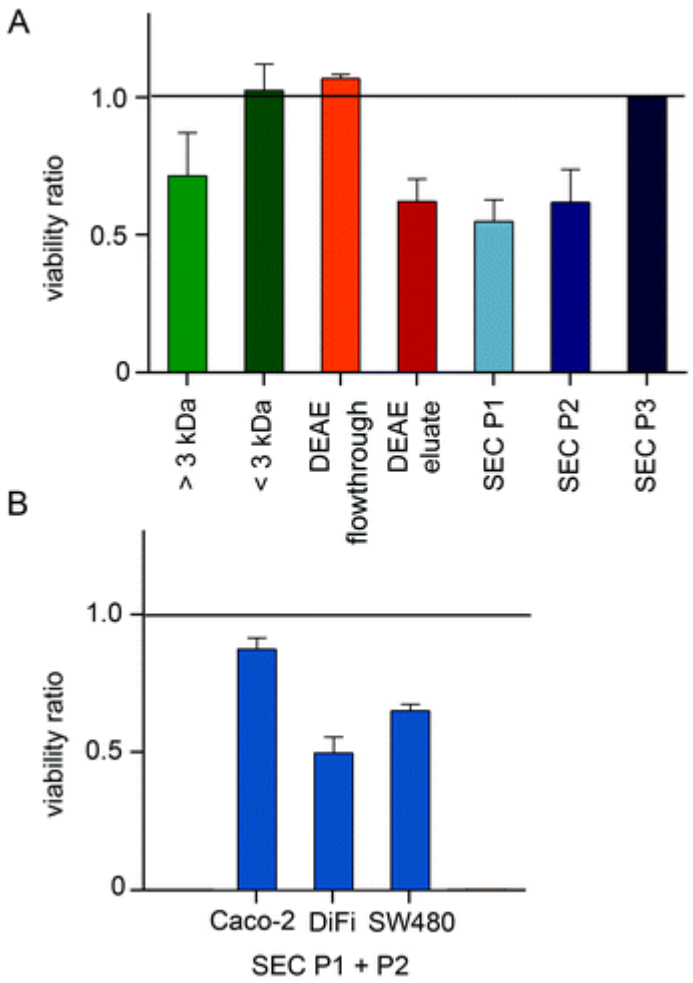

Fig. 5 Viability ratio of E705 compared to CCD841 cells treated with the different fractions obtained by the progressive steps of purification (3 kDa cut-off filter, DEAE chromatography and P1, P2, P3 of the SEC) (A). Viability ratio of Caco-2, DiFi, SW480 compared to CCD841 cells treated with the SEC-purified fraction (P1 plus P2) (B). Values are mean ± SEM.

Protein-dependent selective cytotoxic properties of the extract

The above mentioned purification procedures led to obtain a set of samples with a variety of protein concentrations within the

phytocomplex, so that E705 and CCD841 cells were treated with a wide range of samples with different protein concentrations (from 0 to 115 µg mL−1). To test the effect of protein concentration on the viability of E705 and CCD841 cells a regression model was set up (see Experimental). Results highlighted a clear negative effect of protein concentration on the viability of E705 cells with significant effects already at a concentration around 30 µg mL−1, while in the healthy cell line the effect was much lower and detectable only at doses higher than 90 µg mL−1 (Fig. 6). Therefore, proteins appeared to be the effective component in the selective cytotoxic activity of the extract.

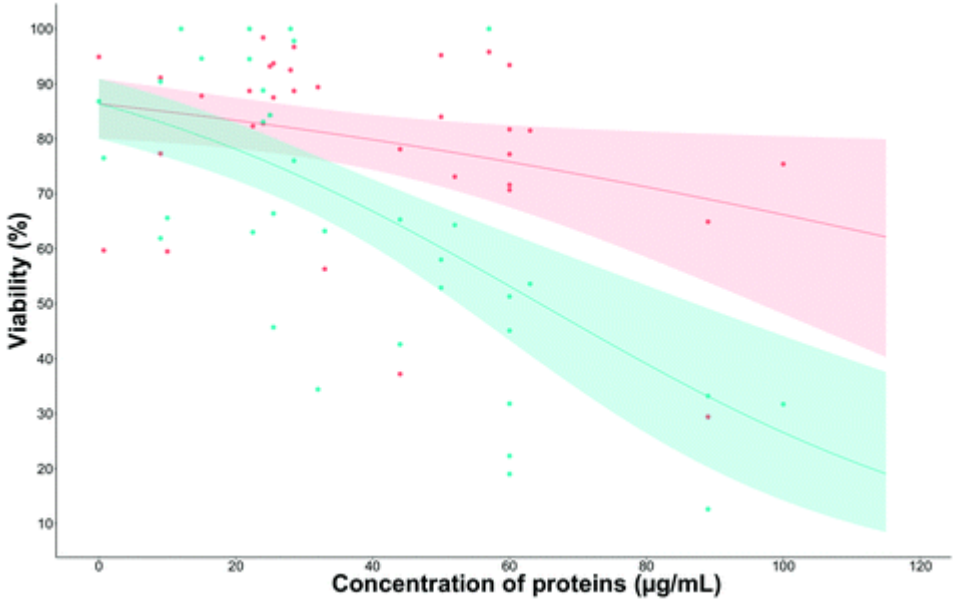

Fig. 6 Model showing the effect of protein amount on CCD841 (red line) and E705 (blue line) cell lines.

Proteomic assessment of bioactive fractions

The protein composition of all the fractions separated by SEC was characterized by LC-MS/MS using a shotgun proteomic approach, a gel free technology that allows to identify all the proteins in a sample by obtaining sequence information without previous purification of each single protein.55 The differential analysis between the active fraction data (P1 plus P2) and fraction P3, which did not show any biological activity, allowed to identify the proteins exclusively present in P1 + P2 fractions, most probably responsible for the bioactivity. The analysis was repeated on two different preparations to verify the reproducibility of the results. The list of these proteins is reported in Table 1.

Table 1 List of the proteins present exclusively in P1 + P2 fractions. The protein composition of all the fractions was determined by a shotgun MS/MS strategy. The Table reports the proteins identified exclusively in P1 and P2 fractions upon differential analysis between P1 plus P2 and P3 mass spectrometry data. AA = number of amino acids, MW = molecular weight

Accession	Description	Score	Coverage	Unique peptides	Peptides	AA	MW kDa	Calc. pI
XP_027903254.1	Serpin-ZX [*Vigna unguiculata*]	54.39	48.35	2	16	424	46.9	7.64
XP_027916766.1	Subtilisin inhibitor 1 [*Vigna unguiculata*]	9.36	43.88	2	2	98	11.1	5.01
XP_027922998.1	Bowman-Birk type seed trypsin and chymotrypsin inhibitor-like [*Vigna unguiculata*]	5.57	18.42	1	1	114	12.4	5.22
XP_027917589.1	Heat shock 70 kDa protein [*Vigna unguiculata*]	38.29	7.4	3	3	649	71.1	5.39

NP_001304197.1	Heat shock cognate 70 kDa protein 2-like [*Vigna radiata*]	17.40	4.17	2	2	648	71.2	5.25
XP_014497815.1	Heat shock 70 kDa protein 17 [*Vigna radiata* var. radiata]	4.35	2.58	1	1	892	99.0	5.60
XP_027923838.1	Class I heat shock protein-like [*Vigna unguiculata*]	3.84	11.43	1	1	140	16.3	6.64
XP_027903882.1	glutaredoxin-like [*Vigna unguiculata*]	21.83	21.67	2	2	180	19.6	8.05
XP_014497624.1	Probable mediator of RNA polymerase II transcription subunit 37c [*Vigna radiata* var. radiata]	12.52	4.16	2	2	649	71.1	5.29
XP_027920371.1	Glycine-rich RNA-binding protein 2, mitochondrial-like [*Vigna unguiculata*]	5.87	9.15	1	1	306	29.9	4.92

Among the different proteins identified, the Bowman-Birk domain trypsin and chymotrypsin inhibitor (BBI) was identified. Many studies are available concerning this family of peptides isolated from pulses (especially lentils and soybean) showing beneficial effects in CRC prevention.[47,56,57] They are small homodimeric peptides of 107 amino acids characterized by high variability among and also within legume species. The most conserved feature is the presence of 4 disulphide bridges within each monomer responsible for their high stability during the digestive processes, so that they are able to resist to pH levels equal to 1.5 without modifications that may affect their bioactivity. This means that the majority of the peptides assumed is able to reach intact the intestine and the colon.[47,58] This study showed that the BBI is maintained after 1-hour boiling of beans before the extraction procedure. Thus, once cooked and eaten it is very likely to reach the colon, where it

is able to carry out its chemopreventive function. A recent work studying the effect of purified BBI from *V. unguiculata* on different breast cancer cell lines showed the internalization of the peptide in cells (probably mediated by endocytosis, since no receptors or carrier proteins were found on the cellular membrane to promote its internalization) and its ability to enable the proteasome 20S functionality, activating a series of processes within cells that lead to apoptosis.[43] BBI internalization could explain the cytotoxic effect against CRC non-responsive cell lines to cetuximab. The main biological function of the Bowman Birk inhibitor peptides in plants is related to defense mechanisms against predation and parasites through the inhibition of the digestion of proteins and peptides, preventing insects feeding.[59,60] The biological activity shown by cowpea BBI against many pests is well known, so that many staple species such as rice and tomato have been genetically modified by using this gene to improve their resistance.[61,62] This aspect sheds new light on the possibility of finding suitable cultivation strategies to enhance the nutraceutical value of cowpea beans *e.g.*, cultivation without the use of pesticides that could be hypothesised to elicit the production of these peptides by plants, therefore coupling healthy nutrition to environmental sustainability.

Conclusions

Legumes are fundamental raw food items supporting human diet, not only as a source of macronutrients such as proteins, starch, fibers and micronutrients (amino acids, vitamins, minerals), but also for their bioactive molecules (mainly polyphenols and peptides) able to provide benefits to human health. Our study focused on one minor African species, *V. unguiculata*, not only for its importance for the economy and the sustenance of African population, but also for its adaptability and ease of cultivation. The results showed a chemopreventive action of *V. unguiculata* beans extract against different colorectal cancer cell lines, without affecting the healthy cell line and its ability to reduce cetuximab

dose in colon cancer therapy. Based on the proteomic characterization and according to the literature the Bowman-Birk serine-protease inhibitor is supposed to be the main active component. The ability of this peptide to resist against boiling and low pH levels (such in the stomach) increases the feasibility of considering legumes such as the cowpea as fundamental supporters to contrast many different forms of CRC in the dietary context.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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9.3 Protective effect of *Vigna unguiculata* extract against aging and neurodegeneration.

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Abstract

Aging and age-related neurodegeneration are among the major challenges in modern medicine because of the progressive increase in the number of elderly in the world population. Nutrition, which has important long-term consequences for health, is an important way to prevent

diseases and achieve healthy aging. The beneficial effects of *Vigna unguiculata* on metabolic disorders have been widely documented. Here, we show that an aqueous extract of *V. unguiculata* beans delays senescence both in *Saccharomyces cerevisiae* and *Drosophila melanogaster*, in a Snf1/AMPK-dependent manner. Consistently, an increased expression of FOXO, SIRT1, NOTCH and heme oxygenase (HO) genes, already known to be required for the longevity extension in *D. melanogaster*, is also shown. Preventing α-synuclein self-assembly is one of the most promising approaches for the treatment of Parkinson's disease (PD), for which aging is a risk factor. *In vitro* aggregation of α-synuclein, its toxicity and membrane localization in yeast and neuroblastoma cells are strongly decreased in the presence of bean extract. In a *Caenorhabditis elegans* model of PD, *V. unguiculata* extract substantially reduces the number of the age-dependent degeneration of the cephalic dopaminergic neurons. Our findings support the role of *V. unguiculata* beans as a functional food in age-related disorders.

Keywords: *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, human α-synuclein, Parkinson's disease (PD)

INTRODUCTION

Nutrients and their metabolites control energy balance, enzymatic activities and genome stability throughout the lifecycle. It is an unequivocal statement that nutritional deficiency as well as excess contribute to the aging process. Dietary restriction is known as the most effective longevity intervention ranging from yeast to primates [1–6]. Several results have suggested new roles of key nutrients in the protection against aging and age-related disorders [5, 7]. Thus, there is an increasing interest in nutrition as a way both to prevent diseases and to reach healthy aging. Much of the current knowledge on the molecular mechanisms of aging comes from lifespan studies on short-lived model organisms, such as the budding yeast *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans* [8]. Specifically,

AMPK (AMP-activated protein kinase), IGF (insulin-like growth factor) and TORC1 (target of rapamycin kinase complex 1) signaling pathways play key functions in regulating aging [9–11]. Neurodegenerative diseases, characterized by aberrant aggregates of the presynaptic protein α-synuclein, are collectively referred to as synucleinopathies, the second most common group of neurodegenerative diseases [12–14]. One of the most common synucleinopathies is Parkinson's disease (PD) and autosomal dominant forms of PD have been linked to mutations in α-synuclein. In PD patients, neurodegeneration is found predominantly in dopaminergic neurons. Despite the advances in the study of these pathologies, the detailed molecular mechanism of neuronal degeneration is still largely unknown. Several studies underline the relevant role of cellular models for a better understanding of the molecular regulation of human pathologies [15]. As such, budding yeast has been extensively employed in models of synucleinopathies [16–19]. In addition, an age-related degeneration of dopaminergic neurons has been shown in wild-type *C. elegans* [20]. Interestingly, neuronal and dendritic loss are accelerated and more severe when human α-synuclein is expressed in dopaminergic neurons in *C. elegans* [21]. *Vigna unguiculata* (L.) Walp. or cowpea is the most relevant *Vigna* species for human food. It is cultivated in tropical and subtropical zones of the world, including Africa, Asia, Latin America and also in some Mediterranean countries [22, 23]. Cowpea seeds are a good source of proteins, which mainly consist of globulins (vicilins or 7S globulins) and, to a lesser extent, albumins, glutelins and prolamins [24]. From a nutritional point of view, there is a high ratio of essential-to-non-essential amino acids, which is over 50%, suggesting the potential capacity of cowpea to cover human nutritional requirements [25, 26]. Moreover, bioactive peptides with antioxidant activity are successfully obtained from enzymatic proteolysis of cowpea proteins, indicating also its potentiality as a functional food [24]. In comparison with other legumes, cowpea has a low-fat content with high level of unsaturated fatty acids and is also characterized by a high proportion of carbohydrates (mainly dietary fibers and resistant starch) [24]. Apart from

the relevant source of essential macronutrients, cowpea also constitutes an interesting source of micronutrients [27]. All these features, together with the presence of minerals (calcium, iron and zinc) and phytochemicals, such as phenolic compounds, are attracting the attention of consumers and researchers, also because of its beneficial properties for health, including anti-diabetic, anti-cancer, anti-hyperlipidemic, anti-inflammatory and anti-hypertensive properties [28]. The aim of the present study is to investigate whether *V. unguiculata* also has anti-aging and neuroprotective effects, exploiting different model organisms to address complementary aspects. We show that an aqueous extract from *V. unguiculata* beans increases lifespan in yeast cells, being dependent on Snf1/AMPK (sucrose-non-fermenting/AMP-activated protein kinase) and Ras/PKA (Rat sarcoma/protein kinase A) pathways. Its pro-longevity feature is also confirmed on the multicellular organism *D. melanogaster*, which is consistent with the increased expression of AMPK-dependent genes associated with fly lifespan extension. Cowpea extract is able to significantly reduce the aggregation of α-synuclein *in vitro* and to attenuate its toxicity both in yeast and neuroblastoma cells. Remarkably, in a nematode model expressing human α-synuclein, the age-dependent degeneration of the dopaminergic neurons is strongly reduced under chronic treatments with *V. unguiculata* extract.

RESULTS

Vigna unguiculata extract extends lifespan in yeast cells

Considering the nutritional properties and positive effects for health of *Vigna unguiculata* [28], we investigated the composition of aqueous bean extracts from *V. unguiculata* in comparison with those obtained from *Cajanus cajan* L. and *Phaseolus vulgaris* L., originating from the Arusha area in Tanzania. *V. unguiculata* extract was characterized by a higher starch amount and less proteins compared to the extracts obtained from the other pulses, while the percentage of total amino acids was comparable among species (Figure 1A–1C). We also confirmed that

cowpea seeds are a good source of amino acids (included the essential ones), as well as of unsaturated fatty acids (more abundant in comparison with the other two beans), confirming its nutritionally desirable features (Supplementary Tables 1, 2) [24].

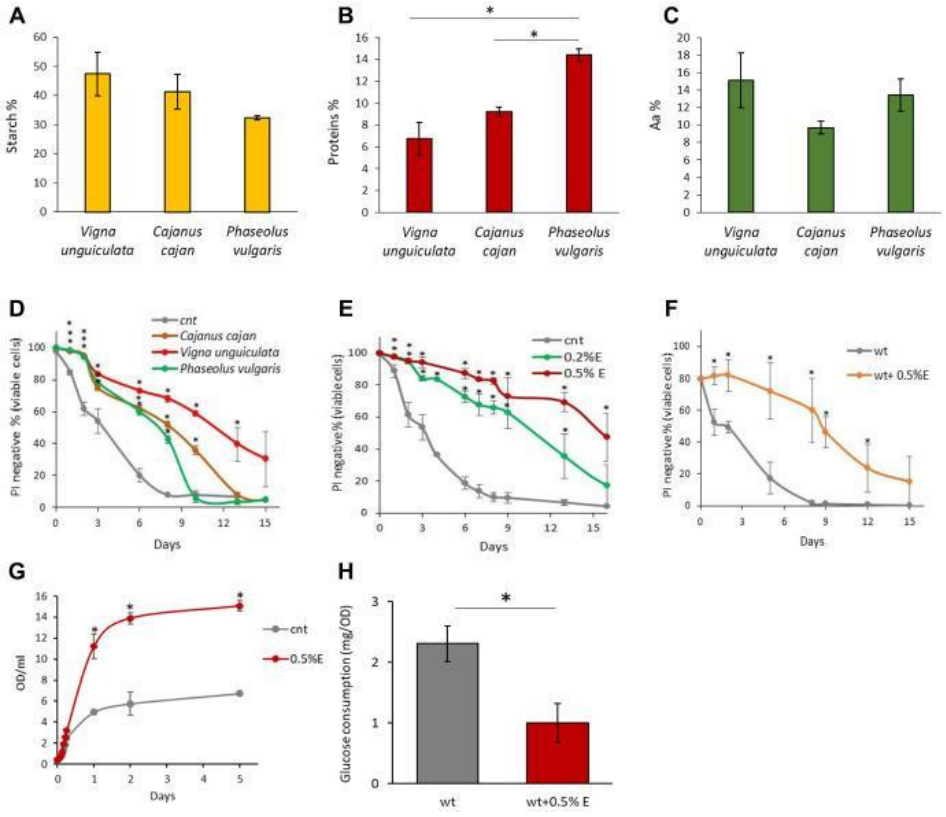

Figure 1

Chemical properties of bean extracts. (**A**) Starch content, (**B**) protein content and (**C**) amino acid content in *V. unguiculata*, *C. cajan* and *P. vulgaris* extracts. *p<0.05. (**D**) CLS of yeast cells grown in the absence or presence of 0.2% *V. unguiculata*, *C. cajan* and *P. vulgaris* extracts. *p<0.05 relative to control cells. (**E**) CLS of yeast cells grown in SD medium containing 2% glucose in the absence or presence of 0.2% or 0.5% *V. unguiculata* extract, added in exponential phase of growth. *p<0.05

relative to control cells. (**F**) CLS of yeast cells grown in SD medium containing 2% glucose in the absence or presence of 0.5% *V. unguiculata* extract, added to cells in stationary phase (and not in exponential phase, as in the other experiments). *p<0.05 relative to control cells. (**G**) Growth curves of yeast cells grown in SD medium containing 2% glucose in the absence or presence of 0.5% extract *V. unguiculata*. *p<0.05 relative to control cells. (**H**) Glucose consumption (mg/OD) of yeast cells grown in SD medium in the absence or presence of 0.5% *V. unguiculata* extract, measured on growth media sampled at multiple time points during exponential phase of growth (0.2-2.5 OD/ml). *p<0.05 relative to control cells.

To explore if these differences could have an impact on the longevity of yeast cells, exponentially growing cells were treated with 0.2% of the extracts from *V. unguiculata*, *C. cajan* or *P. vulgaris* beans and chronological lifespan was monitored by measuring the viability of the cultures throughout time. Although all the extracts increased longevity of yeast cells, the highest response was evident in the presence of *V. unguiculata* one, with a mean lifespan increasing from 3 days to about 9.5 days (Figure 1D, Table 1). On the basis of the above results, we decided to continue our analysis by using only the cowpea extract. A strong dose-response effect on yeast longevity was observed by increasing the concentration of *V. unguiculata* extract in the culture (from 0.2% to 0.5%), since it was able to extend the mean lifespan up to 16 days at the higher concentration (Figure 1E, Table 1). Its anti-aging properties were evident also when the extract was added to "aged" cells, *i.e.* after they had already entered the stationary phase (Figure 1F). Remarkably, cowpea extract not originating from Arusha maintained the same effect, letting us to suppose that the origin of the beans has no relevant impact on its anti-aging features (data not shown).

Table 1

Mean and maximal lifespan of wt cells grown in the presence of the indicated extracts.

lifespan (days)		
wt strain	**mean**	**maximal**
cnt (no extract)	3.05 ± 0.42	9.46 ± 1.47
0.2% *P. vulgaris*	6.42 ± 0.12	11.72 ± 0.05
0.2% *C. cajan*	7.74 ± 0.30	13.53 ± 0.32
0.2% *V. unguiculata*	9.55 ± 0.49	18.55 ± 0.64
0.5% *V. unguiculata*	16.03 ± 3.01	>20

Cell growth was then monitored in the presence of the highest concentration of the extract. Although the growth rate in the presence of 0.5% extract showed only a minor increase in exponential phase, the final biomass of the population was more than doubled in comparison with the control (Figure 1G). On the other hand, the consumption of glucose in the media during the exponential phase of growth was strongly reduced (more than 50%), suggesting that the presence of either starch or proteins induced a decrease of glucose uptake from the medium (Figure 1H). However, the lower glucose consumption in the presence of the extract have no effect on the experimental determination of CLS, which starts after carbon source exhaustion. Importantly, the anti-aging effect of the extract was synergistic with caloric restriction, one of the most effective non-genetic interventions known to promote lifespan extension in several model organisms [10] (Supplementary Figure 1). Overall, the data presented indicate that cowpea aqueous extract extends chronological aging in yeast cells. To increase our knowledge on the composition of *V. unguiculata* extract we performed a proteomic analysis. Interestingly, we identified 174 proteins with a molecular weight ranging from 200 to less than 20 kDa, of which 10% are oxidoreductase, 12% are stress response proteins while 25% are still uncharacterized (Figure 2A, 2B).

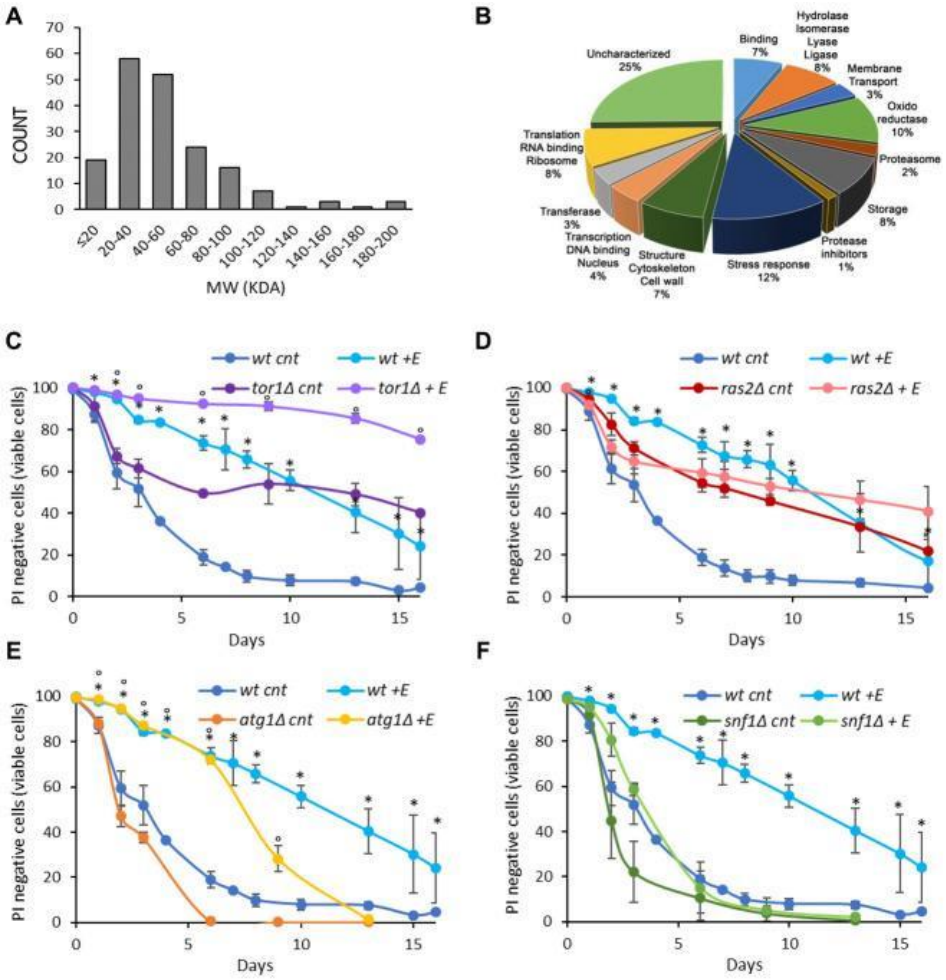

Figure 2

Cowpea extract extends yeast lifespan. (**A**, **B**) Analysis of *V. unguiculata* extract by mass spectrometry using a shotgun proteomic approach to identify all the proteins present in the sample. (A) MW distribution and (B) classification of the proteins identified. The data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017716. (**C**–**F**) CLS of wt and (**C**) *tor1Δ*, (**D**) *ras2Δ*, (**E**) *atg1Δ*, (**F**) *snf1Δ* cells, grown in SD medium containing 2% glucose in the absence or presence of 0.2% *V. unguiculata* extract. *p<0.05 relative to untreated wt cells, °p<0.05 relative to untreated

mutant cells. Curves of wt untreated cells and treated with the extract were repeated in **C**–**F**.

The signaling pathways connected to longevity regulation are well known in yeast. Among them, the Snf1/AMPK (sucrose-non fermenting/AMP-activated protein kinase) and the autophagic pathways are anti-aging pathways, while the Ras2/PKA (Rat sarcoma/protein kinase A) and the TORC1 (target of rapamycin complex 1) pathways are pro-aging ones [8, 9, 29]. To identify through which of them the cowpea extract extended yeast chronological lifespan, we tested its effect on mutants bearing deletion in one of the aforementioned pathways (*snf1Δ*, *atg1Δ*, *ras2Δ*, *tor1Δ*) (Figure 2C–2F, Table 2). The anti-aging effect of 0.2% cowpea extract was still evident in *tor1Δ* and *atg1Δ* strains (Figure 2C, 2E, Table 2), while it was strongly reduced in *ras2Δ* and *snf1Δ* mutants (Figure 2D, 2F, Table 2), indicating that the Ras/PKA and the Snf1/AMPK pathways are involved in mediating the anti-aging effect of cowpea extract in yeast cells.

Table 2

Mean and maximal lifespan of mutant strains grown in the presence of 0.2% *V. unguiculata* extract. Data of wt cells were repeated for clarity.

lifespan (days)				
	mean		**maximal**	
strain	**cnt**	**0.2% E**	**cnt**	**0.2% E**
wt	3.05 ± 0.42	9.55 ± 0.49	9.46 ± 1.47	18.55 ± 0.64
ras2Δ	7.48 ± 0.40	10.48 ± 4.98	18.95 ± 1.34	>20
tor1Δ	5.87 ± 0.23	>20	18.5 ± 0.14	>20
atg1Δ	2.22 ± 0.10	7.35 ± 0.21	4.95 ± 0.21	11.45 ± 0.78

snf1Δ	2.0 ± 0.42	3.35 ± 0.21	4.8 ± 2.40	7.2 ± 1.56

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Vigna unguiculata extract supplementation extends lifespan in *Drosophila melanogaster*

To investigate the pro-longevity effect of the cowpea extract also in a multicellular organism, female Canton S flies were lifelong supplemented with 0.2% or 0.5% *V. unguiculata* extract. A significant marked increase in mean lifespan was observed in flies supplemented with 0.2% bean extract in respect to controls (40.09±1.08 days *vs* 31.82±0.86 days; 25.99% increase), while mean lifespan of flies supplemented with 0.5% bean extract was comparable to that of control flies (Figure 3A). These data are only partially in agreement with the results obtained in yeast cells, where the 0.5% cowpea supplementation was more effective than the 0.2% one. Nevertheless, considering only the survivorship data obtained after a 3 weeks supplementation, a higher survival of flies supplemented with 0.5% in respect to both 0.2% supplement and control was observed (Supplementary Figure 2A, 2B).

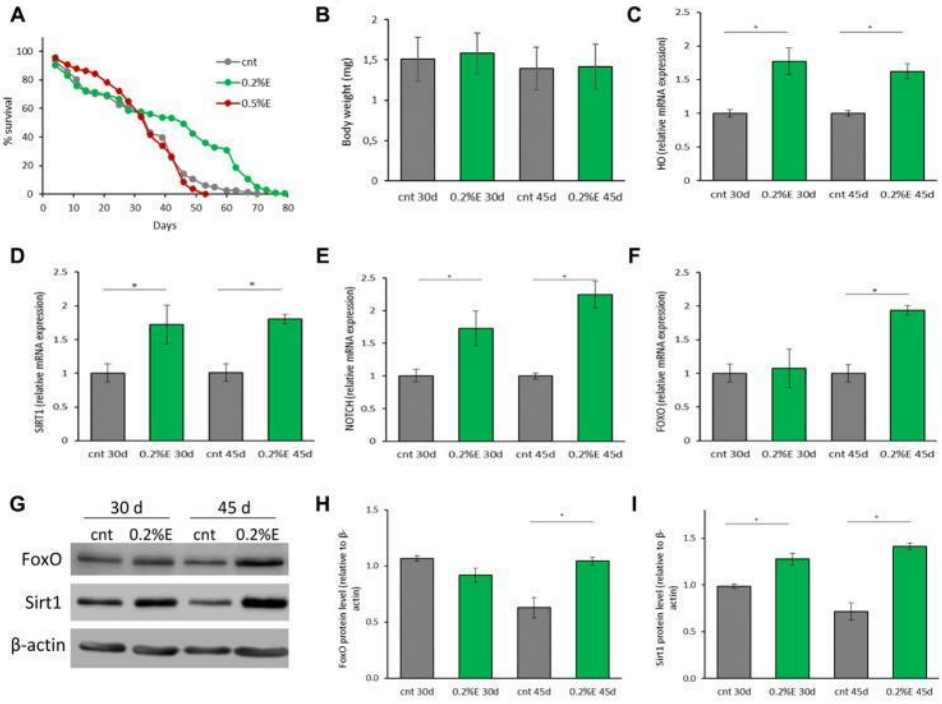

Figure 3

Cowpea extract extends *D. melanogaster* lifespan. (**A**) Survivorship of adult female *D. melanogaster*. Flies were supplemented with 0.2% and 0.5% bean extract lifelong. (**B**) Body weights of *D. melanogaster* supplemented with 0.2% bean extract. Flies were supplemented with 0.2% bean extract for 30 or 45 days. (**C**–**F**) Expression of genes related to longevity and oxidative stress. Flies were supplemented with 0.2% cowpea extract for 30 or 45 days. Total RNA was isolated and the mRNA levels of HO (**C**), SIRT1 (**D**), NOTCH (**E**), FOXO (**F**) were quantified using RT-PCR. (**G**) Western analysis using anti-FoxO and anti-Sirt1 antibodies on proteins extracts from flies supplemented with 0.2% cowpea extract for 30 or 45 days. (**H**–**I**) Densitometric analysis of FoxO and Sirt1 proteins. *p<0.05 with respect to the corresponding controls. To verify whether the increase of the mean lifespan in the presence of 0.2% extract was due to bean extract supplementation itself and not to CR induced by bean extract off-flavor, the body weights of flies were recorded at 30 and 45

days. No differences in fly body weights were observed, suggesting an equal food uptake in control and supplemented groups (Figure 3B). To better clarify, at a molecular level, the positive effect of 0.2% cowpea extract supplementation on *D. melanogaster* lifespan, the expression of genes involved in preserving cellular homeostasis and longevity was investigated. Flies were supplemented with cowpea extract for 30 or 45 days and the expression of genes involved in aging-related signaling pathways (SIRT1 -sirtuin 1-, FOXO -Forkhead box O- and NOTCH) and antioxidant defense systems (HO - heme oxygenase and TRXR - thioredoxin reductase) were measured (Figure 3C–3F). Oxidative stress has been recognized to play a key role in aging [30]. The oxidative stress theory of aging speculates that the functional losses typical of elderly are associated with the accumulation of structural impairments caused by the oxidative damage to macromolecules [31]. HO expression was significantly up-regulated by cowpea extract supplementation after both 30 and 45 days (Figure 3C), while TRXR was not influenced at all (data not shown), suggesting that *V. unguiculata* extract partially modulates the endogenous antioxidant defense system. SIRT1, a member of the class III NAD+-dependent histone deacetylases (HDACs) has been implicated in the extension of longevity in *D. melanogaster* [32]. SIRT1 expression was significantly up-regulated in flies supplemented with cowpea extract after both 30 and 45 days (Figure 3D). Remarkably, also NOTCH expression increased at both time points (Figure 3E), in accordance with findings showing that SIRT1 is a positive modulator of NOTCH [33]. FOXO is a fundamental transcriptional regulator of the insulin pathway modulating growth and proliferation and its increase has been associated with extension of flies lifespan [34]. Although FOXO expression after 30 days of supplementation with *V. unguiculata* was the same as in control flies, cowpea extract triggered a significant up-regulation of FOXO expression at 45 days (Figure 3F). In agreement with the gene expression, the level of FoxO and Sirt1 proteins increased in flies supplemented with cowpea extract (Figure 3G–3I).

Thus, the aging-related signaling pathways of SIRT1, FOXO and NOTCH are involved in mediating the effect of cowpea extract in fruit flies.

Vigna unguiculata extract reduces both α-synuclein toxicity and aggregation *in vitro*

Extensive literature reports the fibrillation-inhibiting effects of plant extracts, including those consumed as part of a healthy diet [35, 36] and others found in traditional medicine [37–40]. α-Synuclein is a presynaptic protein associated with the pathophysiology of synucleinopathies, including Parkinson's disease [12–14], and budding yeast has been extensively employed in models of synucleinopathies [16]. Thus, the effect of *V. unguiculata* extract on the longevity of yeast cells over-expressing the human α-synuclein [41] was evaluated. Interestingly, the addition of cowpea extract to exponentially growing cells strongly reduced the toxic effects of α-synuclein with a significant marked increase in mean lifespan (11.19 ± 2.18 days *vs* 2.22 ± 0.31 days; 404% increase) (Figure 4A, Table 3). Although α-synuclein protein was still present 3 days after the treatment (Supplementary Figure 3), it was less localized to the plasma membrane, as shown by immunofluorescence analysis (Figure 4B, 4C) and cell fractionation (Figure 4D). These data suggest that a different localization of α-synuclein, rather than its protein clearance, could be responsible for the reduced toxicity in the presence of the bean extract.

Table 3

Mean and maximal lifespan of yeast cells bearing pYX242 empty vector or pYX242-SNCA plasmid grown in the absence or presence of 0.5% *V. unguiculata* extract.

	lifespan (days)	
	mean	**maximal**

strain	cnt	0.5% E	cnt	0.5% E
[pYX242]	9.96 ± 0.49	13.3 ± 1.13	>15	>15
[pYX242-SNCA]	2.22 ± 0.31	11.19 ± 2.18	14.21 ± 1.06	>15

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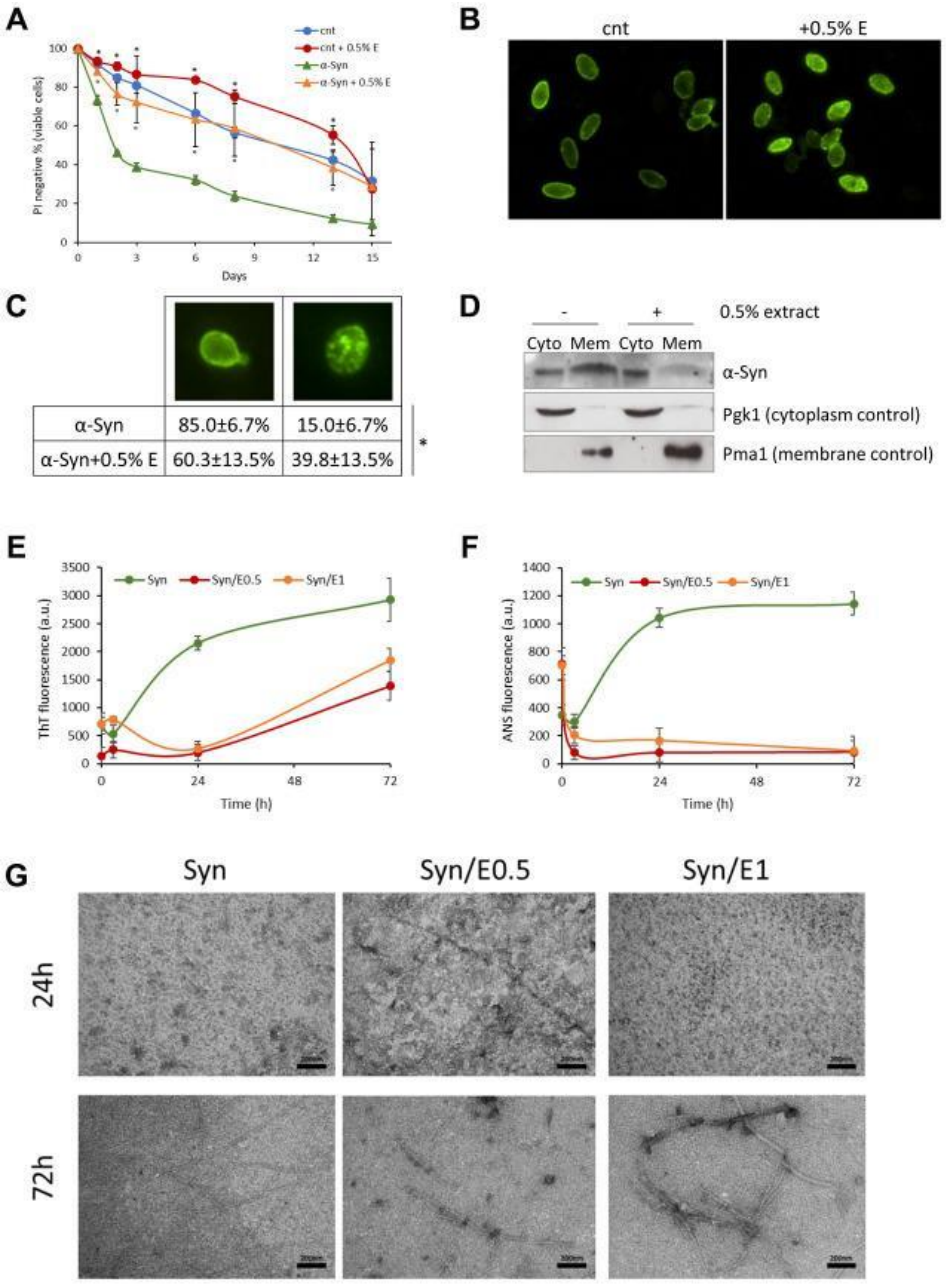

Figure 4

Cowpea extract reduces α-synuclein toxicity and aggregation. (**A**) CLS of yeast cells bearing pYX242 empty vector or pYX242-SNCA plasmid grown

in SD medium containing 2% glucose in the absence or presence of 0.5% *V. unguiculata* extract. *p<0.05 relative to untreated cells bearing the empty vector, °p<0.05 relative to untreated α-synuclein expressing cells. (**B**, **C**) Immunofluorescence showing localization of α-synuclein in cells untreated or treated for 1 day with 0.5% *V. unguiculata* extract. The percentage of cells with α-synuclein localized in the cellular membrane is shown in (**C**). *p<0.05. (**D**) Western analysis using anti-α-synuclein antibody on cytolpasmic and membrane fractions isolated from wt [pYX242-SNCA] cells after 1-day treatment with 0.5% *V. unguiculata* extract. Pgk1 was used as cytoplasmic marker, Pma1 as membrane marker. (**E**, **F**) α-synuclein aggregation process followed by ThT fluorescence (**E**) and ANS binding (**F**) assays. (**G**) TEM pictures taken from α-synuclein aggregation mixture after 24 h and 72 h of incubation in the absence or in the presence of cowpea extract at molar ratio protein:extract 1:0.5 (E0.5) and 1:1 (E1); scale bars are shown. The process of α-synuclein fibrillation was then investigated *in vitro* at two different concentrations of cowpea extract. The increase of ThT and ANS fluorescence emission intensity was used to quantify fibrils formation and conformational change of the protein with or without cowpea extract (Figure 4E, 4F). The presence of *V. unguiculata* extract led to a significant concentration-independent decrease of ThT fluorescence in the α-synuclein aggregation solution, with an increase of the lag time and a decrease of both β-sheet growth rate and final equilibrium levels (Figure 4E). In agreement with a nucleation-dependent polymerization model, α-synuclein exhibited a sigmoidal binding without cowpea extract (Figure 4E). These evidences suggest that *V. unguiculata* extract significantly altered the amyloid aggregation pattern of α-synuclein. Moreover, the ANS binding fluorescence data indicate that the cowpea extract might increase the formation of α-synuclein species with minor solvent exposure of hydrophobic clusters, or it might decrease the binding of ANS to α-synuclein surfaces (Figure 4F). The morphology of α-synuclein aggregates was also studied by TEM analysis. After 24 h of aggregation, the protein, either alone or in the presence of cowpea extract, existed as

globular micelle-like and prefibrillar assemblies (Figure 4G). After 72 h of aggregation in the absence of the extract, α-synuclein samples were mostly mature fibrils. Remarkably, the presence of cowpea extract enriched the samples with short fibrils covered by densely packed globular clusters (Figure 4G). Overall, these findings are consistent with an inhibitory effect of *V. unguiculata* extract on the formation of amyloid fibrils. It has been reported that cytotoxicity of amyloidogenic species largely depends on their biophysical surface properties, which influences their reactivity with the cellular plasma membrane [42–44]. To assess α-synuclein toxicity, we performed MTT assays on the human neuroblastoma SH-SY5Y cell line exposed for 48 h to extracellular α-synuclein aggregates pre-formed *in vitro* in the absence or in the presence of cowpea extract. Coherently, α-synuclein obtained without extract supplementation exhibited the highest cytotoxicity: oligomers (Ol) and fibrils (Fib) showed about 70% and 50% viability, respectively (Figure 5A). In cells incubated with α-synuclein aggregates formed in the presence of the extract, toxicity was reduced and cell viability was about 83% with oligomers (Ol/E0.5) and 86% with fibrils (Fib/E0.5) increasing up to 100% and 95% at the highest concentration of the extract (Figure 5A). Accordingly, ROS level significantly decreased in cells exposed to oligomers and fibrils formed in the presence of cowpea extract (Figure 5B).

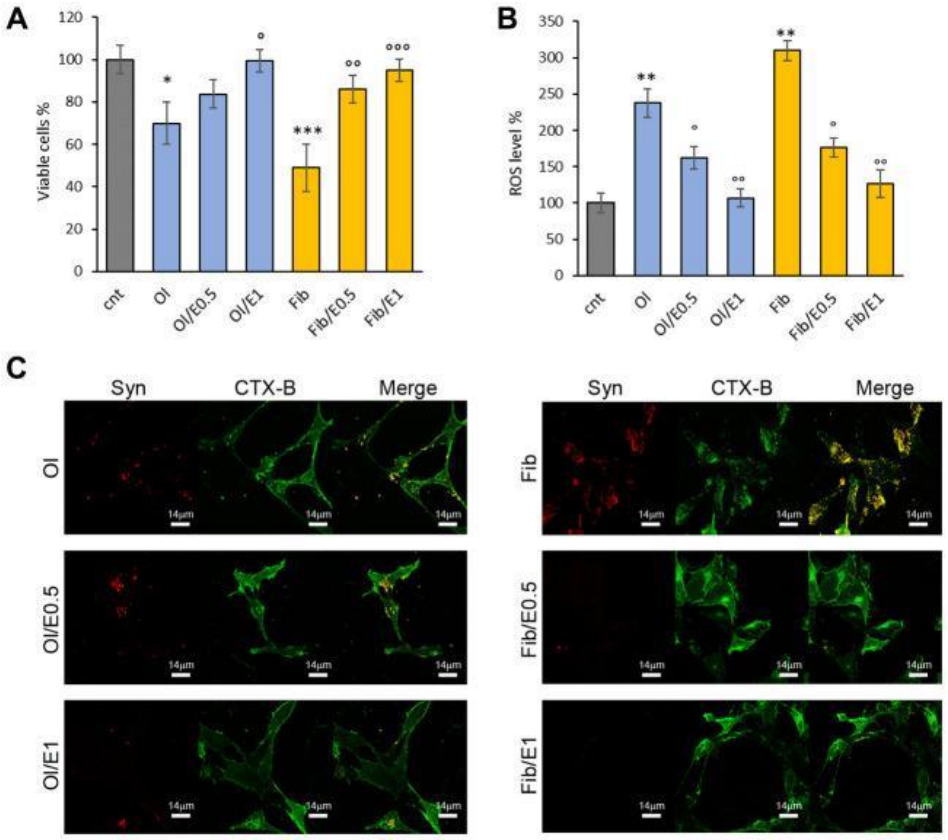

Figure 5

***V. unguiculata* extract reduces α-synuclein toxicity in neuroblastoma cells.** (**A**, **B**) SH-SY5Y cells were grown for 48 h in the absence (cnt) or presence of 5 µM α-synuclein solution obtained after 24 h (oligomers, Ol) and 72 h (fibrills, Fib) of aggregation, without or with extract at molar ratio protein:extract 1:0.5 (E0.5) and 1:1 (E1). (**A**) Cell viability assessed by MTT assay and (**B**) ROS level evaluated by DCFDA fluorescence intensity assays. *p<0.05; **p<0.01; ***p<0.001 *vs* untreated cells. °p<0.05; °°p<0.01 *vs* treated cells with α-synuclein aggregates oligomeric (Ol) and fibrillar (Fib) grown without extract. (**C**) Z-projection of SH-SY5Y cell images by α-synuclein immunostaining (red) and CTX-B plasma membrane staining (green). Scale bars are shown.

These data suggest that in the presence of *V. unguiculata* extract, α-synuclein aggregation is redirected into non-toxic aggregate species. To further explore the potential mechanism for the protective effect of the cowpea extract, the interaction of α-synuclein aggregates with the plasma membrane of neuroblastoma cells was monitored by confocal microscopy. As previously reported [43, 44], a large number of α-synuclein oligomers or fibrils (stained in red) were bound to the cell membrane (stained in green) (Figure 5C). When cells were exposed to α-synuclein aggregates formed in the presence of cowpea extracts, the binding of both oligomers and fibrils to the cellular membranes was drastically reduced (Figure 5C). In conclusion, these data show that the presence of cowpea extract during α-synuclein aggregation decreases the ability of the resulting aggregates to bind the plasma membrane and to raise ROS production and cytotoxicity.

Vigna unguiculata extract reduces α-synuclein induced neurodegeneration in *Caenorhabditis elegans*

In order to evaluate the neuroprotective effects of cowpea extract on a multicellular organism, we turned to the nematode *C. elegans.* The expression of human α-synuclein in *C. elegans* causes the age-dependent degeneration and death of the four cephalic dopaminergic neurons (CEP), a phenotype which can be easily scored using a red fluorescent marker expressed only in those neurons [45]. Consistent with previous reports, we observed an age-related decline in the number of fluorescent dopaminergic neurons expressing human α-synuclein (Supplementary Figure 4). Thus, we investigated the effects of *V. unguiculata* extract both at 0.2% and 0.5%, in 6-day adult animals (Figure 6A, 6B). While in mock treated animals a mean of 3 out of 4 CEP neurons died, in animals exposed to *V. unguiculata* extracts there was a partial rescue of neurodegeneration, with 2 neurons dying in 0.2% extract and only 1 in 0.5%. A similar effect was observed in animals treated with 3 mM valproic acid (positive control) [46].

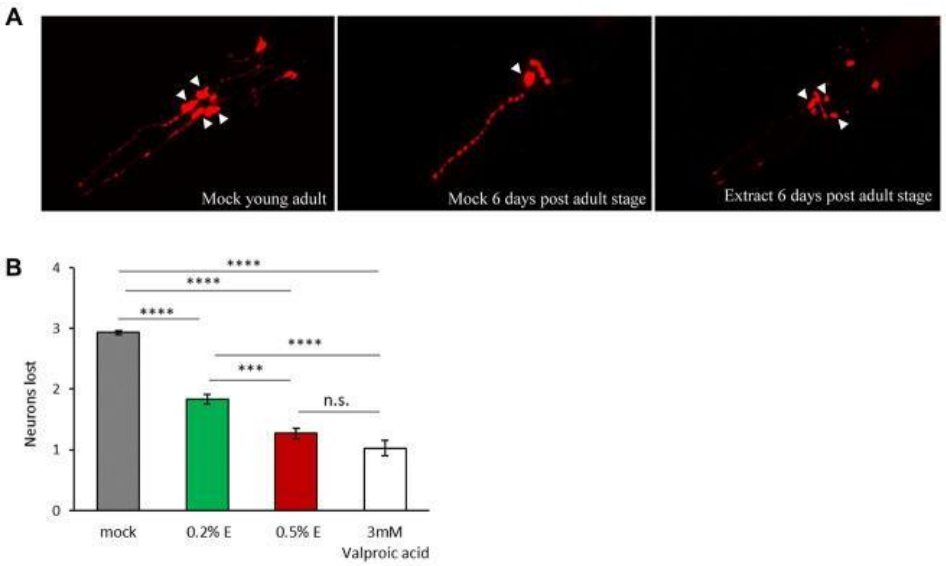

Figure 6

***V. unguiculata* extract is neuroprotective in a dose-dependent manner in a *C. elegans* model of α-synuclein toxicity.** (**A**) The four CEP neurons (indicated by arrowheads), expressing DsRed and human α-synuclein, are viable and with a wild type morphology in young adult animals cultivated in mock conditions (left panel); only one neuron is visible and viable after 6 days from adult stage cultivated in mock conditions (central panel); 0.5% *V. unguiculata* extract partially rescued the neurodegeneration after 6 days from adult stage (right panel). Anterior is to the left. (**B**) Quantification of dopaminergic neuron loss in human α-synuclein expressing animals grown with 0.2% and 0.5% of *V. unguiculata* extract. ***p<0.001, ****p<0.0001. The number of animals scored with mock is 95, with 0.2% extract is 103, with 0.5% extract is 94 and with 3 mM valproic acid 92.

Our results indicate that *V. unguiculata* extract protects CEP dopaminergic neurons from degeneration in a *C. elegans* model of α-synuclein toxicity, in a dose-dependent manner.

DISCUSSION

Cowpea is considered as a source of health-promoting compounds, with a low fat and high protein content, as well as dietary fibers, phenolic compounds and minerals. Consumption of cowpea is associated with reduced risk of gastrointestinal disorders, cardiovascular diseases, hypercholesterolemia, obesity, diabetes and several types of cancer [47]. We now add new important health benefits of cowpea beans, *i.e*. their anti-aging and neuroprotective effects. Indeed, we show that *V. unguiculata* extract extends lifespan, in two different eukaryotic models, such as budding yeast and fruit flies (Figures 1, 3). The extension of longevity requires Snf1/AMPK pathway in yeast (Figure 2F) and induces the upregulation of two downstream proteins of the AMPK pathway, such as FOXO and SIRT1 in *Drosophila* (Figure 3) [48–50]. Strikingly, it has been reported that AMPK and SIRT1 are downregulated with aging and their pharmacological activation is necessary to increase longevity [5]. The anti-senescence properties of cowpea extract are strongly additive with caloric restriction (Supplementary Figure 1), the most effective non-genetic intervention delaying senescence [51], suggesting that *V. unguiculata* beans could display their best effect in terms of aging delay in a proper dietary regimen. The strong neuroprotective features of cowpea extract are conserved in evolutionary distant eukaryotic systems. Indeed, *V. unguiculata* extract decreases α-synuclein toxicity in both yeast and neuroblastoma cells, as well as in a *C. elegans* PD model by partially rescuing the degeneration of cephalic dopaminergic neurons (Figure 6). The anti-aggregation properties of cowpea on α-synuclein is clearly evident (Figure 4E–4G). Along similar lines, the extract decreases the localization of α-synuclein to cell membrane both in the yeast model, in which α-synuclein is intracellularly expressed (Figure 4A–4D), and in neuroblastoma cells, where α-synuclein is added to the medium (Figure 5), also in keeping with the minor solvent exposure of hydrophobic clusters detected by ANS on amyloid assemblies (Figure 4F). These results suggest that *V. unguiculata* extract decreases the neurotoxicity caused by

the intracellular accumulation of α-synuclein aggregates and the cellular damage induced by oligomeric aggregates interacting with the cell membrane by displacing the toxic protein from the lipidic bilayer. Our data are in accordance with recent results showing that inhibition of α-synuclein binding to membranes reduces the toxicity of the protein both in worms and in mice [52, 53]. The aqueous extract of cowpea beans contains starch, amino acids, as well as several different proteins and peptides (Figures 1A–1C, 2A, 2B), while it is probably very poor in phenolic compounds. Although abundance of starch and proteins is generally considered negative from an aging point of view [10], the protection against senescence and neurodegeneration might be the result of a synergistic effect of different elements. Indeed, the nutrient combination of the extract rather than a single component might be responsible for the metabolic reprogramming, which leads to the longevity phenotype. Although the identity of the active components in the extract remains to be investigated, the extraction process strongly mimics the way in which these beans are consumed. Therefore, the anti-aging and neuro-protecting compounds are likely to be conserved during the cooking process. Remarkably, it has been reported that cooking legumes in water increases the insoluble fiber content, protein quality and digestibility, although with a reduction of the content of vitamins and minerals [47]. Therefore, the use of cowpea beans should be encouraged and eventually the identification of the bioactive compounds could lead to the development of specific dietary supplements to support healthy aging and to delay neurodegeneration. Any dietary intervention that has the potential of delaying the progression of age-related diseases could improve the quality of life of the aging population, inducing also an important impact on the economic implications of elderly on the society. Thus, *V. unguiculata* consumption in the global food chain is encouraging since our study suggests that cowpea beans supplementation can prevent age-related disorders. Although data on bioactive compounds from cowpea are still poor, some reports indicate components like peptides may contribute to health benefits derived from cowpea [28]. Remarkably,

several proteins identified by proteomic analysis are still uncharacterized (Figure 2A, 2B). We believe that additional work is necessary to discover the bioactive compounds in cowpea and their interactions to efficiently exploit them in foods, such as snacks and breakfast cereals by targeting benefits to immune function and health gut. Indeed, interesting data show that progression of PD has been frequently associated with dysbiosis of gut microbiota [54, 55]. In conclusion, considering the role of functional food in the management of age-related diseases, we strongly support the intake of *V. unguiculata* beans to reduce senescence, neuroinflammation and the extent of neurodegeneration.

MATERIALS AND METHODS

Extract preparation

V. unguiculata, *C. cajan* and *P. vulgaris* seeds were purchased from two markets (Kilombero and Arusha Central Market) in Arusha, Tanzania (3°22'0.01"S, 36°40'59.99"E). Seeds of each species were boiled for 1 h and left cool down for the subsequent hour. The treatment was performed to mimic the condition of consumption, as described in [56]. Then, seeds were incubated at 50°C overnight till dryness and grinded to obtain a fine powder. 2 g of seed dry powder were suspended in 50 ml of ultrapure MilliQ water. Then, pulses were extracted through a magnetic stirrer at 500 rpm for 5 minutes and centrifuged at 5000 g for 30 min. Supernatant was recovered and freeze-dried.

Chemical and proteomic characterization of the extracts

Starch content

Starch content was evaluated by the enzymatic assay Total Starch AOAC Method 996.1 1 and AACC Method 76.13 (Megazyme ®, Ireland). Briefly, 50 mg of extract were suspended in 200 µl of ethanol 80% v/v and 1 ml of 2 M KOH. Samples were magnetically stirred for 20 min at 4°C. Then, 4

ml of sodium acetate pH=3.8 were added, followed by the addition of 50 µl of α-amylase (8300 U/mL) and 50 µl of amyloglucosidase (AMG, 3300 U/ml). Samples were incubated for 30 min with intermittent mixing on a vortex mixer, then centrifuged for 10 min at 3000 rpm to recover the supernatant. In order to evaluate total starch content, a reaction mixture was prepared as follows in a quartz cell: 1 ml H2O, 25 µl of sample, 50 µl of a buffer solution pH = 7.6, 50 µl NADP+/ATP. The solution was incubated for 3 min at room temperature and then the absorbance was read at 340 nm against the blank. Then, 10 µl of a solution containing hexokinase (HK) and glucose-6-phosphate-dehydrogenase (G6PDH) was added. After an incubation of 5 min at room temperature, the absorbance was read against the blank again at 340 nm. Data are expressed as g of starch per 100 g of extract.

Amino acids content

Amino acids were quantified through a HPLC-DAD method. A 1260 Infinity II LC System (Agilent, USA, 2018) was set up for the analysis. The calibration curve was made up using an amino acid mixed solution (Merck, Germany) in a concentration range between 0.078 mM and 1.25 mM. The column was an Agilent Poroshell HPH C18 (100 x 4.6 mm, 2.7 µm) coupled with a guard column (AdvanceBio Oligo 4.6 x 5 mm, 2.7 µm) and it was kept at 40°C. Mobile phases were: A - 10 mM Na2HPO4 pH=8.2 and B - Acetonitrile:Methanol:Water 45:45:10. The elution program was the following (%B): 0-0.35 min 2%, 13.4 min 57%, 13.5 min 100%, 15.7 min 100%, 15.8 min 2%, 18 min end. Flow rate was constant at 1.5 ml/min. All solvents were HPLC grade, whereas the buffer, solutions and samples were pre-filtered with a 0.22 µm filter. OPA (o-Phthaldialdehyde reagent, Merck, Germany) was chosen as derivatizing agent acting as a fluorophore. Injection volume was 10 µl. The signal used to visualize the fluorescence was set at 338 nm bandwidth 10 nm with a reference wavelength of 390 nm bandwidth 20 nm. All data were displayed and

analyzed on Agilent ChemStation software. Data are expressed as g of amino acids per 100 g of extract.

Proteins content

Total protein content was evaluated by using the Bradford assay as follows: 1 ml of 50% Coomassie-Brilliant Blue Bradford reagent (ThermoFisher, USA) was incubated at room temperature with 2 µl of extract of known concentration for a minute. Absorbance was read against blank at 595 nm and fitted on a calibration curve made up with BSA (Bovine Serum Albumin) in a range between 0 and 6 mg/ml. Data are expressed as g of protein per 100 g of extract.

GC/MS analysis

Before the GC/MS analyses all samples were subjected to a derivatization process, as described below. About 5 mg of each sample were accurately weighed, suspended in 50 µl of 2wt% methoxylamine hydrochloride in pyridine and incubated for 90 min at 37°C. Then, 80 µl of MBDSTFA (N-methyl-N-ter-butyldimethylsilyl-trifluoroacetamide)+1% TBDMCS (tert-butyldimethylchlorosilane) were added and the samples were incubated at 60° C for 30 min. After incubation at room temperature overnight, the samples were analyzed by using a ISQ™ QD Single Quadrupole GC-MS (Thermo Fisher) equipped with a VF-5ms (30 m x 0.25 mm i.d. x 0.25 µm; Agilent Technology). Injection volume: 1 µl. Oven program: 100° C for 2 min; then 6° C/min to 280° C for 15 min; Run Time 42 min. Helium was used as the gas carrier. SS Inlet: Mode Splitless. Inlet temperature: 280° C. Flow 1.0 ml/min. MS transfer line: 270° C. Ion source: 250° C. Ionization mode: electron impact: 70 eV. Acquisition mode: full scan. In order to compare the composition of the extracts, for each analyte identified by GC/MS a target ion (m/z) was extracted by the TIC and the corresponding area was calculated. Supplementary Table 2 reports for each analyte the corresponding target ion used.

Proteomic analysis

The extract was reduced, derivatized and digested with trypsin (protein: protease ratio 20:1) as described in [57] before MS/MS analysis.

Peptides separation was achieved on a Thermo Easy-nLC 1000, with a linear gradient from 95% solvent A (2 % ACN, 0.1% formic acid) to 30% solvent B (80% acetonitrile, 0.1% formic acid) over 60 min, from 30 to 60% solvent B in 5 min and from 60 to 100% solvent B in 2 min at a constant flow rate of 0.25 µl/min, with a single run time of 75 min. MS data were acquired on a Thermo Q-Exactive-HF, with a data-dependent top 15 method, the survey full scan MS spectra (300-1650 m/z) were acquired in the Orbitrap with 60000 resolution, AGC target 3e6, IT 20 ms. For HCD spectra resolution was set to 15000, AGC target 1e5, IT 80 ms; normalized collision energy 28 and isolation width of 1.2 m/z.

Raw label-free MS/MS files from Thermo Xcalibur software (version 4.1) [57] were analyzed using Proteome Discoverer software (version 2.2, Thermo Fisher Scientific) and searched with Sequest algorithm against the proteome of NCBI Phaseoleae (release 05/08/2019). The minimum required peptide length was set to 6 amino acids with carbamidomethylation as fixed modification, Met oxidation and Arg/Gln deamidation as variable modifications.

The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE [58] partner repository with the dataset identifier PXD017716.

Yeast methods

Yeast strains and media

The yeast strains used in this paper are listed in Supplementary Table 3. Cells were grown at 30°C in minimal medium containing 2% glucose as a

carbon source and 0.67% yeast nitrogen base without amino acids, supplemented with 50 mg/l of required amino acids and bases for which the strains were auxotrophic. The natural extracts were dissolved in the medium at a concentration of 0.2% or 0.5% and filtered through 0.22 µm filters.

Chronological lifespan experiments (CLS)

Cell cultures were grown in liquid medium until mid-late exponential phase and then inoculated into flasks containing medium in the presence or absence of the natural extracts (0.2% or 0.5% as indicated in each experiment). Survival was assessed by propidium iodide staining (PI) at the indicated time points with the Cytoflex cytofluorimeter (Beckman Coulter) and analyzed with the Cytoflex software. For some experiments, survival was also confirmed by colony-forming units (CFUs) after 2 days of incubation at 30°C on YEPDA agar plates.

Protein extraction, cell fractionation and immunoblotting

Equal amounts of cells were collected and quenched using TCA 6% and lysed in lysis buffer (6M UREA, 1% SDS, 50 mM Tris-HCl pH7.5, 5 mM EDTA). The cytoplasmic-membrane fractionation experiment was conducted using the MEM-PER kit (Thermo), following the manufacturer's instructions on yeast spheroplasts. Western blot analysis was performed using anti-Synuclein antibody (1:1000, Sigma Aldrich), anti-Pgk1 antibody (1:1000, Molecular Probes, used as loading control and cytoplasmic marker) and anti-Pma1 antibody (1:1500, Abcam, used as membrane marker).

Glucose consumption assay

Extracellular levels of glucose were evaluated on growth media of wt cells exponentially growing in the absence or presence of 0.5% cowpea

extract, using the Megazyme D-glucose-HK assay kit, following the manufacturer's instructions, using an EnSight Plate Reader (Perkin Elmer).

Fluorescence microscopy on yeast

In situ immunofluorescence was performed on formaldehyde-fixed cells and carried using α-synuclein immunostaining (1:2000, Sigma Aldrich) followed by indirect immunofluorescence using rhodamine-conjugated anti-rabbit antibody (1:1000, Pierce Chemical Co). Digital images were taken with a Nikon DS-Qi MC camera mounted on a Nikon Eclipse 600 and controlled by the NIS elements imaging software (Nikon) with an oil 100X 0.5-1.3 PlanFluor oil objective (Nikon).

In vitro aggregation of α-synuclein

α-synuclein was expressed in *Escherichia coli* BL21(DE3) cells transformed with the pET28b/α-synuclein plasmid. The recombinant protein was expressed and purified according to a previously described procedure [43] and further purified by RP-HPLC. The identity and purity of the eluted material were assessed by mass spectrometry**.** Protein samples (250 µM), filtered through a 0.22 µm pore-size filter (Millipore, Bedford, MA, USA) were incubated at 37°C in 20 mM sodium phosphate buffer, pH 7.4 up to 3 days under shaking at 900 rpm with a thermo-mixer in the absence or in the presence of extract by using molar protein/substance ratios of 1:0.5 (E0.5) and 1:1 (E1). Oligomer-enriched or fibril-enriched sample were prepared by incubating a-synuclein for 24 h or 72 h, respectively.

ThT assay

The ThT binding assay was performed according to LeVine [59], using a 25 µM ThT solution in 20 mM sodium phosphate buffer, pH 7.0. Each sample, diluted at a final concentration of 6.25 µM, was transferred into a 96-well half-area, low-binding, clear bottom (200 µl/well) and ThT fluorescence was read at the maximum intensity of fluorescence of 485 nm using a

Biotek Synergy 1H plate reader; buffer fluorescence was subtracted from the fluorescence values of all samples. In controls experiments, a significant interference of the highest concentrations of cowpea extract on ThT fluorescence was observed, so the two molar ratio protein:extract with lowest fluorescence interference were selected (Supplementary Figure 5).

ANS assay

Samples containing aggregating a-synuclein with and without cowpea extract at 250 µM were investigated for their ability to bind 8-anilinonaphthalene-1-sulfonic acid (ANS; Sigma Aldrich, Saint Louis, MO, US). 5 µl of each samples at different times of aggregation was transferred into a 96-well half-area, low-binding, clear bottom (200 µl/well), and ANS (50 µM) fluorescence intensity was read at the binding intensity of fluorescence of 480 nm in a Biotek Synergy 1H plate reader; buffer fluorescence was subtracted from the fluorescence values of all samples. In control experiments, a significant interference of the highest concentrations of the extract on ANS binding fluorescence was observed, so we selected the two molar ratios protein:extract with the lowest fluorescence interference (Supplementary Figure 5).

Transmission electron microscopy (TEM) imaging

5 µl aliquots of α-synuclein aggregated in the presence or in the absence of cowpea extract were withdrawn at different aggregation times, loaded onto a formvar/carbon-coated 400 mesh nickel grids (Agar Scientific, Stansted, UK) and negatively stained with 2.0% (w/v) uranyl acetate (Sigma-Aldrich). The grid was air-dried and examined using a JEM 1010 transmission electron microscope at 80 kV excitation voltage.

Cell culture methods

Cell culture

SH-SY5Y human neuroblastoma cells were cultured at 37 °C in complete medium (50% HAM, 50% DMEM, 10% fetal bovine serum, 3 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin), in a humidified, 5% CO2 incubator.

MTT assay

Cell viability was assessed by the MTT assay optimized for the SH-SY5Y cell line. Briefly, SH-SY5Y cells were seeded into 96-well plates at a density of 10000 cells/well in fresh complete medium and grown for 24 h. Then, cells were exposed for 48 h to 5 µM α-synuclein obtained at different times of aggregation in the presence or in the absence of the *Vigna unguiculata* extract. Cells were also treated with the corresponding concentrations of extract used in the aggregation of α-synuclein and the viability resulted similar to that of untreated control cells. After 48 h of incubation, the culture medium was removed and cells were incubated for 1 h at 37°C in 100 µl serum-free DMEM without phenol red, containing 0.5 mg/ml MTT. Then, 100 µl of cell lysis solution (20% SDS, 50% N,N-dimethylformamide) was added to each well and samples were incubated at 37°C for 2 h to allow complete cell lysis. Absorbance values were measured using iMARK microplate reader (Bio-Rad) at 595 nm. Final absorption values were calculated by averaging each sample in triplicate after blank subtraction. Statistical analysis of the data was performed by using one-way analysis of variance (ANOVA).

ROS determination

Intracellular reactive oxygen species (ROS) were determined using the fluorescent probe 2',7'–dichlorofluorescein diacetate, acetyl ester (CM-H2 DCFDA; Molecular Probes), a cell-permeant indicator for ROS that becomes fluorescent upon removal of the acetate groups by cellular esterases and oxidation. SH-SY5Y cells were plated on 96-well plates at a density of 10000 cells/well and exposed for 48 h to the α-synuclein samples. Then, 10 µM DCFDA in DMEM without phenol red was added to

each well. The fluorescence values at 538 nm were detected after 30 min by Fluoroscan Ascent FL (Thermo-Fisher). Cells were also treated with the corresponding concentrations of extract used in the aggregation of α-synuclein and ROS levels resulted similar to that of untreated control cells. Statistical analysis of the data was performed by using one-way analysis of variance (ANOVA).

Confocal imaging

Subconfluent SH-SY5Y cells grown on glass coverslips were exposed for 48 h to 5 μM (monomer concentration) α-synuclein aggregates grown in the presence or in the absence of cowpea extract at different molar ratios (1:0.5, E0.5; 1:1, E1). Cell membrane labelling was performed by incubating the cells with 10 ng/ml Alexa Fluor 488-conjugated CTX-B (Cholera toxin B-subunit) in cold complete medium for 30 min at room temperature. Then, cells were fixed in 2.0% buffered paraformaldehyde for 6 min and permeabilized by treatment with a 1:1 acetone/ethanol solution for 4 min at room temperature, washed with PBS and blocked with PBS containing 0.5% BSA and 0.2% gelatin. After incubation for 1 h at room temperature with rabbit anti-synuclein polyclonal antibody (1:600 in blocking solution), the cells were washed with PBS for 30 min under stirring and then incubated with Alexa Fluor 568-conjugated anti-rabbit secondary antibody (Molecular Probes) diluted 1:100 in PBS. Finally, cells were washed twice in PBS and once in distilled water to remove non-specifically bound antibodies. Digital images were taken with a confocal Leica TCS SP8 scanning microscope (Leica, Mannheim, Ge) equipped with a HeNe/Ar laser source for fluorescence measurements. The observations were performed using a Leica HC PL Apo CS2 X63 oil immersion objective.

Drosophila melanogaster methods

Fly husbandry and supplementation and longevity assay

Wild type *Drosophila melanogaster* (Canton S) was kindly provided by Dr Daniela Grifoni (University of Bologna, Italy). Flies were maintained at constant temperature (25°C) and humidity (60%) with a 12/12 h light–dark cycle. Flies were reared on Formula 4-24 ® media (Carolina Biological, Burlington, NC, USA). The composition of this diet, as indicated by the manufacturer, is as follows: oat flour, soy flour, wheat flour, other starches, dibasic calcium phosphate, calcium carbonate, citric acid, niocinamide, riboflavin, sodium chloride, sodium iron pyrophosphate, sucrose, thiamine, mononitrate, brewer's yeast, emulsifier preservatives, mold inhibitor, food coloring. The Formula 4-24 diet requires separate application of yeast pellets (*Saccharomyces cerevisiae*) and saturation of this dry media mixture with water. After eclosion, males and females emerged within 1-2 day were allowed to mate freely for two days before female separation into vials containing 1 g Formula 4-24 Instant Drosophila Medium (Carolina) soaked with 4 ml water containing 0.5% or 0.2% bean extract. A total of 20 flies were placed in each vial.

Female flies emerging within a 2-day period were collected under FlyNap (Carolina) anaesthesia. A total of 600 fruit flies were divided into 3 groups: control group, flies supplemented with 0.2% bean extract and flies supplemented with 0.5% bean extract. Flies were transferred into vials containing fresh food every 2-3 days and the number of living flies was counted. This was repeated until all flies had died. Kaplan-Meier survival curves were generated for lifespan assessment.

Measurement of Drosophila body weights

Changes in body weights were used as an indicator of the food intake. Flies were fed on standard diet with and without bean extract for 30 or 45 days. For each condition (0.2% bean extract supplementation and control), five vials containing 20 flies/vial were counted.

Flies in each group were anesthetized by FlyNap (Carolina) and then weighed on a balance. The mean body weights of the flies in each group were calculated.

Gene expression analysis

Total RNA was extracted from the whole bodies of either 30 days or 45 days old flies belonging to the 0.2% group by using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). The 0.2% supplementation has been chosen because it was the one able to significantly increase lifespan in *Drosophila*. All the experiments were performed in triplicate. The yield and purity of the RNA were measured using NanoVue Spectrophotometer (GE Healthcare, Milano, Italy). Only samples with density ratios A260/A280>1.8 were used. cDNA was obtained by reverse transcribing mRNA starting from 1 µg of total RNA using iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer's protocol. The subsequent polymerase chain reaction (PCR) was performed in a total volume of 10 µl containing 2.5 µl (12.5 ng) of cDNA, 5 µl SsoAdvanced Universal SYBR Green Supermix (BIO-RAD), 2 µl of dH20 RNA free and 0.5 µl (500 nM) of each primer. The primers used are reported in Supplementary Table 4 and RPL32 was used as reference gene.

Protein extraction and immunoblotting

Proteins were isolated from the whole bodies of either 30- or 45-day old flies. Proteins were homogenized using lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM dithiothreitol (DTT), 0.002% bromophenol blue) and centrifuged at 12000 g at 4 °C for 5 min. The supernatant was collected and mixed with Sample Buffer, Laemmli 2× Concentrate (Sigma Aldrich). Proteins were then loaded onto 4-20% SDS-PAGE gels followed by transfer onto nitrocellulose membranes and immunoblotted with appropriate antibodies. Anti-Sirt1 (1:1000; Cell Signaling Technology, Beverly, MA), anti-dFoxO (1:1000, Covalab, Villeurbanne, France) and anti-β-actin (1:2000, Invitrogen Carlsbad, CA, USA) antibodies were used

as primary antibodies. The HRP-conjugated anti-mouse IgG and anti-rabbit IgG antibodies were employed as the secondary antibodies (1:10000; Cell Signaling Technology). Targeted proteins were visualized using ClarityTM Western ECL Substrate (BIO-RAD). Densitometric analysis of specific immunolabeled bands was performed using ImageJ software.

Statistical analysis

Each experiment was performed at least three times, and all values are represented as means ± SD. One-way ANOVA was used to compare differences among groups followed by Dunnett's (Prism 5; GraphPad Software, San Diego, CA). Values of $p<0.05$ were considered statistically significant. Survival curves were prepared by Kaplan-Meier survival analysis and analyzed using the OASIS2 software [60].

Caenorhabditis elegans methods

C. elegans strains and treatment with V. unguiculata extract

Standard procedures for *C. elegans* strain maintenance were followed [61]. The strain used in this study, JZF142 [*pdat-1::hαSyn; pdat-1::DsRed*], was kindly provided by Prof. J. Feng (Case Western Reserve University, US) [45]. The *C. elegans* strain was grown on Nematode Growth Medium (NGM) containing agar, seeded with *E.coli* OP50 at 20°C. Lyophilized extract from *V. unguiculata* was solubilized in sterile distilled water at two dilutions, 2% or 5% w/V, and sterilized with 0.22µm filter. α-synuclein expressing animals were exposed to the following treatments: 0.2% or 0.5% of *V. unguiculata* extract, water as negative control (mock) and 3 mM valproic acid (VA) as positive control [46]. *C. elegans* animals at L4 developmental stage were transferred into 12-well plates with NGM agar containing the different conditions as quadruplicates and allowed to become adults and lay eggs. After 14 hours the adults were discarded and the synchronized F1 progeny was allowed to grow in the presence of chronic treatments. F1 animals have been transferred every 3 days on

new plates with treatment, until the day of analysis, to maintain them well fed and separated from the next generation.

The morphology of the four cephalic dopaminergic neurons (CEP neurons) in the F1 treated animals was scored at 6 days post adult stage. The neurodegeneration analysis was performed also on untreated animals at young adult and at 6 days post-adult stage. Animals were mounted and anesthetized with 0.01% tetramisole hydrochloride on 4% agar pads. The neurodegeneration analysis was performed using Zeiss Axioskop microscope (Carl Zeiss). All images were obtained using a Leica SP2 confocal laser scanning microscope (Leica). The spectra used for imaging DsRed were: λ excitation=543 nm and λ emission=580-630 nm. GraphPad Prism software was used for statistical analysis. The statistical significance was determined using Mann Whitney test or One-way ANOVA with Kruskal–Wallis post-test. Data are reported as averages of multiple observations ± SEM.

Go to:

Supplementary Material

Supplementary Figures

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Supplementary Tables

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Footnotes

Contributed by

AUTHOR CONTRIBUTIONS: L.G. and D.P. procured the matrixes, prepared and characterized the extracts. E.F. performed GC/MS analysis. S.N. and G.T. performed proteomic analysis on *V. unguiculata* extract. F.T., L.L., R.M. performed experiments in yeast cells. M.L. performed *in vitro* assays on α-synuclein and experiments on neuroblastoma cells. D.B. performed experiments in *D. melanogaster*. G.O. performed experiments in *C. elegans*. M.B. conceived and provided materials for the experiments and participated to manuscript preparation. C.A., D.B. and S.H. designed experiments in *D. melanogaster* and participated to manuscript preparation. E.D.S. designed experiments in *C. elegans* and participated to manuscript preparation. M.L. got founding and participated to manuscript preparation. P.C. and F.T. designed experiments in yeast cells and wrote the paper. P.C. coordinated the project.

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9.4 Revisiting the Domestication Process of African *Vigna* Species (Fabaceae): Background, Perspectives and Challenges

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Abstract

Legumes are one of the most economically important and biodiverse families in plants recognised as the basis to develop functional foods. Among these, the *Vigna* genus stands out as a good representative because of its relatively recent African origin as well as its outstanding potential. Africa is a great biodiversity centre in which a great number of species are spread, but only three of them, *Vigna unguiculata*, *Vigna subterranea* and *Vigna vexillata*, were successfully domesticated. This review aims at analysing and valorising these species by considering the perspective of human activity and what effects it exerts. For each species, we revised the origin history and gave a focus on where, when and how many times domestication occurred. We provided a brief summary of bioactive compounds naturally occurring in these species that are fundamental for human wellbeing. The great number of wild lineages is a key point to improve landraces since the domestication process caused a loss of gene diversity. Their genomes hide a precious gene pool yet mostly unexplored, and genes lost during human activity can be recovered from the wild lineages and reintroduced in cultivated forms through modern technologies. Finally, we describe how all this information is game-changing to the design of future crops by domesticating *de novo*.

Keywords: ***Vigna*** **genus**; **introgression**; **hybridisation**; **phylogeny**; ***de novo*** **domestication**; **feralisation**; **bioactive compounds**

1. Introduction

Legumes (Fabaceae) are considered one of the most important families of plants for human nutrition, especially considering the rapid growth rate of the world population [**1**]. However, almost all the efforts and resources invested in agriculture during the last century were focused on improving the yield, resistance and quality of a few specific staple crops. Neglected landraces are regarded as having interesting potential, and recent studies have demonstrated that some wild legumes can be an important target to develop modern functional foods because they possess various bioactive molecules that interact positively with human health [**2**,**3**,**4**,**5**]. Among these, members of the *Vigna* genus show a growing social and economic importance in several African regions, especially where the local population is not able to afford animal proteins [**6**,**7**,**8**]. Their seeds are rich in essential amino acids and contain a high concentration of minerals, lipids and vitamins [**9**,**10**].The genus *Vigna* (Savi, 1824), which belongs to the tribe Phaseoleae of the family Fabaceae, includes over 100 species [**11**] distributed in the tropical and subtropical areas of the world [**12**] grouped in five subgenera: *Vigna*, *Ceratotropis*, *Plectotropis*, *Lasiosporon* and *Haydonia* [**13**,**14**,**15**]. Phylogenetic findings propose the age of split between *Phaseolus* and *Vigna* genera at about 8–10 million years (Mya) and the age of split between *Ceratotropis* and *Vigna* subgenera at about 3–4 Mya [**13**,**14**,**15**,**16**,**17**], but the genetic relationships between subgenera are particularly complex and far from being completely solved. Although most domesticated or semi-domesticated species are distributed in Asia, the greatest diversity of the *Vigna* genus is located in Sub-Saharan Africa [**14**,**18**]. *Vigna* subgenus, distributed in Africa, includes about 40 wild and 2 domesticated species, namely cowpea (also called black-eyed peas, chawli and kunde) (*Vigna unguiculata* L.) and Bambara groundnut (*V. subterranea* L.) [**19**] while *Ceratotropis* (Piper) Verdc., distributed in Asia, contains 21 wild and 7 domesticated species used widely for food and

forage, namely mungbean or green gram (*V. radiata* L. Wilczek), black gram (*V. mungo* L. Hepper), moth bean (*V. aconitifolia* Jacq. Maréchal), rice bean (*V. umbellata* Thunb. Ohwi and Ohashi), adzuki bean (*V. angularis* L. Ohwi and Ohashi), creole bean (*V. reflexo-pilosa* Hayata), jungli bean (*V. trilobata* L. Verdc.). [**15**,**20**,**21**,**22**]. Moreover, three species belonging to *Plectrotropis* (Schumach.) are distributed in Africa, including tuber cowpea (*V. vexillata* L.) [**23**]. Most of the African *Vigna* germplasm is based on wild plants and neglected or underutilized landraces, and many of these lineages are declining with a high risk of extinction. The recovery of wild accessions and research devoted to the phylogeny of the genus is therefore essential to prevent genetic erosion and the loss of *Vigna* diversity. Plant domestication is widely recognised as an accelerated evolutionary process driven by a synergistic impact of human and natural selection, occurring in geographically restricted areas from wild progenitors. In legumes, the main modification is the loss of seed pod dehiscence or shattering [**24**,**25**]. The split at the dorsal and ventral sutures of the dry pod and successive release of the seeds occurs due to the desiccation of lignified cells in the pods [**26**]. The shattering habit is related to environmental aridity and persists in many varieties of domesticated *Vigna* species, thereby determining severe yield losses [**27**,**28**]. Additional implementations in *Vigna* domesticated species include an increase in seed or fruit size, change in seed colour, loss of seed dormancy, apical dominance and change in flowering timing [**29**,**30**,**31**,**32**,**33**]. These modifications were inherited more or less effectively in the various vine species currently cultivated, and this is the basis of the agrobiodiversity of this genus. Generally, the current existing crops show lower resistance to biotic and abiotic stress compared to wild relatives, and often they have reached their full yield. The selection of desirable traits and breeding processes to improve crop productivity have caused the depletion of diversity and the increase in the frequency of deleterious genetic variants that are fixed in the genomes of crops [**34**,**35**,**36**]. These constraints have a serious impact on agriculture, limiting the possibility to grow such crops under more extreme

environmental conditions. Thanks to this residual genetic diversity and also to studies performed on *Vigna* species, most of the accessions are well adapted to a wide range of extreme environmental conditions, such as sandy beaches, arid lands and wetlands, harbouring tolerance and resistance genes towards biotic and abiotic stresses. These genetic traits are used for developing new stress-tolerant crops [**37**,**38**,**39**,**40**,**41**,**42**,**43**]. By contrast, less is known about the effects of domestication on the nutritional value of seeds [**7**] even if recent studies have reported that cultivated legumes show a lower carotenoid and protein content in seeds compared with the wild relatives [**44**,**45**]. Where, when and how many times the domestication process of African *Vigna* crops occurred continues to be debated among researchers. Although archaeological remains of *Vigna* indicate that the domestication process in Africa was started recently compared to other field crops [**46**,**47**]. Modern evolutionary models proposed for other crops suggest that the predomestication phase may have lasted several thousands of years [**48**,**49**]. Generally, the centres of origin are also recognized as centres of diversity, and thus these areas require special precautionary measures of conservation [**50**]. Although for many crops the single-origin model is usually the most parsimonious, the hypothesis that provides multiple origins starting from independent founder lineages seems well suited for the crops of *Vigna* originated in Africa [**51**,**52**]. Moreover, despite whether and to what extent introgression influences the domestication process is still underexplored, some studies already show that gene flow between cowpea and its wild relatives may occur. Pervasive introgression can also intensify the feralisation process, promoting the crops to return to a wild environment and causing serious problems for the conservation of biodiversity [**53**]. In this review, we re-examine the available scientific information on the domestication process of three African *Vigna* crops and discuss the future perspectives and challenges in the light of modern technologies in the time of climate change and new parading of conservative agriculture strategies. Another crucial point in exploring natural biodiversity is not only a matter of sustainability but also a matter

of human health. A balanced diet gives extreme benefits to people's wellbeing by properly assuming the correct amount of micro and macro nutrients as well as useful, healthy bioactive compounds. Finding and characterising these compounds is an ambitious challenge for researchers thus we briefly summarise the bioactivity of some compounds, and we discuss how human activity and breeding has impacted the variability of molecules. Although recent genetic studies have led to a deeper understanding of these crops, the continuation of investigating the domestication process through a multidisciplinary approach which includes genomic, transcriptomic, metabolomic and epigenomic analyses is needed to highlight the wide agronomic opportunities related to these species. Moreover, recent techniques of gene editing have opened new and crucial ways to redesign modern crops because traditional genetic improvement is generally limited by the cross-compatibility between species. Thus, because the *de novo* domestication process may represent a turn toward more modern and sustainable agriculture, further efforts are needed to explore the genome diversity of wild germplasm.

2. *Vigna unguiculata* (L.) Walp.

V. unguiculata, which was considered an orphan crop for several decades, has recently become one of the most important legumes in the world. Its name derives from Latin and means "with a small claw", referring to the size of the claw of the petals [**54**] or commonly named as "cowpea" because of its use as fodder for cows [**55**] and black-eyed pea/bean for the black hilum. This crop is largely cultivated, especially in semiarid regions of Africa and Asia where other crops fail to grow [**10**]. Currently, on a global scale, about 15 million hectares are dedicated to *V. unguiculata,* with an annual production of 7 million Mg and an average yield of 0.6 Mg ha−1 [**56**]. The most interesting environmental traits of this species are represented by the generalized low agrochemical input requirements. In fact, this crop shows relatively high adaptation to

drought, especially in comparison to other legumes [**57**] and can fix up to 200 kg N ha−1 [**58**] with a positive soil N balance of up to 92 kg ha−1 [**59**]. Nevertheless, several abiotic and biotic constraints (i.e., low soil fertility, pests, diseases, parasitic weeds, and nematodes) limit the yield [**43**,**60**,**61**]. Moreover, low productivity is often associated with the use of traditional and unimproved varieties, still widely cultivated in Africa [**62**]. This crop has a fundamental role in human nutrition, showing seeds rich in proteins and essential amino acids (i.e., tryptophan and lysine), carbohydrates, folic acid and minerals. Recent studies carried on a large sampling have shown high variability in protein and mineral concentrations, suggesting that some lineages could be potential sources of genes useful to produce new varieties [**63**,**64**,**65**,**66**]. The high number of wild subspecies found exclusively in Africa strongly supports the idea of an African origin. However, intraspecies phylogeny remains far from being completely elucidated [**67**]. The centre of origin of the species is probably located in the southernmost regions of Africa, where most subspecies are found and where most genetic diversity could be still hidden [**68**]. Several taxonomic revisions based on morphological and molecular traits permitted to identify 10 perennial and 1 annual subspecies, the latter split into two varieties: ssp. *unguiculata* var. *unguiculata* (domesticated cowpea) and ssp. *unguiculata* var. *spontanea* (Schweinf.) Pasquet., also known as subsp. *dekindtiana* sensu Verdcourt non Harms [**69**,**70**,**71**,**72**,**73**,**74**,**75**,**76**]. Besides the domesticated cowpea, the *dekindtiana* group includes some obligate short-day wild forms, well adapted to arid environments. While the var. *spontanea* grows especially around cultivated fields and roadsides, and it is recognized as the progenitor of domesticated cowpea [**75**,**77**,**78**,**79**], the subspecies *alba*, *pubescens*, *tenuis*, *stenophylla* and *dekindtiana* are perennial plants [**75**,**76**]. The development of new molecular tools to discriminate among wild, weedy, and cultivated accessions is considered a modern and fundamental target, particularly needed for disentangling the complex taxonomic relationships among subspecies and to discriminate between true wild plants and ferals. Although little is known about the

domestication process, some scientists have hypothesized that ancient cowpea progenitors, such as the modern wild forms, were adapted to dry habitats and grew spontaneously south of the Sahara Desert [**80**]. These plants were gathered, cultivated and dispersed by men near the villages, but they were unsuited for cultivation. Although they did not show high yield, the wild lineages were spread in the humid zones thanks to their pods that remained closed for the humid atmosphere. Through several generations of cultivation, new mutants have arisen, showing interesting domestic traits, including resistance to shattering. Subsequently, humans have selected and helped spread these landraces by exchange and trade activities. Since the oldest archaeological records of domesticated forms found in central Ghana are dated around 1500 BC, the domestication process likely started before that period (**Figure 1**) [**47**,**81**]. However, the precise origin is widely debated, and two independent domestication centres in West and East Africa are proposed by different authors [**68**,**74**,**79**,**82**,**83**,**84**,**85**,**86**].

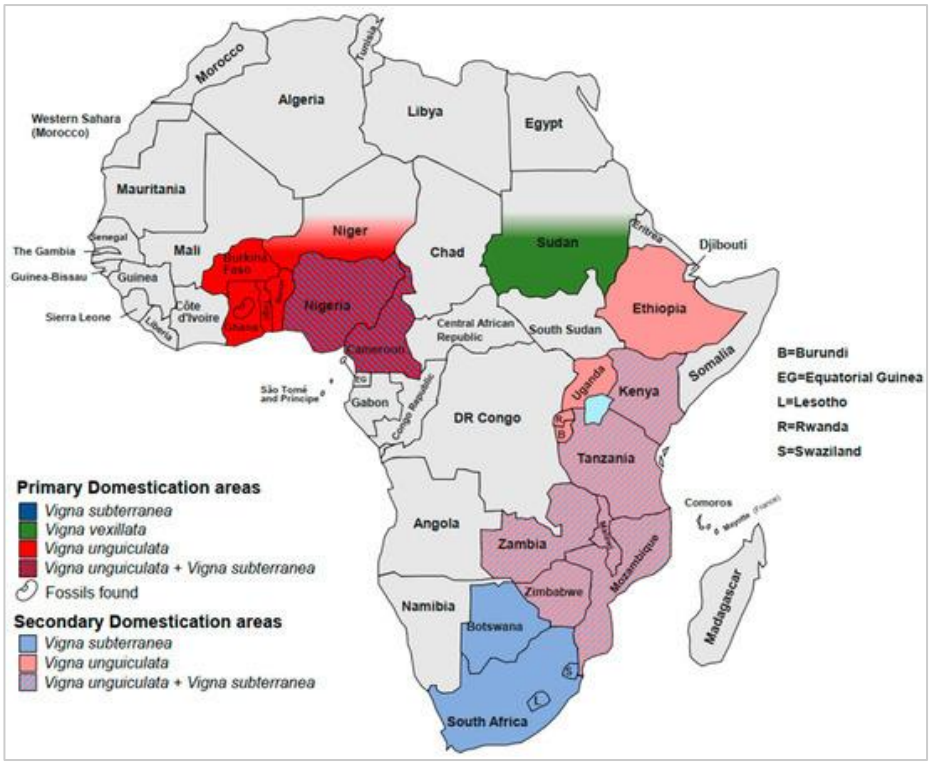

Figure 1. Primary and secondary domestication sites in Africa.

Morphological and DNA markers support the idea that domestication occurred only once, but analyses on whole genomes provide evidence for more independent domestication events in Africa and diversification events out of Africa [**51**,**87**]. Analyses of genetic variability are generally applied to identify the origin of species and the groups of accessions that show high variability in certain geographic areas and are interpreted as the most ancient populations. Although cowpea from West Africa showed a high genetic variability [**88**], cultivated accessions grown in East and West Africa were shown to be most closely related to the respective local wild lineages [**52**,**89**], thereby indicating that domestication could have occurred in both regions. Outside Africa, cultivated cowpea was exposed to different ecological conditions, including new biotic and abiotic stresses that probably have contributed to shaping the genetic structure

of landraces. When cowpea moved through Asian regions (especially in Thailand, China, the Philippines and India), it encountered environments with more humidity and less brightness where the drying of pots and grains was hindered. Some accessions were selected for the use of the immature pods to produce a peculiar form of vegetable called yardlong bean (*V. unguiculata* ssp. *unguiculata* cv. *sesquipedalis*) [**51**,**90**,**91**]. Although Chinese accessions show lower genetic diversity compared to African cowpea, signals of genetic bottlenecks lead to the conclusion that a limited number of relatively recent selection events occurred;however, where the selection process arose is still unknown [**92**]. Moreover, other cultivar groups (e.g., 'Textilis', 'Biflora' or 'Cylindrica', 'Melanophthalmus') are classified by morphological traits [**75**,**93**]. Still, additional genomic analyses should be performed to confirm the genetic relationships and understand how and where these accessions originated [**67**,**85**,**88**,**94**,**95**].

3. *Vigna subterranea* (L.) Verdc.

Vigna subterranea, also named Bambara groundnut, is an indigenous African grain legume. Its common name derives from the groundnut (*Arachis hypogaea* L.) due to the hypogean pods' growth, whereas the "Bambara" name is derived from a Malian tribe [**96**]. Despite its potential in terms of nutritional value and resistance to biotic and abiotic stresses [**97**,**98**], Bambara is cultivated mainly in small farms or in families as a subsistence crop [**99**], and naturally grows in semi-arid regions in Africa. Regarding the origin of the species itself, the domesticated or semi-domesticated *Vigna subterranea* var. *subterranea* was most likely generated from its wild counterpart *Vigna subterranea* var. *spontanea* using both morphological and isozyme data [**100**,**101**]. The origin of this species is hypothesised to be in Mali, in the Timbuktu region [**102**], but the precise centre of origin is still unknown. In fact, there is no evidence of wild lineages in Mali [**103**]. Dalziel, Begemann and Goli [**104**,**105**,**106**]

analysed a lot of morphologic traits such as seed morphology, seed pattern diversity and other diversity indices (number of days to maturity, pod length, number of stems per plant and internode length). They found that the most diversity is located in an area that spans from Jos Plateau and Yola Adamawa (Nigeria) to Garoua (Cameroon). Somta and Olukolu [**107**,**108**] evaluated the phylogeography of several accessions spread in Africa. The markers used (i.e., SSR and DaRT) showed a cluster with higher diversity in the area between Nigeria and Cameroon. The authors confirm the area of origin while suggesting a possible subsequent introduction of Western domesticated accessions in East Africa (**Figure 1**). In contrast, Aliyu et al. [**97**], in an overview of the past two decades of genetic diversity analysis, also proposed that the Southern African region could constitute a divergent time-spaced domestication event. However, the authors suggest that these hypotheses need further examination. In terms of genetic diversity, Bambara has a peculiar behaviour. In fact, many authors studied Bambara's genetics with different techniques to clarify how wide the genetic pool is and how homogeneous the single landraces are. Molosiwa et al. [**109**] evaluated genetic distances between 24 landraces with phenotypic and genetic markers (i.e., SSR and DaRT). The main results report that landraces are different to each other, suggesting the existence of great allelic diversity among the various populations. At the same time, though, single landraces tend to be very homogeneous, and in three generations of inbreeding became pure lines. This is due mainly to its self-pollinating nature [**110**] but also small farmers, who, by breeding the same landraces, also acted as selection drivers [**111**,**112**]. Molosiwa [**113**] selected 12 SSR markers and 5 Bambara accessions to evaluate the potential for creating pure lines, finding that these accessions at the second cycle of selection completely have lost the heterozygosity. All these findings suggest that Bambara has incredible genetic potential. The genetic screening through the different lineages and the consequent discovery of peculiar sites of interest could be the basis for an improvement of crop programs. Moreover, the use of pure lines in agriculture is fundamental not only for the optimisation and

standardisation of agricultural practices but also for the development of breeding programs. Currently, to the best of our knowledge, there are no reports of ongoing improvement or breeding programs for this species. The extremely wide pool of wild and domesticated accessions can be used to create ideal crops that can better withstand climate change as well as being able to grow with low agronomic inputs.

4. *Vigna vexillata* (L.) A. Rich.

Widely distributed in Africa, Asia, America and Australia, *V. vexillata* (Zombi pea) is one the least known and underutilized *Vigna* crops. Likewise, *V. unguiculata*, Zombi pea shows a high morphological diversity probably determined by geological, ecological, climatic and anthropomorphic constraints that also determined exceptional patterns of genetic variability [**19**,**71**]. Eight varieties including *vexillata*, *angustifolia*, *ovata*, *dolichomena*, *yunnanensis*, *plurifora*, *lobatifloria* and *macrosperma* are recognized [**12**,**19**,**23**,**114**,**115**]. Var. *macrosperma* shows typical traits associated with domestication syndrome such as bush-like habit, early flowering and higher seed yield [**116**,**117**]. Moreover, loss of seed dormancy and various degrees of pod shattering were detected in different crop accessions while the wild seeds remained intrinsically dormant [**118**,**119**]. Several authors reported that two forms were domesticated independently (i.e., seed type and tuber type), and some evidence lines suggest that the seed type was domesticated in Sudan, whereas the tuber type was domesticated in India (**Figure 1**) [**120**,**121**,**122**,**123**,**124**]. However, molecular analyses were performed on a limited number of accessions and loci [**124**], and the phylogenetic intra-specific delimitation has resulted in much more complexity than that of other *Vigna* crops [**125**]. Thus, modern genomic analyses are needed to resolve the genetic relationships and confirm the origin of the two forms.Several studies have also shown that the Zombi pea is the result of a long adaptation process to different environmental stress, including

acid, alkaline, saline, drought and wet soils [**115**,**117**,**126**,**127**,**128**]. Moreover, since some accessions were found to be resistant to different viral diseases and parasite insects, widely recognized as major pests of cowpea, this species is an important harbour of resistances to various biotic stresses, particularly useful to improve modern *Vigna* crops [**129**,**130**,**131**,**132**,**133**,**134**].

5. Healthy Natural Compounds for Designing Sustainable Crops

The process of domestication was selected during the early millennia due to all the characteristics that made a species very productive or easier to harvest. Nowadays, a lot of crops varieties that have a great yield and high contents of macronutrients exist, such as carbohydrates or proteins. However, bioactive compounds that are naturally present in the *Vigna* genus were never taken into account. In a world where the main concern is no more denutrition but instead malnutrition, the adoption of crops with high-value nutraceutical compounds becomes a challenge for the next generation. The *Vigna* genus is a great source of small proteins and secondary metabolites with nutraceutical roles in everyday diet. Often agricultural practices themselves could stimulate the production of these compounds, such as hydric stress or no tillage with cover crops fields. However, they could not be sufficient to enhance the output of bioactive molecules. In this perspective, *de novo* domestication programs should consider these compounds to develop future healthy crops. In the next paragraph, we listed and discussed some of these molecules based on the nutraceutical activity they exert against three great world concerns.

5.1. Antioxidant and Anti-Inflammatory Activity

Nowadays, inflammation and oxidative stress are becoming great concerns due to detrimental effects on human health, and diet is a powerful way to protect cells from the rise of reactive oxygen species

(ROS) as well as inflammatory processes. In this view, seeds of cowpea contain different phenols, and other pigments present in the seed coat [**135**] are able to promote antioxidant action; among these, quercetin and flavonols are very well represented [**136**]. Different works [**137**,**138**] demonstrated a clear correlation between antioxidant properties and the colour of the seed coat in different accessions of *Vigna vexillata* and *Vigna subterranea*. Sowndhararajan and Leu [**139**,**140**] identified that *Vigna vexillata* extracts three molecules with strong antioxidant properties. Daidzein, abscisic acid and quercetin were highly active and displayed a pivotal capacity to deny the inflammation pathway. Studies performed on protein extracts of *Vigna subterranea* suggest that different protein fractions exert crucial properties against ROS relevant in cellular metabolism [**141**]. Furthermore, a review by Quan et al. [**142**] summarised how polyphenols and proteins naturally interact, providing a higher antioxidant and anti-inflammatory capacity as a result. The presence of antioxidant compounds is clearly a good starting point for the bioprospecting approach. The research could start from accessions already studied and kept in germplasm banks, with the aim of breeding the most promising ones (e.g., more colourful, thicker coat or better nutrient profile) with domesticated landraces to create variants that are, at the same time, easy to cultivate but with the most interesting characteristics found from the available natural pool. In addition, this could lead to new experiments to understand better the synergic role of the phenolic fraction with bioactive proteins.

5.2. Anti-Tumor Compounds

Concerning the anti-cancer activity, Bowman–Birk inhibitors (BBI), present exclusively in the Fabaceae family and some cereals [**143**], have proven anticancer effects [**144**,**145**]. Panzeri et al. [**146**] demonstrated that aqueous extracts from boiled seeds containing BBI are, as expected, effective against some colorectal cancer cell lines, but the healthy line was not hit by the treatment. Mehdad et al. [**147**] proved its activity on

breast cancer lines, and they were the first to discover a potential intracellular target, the proteasome 20S. Furthermore, they demonstrated cytostatic activity and increased apoptosis in cancer lines, but BBI was ineffective on the healthy mammary epithelial line. It is important to underline that this protein is kept by evolution due to its defensive role; in fact, it inhibits herbivores' digestion by interacting negatively with trypsin and chymotrypsin. Preliminary results obtained via the alignment of sequences downloaded from genebanks (NCBI) showed a high variability of BBI gene in some cowpea accessions, confirming the greatness of natural biodiversity. Unfortunately, little is known about the impact of domestication on the variability of the BBI gene. The domestication process can have acted as a strong constraint causing a bottleneck in the gene pool and reducing the variability of genes and exchange of alleles between cultivated and wild accessions. However, the exploration of haplotypes by sequencing several accessions is needed to verify the effective impact of human activity on gene diversity. Moreover, methods of ancestral sequence reconstruction (ASR) based on phylogenetic inference can predict the existence of stable, soluble, and active variants of proteins. The comparison of the structure of modern proteins with the corresponding ancestral intermediates can highlight functionally important substitutions within proteins and consequently drive the protein engineers to design variants that confer novel or more efficient activities (**Figure 2**). While different case studies are discussed in the literature where ancestral reconstructions were applied in eukaryotes, few instances are available in plants. Since ASR can be used to explore the remote evolutionary past as well as to investigate molecular evolution on shorter timescales, we argue that the proteins expressed in different genera of legumes are particularly well suited for ancestral reconstruction studies.

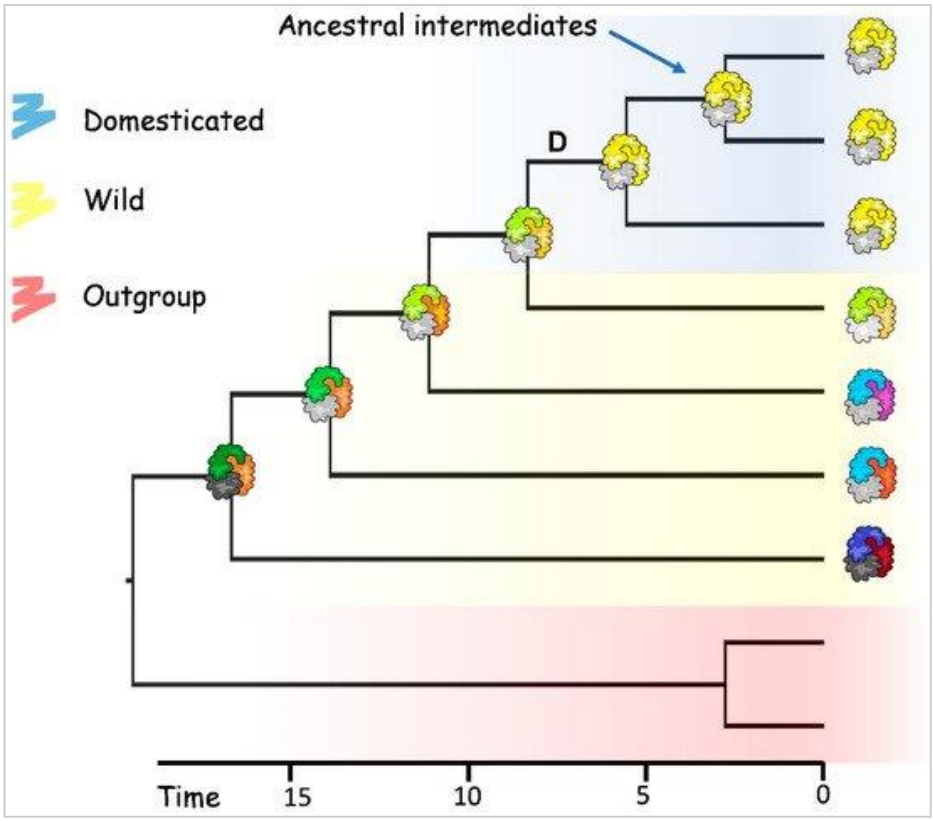

Figure 2. An example of reconstruction of putative ancestral intermediates by inferring the phylogenetic relationship between modern homologs. ASR studies can explore biodiversity to infer the historical evolution of natural proteins. Statistical models of amino acid substitution can be applied to calculate the sequences at internal nodes. Although domestication (D) has produced bottlenecks and reduced the genetic variability, ASR analyses can be applied to wild crop relatives.

Phenolic acids (e.g., gallic acid, ferulic acid, caffeic acid and chlorogenic acid) and flavonols (catechins, kaempferols and quercetins) are groups of molecules that are very active against cancer. Teixera-Guedes et al. [**148**] found some of these molecules in the phenolic fraction of cowpea sprouts. Sprouting is an alternative method to consume food, especially seeds, grains and pulses. As a matter of fact, sprouting refers activating

the metabolism of the dormant seeds and this way, complex reserve molecules are degraded into simpler ones, releasing other molecules and secondary metabolites [**149**]. The authors demonstrated at first the efficacy of the extracts against CRC cell lines; then combined it with 5-Fluorouracil (5-FU). This drug is potent but is susceptible to the occurrence of resistance by the tumour mass [**150**]. Among all these compounds, quercetin is one of the most common, was found to be the main representative of extracts and is well known to be active against different cancer lines [**151**,**152**,**153**]. The capacity to exert different kinds of bioactivities appoints phenolics and small proteins as very potential phyto complexes with an extreme wideness of possible applications. In this paragraph, the fact that extracts can be much more effective than single drugs is underlined. The use of a mixture of bioactive compounds in addition to the chosen drug could help in the treatment of many diseases.

5.3. Anti Hypercholesterolemic

One of the major world concerns is the role of the diet for healthy living. In particular, the main problem is malnutrition, 1.9 billion adults are overweight, and 452 million are underweight [**154**]. These numbers are going to increase during the next few years, so a healthy diet must become a worldwide topic. One way to prevent obesity is to find food or molecules that can lower LDL cholesterol concentration or production. Legumes are known to have a good nutritional profile and possess some interesting anti hypercholesterolemic capacities. For example, in the work of Tan et al. [**155**], *Vigna subterranea* was the object of study to create a powdered drink mix. The authors managed to characterise the extracts and proved the ability to lower the total cholesterol content in a population of rats. The observed effects were comparable to those given by the commercial drug simvastatin, demonstrating a potential commercial formulation usable in everyday life. In addition, Bambara powder fat content was lower than the soybean, while it had more

proteins. Regarding *Vigna unguiculata*, Kanetro [**156**] studied the hypocholesterolemic feature of protein extracts from the sprouts. The tests were performed on rats that mimicked a diabetic condition. This kind of extract established the potential of *Vigna unguiculata* in fighting high cholesterol concentrations. *Vigna unguiculata* was also studied in rabbit models by Janeesh and Abraham [**157**]. Rabbits received a rich fraction polyphenols and flavonoids extracted from the leaves that showed antioxidant capacity, hypolipidemic and anti-atherogenic properties in ill animals. The road opened by the studies reported here is encouraging and already tending to practical applications usable worldwide by combining the natural nutritive features to bioactive compounds present in the seeds. Although many important bioactivities are reported in this paragraph, the actual knowledge is still incomplete. Small proteins and polyphenols were objects of these studies, and their versatility in terms of the panel of bioactivities exerted was highlighted and valorised over and over. A topic that we would like to stress more and encourage research on is the variability of seed coat colours. In fact, human activity has selected a wide range of shapes, textures and pigments in coats (including eye shapes and sizes), allowing us to clearly distinguish seeds of domestication accessions from unattractive seeds of wild lineages. The seed colours are correlated to the presence of tannins and flavonoids [**158**,**159**], and phenolic profiles showed that seed coats contain up to 10 times more flavonoids if compared to whole seeds [**160**]. The seed coat pattern is a fundamental aspect of consumer preference, but in different regions, only some patterns are preferred. On the other hand, local landraces contain a great variability of colours, selected through centuries by human activity, but often this richness remains undervalued [**135**,**137**,**161**]. Our suggestion is to use this kind of information to correlate the colours of seed coats with the proper chemical characterisation regarding previously mentioned bioactivities. Moreover, modern experimental planes should include wild accessions/species and underused landraces because these mostly unexplored taxa could hide important micronutrients. Finally, we

underline that the introduction of new dishes based on a mix of seeds that show different colours could be a new way to assimilate a great variety of nutrients into the diet.

6. Introgression and Feralisation Processes

Through the domestication process, one or more populations that showed desirable traits are selected by humans producing new independent lineages. Farmers have strongly influenced the survival of these cultivated lineages that continued to diverge from wild ancestors because they were affected by different selective pressures. However, crops and their wild relatives can exchange genetic information spontaneously or through human activity. Generally, wild relatives of legumes show undesirable traits, but their genomes can hide a precious gene pool that is mostly untapped that can be recovered and reintroduced in cultivated forms. Introgression of useful genes remains a fundamental way to improve the cultivar [**162**], and successful crosses mediated by humans were acquired, especially in cowpea [**163**]. Although domesticated cowpea is known as an inbred crop, outcrossing is reported suggesting that frequency and distance can vary depending on the environment, climate, subspecies, genotype and insect involved [**73**,**164**,**165**]. In *Vigna unguiculata,* spontaneous introgressive events between wild perennial subspecies of the *dekindtiana* group, including accessions of var. *spontanea*, are widely described observing different morphological traits [**71**,**84**,**166**,**167**,**168**]. Molecular analysis using AFLP [**74**] and internal transcribed spacers [**53**,**169**,**170**] have confirmed the natural propensity to hybridisation between subspecies and have revealed intricate intraspecies phylogenetic relationships. Intragenomic 5S rRNA repeat unit heterogeneity was interpreted as the consequence of extensive hybridisation events [**170**], and recently, plastid DNA sequences have confirmed chloroplast capture events [**76**]. In recent decades, several researchers have tried to produce introgressive lineages

obtaining interesting results and showing that the most important gene pool for breeding programs could be harboured in wild subspecies. Intraspecific hybrids obtained crossing ssp. *unguiculata* with ssp. *pubescens* and cv. *sesquipedalis* with ssp. *tenuis* have shown vigorous growth and partial fertility [**171**,**172**,**173**]. Some authors attributed the incomplete success to chromosomal disturbances that ensue in endosperms and embryos during early seed development when crosses between wild perennial accessions and domesticated cowpeas are performed [**174**]. However, different accessions showed diverse propensity to hybridize, and a recent study suggests that temperature and humidity also have a prevalent role in increasing the success of hybridisation [**175**]. A wild lineage of cowpea (TVNu-1158) collected in the Republic of Congo showed resistance to *Aphis craccivora,* surviving long after infestation [**176**], and was successively crossed with cowpea to produce new lineages [**177**]. Moreover, resistance to *Maruca vitrata* was observed in the wild lineage of ssp. *dekindtiana* (Tvnu 863) from Zimbabwe andresistance to *Clavigralla tomentosicollis* was observed in ssp. *dekindtiana* (TVnu 151) from Ghana; however, literature about their use to produce new cultivars is missing [**178**,**179**]. Limited information is available about the intraspecific introgression of *V. subterranea* and *V. vexillata*. The success of the artificial cross of Bambara is constrained by scarce pollen viability, the small size of the flower and the reduced stigma–anther separation, which improves the transfer of pollen to the stigma but at the same time complicate the emasculation process [**180**,**181**,**182**,**183**]. However, F1 and F2 lines were obtained by crossing *Vigna subterranea* var. *spontanea* (Harms) Pasquet and *Vigna subterranean* var. *subterranea* (L.) Verdc. varieties, allowing us to identify that the main morphological traits to distinguish the two forms (internode length and stems per plant) are regulated by relatively limited numbers of genes [**184**]. Intraspecific introgression success was also obtained by James and Lawn [**185**] who crossed African and Australian accessions of *V. vexillata* with the aim to explain the resistance to mottle carmovirus (CPMoV). Recently, modern hybridisation techniques were

applied to cross var. *macrosperma* cultivated and wild accessions obtaining encouraging results [**186**,**187**]. Unfortunately, scarce findings are achieved by interspecific hybridisation. Differently from Asian taxa, where the compatibility was confirmed in different studies, the African taxa show a cross incompatibility barrier that has so far prevented the introgression of useful genes (e.g., *V. vexillata* × *V. unguiculata*) [**168**,**188**,**189**,**190**]. In recent years, advances in sequencing technologies have allowed the generation of a large number of genomic resources that, if combined with approaches that estimate the rate of gene flow, enable us to detect which lineages are prone to hybridisation. Screening the level of introgression already existing in nature is an important opportunity that can help us to obtain advanced information useful in breeding activity. For example, natural hybrid zones harbour genetic variance and, pervasive and occasional introgressive events are identified in several crops such as kiwi, common bean, soybean, sunflower and grape [**191**,**192**,**193**,**194**,**195**,**196**]. Differently from neutral introgression, which could be lost during successions of generations, adaptive introgression events are maintained by selection, and foreign gene variants introduced by gene flow can increase the fitness of receiver populations as observed in potato, rice and millet [**197**,**198**,**199**]. African *Vigna* species have a potential for introgression that today remains mostly unexplored. *V. unguiculata* and *V. vexillata* show an elevated number of wild lineages that probably have diverged well before the Pleistocene due to climate changes [**71**,**125**]. Several subspecies are adapted to different environments, and Padulosi and Ng [**68**] proposed that the southernmost region of Africa is presumably the origin center for *V. unguiculata* where most subspecies grow, while Pasquet [**71**] indicated that some lineages from Namibia to Zimbabwe are the result of spontaneous introgression events. However, genomic studies are needed to confirm these hypotheses, including in the analyses of populations spread at the margins of species distribution that could hide local adaptation to extreme conditions. Principal component analysis, Bayesian clustering methods (e.g., NEWHYBRIDS, STRUCTURE and ADMIXTURE) and

divergence statistics such as FST are used to explore patterns of divergence in *Vigna* species, but they manifest shortness to provide the effective migration rate. To overcome this limitation, different probabilistic approaches recently developed are able to identify recent and ancient signals of introgression such as tree-based methods (e.g., Treemix), coalescent-with-introgression simulations (e.g., MSci model implemented in BPP), composite-likelihood test (e.g., VolcanoFinder), site frequency spectrum to explicitly model migrations (e.g., ∂a∂i), gene genealogies (e.g., Twisst) and ABBA–BABA statistics [**200**,**201**,**202**,**203**,**204**]. Moreover, only some genomic regions could be involved in gene flow, and thus introgression might be localised in specific chromosomes [**205**]. Since alleles shared through incomplete lineage sorting remain complex to distinguish from alleles shared through introgression and none of the measures described above is without simplifying assumptions, we suggest that different methods should be applied to ascertain the origin of introgression. Although introgression from wild to crops has important economic consequences and many attempts are made to understand the evolutionary dynamics, in recent years, attention to the gene flow from crop to wild is rapidly increasing. Introgression of domesticated alleles can stimulate the evolution of weeds or increase the risk of extinction of wild populations with dramatic evolutionary consequences, as demonstrated in several annual and perennial plants [**206**,**207**,**208**,**209**,**210**,**211**]. Moreover, under specific circumstances, the spread of ferals escaped from cultivation and adapted to wild environments can hardly be contained. Although several authors consider feralisation the opposite process of domestication, few population genomics studies show how these genetic changes occurred in plants [**212**]. Some authors show that multiple de-domestication events have occurred in rice, highlighting that some crops are exceptionally prone to feralisation [**213**,**214**]. The introgression process is probably improved when the wild forms grow along the road margins, villages and fields where domesticated forms are cultivated. To date, few studies have investigated the introgression effects on the wild

populations of African *Vigna* species. Some researchers have proposed that alleles from cowpea may be incorporated into wild forms especially improved by their cohabitation, replacing the original alleles and making new lineages well adapted to wild environments [**215**]. A molecular study based on analysis of isozyme loci showed that outcrossing rates in West Africa range from 1% to 9.5%, confirming possibilities of gene flow from domesticated cowpea to var. *spontanea* [**216**]. The distinction between feral and truly wild lineages is ever more complicated because introgression produces fertile offspring and the small seed-size typical of wild forms is dominant to large seed size [**76**,**217**]. Moreover, var. *spontanea* is represented by both annual and perennial plants, and it is acknowledged that while the annual and inbred habit is an adaptive strategy in dry and warm environments (e.g., in warm tropical savannas), perennials tend to grow in mountainous regions where the environment is often cooler and wetter [**76**]. Annual inbred plants produce more seeds and show a competitive advantage on perennials when they are sympatric in environments. Although few data about introgression are available, we cannot exclude that perennial outcrossed subspecies can be fertilized by cowpea pollen, and consequently, domesticated alleles can be introgressed. Moreover, feralisation can involve adaptive changes in genes related to flowering timing, dormancy and metabolic pathways, which are also unknown. Therefore, several aspects of the feralisation process, including the ability to spread domesticated alleles accross long distances by seeds and pollen through mammals or birds and the predisposition to invade territories where perennial subspecies grow, should be further explored.

7. Domestication-Related Traits and *De Novo* Domestication

As described by Darwin [**218**], most plants subjected to intensive domestication have lost the ability to survive in the wild environment for more than a few generations. Traits selected by humans allow us to

clearly distinguish a domesticated plant from its wild progenitor, and several studies were recently proposed to highlight the genes at the base of these changes. In recent years, modern genomic techniques were applied to *Vigna* germplasm, accelerating research activity and opening new avenues to identify domestication-related traits [**33**,**161**,**219**,**220**,**221**]. Among the main domesticated traits in legumes, the loss of pod shattering and increase of organ size are most relevant for breeding. In cowpea, two main quantitative trait loci (QTLs) were identified for pod shattering, whereas QTLs identified for seed weight, leaf length, leaf width and pod length were located in the same region, suggesting a potential pleiotropy that controls the organ size [**177**]. Lonardi et al., 2019 [**222**] managed to obtain the entire genome sequence in order to analyse and identify the eventual putative syntelog for organ gigantism. They found a region containing a cluster of QTLs controlling pod length, seed size, leaf length and leaf width (CPodl8, CSw8, CLl8, CLw8). Similar results were also observed in *V. vexillata* where the main domestication traits, including seed size, pod size and leaf size, were controlled only by one or a major QTL and some minor QTLs [**33**,**90**,**124**]. More complex is the control of seed dormancy, which is generally managed by water-impermeable layers of cells of the seed coat. In yardlong bean, a vegetable crop that has experienced divergent domestication from cowpea, six QTLs were detected for seed dormancy-related traits [**90**]. The seed coat pattern is an essential trait in cowpea, intensely selected by human preferences that change in the different areas of Africa. For example, pigmentation displays high variability of colours, including varied eye shapes and sizes. Recent studies show that the colour and position of the pigmentation can be defined by expression patterns, and some genes that encode for proteins involved in the flavonoid biosynthesis pathway were identified [**161**]. Moreover, phenotypic observations show that a lack of pigment in flowers is often correlated with a lack of pigment in the seed coat, and a gene was recently proposed to have a dual function in cowpea controlling the colour in both organs [**177**]. As observed for several species, the flower

was involved intensely in the domestication process, and it has a fundamental trait that allows us to distinguish domesticated cowpea from their wild relatives. Recently, innovative studies focused on exploring the genetic basis of floral scent. A group of five O-methyltransferase genes involved in the biosynthesis of melatonin and located within the floral scent QTL region was identified [**221**]. Melatonin is recognized as an essential molecule in several plants used to interact with pollinators. Flowering timing undoubtedly plays a key role in plant adaptation and diffusion of crops because several agronomic traits such as grain quality, plant growth and plant height are directly influenced by this characteristic [**223**]. However, how the domestication process has affected the timing of flowering in legumes is unclear [**224**]. Flowering timing is a complex trait generally regulated by genetic networks. While in *Arabidopsis thaliana* L., the existence of up to 80 loci [**225**] was shown, in a cowpea genome-wide association study (GWAS) seven reliable SNPs were revealed that explained phenotypic variance [**220**]. Important agronomic implications are expected because the candidate genes could be transferred by hybridisation in crops. Early flowering accessions can mature earlier, avoiding periods of drought stress, whereas late-flowering accessions can mature later and extend the vegetative period, thereby increasing biomass production. It is widely recognized that the study of domestication-related traits is a fundamental step that enables us to understand how to design ideal crops for the future. Throughout the process of domestication and successive breeding phases, the genetic diversity of crops was significantly reduced, and this homogeneity is becoming a serious threat. The increase of disease and inability of adaptation to environmental changes that consequently cause an increased use of pesticides and water with a severe impact on the environment are the main issues that affect the sustainability of modern agriculture. Fernie and Yan [**226**] emphasized that wild species contain less deleterious allelic variants than their crops, and Smykal et al. [**227**], in a recent review, reported that modern cultivars have lower levels of key vitamins and micronutrients, suggesting that several wild and semi-

wild African species should be *de novo* domesticated. Unfortunately, few studies of re-domestication are available, but recent advances in gene editing combined with the decryption of pan-genomes are opening new perspectives of manipulation of genes for the creation of modern crops [**228**,**229**]. Gene editing is used to modify the function of genes already existing, incorporate new genes and delete short or large DNA fragments [**230**,**231**]. For instance, undesirable traits can be reduced or removed by intervening in genes that regulate the content of secondary metabolites, accelerating the process of domestication. Otherwise, the life cycle of cowpea could be shifted coming back from annual to perennial, as occurred for *Triticum aestivum* L. [**232**]. Perennial cowpea crop would show deep roots, higher water and nutrients efficiency and would not need to be sown every year. Modern techniques such as CRISPR/Cas9 are applied successfully in several staple food crops. In *Oryza sativa* L., mutations on three yield-related genes have produced more and larger grains and erect panicles [**233**], whereas, in *Solanum pimpinellifolium* L., eight genes were targeted improving architectural traits, day-length insensitivity, the size and shape of fruits and content of vitamins [**234**,**235**]. Moreover, the CRISPR-Cas9 system was also used in cowpea to disrupt the symbiotic nitrogen fixation by the modification of a symbiosis receptor-like kinase (SYMRK) gene, thereby demonstrating that gene editing can be applied to the *Vigna* genus [**236**]. However, this technique requires that the genome is sequenced to identify the ortholog gene that controls the domestication trait [**237**]. African *Vigna* species are an ideal group of plants on which to apply gene-editing techniques and to produce modern crops. A great number of wild species, besides showing resistance to pests and diseases and having high nutritional values, are well adapted to diverse environmental conditions [**9**]. Only Angola, with 28 native *Vigna* species documented, is recognized as one of the most important sources of germplasm in the world [**238**]. *V. monantha* occurs in permanently dry conditions [**42**], whereas *V. marina* and *V. luteola* grow well in saline lands [**9**]. In particular, seedlings of *V. marina* can survive for several weeks in flooded conditions and high NaCl

concentration [**39**], accumulating high levels of salt in leaves, roots and stem [**41**]. However, few farmers currently use these plants because of low yield and strong pod-shattering behaviour, which requires high labour during the harvest. Adaptation to extreme environments often involves multiple genes, whereas domestication-related traits are due to mutations of a single locus that affects the loss of a function. Previously reported domestication-related traits in *Vigna* seem to be controlled by a restricted number of QTLs. Thus, introducing domestication-related mutations into wild species might be preferred rather than modifying multiple genes related to complex adaptation traits. For example, the first steps of re-domestication were achieved by Takahashi [**4**], starting from the accessions of *Vigna stipulacea* (Lam.) Kuntze originated in Asia. The authors obtained one mutant with reduced pod shattering and three mutants with reduced seed dormancy, characterizing the respective SNPs in the candidate genes. *V. stipulacea* was selected for their fast growth, edible seeds andbroad resistance to pests and diseases. Thus, *de novo* crops can be designed to preserve several traits that nature has selected in millions of years. Moreover, in the next few years, the pan-genomes of several economically important crops will be available. The investigation by sequencing multiple individuals, including wild and domesticated accessions, will allow us to acquire full knowledge of variations at the genome level. Since it is widely accepted that the use of few reference genomes is limiting, the pan-genome of the *Vigna* species should be achieved in a short time [**239**,**240**]. Consequently, given the large diversity of wild *Vigna* germplasm spread in Africa and the modern techniques of gene editing, great margins of genetic improvement are expected in the near future.

Author Contributions

Conceptualization, D.P., F.G., W.G.N. and M.L.; investigation, D.P. and F.G.; writing—original draft preparation, D.P. and F.G.; writing—review

and editing, D.P., F.G., W.G.N. and M.L.; visualization, D.P.; supervision, F.G. and M.L. All authors have read and agreed to the published version of the manuscript.

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9.5 Bioactive compounds in legumes: Implications for sustainable nutrition and health in the elderly population

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Highlights

• Food production represents one of the highest impacting activities on our planet, contributing to climate change.

• The concrete transition to a more sustainable diet rich in legumes is required.

• Leguminosae plays a relevant role in human health thanks to their nutritional composition and bioactive compounds.

• Bioactive compounds that play a key role in preventing the development of various chronic diseases related to ageing.

• The bioactive compounds found in legumes regulate glucose metabolism, displaying antioxidant and anti-inflammatory actions.

Abstract

Background

food production represents one of the highest impacting activities on our planet, significantly contributing to climate change. Agriculture is one of the most important drivers of these changes since farming, forestry, and livestock, emit approximately one third of the global total CO2. Moreover, the modern agriculture systems mainly focus on starchy vegetables leading to energy dense and nutrient poor dietary patterns related to chronic diseases, such as NCDs. Elderly are the most vulnerable population portion that experiences even more than others the impact of unhealthy dietary patterns. Therefore, a transition to healthier and more sustainable solutions must be adopted to reach the SDGs promoted by the Agenda 2030, positively impacting the environment and human health. The role of pulses in this context is discussed.

Scope and approach

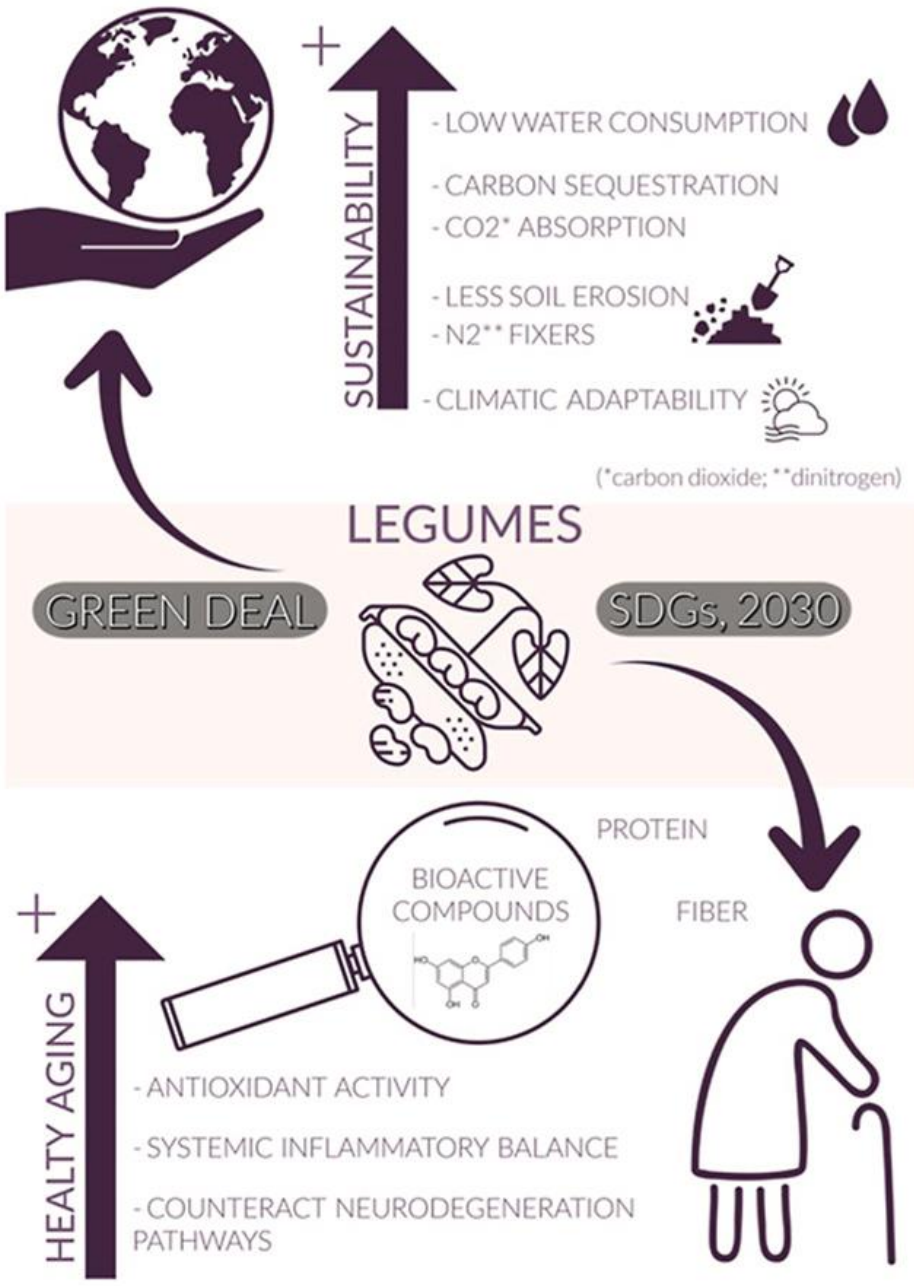
authors focus on the role of Leguminosae considering their potential impact both on health in the ageing population as well as on agri-food chains sustainability.

Key findings and conclusions

there is an increasing awareness on how legumes represent an important source of bioactive compounds (i.e., phenolics, saponins and peptides) with a wide range of healthy activities, all of which share antioxidant properties, essential to prevent or delay oxidative stress and disease in elderly population. With an ever-ageing population at the global level, healthy ageing is an emerging public health priority for securing citizens' quality of life and minimizing healthcare associated costs.

Graphical abstract

The imagine describes the sustainable role of Leguminosae in both Agry-Food System and Health, related to elderly population.



Keywords

Prevention, Healthy life, Phytoextract, Bioactive compounds, NCDs, Ageing population

1. Introduction

Climate change and its effects are expediting, with climate-related calamities accumulating, season after season (Food and Agriculture Orga, 2016). Concerning human nutrition, agriculture is one of the most important drivers of these changes, since innovation brought by the Green Revolution has completely modified process sustainability, leading to an irreversible tendency to adopt conventional and intensive practices (Food and Agriculture Orga, 2016). All of these changes were aimed at maximizing crop yields with the consequent disadvantage of environmental pollution (e.g. agrochemicals, fertilizers), creating negative effects such as greenhouse gases emissions due to adoption of mechanized processing systems. Furthermore, agriculture output, namely livestock, farming and emit nearly one third of the global total CO2 (Food and Agriculture Orga, 2016). Nowadays those agronomic practices are undeniable to meet the market demand but they do not take into account agricultural process sustainability which improvement is at the top of the goals of the 2030 WHO Agenda for Sustainable Development (Sustainable Development Outlook, 2020). As described in the FAO report for the international year of pulses in 2016 (Food and Agriculture Orga, 2016), “the answer to mitigate, adapt and reduce the effects of climate change come in the form of a single seed: the pulse”. In fact, pulses i) need less water than other crops to cultivate; ii) do not compete for water with other crops; iii) play a role in dealing with soil erosion and depletion since some of them are deeply rooted and their slow growth in early stages allows neighbouring crops to take root and thrive; iv) do not require nitrogen fertilizer, fixing themselves; v) improve

soil carbon sequestration, providing absorption of natural CO2 emissions by the earth (Food and Agriculture Orga, 2016). Although FAO focuses on pulses, it should be acknowledged that those plants also offer other edible portions besides seeds and fruits of Fabaceae such as leaves, rich in bioactive and nutritional molecules. Moreover, scientific data suggest that legume consumption reduces the risk of numerous chronic diseases (Becerra-Tomás et al., 2019) and provides a range of essential macro, micronutrients and bioactive metabolites with synergic effect against inflammation, which plays a role in disease onset or progression (Zhu et al., 2018). At the same time, identification of resistant cultivars against abiotic and biotic stresses and development of sustainable field management practices, could address both nutrition and environmental concerns of modern society (Zhu et al., 2018). Accordingly, these plants are a central topic in many international research programs, to promote environmental sustainability development and achieve zero hunger (Sustainable Development Outlook, 2020). In this review, the authors intend to stress the role of Leguminosae in enhancing health for the ageing population by means of sustainability of agri-food chains. In addition to their nutritional value, in terms of protein content, this review will focus on leguminous micronutrients, bioactive compound contents and their related health benefits. These considerations promote intake of legumes in plant-based diets and support the adoption of legumes in modern day agricultural systems, both in developing and industrialized countries to pursue long-term health.

2. Why is legume cultivation more sustainable than other major crops?

Today, the principal source of proteins in western diet is red meat (Willett et al., 2019). The consumption of meat at the global level is higher than other protein sources (Legumes, Fish, Total dairy, Poultry, Eggs), and North America stands out at the top of this ranking, followed by Europe and central Asia (Willett et al., 2019). This has negative effects not only on human health but also on the environment since livestock practices

are among the main sources of greenhouse gases (Willett et al., 2019). Transition to healthy dietary patterns by 2050 will require substantial dietary modifications, with more than 50% reduction in global ingestion of processed food, animal source foods (ASF) and more than 100% consumption increase in plant based foods, including nuts, fruit and vegetables (Willett et al., 2019). Despite the well-documented health benefits of legumes consumption, their actual intake remains low (Polak et al., 2015). Indeed, in many industrialized countries legume consumption does not reach the minimum daily-recommended intake (Polak et al., 2015). According to the National Health and Nutrition Examination Survey (NHANES) data, only about 8% of adults consume dry legumes and peas, on any given day (6https://www.ncbi.nlm.nih.gov/pubmed/?term=Campbell%20A%5BAuthor%5D&cauthor=true&cauthor_uid=26487796). Furthermore, from 1970 to 2013 legumes consumption in Europe has been subjected to a constant decrease due to the agronomical criticisms (i.e. low productivity, low economic competitiveness) and the tendency to adopt cereal crops characterized by a high market demand (Zander et al., 2016). However, in recent years people's food preferences tend to be more aware about the nutraceutical value of consumed foods (Agnoli et al., 2017). In general they tend to vary their dietary patterns depending on the knowledge of the presence of healthy nutraceuticals and legumes stand out for these features (SAWICKA et al., 2019). According to FAO stats, (Food and Agriculture Orga, 2016), the overall global production of pulses (seeds of leguminous) exceeds 92 million tonnes of which Asia contributes to more than 42 million tonnes. Today, the legume sector appears to be suitable for further growth and technical innovation (Disruption at the dinner, 2019; Wolk, 2017). For example, the market growth of meat-like plant-based products is estimated to rise from $ 4.6 billion in 2018 to $85 billion in 2030 (Disruption at the dinner, 2019). In May 2019, Impossible Foods, partnered with Burger King, presented the plant-based beefless “Impossible Whopper” and in January 2020, the meatless “Impossible Pork” and “Impossible Sausage” were further added to the restaurant

menu (Lucas, 2020). Kroger, the leading chain of grocery stores in North America, also presented the “ground beef” based on pea protein, “burger patties'', and other similar products without meat, under its Simple Truth Emerge line (Lucas, 2020). These pioneering market launches have led to a tendency, expected to increase, to test non-meat foodstuffs like legumes by numerous other fast-food chains (Lucas, 2020). The concrete transition to a more sustainable diet rich in legumes requires a substantial change of the typical “western” dietary habits, and food choices (also supported by industrial stakeholders) as well as the suitable strategies to enhance legumes cultivation, distribution and consumption (Wolk, 2017). This underlines once again how the concept of sustainability in today's food system is inseparable from that of human health (Willett et al., 2019) and Leguminosae well respond to both of these social needs. We know that legumes show many environmental sustainability advantages such as i) low agrochemical demand (fertilizers); ii) ability to grow up in harsh conditions like drought and by means of conservation agriculture (i.e., reduced or minimum tillage, cover crops maintenance) (Ngwira et al., 2012); iii) adaptability to different climatic conditions of different continents and iv) suitability to be involved in intercropping approaches with common cash crops, such as maize or wheat (Ngwira et al., 2012) with a consequent reduction of soil consumption, environmental pressure and plant damages (Hauggaard-Nielsen et al., 2008). Moreover, yield performances of several pulses are good both in intensive and conservation management (Kassam et al., 2009; Parihar et al., 2016), without affecting their nutritional value actually showing improvement in terms of nutritional value (Etemadi et al., 2018; Guzzetti et al., 2020). These characteristics, combined with the tendency to reduce soil disturbance and promote organic agriculture, can undoubtedly favour the spread of legumes in different countries. However, as previously mentioned also social, economic and health levers are needed to enhance the consumption of legumes in human diet. Health is deeply tangled with food production systems and food consumer perception, therefore this could act as a driver to select the most promising legumes, to enhance

local production and to improve the food distribution and consumption strategies (Etemadi et al., 2018). With this in mind, an in-depth study of the micronutrients present in legumes, not only in seeds and fruits but also in daughters, could suggest new forms of consumption, paving the way for food supplements and fortified foods as well as to propose a circular economy process to valorise the agricultural by-products for human health (Nugent et al., 2018). Furthermore, new bioactive compounds identification with beneficial or even therapeutic functions represents a lever of considerable importance also in relation to well-being and prevention of disease, especially in old age (Bruins et al., 2019; Nugent et al., 2018, 2020; Wichansawakun & Buttar, 2019). The advantage of plant extracts, compared to single isolated compounds or synthetic products lies in the complexity of the phyto-complexes and in the multiple actions that different molecules may carry out (Bruins et al., 2019; Wichansawakun & Buttar, 2019). Moreover, it is necessary to rethink the methods of habitual intake of these products, trying to meet both modern consumer taste and lifestyle, which leaves little time for meals preparation (Nugent et al., 2020). In this context, processing technologies can provide an important benefit both for primary matrices such as seeds and fruits, and for secondary products such as leaves, which have always been considered unused waste that can become a valuable resource (Nugent et al., 2020). Finally although globally more than 1000 of legumes are known to be grown, only 20 belonging of genus *Cicer*, *Glycine*, *Vigna*, *Lens*, *Phaseolus* and *Pisum* are cultivated for consumption (Rao, 2002); therefore an extensive analysis and valorization of legumes biodiversity should be performed to expand the genetic and metabolic resources and to improve the nutritional value of this family.

3. Health benefits of legume consumption

It is well known that legumes are an excellent source of macronutrients, such as proteins, fibers and complex carbohydrates. These features make them a reliable dietary item since:

i) they are slowly digested, compared to the starch-richer cereals and tubers, thus providing a feeling of satiety; ii) they help to control glucose level in blood by reducing spikes after mealtimes thanks to the requirements for fiber and protein components digestion. This is of particular concern in ageing populations, notoriously more glucose intolerant and insulin-resistant (Tharanathan & Mahadevamma, 2003). Moreover, legume dietary fibers have been shown to exert prebiotic properties in cellular, rodents and human models and are SCFAs precursors in the gut throughout fermentation processes (Morel et al., 2015). In particular, α-GOS belong to the soluble polysaccharides class made of galactosidic units acting as appetite regulators, decreasing blood glucose and cholesterol levels and inducing proliferation of beneficial microbes in the gut, such as Lactobacillus and Bifidobacterium (Dai et al., 2017; Morel et al., 2015). However, some soluble oligosaccharides found in legumes such as α-galactosides, raffinose-family oligosaccharides (RFOs), verbascose, ajugose, ciceritol and stachyose pass through the stomach undigested, into the intestines. The small intestine of monogastric organisms, such as humans, does not produce the enzyme necessary to convert these complex sugars into their simpler constituent ones. Thus, these undigested sugars are fermented by colonic bacteria, such as *Escherischia coli*, *Enterococcus faecium*, *Streptococcus macedonius*, *Streptococcus pateuranius*, *Enterococcus avium*, (Ranganathan, 2020). The by-products of this degradation process are hydrogen, carbon dioxide, methane, and sometimes sulfur, which can cause bloating, cramping, and flatulence (Winham & Hutchins, 2011). As far as micronutrients are concerned, a considerable number of vitamins, such as those belonging to the B complex (including folates, thiamin and niacin), are highly represented in legumes, together with some important minerals such as potassium, phosphorus, calcium, magnesium, iron and zinc (de Jager, 2013). These micronutrients are fundamental to many cellular metabolisms and are excellent antioxidants that slow-down natural ageing processes (Höhn et al., 2017). Moreover, potassium and magnesium also act in controlling blood pressure, reducing the risk of

heart diseases and, together with calcium, contribute to reducing risk of bone fragility (Höhn et al., 2017; Murphy et al., 2018; Tharanathan & Mahadevamma, 2003). The beneficial role of legumes for human health also resides in their bioactive molecules (Murphy et al., 2018) of which phenolic compounds, saponins, peptides and small proteins are the most relevant (Wink, 2013). Some of these are ubiquitous in the family, while others are typical of some genera or species and their synthesis is elicited by plant growing conditions (e.g., development stage, amount of light, and water availability) (Sparvoli et al., 2015). Phenolic compounds are probably the most studied category of phytochemicals Within legumes, the most represented phenolic classes are flavonoids, tannins, stilbenes and phenolic acids. Their biosynthesis occurs with a particular extent in the seed coat and in the leaves (Heleno et al., 2015). These compounds exert a wide range of beneficial properties, including metabolism, homeostasis and cell proliferation modulation. Moreover, they have antioxidant properties, which are essential for the prevention of oxidative stress conditions and diseases (Zhao et al., 2014). Flavonoids are probably the most common bioactive compounds of legumes, especially in those having colored seed coats (Aguilera et al., 2011; Rolnik et al., 2020; Żuchowski et al., 2014) and are the main class of polyphenols showing the highest antioxidant potential. This group encompasses anthocyanins and anthoxanthins, in addition they exhibit free radical scavenging capacity, anti-inflammatory and anticancer properties besides a positive impact on immune response (Cena & Chieppa, 2020). Tannins are another class of polyphenols that are common in the seed coat of many legumes such as beans (Jin et al., 2012). Often, these phytochemicals are present as condensed tannins (e.g., catechins), which are considered anti-nutritional compounds since they affect protein digestibility, decreasing the absorption of minerals and some vitamins (Jin et al., 2012). However, in the last years, several health benefits have been attributed to the intake of tannins, ranging from antioxidants to the inhibition of lipid peroxidation and antimicrobial activities (Silva & Pogačnik, 2020). In addition to polyphenols, legumes are also rich in peptides and small

proteins that display many biological activities applicable as nutraceuticals and/or therapeutic agents (Jeong et al., 2007, 2009). In the last years, these small proteins have been investigated *in vitro* and *in vivo* to assess their beneficial properties and their potential role in prevention of chronic degenerative diseases (e.g., throughout reduction of inflammatory process, immunomodulatory and anticancer properties). (Jeong et al., 2007, 2009; Park et al., 2005). Some of the most studied bioactive peptides of legumes belong to the albumin and globulin fractions, as well as to many still uncharacterized non-digestible peptide fractions. Among the most promising molecular cancer-preventive peptides there is lunasin, firstly isolated from soy and consisting of 43 amino acids and a molecular weight of 5.5 kDa (Jeong et al., 2007, 2009; Park et al., 2005). Its bioactive properties rely on the ability to induce an arrest of cell division of cancer cells but not of healthy ones (Park et al., 2005). *In vitro* tests suggested that this small peptide is able to inhibit core histone acetylation in mammalian cells and to protect DNA from oxidative damage (Hsieh et al., 2018). Lunasin is also able to reduce oxidative stress and to inhibit the release of pro-inflammatory cytokines and interleukin-6 (Hsieh et al., 2018). Another promising bioactive compound is known as Bowman-Birk inhibitor (BBI). This is a small trypsin-chymotrypsin inhibitor produced by plants as a defence against herbivores (mainly insects) (Mehdad et al., 2016). It is a small molecule of 70–80 amino acids found for the very first time in soy and subsequently in many other legume species (Mehdad et al., 2016). BBI is a promising anticancer agent, which showed a proteasome 20 S inhibition action in breast cancer cells (Mehdad et al., 2016). Although the activity mechanism played by this inhibitor has not been fully characterized yet, experimental studies suggested that this peptide induces cell apoptosis and acts as a suppressor of CDK2 with the consequent arrest of cell cycle in G1/S phase (Chen et al., 2005; Saito et al., 2007). Finally, a well-studied family of legume proteins is represented by lectins whose peculiar binding ability makes them good candidates as carriers for target drug delivery. For example, it is well known that carbohydrates present on the cancer cell

membrane are involved in recognition processes and lectins could be used to transport and release anti-cancer drugs during the different phases of tumour progression (Boland et al., 1992). Overall, due to the wide variety of bioactive compounds occurring in legumes, research activities have been promoted both on common species (Table 1A) and on less known local taxa ones (Table 1B) to screen specific compounds as well as to study the entire phyto complexes (Boland et al., 1992).

Table 1A. Bioactive properties of common legume crops.

Species	Bioactive properties	Molecules involved	References
Cicer arietinum L. 	Antioxidant	Total extract, peptides, proteins, phenolic compounds, fibers	Faridy et al., 2020 (Faridy et al., 2020)
	Hypoglycemic and anti-diabetes	Genistein, formononetin, biochanin A	Lin et al., 2020 (Lin et al., 2020)
	Antimicrobial	Lectins	Gautam et al., 2018 (Gautam et al., 2018)
	Glucose metabolism modulation, body weight regulation and anti-diabetes	No one in particular (focus on legume based meals)	Becerra-Tomàs et al., 2018 (Becerra-Tomás et al., 2018)
Phaseolus vulgaris L. 	Anti-diabetes, anti-hypertensive, antioxidant	Digested peptides of seed globulin fractions	Garcia et al., 2020 (De Fátima Garcia et al., 2020)
	Prevention of cardiovascular disease	Peptides	Ngoh et al., 2017 (Ngoh et al., 2017)
	Hypocholesterolemic, prebiotic and fermentation modulator	Resistant starch and dietary fibers	Kilua et al., 2020 (Kilua et al., 2020)

	Anti-inflammatory, hypolipidemic and hypocholesterolemic, antioxidant	Bioactive hydrolyzed peptides	Gomes et al., 2020 (Gomes et al., 2020)
	Glucose metabolism modulation, body weight regulation and anti-diabetes	No one in particular (focus on legume based meals)	Becerra-Tomàs et al., 2018 (Becerra-Tomás et al., 2018)
Pisum sativum L.	Protease inhibition	Bowman-Birk protease inhibitor	Clemente et al., 2018 (Clemente et al., 2012)
	Modulation of intestinal bacteria	Protein fraction	Ge et al., 2020 (Ge et al., 2020)
	Glucose metabolism modulation, body weight regulation and anti-diabetes	No one in particular (focus on legume based meals)	Becerra-Tomàs et al., 2018 (Becerra-Tomás et al., 2018)
Lens culinaris L.	Prebiotic, hypocholesterolemic, fecal bile acids and SCFAs enhancer	Soyasaponins (group B)	Micioni Di Bonaventura et al., 2017 (Micioni Di Bonaventura et al., 2017)
	Anticancer potential	Seed aqueous extract conjugated to nanoparticles	Ahmeda et al., 2020 (Ahmeda et al., 2020)
	Glucose metabolism modulation, body weight regulation and anti-diabetes	No one in particular (focus on legume based meals)	Becerra-Tomàs et al., 2018 (Becerra-Tomás et al., 2018)
Glycine max L.	Inhibition of proliferation, anti-inflammatory	Peptides derived from *in vitro* simulated digestion	Gonzalez-Montoya et al., 2018 (González-Montoya et al., 2018)
	Osteoporosis prevention	Isoflavones	Taku et al., 2011 (Taku et al., 2011)
	anti-inflammatory	Glucosylceramide and steroidal glucoside	Mizushina et al., 2012 (Mizushina et al., 2012)

Vicia faba L. 	Anti-oxidant, anti-biofilm and tyrosinase inhibition	Peptides	Karkouch et al., 2017 (65)
	Anti-carcinogenic and hypocholesterolemic	Protein hydrolysates	Leon-Spinosa et al., 2016 (León-Espinosa et al., 2016)
	Antimicrobial, antioxidant, anti-diabetes	Pods alcoholic extract	Mejri et al., 2018 (Mejri et al., 2018)
Lupinus spp.	Anticancer	Alkaloids	Liu, 2009 (Liu, 2009)
	Anti-inflammatory	Protein hydrolysates	Millán-Linares et al., 2014 (Del Carmen Millán-Linares et al., 2014)
Arachis hypogea L.	Hypolipidemic	Polyphenolic extract of peanut skin	Bansode et al., 2012 (Bansode et al., 2012)
	Antioxidant and antimicrobial	Spray dried extracts	Valle Calomeni et al., 2017 (do Valle Calomeni et al., 2017)

Table 1A description: non-exhaustive summary of main bioactive properties of common legume crops.

Table 1b. Bioactive properties of neglected and underutilized legume species.

Species	**Distribution area**	**Bioactive properties**	**Molecules involved**	**Reference**
	Africa and India	Anti-ageing and anti-neurodegenerative	Seed aqueous extract	Tripodi et al., 2020 (Tripodi et al., 2020)

Vigna spp. 		Anticancer	Purified extracts containing Bowman-Birk inhibitors	Panzeri & Guzzetti, 2020 (Panzeri et al., 2020)
		Antihypertensive and antioxidant	Peptides	Arise et al., 2017 (Arise et al., 2017)
		Hypocholesterolemic	Powder mix with soybean	Tan et al., 2020 (Tan et al., 2020)
		Anti-diabetes, anti-hypertensive, antioxidant	Digested peptides of seed globulin fractions	Garcia et al., 2020 (De Fátima Garcia et al., 2020)
Cajanus cajan L. 	Africa and India	Anti-inflammatory and cytotoxic	Cajaninstilbene acid and pinosylvin monomethylether	Schuster et al., 2016 (Schuster et al., 2016)
		Apoptosis inducer	Cajanol	Luo et al., 2010 (Luo et al., 2010)
Lablab purpureus (L.) Sweet 	India	Anti-obesity	Chikusetsu Saponin IVa	Yin et al., 2018 (Yin et al., 2018)
Lathyrus spp. 	Asia and West Africa	Antioxidant, enzyme inhibitory and cytotoxic	Extracts	Llorent-Martinez et al., 2017 (Llorent-Martínez et al., 2017)
		Anti-elastase	Bowman-Birk inhibitors	Rocco et al., 2011 (Rocco et al., 2011)

Table 1B description: non-exhaustive summary of main bioactive properties of neglected and underutilized legume species.

Table 1A shows a not exhaustive list of the most common legume species and their health properties based on scientific evaluation. It can be noted that in some cases specific molecules classes and cellular targets involved in the responses have been identified; however, in other cases a biological response was induced by the total extract. Considering the multitude of bioactivities described for the most cultivated and the minor legume species and cultivars, it is evident that these plants constitute a key source for both diet and new nutraceuticals agents. However anti-nutritional factors or food toxicants in legume phyto-complexes must also be addressed (Singh et al., 2017). Usually, these metabolites are associated with plant defence mechanisms against biotic and abiotic conditions (Murphy et al., 2018). These include polyphenols, alkaloids (Singh et al., 2017) enzyme inhibitors, lectins, phytates and oxalates. Depending on their content in raw seed or flour, the ingestion of lectins may cause adverse effects including growth suppression, bloating, vomiting, diarrhoea, and red blood cell agglutination (Singh et al., 2017). Nevertheless, evidence shows that inactivation of lectins occurs if food is treated at 100 °C or more for at least 30 min (Panacer & Whorwell, 2019) which is below the average time cooking for legumes. Therefore, it is likely that their occurrence in a legume-based diet would not be responsible for detrimental dietary effects (Lucius, 2020). Finally, the issue of anti-nutrients is very complex and ambivalent since many compounds considered as anti-nutrients are the same ones that, when suitably modulated, can cause beneficial or therapeutic effects, and usually differential effects depend on the doses. For instance, alkaloids and saponins can cause disturbances in the central nervous system and digestive, reproductive and immunological disorders (Lucius, 2020), but, on the other hand, saponins can act as cancer-preventive agents, decrease the level of blood cholesterol and lower blood glucose response. In addition, anti-inflammatory, hypocholesterolemic and immune-

stimulatory activities of saponins have also been described (De Fátima Garcia et al., 2020).

4. Legume consumption and elderly population health

Nowadays the world's population is ageing because of an increased global life expectancy and the incidence of Non-communicable diseases (NCDs), including metabolic, cardiovascular, neurodegenerative, inflammatory bowel diseases and many types of cancer, is increasing accounting for about 63% of all global deaths (Santoro et al., 2020). It is important, therefore, to focus on the elderly population group to ensure a healthy aging process. As stated by ESPEN (Volkert et al., 2019) guideline on clinical nutrition and hydration in geriatrics, the main aim of geriatric medicine is to optimize functional status of the older individuals to ensure greatest possible autonomy and best possible quality of life. Epidemiological data show that an adequate diet plays a pivotal role in healthy ageing (Prinelli et al., 2019) as the elderly population is more vulnerable to inadequate nutrition than the younger population and dietary intake is determined by a combination of several medical and socio-economic aspects (Brownie, 2006). In this contest, several studies described how a plant based dietary pattern undoubtedly contributes to promoting healthy eating, diet. As mentioned above, legumes contain several nutrients and healthy bioactive components able to prevent the NCDs development (Prinelli et al., 2019). Even if, to date, most evidence resulted from *in vivo* and *in vitro* studies, as previously described, an habitual consumption of legumes during one's life, may have a prevention role in the onset of NCDs (Bruins et al., 2019). Very recently, Caballero and colleagues (Caballero et al., 2020) conducted a prospective study with the purpose to evaluate in the elderly population the association of legumes/traditional legume-based recipes consumption, with a multidimensional health index, named *Deficit Accumulation Index* (DAI), considered an indicator of unhealthy ageing. The authors concluded that consumption of legumes should be part of a healthy dietary pattern in

aging (Caballero et al., 2020). The Food Habits in Later Life (FHILL) multi-cohort study (Japan, Sweden, Greece and Australia) conducted on 785 participants aged> 70 reported that legume intake was a predictor of survival regardless of ethnicity, showing 7%–8% of reduction in mortality risk rate for each 20 g increase in daily intake (Darmadi-Blackberry et al., 2004). Similarly, a meta-analysis of six prospective cohort studies described a weak and inverse association between the intake of legumes and the risk of all-cause mortality (Relative Risk: 0.96; 95% Confidence Interval: 0.94–1.00) (Schwingshackl et al., 2017). As we have already underlined, the nutraceutical components of legumes are numerous and the beneficial effect on health applies to all groups, particularly to the elderly. In fact, the consumption of legumes could positively impact essential processes, including cellular oxidation and inflammatory processes, frequently impaired in ageing (Lardiés-Sánchez & Sanz-París, 2017).

4.1. Cellular oxidation and ageing phenomena

Ageing process is the greatest risk factor for the development of neurodegenerative and neurocognitive diseases, where oxidative stress plays a central role (Phillipson, 2014). Recently, it has been published a study showing the protective effect of *Vigna unguiculata* beans against ageing and neurodegeneration where oxidative stress and neuroinflammation have been demonstrated to play a transversal role in both α-synuclein abnormal aggregation and ageing (Phillipson, 2014). Indeed, *V. unguiculata* extract decreases α-synuclein toxicity and in a *C. elegans* Parkinson Disease model partially rescues the degeneration of cephalic dopaminergic neurons (Tripodi et al., 2020), supporting the role of *V. unguiculata* beans as a functional food in age-related disorders. It is well-documented that both seeds and leaves of legumes are a source of several molecules with antioxidants properties (Martín-Cabrejas, 2019). Davis and colleagues (Davis et al., 2020) described the role of the anthocyanins, a class of dietary polyphenols, contained in black beans in

improving the cellular antioxidant activity, including metal sequestration, cell signal modulation processes, and the expression of antioxidant genes (Moreno-Valdespino et al., 2020). Saponins are able to actively capture free radicals and at the same time stimulate enzymes with antioxidant properties thus counteracting the formation of the metal ion-free radical complex (Moreno-Valdespino et al., 2020). Furthermore, legumes represent a good source of tocopherols and carotenoids, fat-soluble molecules, which exert their antioxidant activity by scavenging peroxyl radicals, thus preventing lipid peroxidation (Moreno-Valdespino et al., 2020). Finally, oxidative status is also modulated by protein fraction and fiber, through the activity exerted by the gut microbiota (Martín-Cabrejas, 2019). However, the conjugated forms of these antioxidants molecules change after the legume processing (Martín-Cabrejas, 2019). For example, during boiling procedures, which is one of the most popular methods of processing legumes, some phytochemicals can seep into the cooking water or be altered by the heating (Martín-Cabrejas, 2019). For this reason legumes have been used as raw material for the production of extracts with antioxidant properties, tested *in vitro* and *in vivo* (Martín-Cabrejas, 2019).

4.2. Inflammatory processes

Numerous *in vivo* and *in vitro* studies have correlated the health benefits of legumes to their content of bioactive compounds, such as polyphenols, saponins, proteins and peptides capable of remodelling the systemic inflammatory balance in the elderly by slowing the production of inflammatory molecules and simultaneously enhancing anti-inflammatory responses (Martucci et al., 2017). Indeed, Nah et al. recently documented that peptides can regulate several markers of inflammation, including prostaglandin E2 (PGE2), nitric oxide (NO), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX- 2), cytokines, and chemokines (Nah et al., 2008). Again, soyasaponins, that are found in soybeans and other legumes, such as common beans, adzuki

bean, green peas and lentils inhibit the production of the pro-inflammatory cytokines (TNF-α and MCP-1, PGE2 and NO), the inflammatory enzymes (COX-2 and iNOS) and the degradation of IκB-α (an inhibitor of NF-κB, in LPS-stimulated macrophages) as previously described by Nah and colleagues (Nah et al., 2008).It is well documented in the literature how the role of the legumes's consumption in preventing the onset of NCDs and improving health in the elderly (Bruins et al., 2019) Intake of certain types of nutrients, higher in plant-based foods, including legumes, positively influences health and promotes the prevention of common non-communicable diseases (NCDs) (Cena & Calder, 2020). Concerning sugar metabolism, Recently the comprehensive study PREDIMED (PREvención con DIeta MEDiterránea) conducted on 3349 participants (age range 60–80 and 55–80 years, respectively in women and men), suggested that the frequent legumes consumption (especially lentils), may provide benefits in the prevention of type 2 diabetes mellitus in older adults showing an decreased cardiovascular disease risk (Becerra-Tomás et al., 2018). Similarly, other evaluations performed on consumption of legumes at medium-to-long-term in individuals with prediabetes or diabetes mellitus, suggested a positive effect on markers of glycaemic control (Bielefeld et al., 2020). A possible explanation of the beneficial role of legume consumption on glucose metabolism is provided by the agrobiodiversity analysis showing hypoglycemic activity of naturally occurring peptides and protein hydrolysates (Brownie, 2006), in addition to inhibitory action on key enzymes, such as α-amylase, α-glucosidase and Dipeptidyl-peptidase IV, involved in sugar metabolism (Bielefeld et al., 2020). Furthermore, legumes are also rich in magnesium, an essential micronutrient and cofactor for enzymes that are involved in glucose and insulin metabolism (Bielefeld et al., 2020). Although not all the mechanisms of the nutrients and biocompounds contained in legumes are known, research is building evidence of their key role in not only on glucose metabolism but also on body weight regulation, both intimately bound and frequently impaired in the elderly, (Bielefeld et al., 2020). The prevalence of obesity in ageing population is increasing and in

those aged 65 and older is frequently combined with sarcopenia (Batsis & Villareal, 2018). This association leads to functional impairment, increased mortality and reduction in quality of life. Age-related body composition changes with loss of muscle mass and increase of fat mass are due to multiple factors and treatments include also diet which must ensure adequate protein intake and quality of proteins to counteract weight loss-induced sarcopenia (Batsis & Villareal, 2018). Moreover dietary protein not only impacts on muscle-protein synthesis but also on the gut microbiome and evidence suggests that plant-based proteins, alongside with increased dietary fiber intake, promote gut microbiota eubiosis with an increase in satiety, besides protein synthesis and the over all metabolic effects discussed above (Prokopidis et al., 2020). More and more results indicate that legumes are important in regulating body weight, drawing inspiration from *in vitro* studies, focused on the inhibition activity of common bean peptides on lipid accumulation in 3T3-L1 cells (Toledo et al., 2016), therefore future exploitation is expected. Legumes could be a base for the development of many functional foods to promote human health.

5. Nutrition transition: legumes to prevent neurodegenerative phenomenon

Mazza and colleagues (Mazza et al., 2017) studied the association between food choices among the elderly Italian population and cognitive function, highlighting the advantages of a dietary pattern based on plant proteins and legumes consumption. Legume's beneficial effect on cognitive performance endured regardless of age at enrolment, lifestyle habits like smoking and sex (Darmadi-Blackberry et al., 2004). The role of legumes on cognitive function is still not fully understood, although some evidence suggests that their neuroprotective role could be related to improvement of insulin sensitivity (Mazza et al., 2017). On the other hand consumption of legumes is usually associated with dietary patterns rich in vegetables known for their positive impact on homocysteine levels

which contributes to cognitive impairment (Mazza et al., 2017; Polak et al., 2015). Besides a growing body of evidence suggests that bioactive compounds of legumes contribute to prevention of NCDs (Martín-Cabrejas, 2019). Legumes are an excellent source of antioxidants molecules helpful in counteracting the natural ageing processes, including inflammation and oxidative stress (Franceschi et al., 2018). Recently, the Global Burden of Disease indicated 60 g/d (50–70 g/d) as the optimal mean daily consumption of legumes for beneficial health outcomes (Polak et al., 2015; Willett et al., 2019). However, despite the well-known properties of legumes, Caballero and colleagues (Caballero et al., 2020), in the Seniors-ENRICA cohort on 2505 individuals aged ≥ 60 years, reported that 78.4% of subjects came close to the optimal mean daily intake as reported above (mean intake 57,9 g/d). Furthermore legume consumption tends to decrease with age (Willett et al., 2019) and varies according to cultural background, culinary education (Polak et al., 2015) and geographical location, showing greater propensity to higher intake of legumes in the Mediterranean region (8–23 g/d) and less in Northern Europe (<5 g/d) (Polak et al., 2015).

6. Practical advice for legume consumption

The 2020–2025 Dietary Guidelines for Americans (DGA) (U.S. Department of AgricultureU.S. Department of Health and Human Services, 2020) emphasizes the benefits of a plant-based diet for better health. These recommendations include consumption of legumes several times per week. Unfortunately, many consumers avoid eating legumes because they fear excessive intestinal gas or flatulence. Increased flatulence is an expected outcome among some people, especially the elderly who often suffer from intestinal bloating and discomfort, disturbances attributed to the consumption of fiber and legumes, which are therefore reduced However, health care professionals, including dieticians, should inform subjects that intestinal discomfort will decrease over time if legume consumption is gradually increased and continued and that adequate

consumption outweighs the potential for transitory discomfort (Winham & Hutchins, 2011). Individual variation in response to different bean types should be considered and psychological anticipation of flatulence problems addressed. The potential of increased flatulence should be managed with practical advice including increasing legume intake slowly, make sure to drink as much water, as recommended, soaking dry legumes before cooking changing the water several times, adding herbs when cooking, try different bean varieties to determine if certain types produce greater desirable or undesirable symptoms than others (Winham & Hutchins, 2011).

7. Conclusions

Legumes as cereal grains have been part of human history for over 10,000 years. The history of plant domestication has told us that prehistoric men were focused more on efficient sources of energy as starches and sugars, but nowadays the latter have certainly surpassed legumes in terms of energy density, economic yield and unsustainability. The Green Revolution has further increased this gap by building the basis of the modern trend of diets that are neither sustainable nor healthy, leading to frequent micronutrient inadequacies and deficiencies (MNDs) all over the world regardless of the countries' development stage. MNDs have enormous influence on the health of vulnerable people like older adults posing them at higher risk of NCDs. In this context legumes become important both for the present and the future, providing bioactive compounds besides valuable proteins and soluble fiber and can also be thought of as new promising novel food sources (Polak et al., 2015). We have moved from considering nutrition as a preventive tool to acknowledging it as a disease management one and legume bioactives are recognized to be useful to fight chronic degenerative diseases throughout their multiple and synergic actions, including metabolic, antioxidant, ant-inflammatory and regulatory functions. However we should try not to incur in the mistakes of the past when plants genetic

improvement was focused to provide only one or few components, losing many secondary compounds that may act preventing metabolic alterations and pathological conditions. In addition, it would be important to consider crops as a whole by assessing both the edible portions and the so-called by-product that could be used for extracting bioactive compounds. This is true for legumes too that are primarily consumed as seeds in Western countries, while in other countries (e.g., Africa, India etc.), consumption of leaves, either fresh or boiled, is also common. Legumes leaves are proven to be a great source of bioactive compounds and micronutrients, and therefore may become valuable allies in the promotion of a healthy plant based diet aimed at delaying nutrition-related NCDs (Polak et al., 2015). This is in line with the strategies of the European green deal and especially with the principles of the Circular Economy and may be achieved by trying to have a global vision of the nutritional sources. Noteworthy legumes are central components of several health promoting dietary patterns around the world, embedded in local tradition or developed to prevent disease and/or positively influence health (Cena & Calder, 2020). Scientific evidence suggests that it is more effective to focus on food policies that encourage the consumption of diet components, such as legumes, which is currently below the optimal level, rather than focusing on countering the consumption of foods high in sugars and fat (Polak et al., 2015). This needs broad interventions on the food system starting from production. Production of legumes should be enhanced both by urban and peri-urban agriculture with the aim of promoting distribution and consumption across nations, promoting a sustainable and proximity production chain (Galimberti et al., 2020). In this context, smallholder food producers play a key role in feeding cities, contributing to build synergies across food security, ecosystem services, sustainability, with the ultimate goal of achieving adequate nutritional status, to build resilience and well-being (Galimberti et al., 2020).

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Declaration of competing interest

None.

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10. Manuscripts in preparation

In this chapter are presented works that have been carried on during the last PhD thesis period that show interesting data, but are not concluded or need pivotal experiments to be considered finished and then submitted to journals.

10.1 Exploration of Natural Genetic Diversity and Computational Analyses of Bowman-Birk Protease Inhibitors in *Vigna unguiculata*

Background of the work

Vigna unguiculata is a very biodiverse African legume species. This species is very relevant in rural and small farm contexts due to its adaptability and little cultivation demands. However, the great majority of landraces is still wild and untouched by human activity. The exploration of potential bioactive compounds into wild and domesticated accessions is fundamental to find new or better features for further analyses or improvement programmes. Bowman-Birk protease inhibitors are defensive proteins for legumes, but are chosen as target compounds due to the proven efficacy in facing different types of human diseases.

Aim of the work

The objective of this work is to evaluate the natural genetic diversity of the gene encoding a Trypsin-Trypsin BBI isoform (TT-BBI). In particular, how this diversity is diffused and diversified in wild, spontaneous and domesticated accessions to identify protein isoforms with differential calculated bioactivities and potential applications for human health.

Approaches involved

- 185 seed accessions coming mainly from Africa are retrieved from 3 different germplasm seedbanks, covering the great majority of *Vigna unguiculata* subspecies and landraces.

- Molecular biology techniques are employed to identify, amplify and sequence the target BBI gene.
- Phylogenetic approaches are used to estimate diversity parameters and relationship between haplotypes and isoforms found. Subsequently, analyses of episodic and diffused selection are performed.
- Computational analyses of interaction energy and dynamic structure are applied to identify isoforms with better interacting capability.

Preliminary Results

Diversity evaluation

A total of 472 sequences were found from 465 genotypes of 185 accessions. 24 haplotypes and 13 corresponding isoforms were discovered. In Tab. 1 are summarised haplotypes found, their frequency and the corresponding isoform. Calculated diversity indexes: 19 polymorphic sites, of which 18 were Parsimony-Informative Sites (Bases positions: 57, 79, 80, 84, 89, 100, 113, 120, 127, 138, 150, 155, 180, 192, 213, 228, 245 e 261). Nucleotide diversity detected was 0.00398 and a haplotype diversity of 0.655.

1	252	1
2	98	2
4	4	
5	24	
6	2	
7	15	
8	3	
10	5	
11	1	
18	2	
3	1	3
15	17	
16	8	
9	12	4
19	5	

12	4	5
13	2	6
14	1	7
17	1	8
20	4	9
21	1	10
22	7	11
23	2	12
24	1	13

Tab. 1: Haplotypes found, frequency and corresponding protein isoform.

Haplotypes-Isoforms relationships

A B

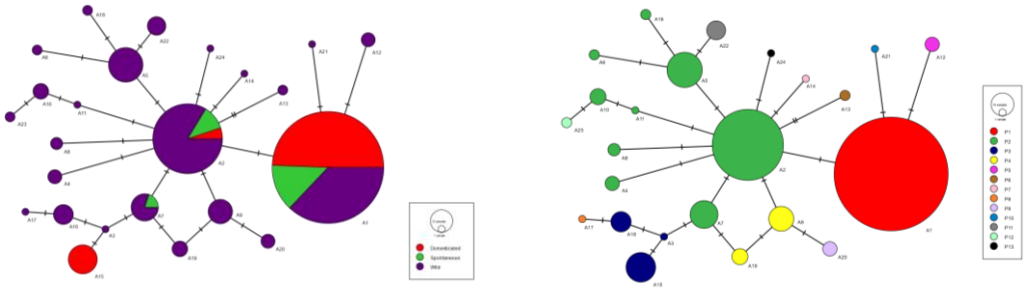

Fig. 1: A) Network of haplotypes and distribution within accession type (purple = wild, green = spontaneous, red = domesticated). B) Network of haplotypes and corresponding isoform (every colour is a different isoform)

Networks shed a light on the relationships between haplotypes based on the accession type and the corresponding isoform. In the first network (Fig. 1A) is observed a great dominance of wild-exclusive haplotypes, while domesticated ones are only confined to 3 haplotypes. Haplotype 2 results to be the most central one with 9 branches derived from it. The second network (Fig. 1B) investigates how isoforms are distributed among haplotypes. In this case it is clear that the isoform 2 (mostly from

wild accessions) is very spreaded among haplotypes showing a tendency to maintain this isoform.

Positive selection analyses

In order to find sites subject to positive selection, FUBAR and Site Model Test analyses were performed. FUBAR analysis detected 1 site under positive selection and 5 purifying selections (Tab. 2). The Site Model Test evaluates only positive sites by confronting models basing on Maximum-Likelihood. In this case, 3 positive sites are detected, one of which is the same site revealed by FUBAR, but they do not reach the acceptance criteria (Tab. 3).

Site	α	β	β-α	Prob[α>β]	Prob[α<β]	BayesFactor[α<β]	Amino Acid
28	16.654	0.637	-16.017	0.912	0.069	0.089	S
34	1.414	19.057	17.643	0.028	0.951	23.377	S/T
40	27.918	0.660	-27.258	0.993	0.004	0.005	E
60	16.657	0.669	-15.988	0.910	0.071	0.092	H
64	26.610	0.707	-25.903	0.991	0.005	0.006	I
71	17.080	0.782	-16.297	0.972	0.016	0.020	S

Tab. 2: FUBAR analysis outcome

Site model (SM)									
Model	**np**	**Ln L**	**Estimates of parameters**				**Model compared**	**LRT P-value**	**Positive sites**
M8	50	-612.55510 4	p0=0.81708 (p1=0.18292)	p=0.00730 ω=1.77758	q=2.11833		M7 vs.M8	0.554450359	27 G 0.766 34 S 0.816 43 E 0.737
M7	48	-613.14488 2	p=	0.01253	q=	0.02596			-

Tab. 3: Site Model analysis outcome

Isoforms *in silico* interaction evaluation

To understand the potential activity of each isoform, computational analyses were realised (Fig. 2). Three isoforms seem to be better in interacting with trypsin, P13, P9 and P3 (the higher the value, the more

stable the interaction). Instead P4 resulted worse in interacting with the target.

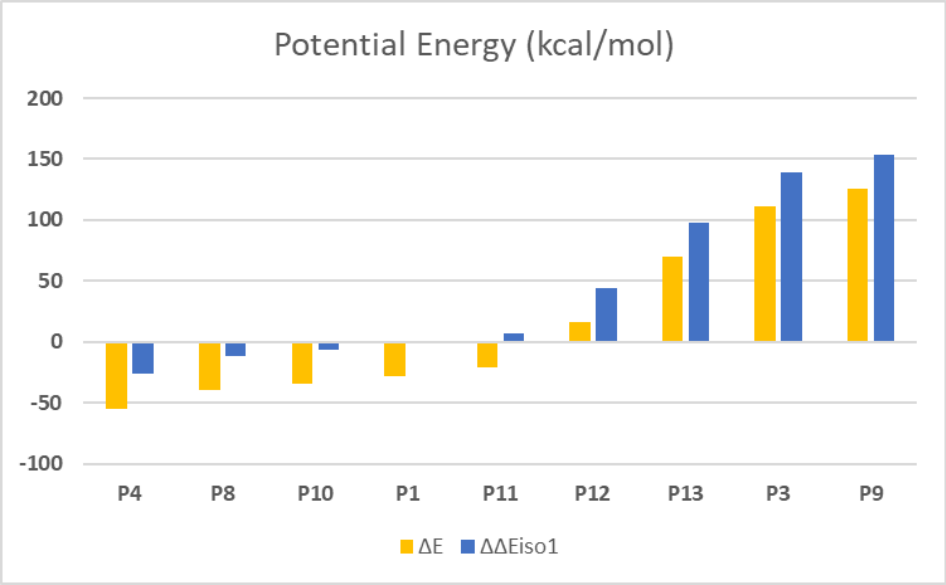

Fig. 2: Calculated interaction energies between BBI isoform and trypsin

Discussion

The exploration of natural genetic diversity of the TT-BBI gene showed a great amount of haplotypes, many of which derived exclusively from wild accession. Therefore, every deduced isoform is represented by at least one wild accession, remarking the importance of wild gene pool for plant species. The network analyses showed two distant clusters with domesticated accession, making us hypothesise the occurrence of two different domestication events. Selection analyses showed the existence of sites under selective process. The site 34 is under positive selection and, even if it falls on the propeptide region, its mutation could be relevant for correct protein folding. Negative selection is detected on other sites, probably due to their structural role. Finally, isoforms were tested *in silico* to evaluate the binding energy with the trypsin physiological target. In this case, we identified at least three isoforms that interacted better and suggesting a more potent or lasting inhibition activity. BBIs bioactivity is strictly linked to their interactivity with

physiological targets: in our case, biodiversity can provide isoforms that could exert better performance in health-promoting capabilities.

10.2 Bowman-Birk Protease Inhibitors from *Vigna unguiculata*: Purification and Deep Characterisation

Background of the work

Bioactive compounds are a group of secondary plant metabolites that show remarkable health-promoting activities. The protein fraction in legumes is not only important for a balanced diet, but contains valuable peptides and small proteins. Among these, the Bowman-Birk protease inhibitor (BBIs) family stands out for their panel of confirmed bioactivities. Although BBIs are very studied in legume crops, there is little knowledge in African Indigenous Vegetables, such as *Vigna unguiculata*.

Aim of the work

This work has the objective to develop an optimised extraction and purification procedure to obtain pure isoforms from seeds. Secondly, isoforms need to be characterised in terms of trypsin and chymotrypsin inhibition activity and singularly sequenced and identified.

Approaches involved

- Proteins of *Vigna unguiculata* seeds are extracted and separated in three protein macro groups: Legumins, Vicilins and Albumins. The albumin fraction is the one containing BBIs.
- Size-exclusion and cation exchange chromatographies are applied and customised to purify BBIs from albumins.
- Trypsin and Chymotrypsin inhibition activities are tested *in vitro* with albumin fraction and purified isoforms.
- Peptide mass fingerprinting and precise molecular weight are calculated for the purified isoforms through mass-spectrometry analyses.

Preliminary Results

Extraction yield

From a starting material of 100 g of seeds, about 1 g of albumins is recovered. Albumin fraction has 97% of purity.

Purification step - Size Exclusion Chromatography

Albumin fraction is firstly loaded into a size-exclusion column to separate contained protein basing on the molecular weight. Trypsin inhibition assays are performed to follow the presence of BBIs (red line, Fig. 1).

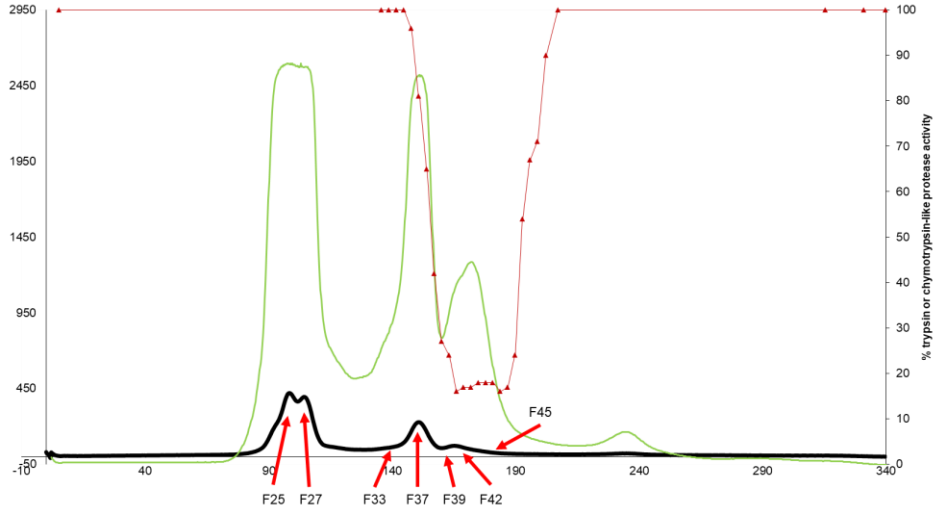

Fig. 1: size exclusion chromatography profile (green line = 214 nm, black line = 280 nm, red line = trypsin inhibition screening) and collected fraction for SDS-Page Gel Electrophoresis.

Collected fractions are then loaded into an SDS-Page Gel electrophoresis, Fractions 42 and 45 present Trypsin inhibition activity and, in the gel, show a protein band at the expected BBIs molecular weight (around 8-9 kDa, Fig. 2).

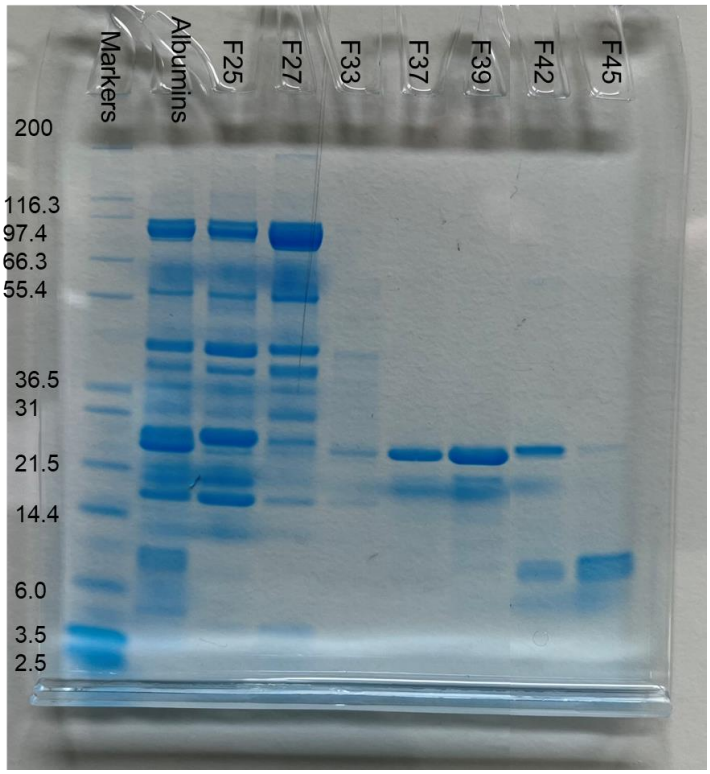

Fig. 2: SDS-Page Gel electrophoresis on collected fraction of size-exclusion chromatography

Purification step - Cation Exchange Chromatography

Fractions showing inhibitory activity from the size-exclusion are collected and concentrated. Then, are loaded into a cation-exchange column to separate single isoforms basing on net protein charges. The trypsin inhibition screening showed the presence of eight different separated peaks, each of which corresponds to a BBI isoform (Fig. 3). Among these, two peaks also presented chymotrypsin inhibition activity (Trypsin-Chymotrypsin isoforms) while the other only showed trypsin inhibition (Trypsin-Trypsin isoforms).

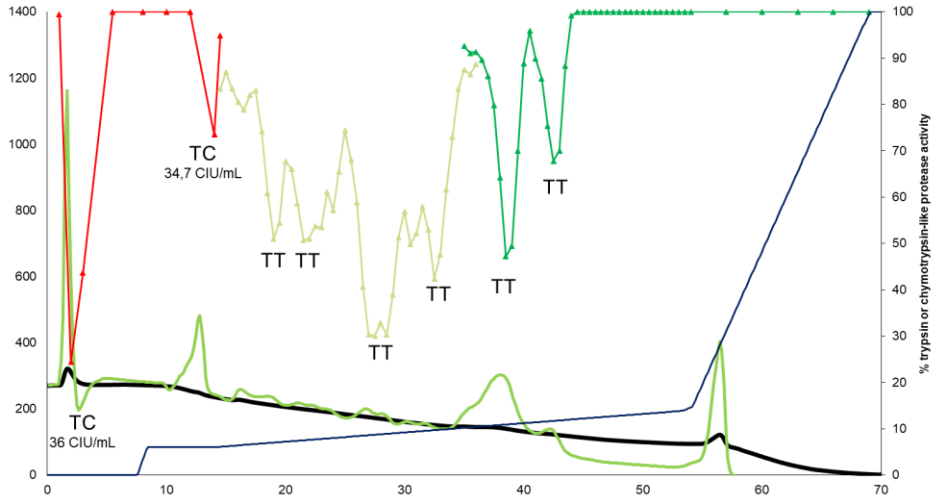

Fig. 3: cation-exchange chromatography profile (green line = 214 nm, black line = 280 nm, red line = trypsin inhibition screening). Isoform are indicated with TC for Trypsin-Chymotrypsin BBI and TT for Trypsin-Trypsin BBI. For TC-BBI also quantification of inhibited chymotrypsin unit per mL is displayed.

Characterisation of a Trypsin-Chymotrypsin isoform

The Trypsin-Chymotrypsin isoform (the first peak in Fig. 3) was isolated and further characterised by precise mass and sequence with mass peptide fingerprinting. In Fig. 4A is shown the purified peak on a SDS-Page gel electrophoresis while in Fig. 4B are presented the mass profile and molecular weight. The non-denaturing conditions of the mass calculation permitted to detect multimerization statuses of this isoform: monomers, dimers and trimers are shown. The peptide sequence is presented in Fig. 4C, domains have been sequenced thus confirming the isoform.

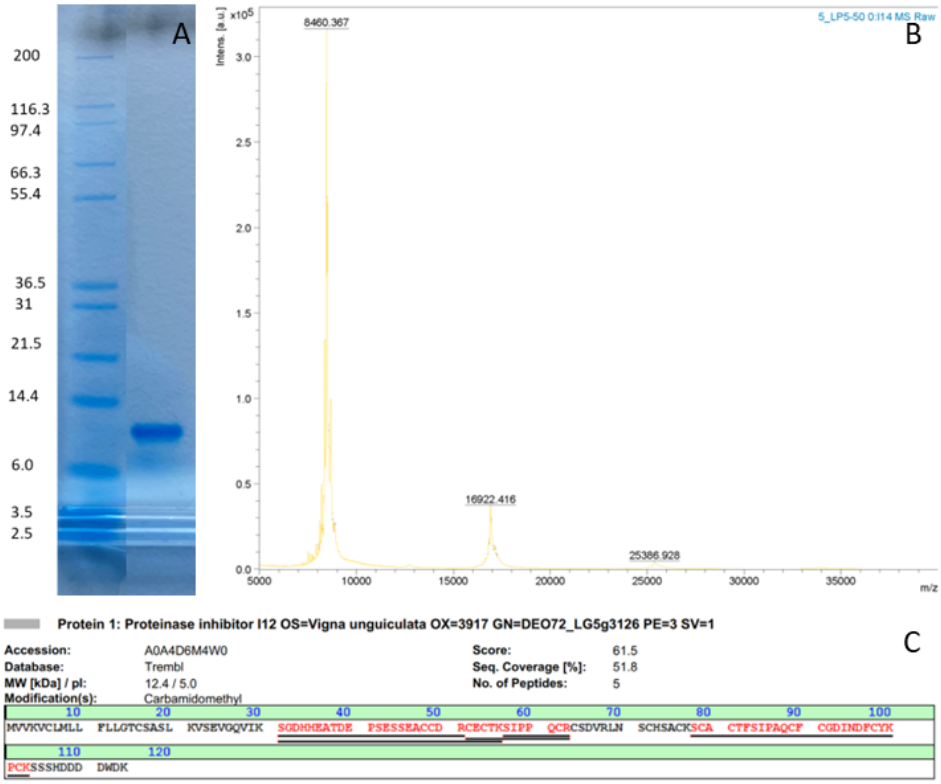

Fig. 4: A) SDS-Page Gel Electrophoresis , B) Precise mass and multimerization status, C) Peptide sequencing of TC-BBI fraction.

Discussion

Bowman-Birk protease inhibitors are relevant molecules with high added value for diets due to their health-promoting properties. The characterisation deepening is fundamental to identify correctly and univocally isoforms and understand their interacting features. In this work, we managed to extract proteins at a high purity level allowing further identification. The combination of chromatographic techniques displayed a sufficient separation capacity, dividing single isoforms from each other. Unexpectedly, from molecular weight analyses a trimer BBI structure was reported. The peptide mass fingerprinting made it possible to confirm at a sequence level the identity of the Trypsin-Chymotrypsin isoform. Further characterisation is needed to grant the full panel of identified isoforms and compare it with other legume profiles to find the most suitable for assessing most beneficial BBIs.

11. Conclusions

This thesis evaluated different aspects of the potentiality of a known, but still not fully employed plant species, *Vigna unguiculata*. Natural biodiversity and, especially, wild relatives are crucial to expand the current knowledge and exploit it to develop new cultivars with optimal nutritional profile and containing high value bioactive molecules. In this conclusive chapter the main outputs of this PhD thesis are summarised and discussed along with future perspectives and take-home messages.

11.1 Sustainability potential of *Vigna unguiculata*

During this thesis work, one of the main issues addressed was the exploration of natural biodiversity and sustainable exploitation for human health. The research in this field is quickly accelerating to match the increasing need in terms of high quality foods, keeping an eye on the entire production chain. Despite the well known nutritional properties of legume, their intake is actually lower than the recommended (Polak et al., 2015, Chapter 9.5). The choice of *Vigna unguiculata* as object of the project was mainly due to the fact that this legume is already adapted to stressful conditions but with a little literature that gave a peak on the extreme potential of this species. Our first work part (Chapter 9.1) was then developed to confirm and evaluate growth and production related parameters of *Vigna unguiculata* through ad-hoc field experiments. The outcome of the work was promising, we detected no significant differences between the various conditions plants were stressed with and demonstrated the suitability of this species to deepen our research. In particular, the nutritional profile and yield performances were not affected by the cultivation treatments, being similar in conventional and conservation land management. This is important information to encourage the spread of legume cultivation in different countries and

primarily of already adapted cultivars. Many current crops are in fact much more affected by environmental changes.

The main conclusions of this phase suggest that:

1) *V. unguiculata* lends itself to low environmental impact crops, i.e. it does not require deep ploughing and therefore allows to preserve the texture of the soil, reduce percolation phenomena and excessive loss of water and nutrients. It is a species with low water demand and this is a significant factor both for cultivation costs and for its resistance to drought phenomena also deriving from ongoing climate changes.

2) Although there are numerous international initiatives aimed at safeguarding the African accessions of *V. unguiculata*, there is a modest availability of certified seeds. Often, the single accessions have a high interspecific genetic variability also due to heterozygosity. This is an advantage for breeding programs but a problem for large-scale crops that require adequate standardisation of growth and harvest. It is therefore necessary to invest in local seed servers and African propagation centres to differentiate the most promising cultivars and also characterise their main agronomic features. Only through this process will it be possible to promote the cultivation of valuable African varieties on a global scale.

3) The edible portions of the plant are not just the seeds. African culture offers many recipes for consuming the leaves both cooked and cooked. This aspect is interesting as it allows to enhance the vegetative parts of the plant and not just the seed. To promote this practice, however, it would be essential to further investigate the nutritional and nutraceutical value of vine leaves in different stages of development and growing conditions.

11.2 Bowman-Birk inhibitors diversity in *Vigna unguiculata*

Bowman-Birk protease inhibitors are ending up in the spotlight for their nutraceutical properties , but little is known about their genetic diversity. Wild lineages or relatives keep the great majority of diversity, while domesticated crops or variants tend to be much more homogeneous (Maxted and Kell, 2009). In my work the evaluation of natural diversity was initially accomplished through a gene sequence screening of 185 accessions of *Vigna unguiculata*, mainly chosen in the African continent because it comprises the great majority of subspecies, varieties and cultivars (Chapter 10.1). In addition, being Africa the cradle of diversification of *Vigna unguiculata*, it has plenty of wild and untapped landraces (Chapters 9.4 and 10.1). As expected, we found a high number of gene haplotypes (24) and more than 50% of these (13) could be translated into different isoforms. As expected, expanding the screening into wild accession diversity increases exceptionally. The genetic screening and network analyses revealed one of the main domestication effects, the bottleneck. In fact, domesticated cultivars were represented by only 3 different haplotypes out of 24 total ones. On the other hand, this data permitted us to hypothesise two distinct domestication events: the first and, probably, the older one represented by haplotype 1 and the second one, found on a limited number of accessions, represented by haplotype 15. However, the most interesting results regard the wild accessions. Haplotypes that fall into wild accessions were 23 out of 24 found and all isoforms were found into wild landraces. Computational analyses on interacting energy with trypsin target showed interesting results. Differently from the expected, the reference isoform (P1), found in the greatest number of cases, especially domesticated, was not the best isoform. Some amino acid mutations revealed to improve the overall stability of the BBI in interaction with trypsin: we managed to calculate that some isoforms (P9, P3 and P13) are better interactors (see Chapter 10.1, Fig. 3). These isoforms, P9 and P13, are found exclusively in wild

accessions while P3 can be found both in domesticated and wild accessions (see Chapter 10.1, Fig. 1B). The reason for this improvement has to be fully elucidated, but two hypotheses can be formulated: mutation stabilises the protein during the interaction or rises the affinity with the ligand. Further calculations and *in vitro* analyses are necessary to better understand the mechanism. Our work demonstrates that wild diversity could hide better genes or better features that could be exploited to improve current cultivar crops. Could it be an elite trait? Very recently, scientists started to talk about a new approach to overcome traditional domestication techniques to generate "super cultivars" that possess the best characteristics possible. Until a few years ago, domestication and cultivar development was a trial and error approach by breeding or mild editing. *De-novo domestication* approach should completely surpass all the problems in favour of a targeted modus operandi that could mimic in a few years what natural and artificial selection made possible in hundreds, if not thousands, of years. Differently from conventional techniques, laboratory driven data, big data, literature and databases are the starting point and not the final stage. All information we can recover from these sources could be applied with targeted breeding, editing and then field trials. Further experiments and laboratory validations are needed, but, of course, this is very encouraging and bioactive compounds could be crucial for new upgraded cultivars.

The main conclusions of this phase allow us to suggest that:

1) Knowledge of the diversity of wild varieties and indigenous cultivars offers significant opportunities for precision bioprospecting which aims to identify the most active forms of biomolecules and to produce basic knowledge for *de novo* domestication projects;

2) Despite the bottleneck effect, the observed BBI isoforms show a fair genetic variability and mutations in some parts of the protein that could translate into functional variations. It will be interesting to understand both the effect of isoform BBIs both on target enzymes (trypsin and chymotrypsin), and on cells in vitro and subsequently in vivo.

3) Although our research has been addressed to the BBI, it should be noted that legumes are particularly rich in interesting compounds that could be useful both for human well-being and for ecosystems (e.g. molecules for defence against pathogens, biostimulants, etc.)

11.3 Bowman-Birk inhibitors as promising nutraceutical compounds

The last questions this work wanted to answer are: Are there interesting bioactivities? What is the role of BBIs? I succeeded in demonstrating that extracts from *Vigna unguiculata* containing BBIs can exert a wide panel of bioactivities (Chapters 9.2 and 9.3). We then managed to further deepen scientific knowledge by partially purifying and characterising the BBI isoforms from seeds (Chapter 9.2). Interestingly, we provided proof of interactions with an undescribed target, the Epidermal Growth Factor Receptor (EGFR, Chapter 9.2). Phosphorylated EGFR (pEGFR) is a marker of cancer growth and survival and can be subject to mutation that make it over-expressed or permanently active. In our work we also demonstrated that cowpea extracts can lower concentrations of pEGFR, interfering with cancer vital processing. EGFR is targeted by Cetuximab, a monoclonal antibody employed as the election drug in patients with mutated EGFR cancer profile. This kind of treatment though can become rapidly ineffective due to the development of drug resistance strategies. An interesting finding we illustrated is that our extracts were able to supplement the Cetuximab activity. In fact, we obtained the same viability reduction by combining drug and extract but with much lower dosage, suggesting a synergic interaction between them. Further studies are needed to clarify how they can interact and if this combination could reduce drug resistance occurrence. With much probability, molecules extracted, including BBIs, can interact with a set of molecular targets, many of which are still unknown. Literature is quite concordant that BBIs target Proteasome 20S (Mehdad et al., 2016), but since it is a huge molecular complex, the precise interaction area is to be determined.

Furthermore, we demonstrated bioactivities other than antitumour capacity by studying (Chapter 9.3) ageing and neurodegeneration models. These are serious concerns due to the increment of life in industrialised countries. Finding molecules that could slow age-related cellular processes is an issue that needs a strong and rapid response. For this purpose, we designed a complex series of experiments that could evaluate *Vigna unguiculata* extracts in models, both *in vitro* and *in vivo*, representing distant evolutionary groups and with peculiar ageing and neurodegenerative phenotypes. The chosen models were *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and a human neuroblastoma cell line. Cowpea extracts acted very efficiently and responded coherently in every model. They were able to exert neuroprotective effects and interfered with α-synuclein aggregation. These proteins are responsible for Parkinson's disease and our extracts delayed their aggregation both in *Saccharomyces cerevisiae* and the human neuroblastoma cell line. By exerting this activity, *Vigna unguiculata* extracts decreased significantly neurotoxicity caused by α-synuclein aggregation and also reduced the consequent intracellular damages, demonstrating a high protective potential for a grievous age-related disease.
All these data suggest that further research is mandatory to fully comprehend the processes involved in the various bioactivities. However, the main conclusions of this phase allow us to suggest that:

1) combined computational approaches and biochemical and cellular tests *in vitro* make it possible to evaluate the activity of a compound and the synergy of this in a mixture of natural molecules (phytoextract). To confirm the effectiveness of the molecules it is essential to study stability, bioavailability and above all adequate routes of administration in more complex model systems (from organoids to animal models)
2) an in-depth study on the molecular mechanisms of action of the BBI will be able to clarify whether there is a direct action on a cellular receptor

or if the effect is linked to certain properties of activating cellular defence pathways (i.e. pathways of stress signals, systemic cell repair, etc.)

11.4 Take-home messages

Biodiversity loss: a worldwide problem

The diversity of life on Earth delivers essential contributions to human life: crop pollination, climate regulation, water and air filtration, soil formation and disaster risk mitigation. However, biodiversity is dramatically declining, mainly due to human-induced pressure and this trend is expected to grow fastly, unless broad policy actions are taken to stem the drivers of this deterioration. “Expected future climate change related temperature increases may threaten one in six species at global level.” (European Parliament, Preparing the post-2020 biodiversity framework). The loss of biodiversity is now a realistic primary concern in Italy and the whole Mediterranean regional area. The evidence of the problem is now widely perceived as urgent that the Italian Parliament has recently (February 2022) approved two articles of its Constitution Chart (9 and 41) in order to introduce the central role of biodiversity as a constitutional value that deserves specific protection for public interest. This results in a call for action for the scientific community in order to develop knowledge and enable technologies to address the challenge and respect the fundamental Constitutional change

The EU with the Green Deal is acting directly on biodiversity conservation and high quality food through targeted actions, for example the 2030 Biodiversity strategy. In a more global vision, the United Nations Members have generated and signed in 2015 the 2030 Sustainable Development Goals Agenda, which enlists 17 major goals (UN SDGs, 2015) to organise a worldwide joint response to 17 issues. Since early 2000, an international day has been dedicated to biodiversity, falling on 22nd of May. Every year, this day is devoted to a precise objective to create awareness among citizens all over the world. This year the topic was

“Building a shared future for all life” and the convention itself proposed 22 actions that everyone can take to improve or protect biodiversity.
During this thesis project we aimed to address this challenge by studying biodiversity in its complexity by evaluating the lowest level or the genetic level and investigating the metabolic and molecular diversity of emblematic plants.
The most striking messages emerging from this three-year experimental work are:
- The Fabaceae family offers both macronutrients and bioactive molecules but, above all, there are numerous selvatic, indigenous and proto-domestic species that grow even in extreme conditions, such as those of sub-Saharan Africa. Our experiments have shown that *V. unguiculata* is well suited to the Mediterranean climate and to any phenomena induced by water stress. This suggests that it is possible to implement our agriculture by enhancing the current cultivars, by practising *de-novo* domestication actions but also by enhancing the local germplasm of precious areas of the planet where the green revolution has not caused vegetal and varietal erosion by promoting only hyper-productive species and agronomically demanding. The alliance between biodiversity and agrobiodiversity is not a foregone conclusion. In the past, monocultures required the absence of any other species considered pests. Nowadays, not only are intercropping approaches practised, but the wild species serve as a protective cover for the land in the response phase. Inspired by the strategies of conservative agriculture with low mechanisation and by exploiting more rustic species such as the African indigenous vegetables, it would be possible to obtain a renewal of Mediterranean agriculture by reducing environmental stress.

- Plants offer a multitude of bioactive compounds resulting from a long evolutionary process that led them to interact with the animal and microbial world to defend themselves but also to exploit pollinators, dispersers and transformers. It is therefore evident that many secondary metabolites of plants affect animal biological receptors and among these

is also included human populations. In many cases, evolution has favoured the development of variants of bioactive molecules, as in the case of the BBIs family that demonstrate the ability to bind simultaneously and independently to trypsin and chymotrypsin. We do not yet know well what the molecular mechanism is underlying the anti-cancer action exerted by BBIs, it could be the recognition of a specific receptor or a complex but what is relevant is the possible synergistic action of these factors even with chemotherapy drugs. One of the most important problems of anticancer therapies are relapsing tumours that are able to evade the action of the drug. A possible strategy is the adoption of a mixture of drugs and bioactive molecules capable of hitting different targets, reducing the onset probability of resistant cancer cells. The combinatorial action between cetuximab and extracts, containing BBI, exerted on some cell lines opens up the possibility of exploiting this protein as a support element for the drug.

- Finally, the relationships with the African countries, with the germplasm collections and with the local farmers was a fundamental part of the doctoral experience as it allowed to build an operational research process based on the analysis of varieties and cultivars of real interest to diversi African countries such as Tanzania and Kenya, both suitable for the Mediterranean basin. In this sense, I believe that a process of Innovative and Responsible Research (RRI) is essential both to sharpen the starting technical information and to direct research actions towards concrete themes. Some factors such as resistance to water stress rather than the importance of having more homogeneous seeds to increase yields represents a key issue that we have addressed both with field experiments and with molecular biology and phylogeny approaches. A fundamental part of my PhD project will therefore be the return of information to African communities so that research data can be transformed into social value.

12. Appendix

During this PhD thesis, I contributed to other studies related to the fields of bioactive compounds and phylogenetics. I also contributed to other investigations, not directly related to this issue. A list of these articles is reported below in chronological order.

1. Guzzetti, L., Panzeri, D., Ulaszewska, M., Sacco, G., Forcella, M., Fusi, P., ... & Labra, M. (2021). Assessment of dietary bioactive phenolic compounds and agricultural sustainability of an African leafy vegetable Corchorus Olitorius L. *Frontiers in nutrition*, 383.

Contributions: in this work I participated during the field activities, laboratory experiments and manuscript preparation and revision.

2. Taskin, E., Boselli, R., Fiorini, A., Misci, C., Ardenti, F., Bandini, F., ... & Puglisi, E. (2021). Combined impact of no-till and cover crops with or without short-term water stress as revealed by physicochemical and microbiological indicators. *Biology*, *10*(1), 23.

Contributions: in this work I participated during the field activities.

3. Pagliari, S., Giustra, C. M., Magoni, C., Celano, R., Fusi, P., Forcella, M., ... & Labra, M. (2022). Optimization of ultrasound-assisted extraction of naturally occurring glucosinolates from by-products of Camelina sativa L. and their effect on human colorectal cancer cell line. *Frontiers in nutrition*, *9*.

Contributions: in this work I contributed with explorative experiments and statistical analyses.

4. Zecca, G., Panzeri, D., Grassi, F. (2022). Detecting signals of adaptive evolution in grape plastomes with a focus on the Cretaceous-Paleogene

(K/Pg) transition. *Annals of Botany*. (Submitted, under second revision phase)

Contributions: in this work I contributed with manuscript editing and revision.

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