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Study and development of maize cultivars rich in flavonoids

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General introduction

According to the archaeological findings, maize (*Zea mays* L.) started to be cultivated between 7000 and 10000 years ago in Mexico (Piperno et al., 2009; Ranere et al., 2009; Van Heerwaarden et al., 2011; Ranum et al., 2014). European explorers introduced maize to Europe since the end of the XV century and only later it was adopted in Asia and Africa (Brown and Darrah, 2002; Gibson and Benson, 2002; Matsuoka et al., 2002; Vollbrecht and Sigmon, 2005; Mir et al., 2013; Ranum et al., 2014). Maize represented at first a botanical curiosity, but it rapidly become a staple food for the local population in all these countries, thanks to its higher yields compared to the other cereals and to its flexibility of usage. The process of adaptation to different environments and growing conditions, together with human selection, led to the diversification of hundreds different landraces (or farmer's varieties) maintained as open pollination varieties (Messedaglia, 1924; Brandolini, 1958; Brandolini and Brandolini, 2009; Mir et al., 2013).
Modern hybrids introduction, after World War II led to the gradual disappearance of the less productive local varieties (Brandolini and Brandolini, 2009). Nowadays ancient landraces represent a potential source of genetic variability and genotypes adapted to low input cropping systems, in a context of sustainable or subsistence agriculture (Kuhnen et al., 2011; Prasanna, 2012). Hence the characterization of the ancient landraces is very important for their conscious protection and valorization, not only considering the possible economic interest of the farmers, but also because they can be considered a useful tool in future breeding programs (Liu et al., 2003; Vigouroux et al., 2008; Warburton et al., 2008; Mir et al., 2013).
The preservation of maize biodiversity appears crucial given the importance of this species for global food security, especially considering a biofortification approach, aimed to develop improved maize varieties characterized by an high nutritional power, that could be considered as real functional foods for the poorest population living in developing countries, whose diet is mainly based on cereals and on derived products.
In fact corn still represents a staple food for many populations: maize tortillas provide about 50%-65% of human energy intake in Peru, Bolivia and in rural areas of Mexico (Ávila-Curiel et al., 1997; Villalpando, 2004; Petroni et al., 2014) (Fig. 1). In Africa are located 16 among the 22 countries in which corn represents the main source of energy

in the diet (Dowswell et al., 1996; Nuss and Tanumihardjo, 2011; Ranum et al., 2014) (Fig. 1); in particular in Sub-Saharian countries maize consumption is comparable to that of rice in Asia (Oldewage-Theron et al., 2005; Gouse et al., 2006; Nuss and Tanumihardjo, 2011).

To allow optimal growth and an healthy life, avoiding nutrient deficiencies, humans have to consume proteins, carbohydrates and lipids in large quantities, and small concentrations of microelements (Welch and Graham, 2004).

Maize is a good source of starch (72%-80%) and protein (10%-15%); it also provides lipids (4-5%) (Nuss and Tanumihardjo, 2010; Panzeri et al., 2011), fiber, macronutrients and micronutrients (*e.g.:* Na, Mg, P, K, Ca, Fe, Zn) (Rodriguez-Amaya et al., 2008; Brandolini and Brandolini, 2009; Ranum et al., 2014). Furthermore several bioactive molecules, such as carotenoids and flavonoids are produced by plants secondary metabolism (Escribano-Bailon et al., 2004; Kuhnen et al., 2011); their regular consumption is associated to a reduced risk of chronic diseases, mainly linked to their antioxidant activity.

Corn usually contains appreciable amounts of carotenoids (Panfili et al., 2004; Messias et al., 2015) accumulated mainly in the endosperm (80%) (Fig.2), conferring a yellow-orange pigmentation to the kernel, depending on their concentration.

Several flavonoids compounds can be accumulated in seeds and in other plant tissues; anthocyanins and phlobaphenes confer them a red-purple, blue or brown pigmentation.

Pigments from corn were traditionally used by pre-Columbian civilizations, and are still used today in some areas for tissues staining (Escribano-Bailon et al., 2004; Melo, 2008; Roquero, 2008; Gamarra, et al., 2009; Zaffino et al., 2015). Anthocyanins have been used as dyeing matter since the Roman period (Cardon, 2007; Pina et al., 2012) and the recent interest for these molecules is partially due to the possibility of using them as natural food colorants and in food supplements production (Buchweitz et al., 2013; Song, et al., 2013; Zaffino et al., 2015).

Anthocyanins can be accumulated in the seed aleurone layer or in the seed pericarp (Fig. 2); among them cyanidin, pelargonidin, and peonidin glycosides are mainly accumulated (Cuevas Montilla et al., 2011; Tsuda, 2012; Žilić et al., 2012). Phlobaphenes can be accumulated only in the pericarp layer of the seeds (Fig. 2). Other phenolic compounds such as flavonols, flavones and phenolic acids are accumulated also in colorless maize varieties, but their total amount is usually higher in colored varieties.

Anthocyanins are water-soluble pigments conferring a red, purple or blue pigmentation to different plant tissues (Escribano-Bailon et al., 2004); their chemical structure consisting of two phenyl rings and an heterocyclic ring, forming a skeleton with fifteen carbon atoms (C6-C3-C6) is based on the one of the flavylium ion (2-phenylbenzopyrylium) (Zaffino et al., 2015). In plants tissues the anthocyanidin molecules (aglycones) are usually coupled with a sugar (generally 3-glucoside) producing more than 400 different anthocyanins through structural variations (*e.g.:* B-ring substitution, methylation, glycosilation and acylation) (Wrolstad, 2004).

These secondary metabolites have important roles in plants tissues: anthocyanins are involved in the recruitment of pollinators and seeds dispersers (as pigments in flowers and fruits), in male fertility (Mo et al., 1992; Ylstra et al., 1992), in UV protection (Wingender et al., 1990; Li et al., 1993; Kootstra, 1994; Stapleton and Walbot, 1994), in disease resistance, and in response to heat and cold stress (Christie et al., 1994; Pietrini et al., 2002; Mahmood et al., 2014); hence their accumulation in plant tissues may be a marker of different stresses able to induce oxidative damages (Winkel-Shirley, 2002; Kuhnen et al., 2011). Anthocyanins accumulation increases temperature in plants expose to sunlight hastening plant maturity (Barthakur, 1974), and they are also able to act as metal-chelating agents, protecting plants against the effects of metal toxicity under excess of edaphic metal ions thanks to the 3',4'-O-dihydroxyl group in the *B* ring characterizing flavonoids skeleton (Hondo et al., 1992; Hale et al., 2001; Hale et al., 2002; Landi et al., 2015).

The consumption of anthocyanins and other flavonoids is associated to many beneficial effects on health, especially in the prevention of cancer, cardiovascular diseases, myocardial infarction, age-related neurodegenerative diseases, obesity and type 2 diabetes (Renaud and De Lorgeril, 1992; Liu et al., 1999; Meyer et al., 2000; Hagiwara et al., 2001; Tsuda et al., 2003; Fukamachi et al., 2008; Li et al., 2012; Tsuda, 2012; Cassidy et al., 2013; Martin et al., 2013; Woo et al., 2014; Guo and Ling, 2015; Urias-Lugo et al., 2015); their anti-inflammatory and hypoglycemic properties (Hagiwara et al., 2001; Tsuda et al., 2003; Wang and Stoner, 2008; Kuhnen et al., 2011), and their ability to reduce allergen immunoreactivity appear particularly interesting too (Taddei et al., 2013). The great part of these beneficial effects can be ascribed to flavonoids well known antioxidant activity (Wang et al., 1997; Adom and Liu, 2002; Prior, 2003; Abdel-Aal et al., 2006; Rodriguez et al., 2013; Lago et al., 2014; Petroni et al., 2014).

Phlobaphenes represent another class of pigments accumulated in maize kernel: these water-insoluble phenolic compounds constituted by polymers of the flavan-4-ols apiforol or luteoforol (derived from 3-deoxy flavonoids), are produced by a specific branch of the flavonoids pathway together with anthocyanins (derived from 3-hydroxy flavonoids) (Sharma et al., 2012; Petroni et al., 2014).

Phlobaphenes are accumulated in a limited number of tissues, such as seed pericarp (Fig. 2), and cob glumes (Grotewold et al., 1991; Grotewold et al., 1994; Ferreyra et al., 2010; Casas et al., 2014), conferring them a typical red-brown pigmentation, sometimes very dark. Venturini and coauthors (Venturini et al., 2016) reported a positive effect of phlobaphenes against *Fusarium* Ear Rot and against the consequent fumonisin accumulation in maize kernel. These molecules are thought to harden maize pericarp (Treutter, 2006), acting as a physical barrier against fungal infection (Venturini et al., 2016); they are also supposed to inactivate fungal proteins by complexing them with nucleophilic aminoacids (Treutter, 2006) and to block fumonisin production inhibiting the enzymes involved in their biosynthesis (Kim et al., 2006 Pilu et al., 2011; Sampietro et al., 2013; Venturini et al., 2015; Venturini et al., 2016). Furthermore the accumulation of maysin, a flavone defence compound able to reduce larvae development, that is linked to flavonoids accumulation in the pericarp, seems to reduce ears damages and fungal infections (Byrne et al., 1996; Sharma et al., 2012; Venturini et al., 2016).

Phlobapenes are accumulated in several ancient landraces traditionally cultivated in the Padana Plain and in mountainous regions of Northern Italy, that are supposed to be among the first maize varieties introduced in Europe after Americas discovery. The cultivation of varieties rich in phlobaphenes in these areas suggests a selective pressure of the farmers against fungal contamination that led to prefer these varieties respect to the colorless ones.

Even if the most of the colourless maize varieties retain the ability to weakly pigment different tissues, enhanced by biotic or abiotic stresses (Petroni et al., 2014), coloured maize varieties differ from the colourless ones because of the presence of “strong” dominant alleles of the regulatory genes, able to up-regulate the structural genes involved in flavonoids biosynthesis.

Two classes of regulatory genes coordinately regulate the anthocyanin pathway: the *MYB* and the *bHLH* gene families (Dooner et al., 1991; Shen and Petolino, 2006).

c1 (*colored aleurone1*) and *pl1* (*purple plant1*) genes encode proteins with sequence homology to the DNA-binding domains of the *MYB* related oncoproteins (Paz-Ares et al., 1987; Pilu et al., 2003); instead *r1* (*red color1*) and *b1* (*booster1*) are the main regulatory genes belonging to the *bHLH* gene family (*R1*, *B1*, *Sn1*, *Lc1*, *Hopi1*), encoding proteins with sequence homology to the basic Helix-Loop-Helix DNA binding domain of the *MYC* oncoproteins (Chandler et al., 1989; Dooner et al., 1991; Pilu et al., 2003; Petroni et al., 2014).

The pattern of pigmentation in plant tissues greatly depends on the combination of the different alleles of these regulatory genes, nevertheless anthocyanins accumulation is greatly influenced by the genetic background and by the environmental conditions, showing great differences in different growing seasons.

Considering phlobaphenes, their biosynthesis is regulated by the R2R3-*MYB* transcription factor *P1* (*pericarp color1*) (Styles and Ceska, 1977; Chopra et al., 1996) regulating *C2*, *Chi1,* and *A1* structural genes transcription (Styles and Ceska, 1989; Grotewold et al., 1991).

The growing interest for foods rich in flavonoids and other bioactive molecules, whose regular consumption is associated to a reduced risk of chronic diseases, lead geneticists to focus their attention on pigmented maize varieties: until now breeders focused their attention mainly on yields, without considering the presence of phytochemicals important for both plant protection and human nutrition and many other nutritional aspects (Bailey and Bailey, 1938; Huang et al., 2002; Casas et al., 2014).

Purple corn is traditionally cultivated in Peru and Bolivia, where it is used to produce a wide variety of meals and the “Chicha Morada” traditional drink (Schwarz et al., 2003).

Pigmented maize varieties are widely used also in other countries such as Mexico, Guatemala, Arizona, Colorado, New Mexico and Texas (Betran et al., 2001; Urias-Peraldì et al., 2013).

The great part of the pigmented maize varieties weren’t adopted outward their centre of origin during maize worldwide diffusion, partly because of the incapacity of tropical origin varieties to set seeds in environmental conditions characterized by a longer photoperiod and a colder climate as the European ones, and also because of cultural reasons that led to prefer yellow or white maize for flour production. Hence only a few coloured varieties where adopted in Europe, mainly where maize was first introduced (*e.g.* Spain and Italy) (Anderson and Cutler, 1942; Bianchi et al., 1963; Brandolini and Brandolini, 2009; Petroni et al., 2014).

Flavonoids, such as anthocyanins and phlobaphenes, could be increased or introduced in improved maize varieties to obtain real functional foods able to exert an higher antioxidant activity increasing beneficial effects on human and animal health with respect to the colourless hybrids actually cultivated.

Three different strategies can be followed to obtain maize varieties rich in anthocyanins and other flavonoids adapted to the European growing conditions without recurring to a biotechnological approach.

- The easiest strategy is represented by the rediscovery of ancient pigmented landraces that are still cultivated in restricted areas or maintained ex situ in germplasm banks; nutritional value and yields of these varieties can be further improved through the selection of specific characters, but they can also be considered as a source of genetic variability for the development of coloured inbreed lines and hybrids.
- A second strategy is represented by the possibility of developing new varieties characterized by a high anthocyanin content crossing colourless varieties and coloured varieties already adapt to the European growing conditions (climate and photoperiod), using them as source of the dominant alleles of the main regulatory genes of the anthocyanin biosynthesis in breeding programs based on backcrosses and selection. In this case colourless varieties are used as recurrent parent.

Using this strategy it is possible to obtain coloured varieties near-isogenic with respect to the colourless recurrent parent (Lago et al., 2013; Lago et al 2014) but able to confer higher nutraceutical benefits.

- A third strategy can be followed to obtain varieties accumulating very high amounts of anthocyanins in seeds pericarp layer: using tropical or subtropical origin varieties (*e.g.:* Maìz Morado) as pollen donors it is possible to introduce in other varieties very “strong” alleles of the regulatory genes (in particular *Booster1, B1,* and *Purple plant1*, *Pl1*) through breeding programs based on pedigree selection schemes, selecting the progeny for the anthocyanins content and for the adaptation to the photoperiod in each cultivation cycle. In fact tropical varieties cannot be directly cultivated at our latitude as they are unable to reach maturity and set seeds because adapted to a shorter photoperiod (Petroni et al., 2014).

The use of transgenic techniques and of modern genome editing tools, particularly RNA-guided endonucleases (RGENs), represent an interesting possibility also for what concern the obtainment of flavonoids enriched varieties, making this process more rapid;

nevertheless these techniques weren't taken into consideration in this work because of the lack of global acceptance of genetic engineering and because of the restrictive and costly GMO regulations that make difficult to commercialize the varieties so obtained (Kanchiswamy et al., 2015).

Since their commercial introduction in the 1990s, GM food crops weren't adopted in many countries, including most of the European ones (except in Spain, Portugal, Czech Republic, Slovakia and Romania) (Lucht, 2015; Ishii and Araki, 2016), New Zealand and Japan (Ishii and Araki, 2016); however, even in permissive countries, these products have not been accepted by the whole population.

Nowadays genome editing, represented by the CRISPR/Cas9 system, can provide transgene-free gene modifications, avoiding the potential adverse effects that are among the major concerns for consumers; but their negative attitude, associated with insufficient knowledge toward genetically modified organisms (GMOs), could lead to consider the transgene-free crops comparable to conventional GM crops; hence consumer acceptance of genome edited crops doesn't appear optimistic (Ishii and Araki, 2016).

Furthermore considering the development of pigmented maize varieties, the breeding process, adopting conventional techniques, doesn't appear particularly difficult because flavonoids content is mainly determined by a few regulatory genes, and visual observation together with the use of molecular markers can greatly help in making this process faster without recurring to genetic engineering.

Summary of the thesis work

With the aim of finding maize varieties that could represent an interesting source of bioactive compounds such as carotenoids and flavonoids we studied and characterized two ancient pigmented maize varieties traditionally cultivated in Europe, according to the first strategy.

In the article "Study and Characterization of an Ancient European Flint White Maize Rich in Anthocyanins: Millo Corvo from Galicia", the blue maize cultivar "Millo Corvo" (Fig. 3A) typical of the Spanish region of Galicia was found to accumulate high amounts of anthocyanins (83.4 mg/100g flour) in seeds aleurone layer (the outer layer of the endosperm) (Fig. 2), due to the presence of a dominant allele of the *R1* (*red color1*) gene (bin 10.06), as demonstrated by mapping and sequencing data. Due to the fact that the aleurone layer is a triploid tissue, the *r1* gene shows a typical dosage effect. TLC (Thin Layer Chromatography) and HPLC (High Performance Liquid Chromatografy) analysis showed that cyanidin is the main anthocyanin accumulated in this cultivar, as well as in the great part of pigmented maize varieties. Cyanidin 3-glucoside is considered among the anthocyanins one of the most effective in cancer prevention (Long et al., 2013).

Millo Corvo was found to lack carotenoids, but it showed an higher antioxidant power compared to yellow and white maize used as control, that could be ascribed to the presence of anthocyanins.

In the article "Genetic studies regarding the control of seed pigmentation of an ancient European pointed maize (*Zea mays* L.) rich in phlobaphenes: the "Nero Spinoso" from the Camonica valley" we studied and characterized the ancient cultivar "Nero Spinoso" (Fig. 3B), whose peculiarities are the pointed shape of the seeds and their dark brown-black pigmentation.

Spectrophotometric and TLC analysis showed that this variety accumulates very high amounts of phlobaphenes (320 A_{510}/100 g flour). The involvement of the monogenic dominant gene *pericarp colour1* (*P1*) in seeds pigmentation was demonstrated by mapping and sequencing data: a perfect cosegregation between a *PHI095* polymorphism (SSR marker inside the *P1* gene) and the trait ''pigmented ear'' was observed, and a 99% identity (334/336 bp) was found between a portion of the *P1* gene and the *P1-rw1077* allele previously sequenced.

Thanks to the high flavonoids content, these varieties could be considered as real functional foods, able to increase the amount of antioxidants introduced with the diet, thus representing also a useful tool, as a source of genetic variability, for future breeding programs. Furthermore the selection of varieties rich in phlobaphenes and other flavonoids could represent an interesting opportunity not only because of their direct beneficial effects on human health, but also because of their role in protecting maize plants against fungal infections (Venturini et al., 2016), thus increasing the quality of the kernel and its healthiness, that often represent a relevant problem for the whole production chain.

Considering that, at our knowledge, there are no ancient varieties in Europe carrying dominant alleles of the *purple plant1* gene (*Pl1*) (bin 6.04), able to induce the accumulation of high amount of anthocyanins in the seed pericarp layer (Fig. 2, Fig. 3C), we used the pigmented inbred line Reduno, already included in the register of the Community Plant Variety Office (CPVO), (N° EU 33449) and carrying dominant alleles of the *B1* and *Pl1* regulatory genes from North American origin, as pollen donor (non-recurrent parent) in a breeding program based on backcrosses and selection, aimed to develop a sugary corn line rich in anthocyanins from a colorless one used as recurrent parent, according to the second strategy previously reported. The results of this breeding program were reported in the article "Development and characterization of a coloured sweet corn line as a new functional food". Our data showed that the accumulation of other phenolic compounds is pushed up together with anthocyanins, as they share a part of the same biosynthetic pathway, giving to the new coloured sugary line a higher antiradical scavenging activity compared to the colorless control.

Considering that canned sweet corn undergoes an heat treatment before being consumed, we compared the effect of two different cooking processes on flavonoids content. In fact duration and temperature of cooking procedures/pre-treatments are known to affect the final content of flavonoids/antioxidant capacity reducing in a not-negligible manner the total amount of anthocyanins (Chatthongpisut et al., 2015) and leading to the release of the bound flavonoids fraction.

Bound flavonoids are accumulated mainly in the pericarp due to the presence of complex polysaccharides (Das and Singh, 2016), representing about the 60-80% of the total amount present in corn kernels (Adom and Liu, 2002; Urias-Peraldì et al., 2013; Messias et al., 2015).

The cooking process, especially the strongest treatment, drastically decreased, as expected, the amount of anthocyanins, without changing molecules chemical structure, reducing also the total antioxidant power.

This coloured sugary corn can be considered a new functional food that is potentially able to increase the daily intake of antioxidant compounds in the diet of many people thanks to sweet corn worldwide consumers appreciation.

Considering that some classes of flavonoids (*e.g.* flavonols and phenolic acids) can be accumulated in maize seeds without conferring them a distinguishable pigmentation, we sampled two unpigmented maize varieties, one white and one yellow, directly from the farmers in the rural region of Qwa-Qwa (Free State Province) in South Africa (Fig. 3D). Looking for flavonoids we founded that these varieties accumulate a limited amount of flavonols and phenolic acids, comparable to the one observed in the B73 colourless inbred line used as standard. We characterized these maize varieties from the phenotypical and nutritional points of view: calorific value, oil, protein, starch, minerals and carotenoids content were determined, together with free and phytic P, finding that these varieties have low protein and Fe content in comparison to the ones used as control. As expected the white variety was characterized by a very low level of carotenoids, and even if it showed a quite higher content of free P, our data suggest that there are no nutritional reasons to prefer this white variety for human consumption to the yellow one or to other maize varieties.

Nevertheless the white variety appears interesting from a scientific point of view because of the very large dimensions of the seeds and of the high variability observed among plants. Our data were reported in the article "Nutritional and phenotypical characterization of two South African maize (*Zea mays* L.) varieties sampled in the Qwa-Qwa region".

Biofortification represents a sustainable strategy to improve human nutrition where the population, as the South African one, suffers from nutritional deficiencies (Pfeiffer and McClafferty, 2007; Bouis and Welch, 2010), by enhancing the nutritional value of staple crops using modern breeding techiniques, with the aim of guarantying to the population at least the minimum intake of nutrients needed to improve health, avoiding deficiencies. Maize, as a staple food, appears a good candidate for biofortification strategies thanks to its wide diffusion, consumers appreciation and to the low production costs (Graham and Rosser, 2000; Bai et al., 2011; Mellado-Ortega and Hornero-Méndez, 2015).

Hence the nutritional value of the two south African varieties studied in this work could be improved increasing carotenoids and vitamins content, as well as the content of minerals such as zinc, iron and phosphorous, thus obtaining a cheap and easily accessible functional food. Furthermore increasing flavonoids content it could be possible to henance antioxidant intake in the poorest fractions of the rural South African population.

Figures

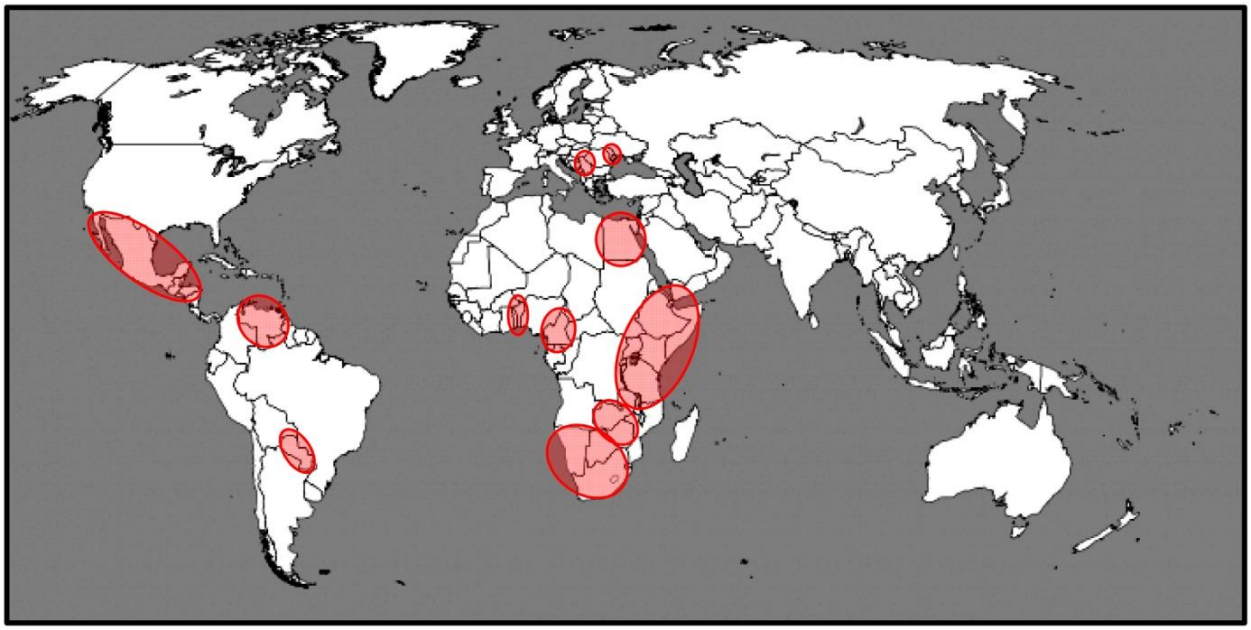

Figure 1. Countries in which maize represents the main source of energy in human diet. From CIMMYT, 2011. "MAIZE - Global Alliance for Improving Food Security and the Livelihoods of the Resource-poor in the Developing World".

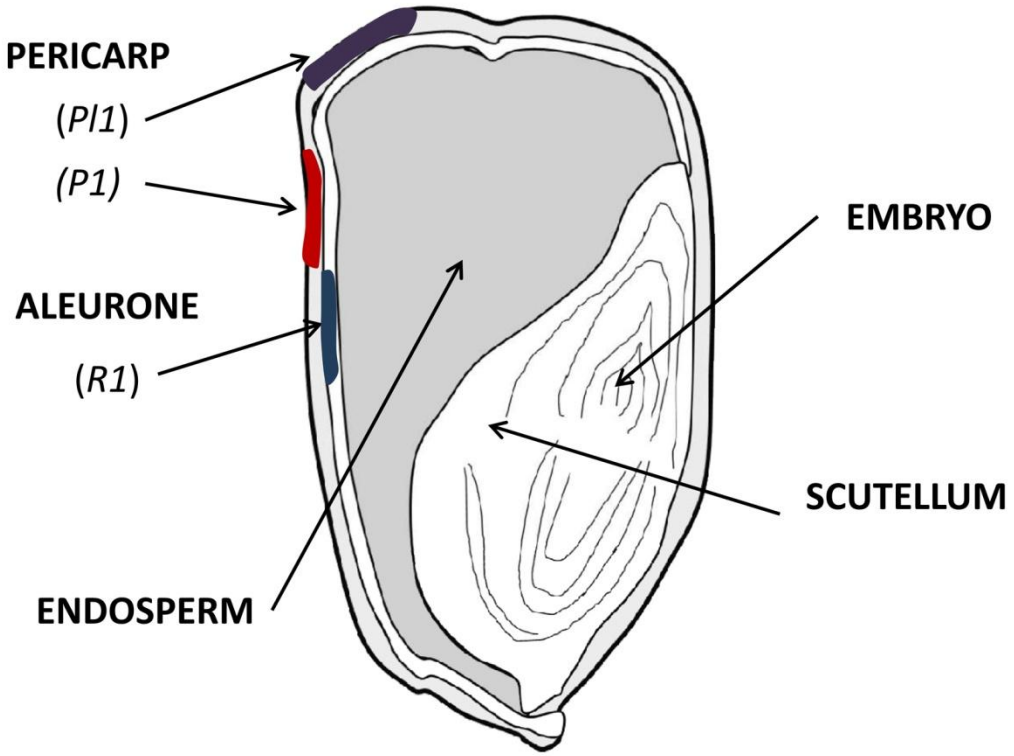

Figure 2. Transversal section of a maize seed.

The main tissues of the seed are indicated together with the main regulatory genes able to induce an high accumulation of flavonoids in pigmented maize varieties. *R1* confers a blue pigmentation to the aleurone layer through the accumulation of anthocyanins; the same molecules confer a purple-black pigmentation to the seed pericarp in presence of *Pl1* alleles, instead *P1* induces phlobaphenes accumulation in the pericarp layer, conferring a red-brown pigmentation, sometimes very dark.

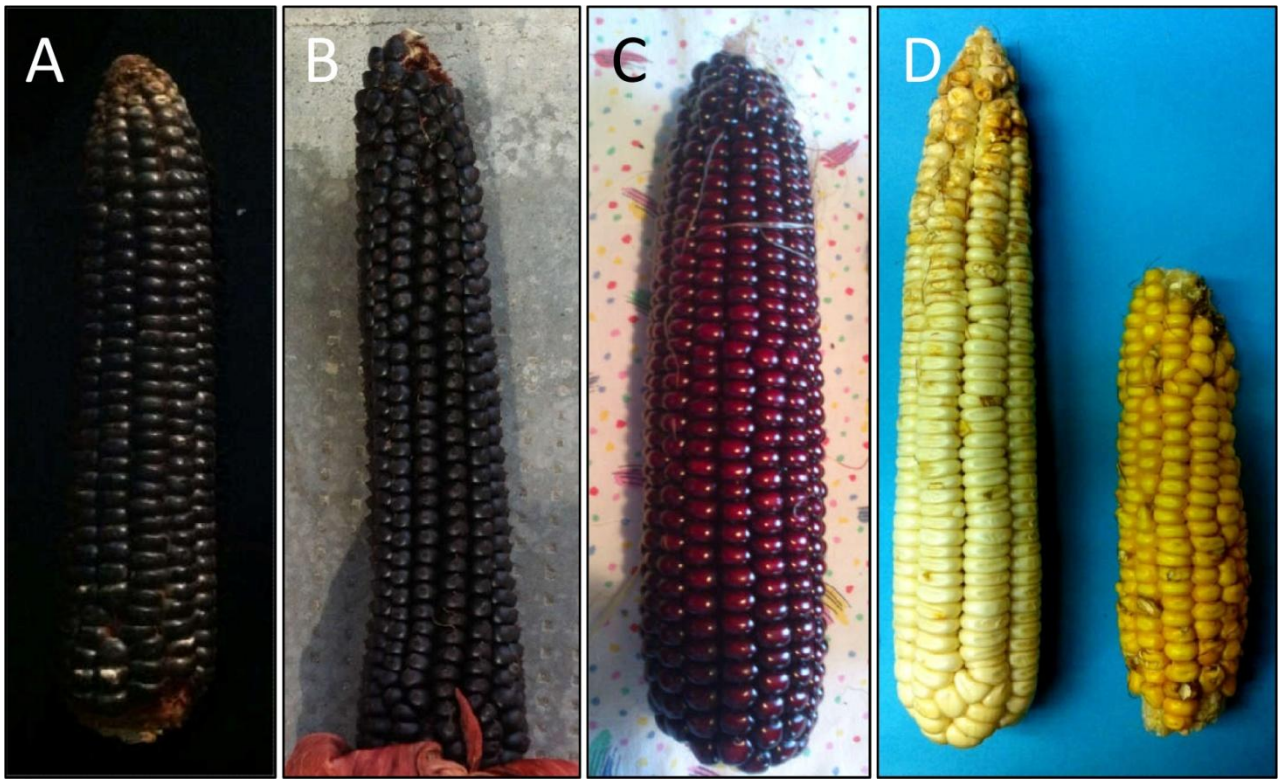

Figure 3. Biodiversity regarding maize pigmentation.

Millo Corvo, accumulating anthocyanins in the aleurone layer (A). Nero Spinoso accumulating phlobaphenes in the pericarp layer (B). Colored sweet corn accumulating anthocyanins in the pericarp layer (C). South african maize varieties, without carotenoids on the left, and accumulating carotenoids in the endosperm on the right (D).

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Study and Characterization of an Ancient European Flint White Maize Rich in Anthocyanins: Millo Corvo from Galicia

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Abstract

In the second half of the last century, the American dent hybrids began to be widely grown, leading to the disappearance or marginalization of the less productive traditional varieties. Nowadays the characterization of traditional landraces can help breeders to discover precious alleles that could be useful for modern genetic improvement and allow a correct conservation of these open pollinated varieties (opv_s). In this work we characterized the ancient coloured cultivar "Millo Corvo" typical of the Spanish region of Galicia. We showed that this cultivar accumulates high amounts of anthocyanins (83.4 mg/100g flour), and by TLC (Thin Layer Chromatography) and HPLC (High Performance Liquid Chromatografy) analysis, we demonstrated that they mainly consisted of cyanidin. Mapping and sequencing data demonstrate that anthocyanin pigmentation is due to the presence of the *red color1* gene (*r1*), a transcription factor driving the accumulation of this pigment in the aleurone layer. Further chemical analysis showed that the kernels lacks in carotenoids. Finally a DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging ability test showed that Millo Corvo, even though lacking carotenoids, has a high antioxidant ability, and could be considered as a functional food due to the presence of anthocyanins.

Introduction

The beginning of maize (*Zea mays* ssp. *mays*) domestication has been dated to around 8700 years before the present in Mexico (Matsuoka et al., 2002; Piperno et al., 2009; Ranere et al., 2009; van Heerwaarden et al., 2011). Then the progressive spread of the cultivated crop into the tropical regions and throughout the Americas in the following thousands of years (Grobman, 1982; Piperno et al., 2000; Pearsall et al., 2004; Piperno, 2006; Pohl et al., 2007; Zarrillo et al., 2008; Mir et al., 2013) allowed hundreds of landraces to adapt and to evolve to suit different environments through human cultivation (Shen and Petolino, 2006). After the discovery of the Americas by Europeans, three main maize sources: corn from the American east coast with higher latitude adaptation (Eschholz et al., 2010), the photoperiod insensitive CATETO types (Eschholz et al., 2010) and the Pearl White (Brandolini and Brandolini, 2009; Eschholz et al., 2010) played a very important role for the adaptation of maize to Europe. The hybridization of these different corn sources, together with the effects of photoperiod, temperature, humidity and altitude of the different environments allowed the constitution and the differentiation of local European varieties and landraces (Brandolini and Brandolini, 2009; Eschholz et al., 2010). Hundreds of new landraces have been created in the past 500 years (Dubreuil et al., 2006; Mir et al., 2013).

During this process the farmers' work of selection, based on specific needs for use and cultivation has been important too: they maintained the landraces as open pollinated populations, creating a collection of corn plants with high heterozygosity and heterogeneity, which represented a very important source of variability and of alleles with high adaptation to the local environments. However in the second half of the last century dent hybrids began to be widely grown in Europe in place of the traditional varieties: these commercial maize cultivars guaranteed superior productivity in response to the need for higher yields (Hallauer et al., 1988; Brandolini and Brandolini, 2009). In recent years, renewed interest for the ancient cultivars has been increasing due to the new vision of agricultural systems not only based on yield performance but also on sustainability and the quality of the products. In this work we characterized an ancient colored landrace, the "Millo Corvo", cultivated in the Spanish region of Galicia and used to produce a variety of foods. The peculiarity of Millo Corvo is the distinctive dark blue/black coloration of the kernels that confers a typical blue coloration to the bread

cooked using this flour. Maize is able to accumulate pigments in the seeds: carotenoids, that confer the typical yellow to orange color and more rarely anthocyanins, conferring a red, purple, blue and black coloration, associated with antioxidant power, thought to be highly beneficial for human health (Rodriguez et al., 2013). Carotenoids are hydrophobic C40 isoprenoids that are synthesized in amyloplasts (Kirk and Tinley-Bassett, 1978). In maize endosperm those present are mainly lutein and zeaxanthin. In yellow maize there are more than 30 loci involved in the biosynthesis of carotenoids and the main class of mutations that reduce or deplete carotenoids are the y_s conferring white or pale yellow endosperm (Chander et al., 2008). In various developing countries white maize is consumed in human diet, even though it is now well understood that Vitamin A, derived from carotenoids, is essential for human health. In fact the World Health Organization estimates that hundreds of millions of people (in particular children) worldwide suffer from vitamin A deficiency (VAD) (West et al., 2002).
The anthocyanin biosynthetic pathway in maize is known to be controlled by at least two classes of regulatory genes, both of which are required for tissue specific pigmentation of plant and seeds (Shen and Petolino, 2006). The *R1/B1* family encodes proteins with sequence homology to the basic helix-loop-helix (bHLH) DNA binding domain of the MYC oncoproteins (Grotewold et al., 2000), while the *C1/Pl1* family encodes proteins with sequence homology to the DNA-binding domains of the MYB-related oncoproteins (Paz-Ares et al., 1987; Pilu et al., 2003); the presence of one member of each family and their interaction allow the activation of the approximately 20 structural gene required for anthocyanin pigment production (Dooner et al., 1991). In nature many different alleles of these regulatory genes exist, each one driving a tissue-specific coloration (Pilu et al., 2012).

Materials and methods

Plant and sampling material

The Millo Corvo maize variety (from the Spanish region of Galicia), the B73 inbred line (provided by Stock Center Resources of MaizeGDB, http://www.maizegdb.org/stock.php), the Scagliolo variety (from Carenno, LC, VA1210) and the Ottofile variety (from Zinasco, PV, VA61) were cultivated in the experimental field of the University of Milan located in Landriano (PV), Italy (N 45°180, E 9°150).

For all genotypes tested, about 100 seeds were sown in adjacent rows, under the same agronomic conditions. These plants were selfed and the ears obtained were harvested at the same time at the end of the season. About 70 ears of Millo Corvo were shelled and the seeds obtained mixed to create a single bulk used for the determination of anthocyanins, flavonols and phenolic acid. The same was done for the B73 inbred line used as colorless control. For the anti radical power (ARP) determination we used the Millo Corvo seeds bulk described above, a Scagliolo seeds bulk obtained in the same way and the segregant *yy* seeds (without carotenoids) obtained by selfing the progeny Millo Corvo x B73.

Milling

Flour samples were obtained using a ball mill (Retsch MM200, Retsch GmbH Germany). Seeds (cleaned from the glumes) were ground for 5 min at 21 oscillations s^{-1} frequency.

Spectrophotometer determination of anthocyanins, flavonols and phenolic acids

Five mg of flour were first boiled with 100 µL of distilled water for 30 minutes and then left in an overnight agitation with 1 mL of the extraction buffer (1% HCl, 95% ethanol). After another agitation time of 2 hours with 500 µL of extraction buffer, the supernatants were collected together and centrifuged for 30 minutes. Their absorbance was determined spectrophotometrically at 530 nm for anthocyanins, at 350 nm for flavonols and at 280 nm for phenolic acids (Petroni et al., 2010). The amount of anthocyanins was calculated as cyanidin 3-glucoside equivalents (molar extinction coefficient (ε) 26900 L m^{-1} mol^{-1}, M.W. 484.82), flavonols content as quercetin 3-glucoside equivalents (ε 21877 L m^{-1} mol^{-1}, M. W. 464.38) and the amount of phenolics as ferulic acid equivalents (ε 14700 L m^{-1} mol^{-1}, M.W. 194.18). The analyses were conducted four times for each genotype, and the confidence interval (C.I.) at 95% was calculated.

Qualitative determination of anthocyanins: TLC (Thin Layer Chromatography) and HPLC (High Performance Liquid Chromatography)

The fine powder of the pericarp and aleurone layers of the Millo Corvo kernels (obtained using a manual electric drill) was boiled at 100°C in 2 mL of 2N HCl for 40 minutes. After adding 1 mL of isoamyl alcohol, the upper phase was dried and suspended in EtOH 95% and HCl 1% for the TLC analysis and in methanol for the HPLC run. For TLC analysis, cyanidin, pelargonidin and delphinidin standards were loaded together with the extracts on a pre-coated plastic sheet Polygram Cel 300, Macherey-Nagel) for TLC using formic acid:HCl:water 5:2:3 as solvent. Developed plates were dried and pictured with a digital camera (A430 Canon) using both white and UV illumination. For HPLC 20 µL of the sample were injected in an HPLC Kontron Instrument 420 system equipped with a C18 column Zorbax ODS column, 250 mm X 4.6 mm, 5 µm, Teknokroma (Agilent Technologies, Santa Clara, CA, USA) and the absorbance at 530 nm was monitored.
Anthocyanins quantification was performed by the method used by Astadi (Astadi et al., 2009); the HPLC conditions were as follows: from min 0 to 8 min, solvent A (10% formic acid) from 96 to 85%, solvent B (100% Acetonitrile) from 4 to 15%; from min 8 to 25, solvent B was kept at 15%; from min 25 to 27, solvent A 20%, solvent B 80%; from min 27 to 30, solvent A 80%, solvent B 20%. The flow rate was 1 mL/min.

Qualitative determination of seed carotenoids: HPLC

After incubating 1 g of maize flour in 3 mL of hexane/acetone 1:1 solution with 100 mg/mL of BHT for 30 min at room temperature, the sample was dried by means of a speedvac and the pellet was dissolved in 3 mL of hexane and washed three times with 4 mL of distilled water in order to remove the hydrophilic compounds. Sample extracts were concentrated by speedvac and immediately analysed. Carotenoids were assayed by an HPLC method adapted from that described by Tukaj and collegues (Tukaj et al., 2003) using a Kontron Instrument 420 system, equipped with C18 reverse-phase Zorbax ODS column, 250 X 4.6 mm, 5 µm (Agilent Technologies, Santa Clara, CA, USA). The solvent initially consisted of 60% solvent A (methanol-ammonium acetate 80/20 v/v) and 40% solvent B (methanol/acetone, 80/20 v/v), which finally was brought to 0% solvent A and 100% solvent B over a period of 20 min, and fluxed under these conditions for 5

additional minutes. The column was subsequently returned to its original mobile phase (60% solvent A and 40% solvent B) over the next 5 minutes, and fluxed under these conditions for 5 additional minutes prior to the injection of a new sample. The solvent flow rate was 1 mL min^{-1}.

Mapping

Millo Corvo was mapped in segregating F2 populations using the *bnlg1028* simple sequence repeat (SSR) marker chosen on chromosome 10 (bin 10.06) from MaizeGDB (http://www.maizegdb.org). A total of 85 F2 seeds (obtained by selfing the progeny of the cross B73 x Millo Corvo) were screened for color and each flour was used for DNA extraction (Dellaporta et al., 1983). Polymerase chain reactions were performed in a final volume of 10 µL and the reactions were carried out as follows: 94°C for 2 min, 35 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final step at 72°C for 5 min. The amplified fragments were resolved on 3% agarose gels. Recombinant values were converted to map distance using MAPMAKER3 (Lander et al., 1997).

Histological analysis of Millo Corvo seeds

For light microscopy studies, coloured Millo Corvo and colourless B73 seeds were imbibed in water overnight and fixed in freshly prepared 4% paraformaldehyde (Sigma P4168) in PBS (130mM NaCl, 7mM Na_2HPO_4, 3mM $NaH_2PO_4 \cdot H_2O$) at 4°C overnight, then rinsed in 0.85% NaCl and transferred in 70% ethanol at 4°C until being processed.
Following successive dehydration in ethanol series and embedding in Paraplast Plus (Sigma P3683), 15 µm thick sections were cut and serially arranged on microscope slides. To preserve anthocyanin pigments *in situ*, sections were mounted on slides using tert-butyl alcohol instead of water. Images were taken using a Zeiss IMAGE R.D1 microscope equipped with an AxioCam MRc1 camera.

Amplification and sequencing

The presence of the *R-g* allele involved in the coloration of the Millo Corvo kernels has been determined by sequencing: genomic DNA was amplified by high fidelity PCR (Pfu polymerase; Stratagene, La Jolla, CA, USA) using the specific primers OR31 (5'-ATGGCTTCATGGGGCTT AGATAC-3') and OR32 (5'-GAATGCAACCAAACACCTTATGCC-3') for *R1* gene (Consonni et al., 1997). Four sequences coming from independent amplification

were sequenced in outsourcing. To deduce the consensus DNA sequence we used the freely available computer software CLUSTALW (http://www.ebi.ac.uk/clustalw/). To study the sequence obtained we used BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Anti Radical Power (ARP) determination

The antioxidant ability of the pigments was inferred by the comparison of the Anti Radical Power (ARP) possessed by the white and colored kernels of a Millo Corvo segregating synthetic population, using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging activity method (Cevallos-Casals and Cisneros-Zevallos, 2003).
Acetone 70% (acetone:water 70:30 v/v) was added to an aliquot of the fine powder, keeping the ratio 1:4 (w/v). The mixture was shaken at 4°C in the dark for 3 hours, then centrifuged to collect the clean extracts. A 0.12 mM ethanolic DPPH solution was added to increasing aliquots of each sample and the final volume adjusted to 2.50 mL. The absorbance of the discolorations of the DPPH in ethanol and of the samples were measured at 516 nm after incubation for 2 hours at room temperature in the dark, until the reaction reached the steady state. The percentage of scavenged DPPH values was calculated and then plotted against the extract volumes so as to calculate by interpolation the amount of extract required to consume 50% of the initial DPPH amount (Lago et al., 2014). The ARP is the reciprocal of this value (Doria et al., 2009). The analyses were conducted three times for each genotype.

Results

Phenotypic characterization of the Millo Corvo landrace

The Millo Corvo traditional open pollinated variety was cultivated in the field at Landriano (PV) from April to September, during this period some agronomic traits were measured (Table 1). The plants reached maturity in about 90 days after sowing in this environment. The plants were, on average, 248.36 cm in height, with the ears positioned at 105.09 cm from the soil. The ears were of cylindrical-conical shape with 12 rows, measuring 16.26 cm in length with a cob diameter of 2.75 cm (Table 1). The kernels were flint type and pigmented, with an average weight of 0.319 g (Table 1); each ear weighed about 109.26 g for an estimated yield of about 6–7 tons per hectare (sowing 6–7 seeds per square meter). As control Ottofile and Scagliolo varieties, out of

more than 700 catalogued open pollinated traditional Italian flint maize, were cultivated and measured in the same conditions (Table 1).

Characterization of seed pigment: anthocyanins, flavonols and phenolic acids

The important peculiarity of this variety is surely the seed color, that would seem to be the only pigmented tissue with the exception of the seedling (Fig. 1 and Fig. S1). It is well known that maize plants can accumulate anthocyanins and for this reason we conjectured that the pigments observed in the Millo Corvo seeds were flavonoids and in particular anthocyanins. Table 2 shows the spectrophotometric results on the amounts of anthocyanins, flavonols and phenolic acids present in the seed flour of the Millo Corvo, in comparison to the colorless B73 inbred line: we found respectively 83.45 mg/100g of flour, 74.21 mg/100g, 216.63 mg/100g in the Millo Corvo variety, while 3 mg/100g, 66 mg/100g, 113 mg/100g in the B73 line. We used the B73 inbred line to represent all the classical yellow maize cultivars where the pigments are not present or present as trace. Anthocyanins are a very wide group of pigments, so to better characterize them, we performed Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). We did not analyze from a qualitative point of view the colorless controls (B73, Scagliolo and Ottofile) because the very low amount of anthocyanins as shown for the classical yellow inbred line B73. In Fig. 2B the TLC plate shows that the main spot present in the Millo Corvo extract is due to the cyanidin molecule: according to the standards loaded in the plate and considering that the absorption peak of the anthocyanins extract at 550 nm is close to the typical peak of cyanidin at 545 nm (Fig. 2C). Another little spot, poorly visible and not identified, has been detected with a run length higher than those of the standards (Fig 2B). The following HPLC analysis (Fig. 3) confirmed that cyanidin is the most abundant anthocyanin in Millo Corvo, representing 65.90% of the total anthocyanidin molecules; this analysis also detected 31.40% of peonidin, 1.96% of pelargonidin (Fig. S2).

Characterization of seed pigment: carotenoids

Unexpectedly, in the Millo Corvo seeds' flour, the HPLC analysis found that the amount of carotenoids present was under the detectable threshold. We hypothesized that the Millo Corvo cultivar carried a recessive homozygous mutation belonging to the white

endosperm class (y_s) whose phenotype effect was hidden because of the anthocyanins accumulation. To confirm this hypothesis we used a hand drill to mill the seeds' surface, where the anthocyanins were accumulated: as expected we demonstrated the absence of carotenoid in the inner layer of the seed, which appeared completely white (Fig. 4A) as reported for the y_s recessive mutations (Wurtzel et al., 2012). Furthermore, as expected for a recessive mutation, crossing the Millo Corvo with the B73 line (able to produce carotenoids) we obtained yellow F1 seeds after surface milling (Fig 4B). These results were further strengthened by studying the F2 segregating progeny for the *yy* seeds and the following F3 ears obtained selecting and sowing the *yy* seeds (Fig. 4C).

Genetic constitution and heritability of the colored seed trait

It is well known that anthocyanins can be accumulated in the pericarp layer, a tissue of maternal origin, or in the aleurone, the outer layer of the seed endosperm (Dooner et al., 1991). With the aim to identify the tissues where anthocyanins were accumulated and to understand the heritability of the trait "seed pigmentation" in the Millo Corvo variety we studied the progeny of an F2 population obtained as described in the previous paragraph. As shown in Fig. 4, the pigmentation of the F1 seeds (obtained using the B73 plant as female and Millo Corvo as pollen donor) was weaker compared to the Millo Corvo. This finding suggested a dosage effect typical of pigments accumulated in aleurone layers (aleurone is a triploid tissue): in Millo Corvo aleurone, three doses of genes involved in the pigmentation seemed to be present, whilst in the F1 there was only one. Furthermore the presence of the pigment in F1 seeds excluded the possibility that it was a pericarp pigmentation that would appear only in the next generation (being a tissue of maternal origin). Selfing F1 plants we obtained an F2 progeny segregating 3:1 for seed color (Fig. S3), confirming that the pigmentation is under the control of a monogenic dominant character that drives the accumulation of anthocyanins (mainly cyanidin) in the aleurone layer. The genetic data were confirmed by histological analysis of transverse sections of mature seeds, showing the pigmentation only in the aleurone layer (Fig. 5). This evidence led us to think that the regulatory *r1* gene may be responsible for the seed anthocyanin biosynthesis. To strengthen this finding we mapped the character "seed pigmentation" using SSR markers to confirm the presence of the "colored seed" trait on the long arm of chromosome ten where *r1* locus maps (bin 10.06). We used genomic DNA obtained by F2 mapping populations of 85 F2 seeds

screened for color and genotyped using the *bnlg1028* simple sequence repeat (SSR) marker mapping on chromosome 10 (bin 10.06). We found an association of 3.4 cM between the trait "seed color" and *bnlg1028*.

Molecular analysis of the R1 gene

R1 gene is a complex locus composed of two distinct components: the *S1* and *S2* component driving the pigmentation of the seed and the *P* component driving the pigmentation of the plant tissues (Robbins et al., 1991). When an allele at the *r1* locus carries both the components it is named *R-r*. If intrachromosomal rearrangement occurs (typical in complex genes) and the allele loses *S* components, *r-r* alleles are formed; when the *P* component is lost, alleles of class *R-g* are formed, and when both are lost we have *r-g* alleles unable to confer any plant pigmentation (Robbins et al., 1991; Walker et al., 1995; Pilu et al., 2012). In the case of Millo Corvo the pattern of pigmentation of the *R1* gene is similar to that of *R-g* (Table 3) in fact in our case we have the seed colored and the plant colorless with the exception of the seedling (Fig. S1). To confirm these data we sequenced a 3' portion of *R1* gene using specific primers (see Material and Methods chapter). The sequencing of 4 independent amplicons and the following alignment with the CLUSTALW program allowed us to obtain a consensus sequence of 454 nucleotides (GenBank accession number: BankIt1769632 Seq1 KP056782) used for the research by the BLASTN program. The results obtained confirmed the presence of an *R-g* allele in the Millo Corvo cultivar, in fact we found significant alignments with the sequence NM_001112603.1, the seed color component at *R1* (*S*) mRNA of *Zea mays* (Fig. S4).

Antioxidant ability of the Millo Corvo flour

To detect the antioxidant ability conferred by the anthocyanin molecules, a DPPH assay was performed on the flour obtained from the Millo Corvo seeds (containing anthocyanins but not carotenoids) and from F2 white segregating seeds (without anthocyanins and carotenoids). We also analyzed as control the yellow Scagliolo variety, a popular Italian polenta variety (containing only carotenoids). The percentage of scavenged DPPH values were calculated and then plotted against the extracted volumes (Fig 6). The antioxidant results were expressed as AntiRadical Power (ARP) as suggested by Doria and colleagues (Doria et al., 2009). The colored Millo Corvo seeds showed the

highest antioxidant ability with 0.06 of ARP, the Scagliolo variety had a value of 0.04, while the F2 white seeds showed the lowest antioxidant power with 0.03 as expected, since they lack carotenoids and anthocyanins.

Discussion

Starting from the last century, the increased needs for corn have focused the farmers' attention on the dent hybrids with high yields, displacing the maize landraces with the risk of losing their sources of genetic variability. Now the institutions and the companies responsible for conducting maize genetic improvement are starting to study the ancient landraces across the continents with the aim of identifying and using novel alleles and haplotypes in a context of low input and sustainable agriculture (Prasanna, 2012). In this scenario the study of the immense maize genetic diversity present around the world has a big limiting factor in the requirement for conscious protection of open pollinated varieties and their precise characterization. For these reasons the Millo Corvo ancient landrace from the Galician Spanish region has been studied and characterized. The most obvious characteristic of this cultivar is the blue/black pigmentation of the seed (Fig. 1D) which differentiates it for example from Ottofile (Fig. 1E) and Scagliolo (Fig. 1F) traditional Italian cultivars, which as well as most other maize varieties don't accumulate high amounts of flavonoids.

It is well known that red/black coloration of maize kernels is due to the accumulation of flavonoids and in particular anthocyanins (Žilić et al., 2012) and with the aim to quantify these pigments a spectrophotometric quantification of the main class of molecules was performed. As reported in Table 2, significant differences were found, as expected, for anthocyanins and phenolic acids amounts whilst no difference was noted for the flavonols content in comparison to the well characterized B73 inbred line used as typical control of all the colorless varieties. These data are in agreement with the work of Lopez-Martinez and colleagues who found a range between 76 and 869 mg/100g of anthocyanins in 18 colored landraces of Mexican maize (Lopez-Martinez et al., 2009). With the aim to characterize the anthocyanins present we carried out TLC and HPLC analysis of the pigment. The TLC plate indicated the presence of the cyanidin molecule (Fig. 2), confirmed by the HPLC analysis (Fig. 3 and Fig. S2). The second spot, poorly visibly in the TLC plate, is probably due to the presence of peonidin, quantified as 31.4%

by the HPLC analysis (Fig. S2). Several reports have shown that cyanidin, pelargonidin, and peonidin glycosides are the main anthocyanins present in maize kernels (Cuevas Montilla et al., 2011; Tsuda, 2012; Žilić et al., 2012), among which cyanidin 3-glucoside is the most abundant one in the dark red, dark blue, light blue and multicolor maize kernels (Žilić et al., 2012). The presence of anthocyanins in cereals is generally associated with a stronger antioxidant activity and the higher amounts of these phenolic compounds seem to directly contribute to higher antioxidant power (Lopez Martinez et al., 2009; Žilić et al., 2012). The presence of these molecules in the diet is important in the prevention of chronic diseases such as cardiovascular disease, cancer, respiratory disease, diabetes and obesity as shown in numerous papers (reviewed by Tsuda) (Tsuda, 2012). Considering that the yellow and white corn varieties do not accumulate anthocyanins in the kernel or only in trace amounts, we can consider the Millo Corvo cultivar a proper functional food.

Furthermore the high percentage of cyanidin present (about 66%) in this variety (Fig. S2) represents an important feature because several papers reported the specific beneficial effect of the cyanidin in the diet of animal models. In particular a work by Toufektsian et al. in 2008 reported that chronic dietary intake of a synthetic maize population rich in cyanidin (about the same quantitative present in the Millo Corvo) protected the rat heart against ischemia-reperfusion injury (Toufektsian et al., 2008). In this work we also characterized in detail the genetic basis of pigment accumulation showing that the trait "seed colored" is a monogenic dominant character (Fig. S3). Furthermore histological analysis conducted preserving the pigment present in the fresh tissue showed that the pigment is accumulated in the aleurone layer (Fig. 5). Taken together the results obtained suggested that in this cultivar there was present an allele of the *R1* regulatory gene of anthocyanin biosynthesis, because typically the *r1* and *c1* genes control the aleurone seed pigmentation whilst *b1 pl1* genes control the vegetative tissue (in the seed the pericarp layer is of maternal origin). Further a strong evidence to support our hypothesis was given by the mapping: we demonstrated that the "colored seed" trait maps on the long arm of chromosome 10, where the *r1* gene maps. Further investigations were made to assess which kind of *r1* allele was present in Millo Corvo variety. Comparing the data obtained from tissue specificity pigmentation of Millo Corvo (Fig. S1) with the data on the four principal classes of *r1* alleles (Table 3) we inferred the presence of an allele of *R-g* class. The r1 gene is a complex locus, made up of three components *P, S1, S2* which arose by gene duplication (Walker et al., 1995). This

complex locus undergoes with high frequencies (overall frequency of 6.2 x 10^{-4}) genetic rearrangement by intrachromosomal recombination between *P* and *S* units, which results in the loss of one *R-r* component and generates the big genetic variability present at this locus (Dooner and Kermicle, 1971). The allele in which all these three components are functional is called *R-r, R-g* if the *P* component is missing, *r-r* if both the *S* components are missing and *r-g* if all the three components are missing.

As we reported, each of these alleles has a specific tissue specificity for the synthesis of the anthocyanins, and given the phenotypic data acquired on Millo Corvo plants in the field and the histological analysis, it can be supposed that the anthocyanin biosynthesis in Millo Corvo is regulated by an *R-g* allele type. This hypothesis has been further confirmed by the sequencing and the following alignment analysis by BLAST program (Fig. S4). Further work will be necessary to better characterize this new allele at the *r1* locus from a molecular point of view, also because of its capacity to accumulate pigment in the seedling tissue that usually is not pigmented in the presence of *R-g* alleles.

Another important source of hydrophobic dietary antioxidants and pigment in maize are carotenoids. Generally carotenoids, and in particular lutein and zeaxanthin, are present in maize varieties with yellow to orange coloration (Kurilich and Juvik, 1999; Žilić et al., 2012). For example, Berardo et al. (Berardo et al., 2004) found in average around 42.07 mg/kg of total carotenoids in an Italian polenta corn collection; among them, however, there were a few white varieties in which carotenoids were not synthesized. On the other hand in many developing countries around the world the utilization of white maize landraces is widespread, for reasons that so far are not well understood. In fact an adequate daily consumption of carotenoids is essential for human health: its deficiency may cause blindness, increased infectious morbidity and mortality, growth retardation, and anemia (Sommer and Davidson, 2002; Žilić et al., 2012), as already experienced in Africa where white corn is the main staple food (World Health Organization, 2002; Žilić et al., 2012).

Our HPLC analysis showed that the Millo Corvo cultivar lacks carotenoids and that this character is controlled by a monogenic recessive mutation, as shown by the study of F1, F2 and F3 progenies (Fig. 4). White endosperm is an ancient trait shared with teosinte, the wild progenitor of maize, caused by y_s recessive mutations impairing carotenoids biosynthesis (Buckner et al., 1996). It seems likely that Pyrenean-Galician landraces have been developed through hybridization with the Northern US flints introduced into

Europe in the sixteenth century from the north of France (Camus-Kulandaivelu et al., 2006; Dubreuil et al., 2006; Mir et al., 2013) and we can conjecture that this last parental contribution brought the *y* allele which has been fixed in the following generations. To further characterize the Millo Corvo variety we measured the antioxidant ability of its flour containing anthocyanins and lacking carotenoids in comparison with a Scagliolo cultivar used as control (an Italian polenta maize variety containing carotenoids) and compared the data obtained with an F2 segregating white seed lacking both anthocyanins and carotenoids (Fig. 6). The results obtained showed the highest ARP value (0.06) in the dark blue kernels of Millo Corvo and the lowest ARP value (0.03) in the white kernels, while the yellow–orange cultivar Scagliolo showed an intermediate ARP value of 0.04. These data showed that Millo Corvo even though lacking carotenoid has a higher antioxidant ability, due to the presence of anthocyanins, compared with a classical yellow orange cultivar such as Italian Scagliolo polenta maize.
To conclude, this ancient cultivar represents an historic landrace that could be a useful tool in future breeding programs and a promise for the development of functional foods or natural colorants.

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Author contributions

Conceived and designed the experiments: RP CL. Performed the experiments: CL ML E. Cassani E. Cantaluppi ED EN RP. Analyzed the data: RP CL EN AG. Contributed reagents/materials/analysis tools: RP AG. Wrote the paper: CL RP AG.

Figures

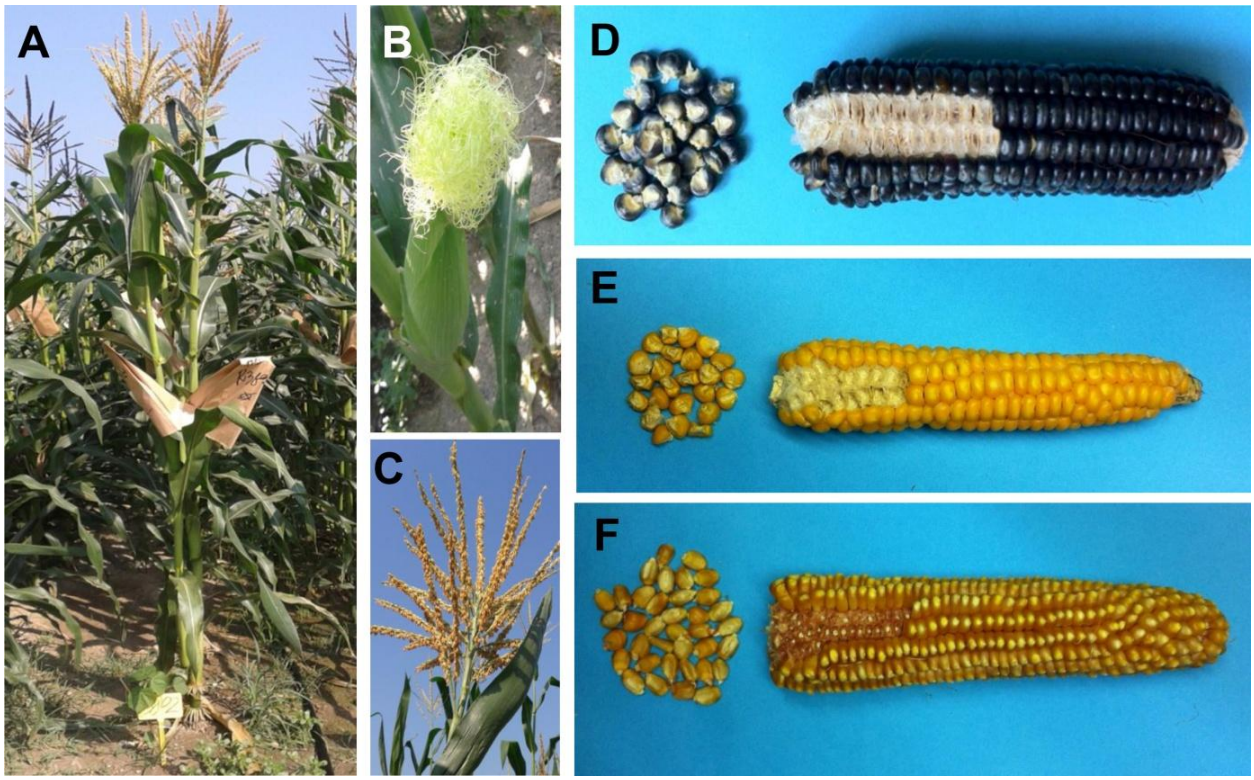

Figure 1. Phenotype of the Millo Corvo maize cultivar and ears compared to two other maize traditional cultivars.

(A) Plant at maturity, (B) immature ear with silks, (C) tassel and (D) ear of Millo Corvo cultivar, (E) ear of Ottofile cultivar and (F) ear of Scagliolo cultivar.

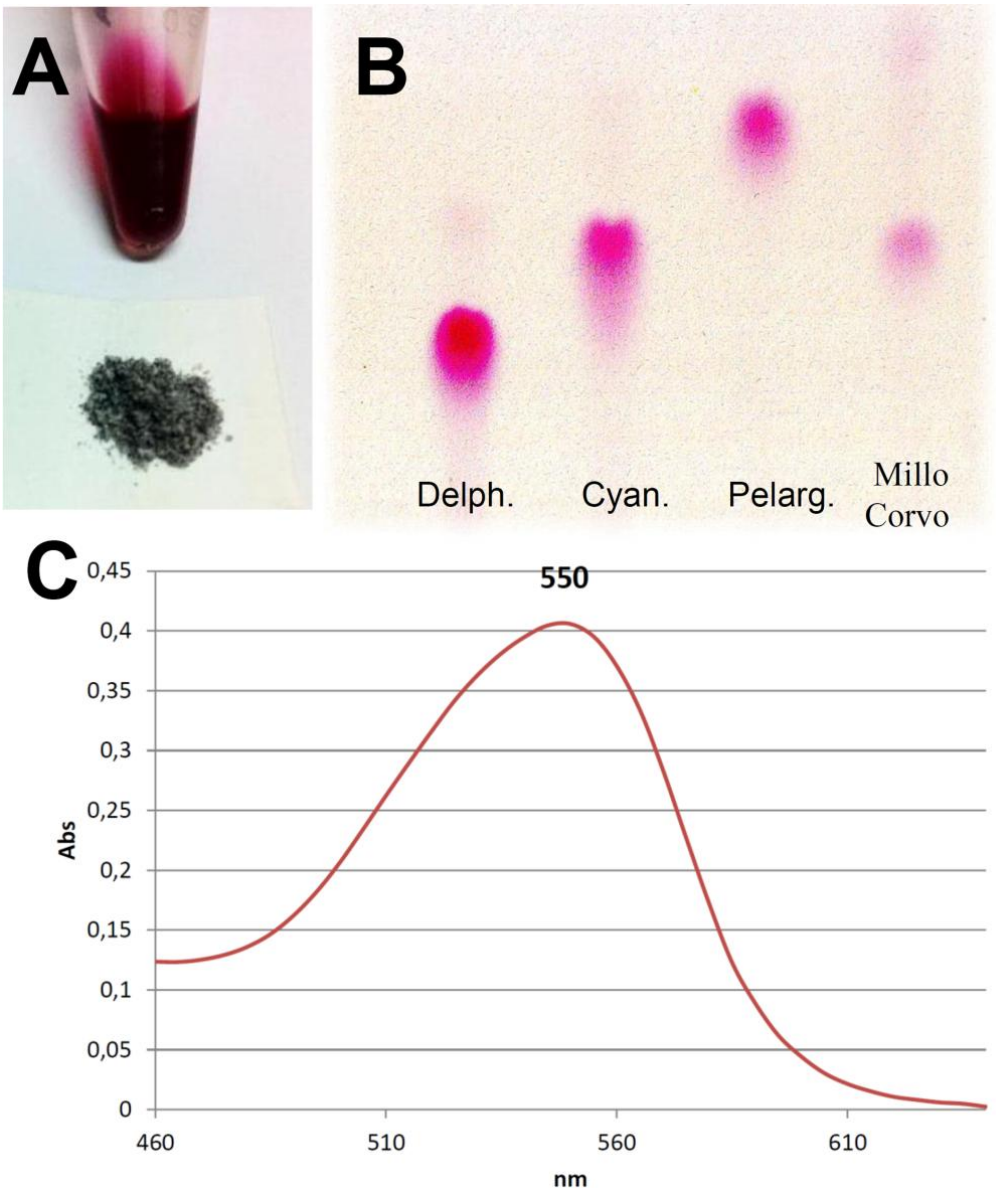

Figure 2. Anthocyanin characterization.

Anthocyanins alcoholic extract from the powder (A, above) obtained by milling the surface of the Millo Corvo kernels (A, below). TLC analysis (B) and absorbance spectrum of the extract (C). The standard used for the TLC analysis were: cyanidin (cyan.), delphinidin (delph.) and pelargonidin (pelarg.).

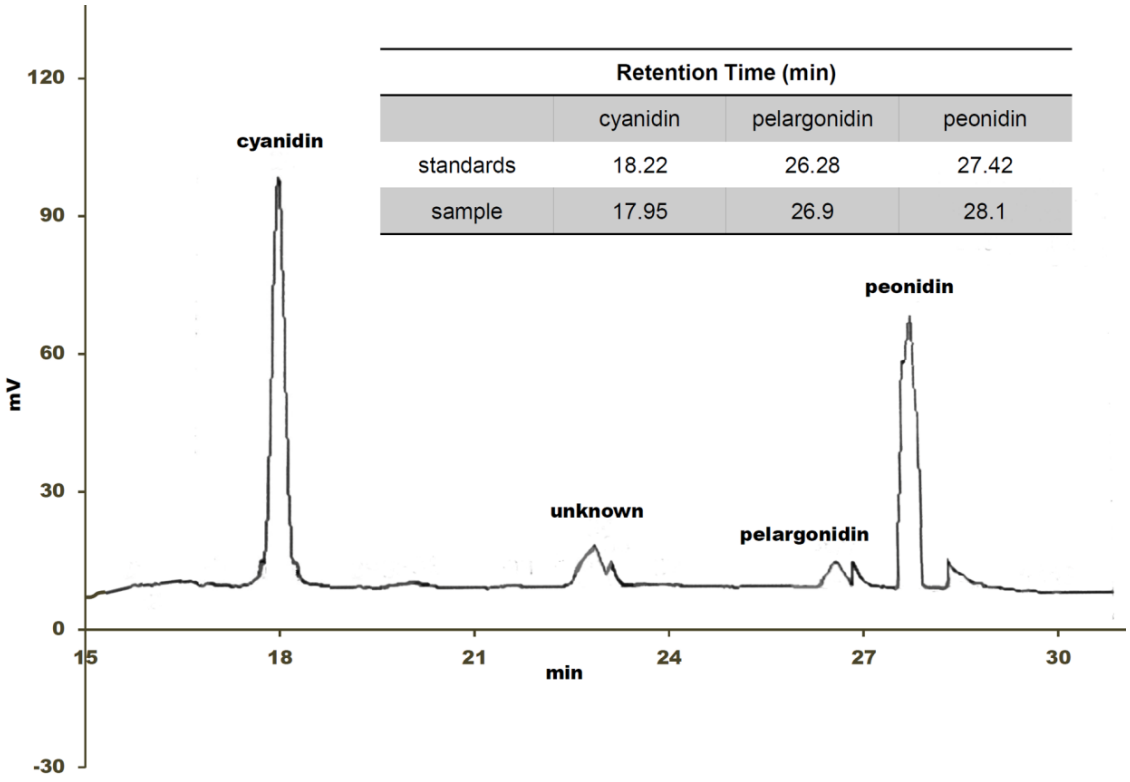

Figure 3. HPLC analysis.

HPLC chromatogram of the anthocyanins extracted from the Millo Corvo seeds and the corresponding retention times compared to the standards cyanidin, pelargonidin and peonidin.

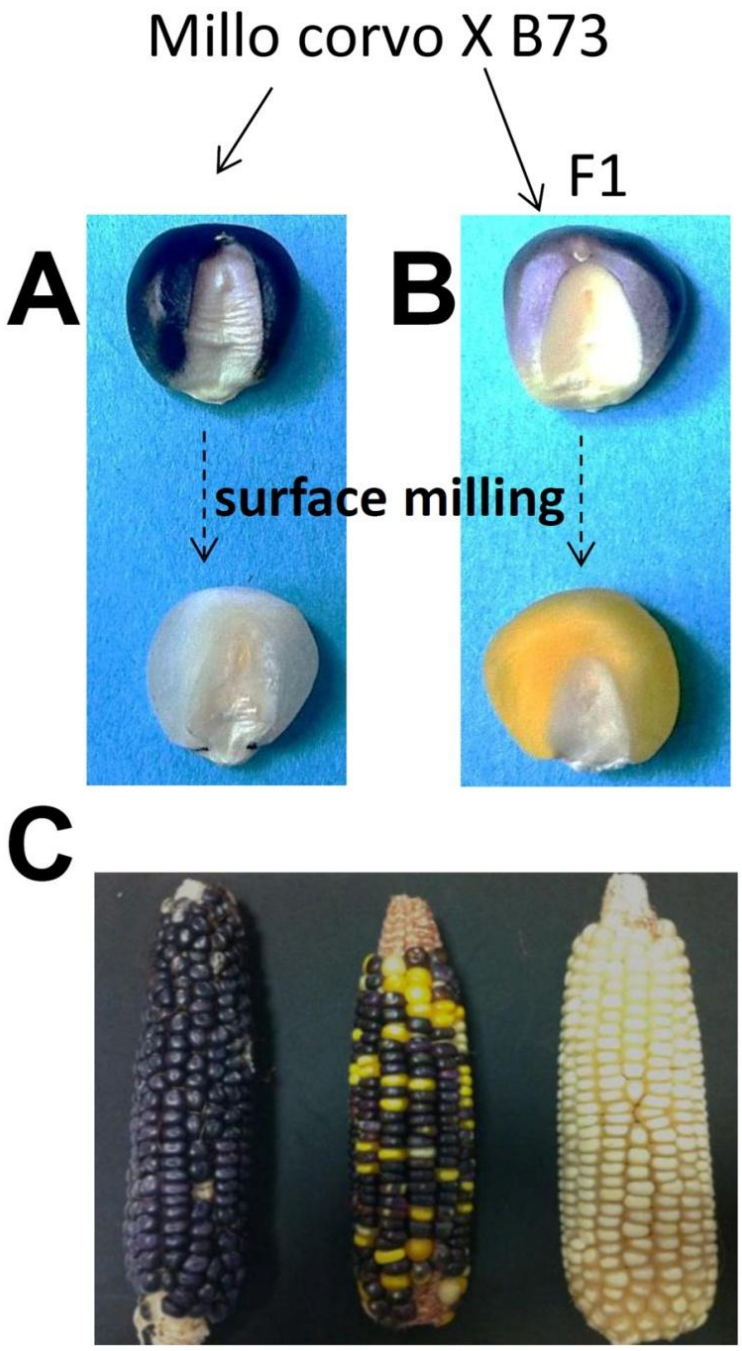

Figure 4. Carotenoid assay by surface milling and following F2 and F3 *yy* segregation. Surface milling of the Millo Corvo seeds (A) and of the F1 seeds obtained by crossing with B73 inbred line (B). Millo Corvo ear, F2 segregating ear and a F3 ear obtained selecting and sowing F2 seeds without carotenoids (C, from left to right).

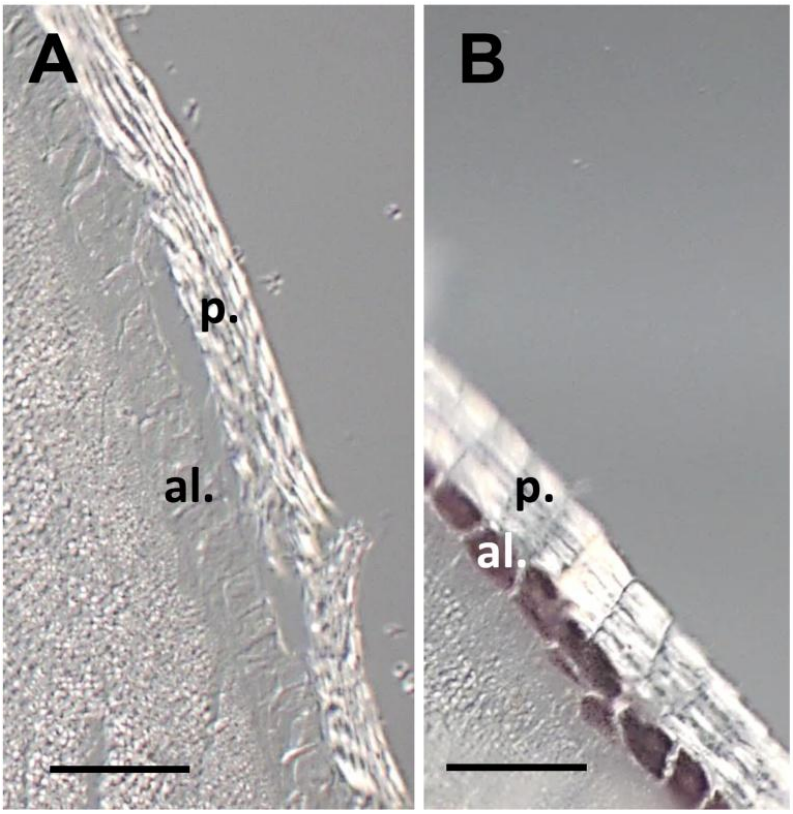

Figure 5. Histological analyses of seeds preserving anthocyanin pigments in situ. B73 colourless seed used as control (A) and Millo Corvo seed (B).
al. aleurone layer; p. pericarp layer. Bar = 100 μm.

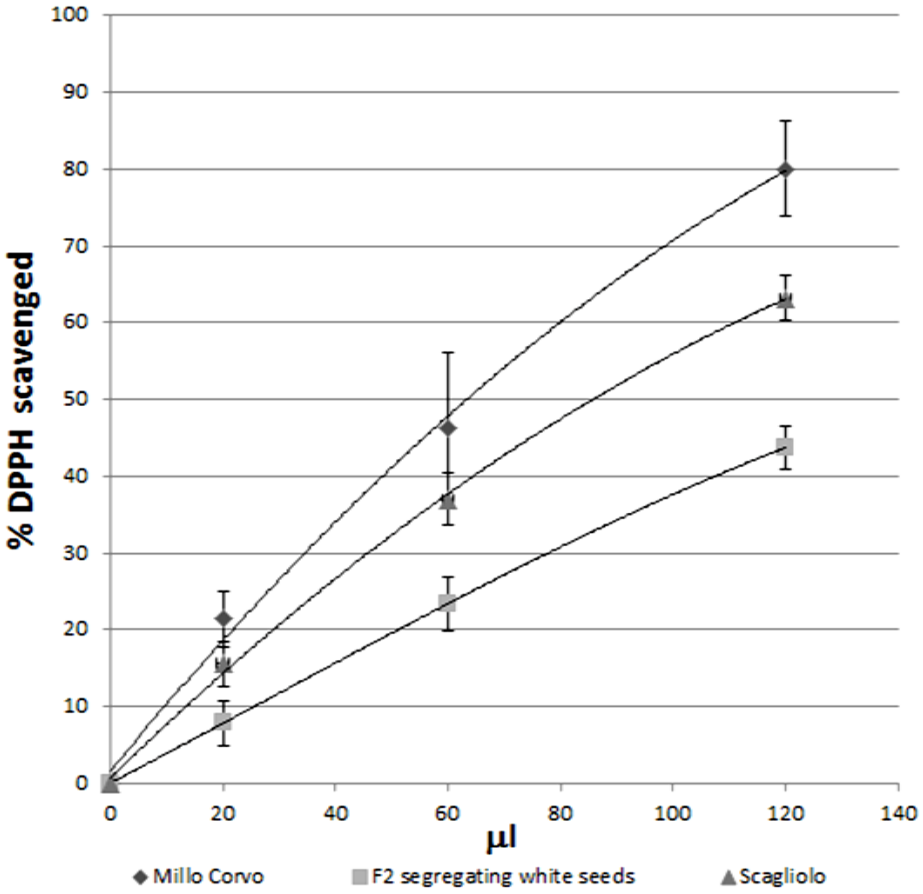

Figure 6. DPPH radical scavenging test.

Comparison of the antioxidant ability in the DPPH radical scavenging test of Millo Corvo (accumulating anthocyanins and no carotenoids), F2 segregating white seed (lacking anthocyanins and carotenoids) and Scagliolo cultivar (accumulating carotenoids) flours. Error bars represent S.D. (n=3).

Tables

Table 1. Agronomic parameters of Millo Corvo, Ottofile and Scagliolo cultivars cultivated at Landriano (PV). Confidence Intervals at 95% are shown, n > 50.

Parameters	Millo Corvo	Ottofile	Scagliolo
Plant height (cm)	248.36 ± 10.90	198.17± 12.30	172± 16.30
Ear height (cm)	105.08 ± 6.04	112.35± 8.30	64± 5.30
Ear length (cm)	16.26 ± 0.77	21.49± 1.24	16.35± 1.38
Cob diameter (cm)	2.75 ± 0.21	1.93± 0.28	2.23± 0.27
Kernels weight per ear (g)	109.25 ± 26.02	54.14±13.74	99.19±17.66
Seed weight (g)	0.31 ± 0.02	0.26± 0.08	0.24± 0.12
No. of rows	12	8	12

Table 2. Spectrophotometric quantification of anthocyanins, flavonols and phenolic acids, quantified as mg cyanidin-3-glucoside equivalents, quercetin 3-glucoside equivalents and ferulic acid equivalents respectively per 100 g of dry seed flour. The analyses were conducted four times for each genotype, and the confidence interval at 95% was calculated.

	Anthocyanins (mg/100g)	Flavonols (mg/100g)	Phenolic Acids (mg/100g)
Millo Corvo	83.45 ± 11.44	74.21 ± 17.83	216.63 ± 29.05
B73	3 ± 1	66 ± 10	113 ± 0.2

Table 3. Tissue specific expression of the main classes of *r1* alleles.

Allele	Pigmentation	
	Seed	Plant
R-r	+	+
R-g	+	-
r-r	-	+
r-g	-	-

Supporting information

S1. Tissues in which pigments are accumulated in the Millo Corvo cultivar.

Tissue	Pigmentation
Seedling	+
Roots	-
Stem	-
Anthers	-
Silks	-
Husks	-
Cob	-
Seed	+

SI2. Partition of the anthocyanidins present in the extracts of the Millo Corvo kernels , according to the HPLC analysis.

Anthocyanidin	%
Cyanidin	65.90
Peonidin	31.40
Pelargonidin	1.95

SI3. Segregation of the "seed color" trait observed in the F_2 progeny obtained by selfing Millo Corvo x B73 plants. The expected segregation values for color trait was 3:1 in the case of the presence of a single dominant gene driving the pigmentation.

cross	segregation		$\chi 2$ value	p
	colored	colorless		
(Millo Corvo x B73) selfed	460	171	1.48	0.3-0.2

SI4. Partial sequencing analysis of *r1* Millo Corvo allele. Alignment obtained by BLASTN program using as query the consensus sequence of 454 nucleotide at the 3' portion of *r1* gene.

Blast hit: *Zea mays* seed color component at *R1 (S)*, mRNA
Sequence ID: ref|NM_001112603.1|Length: 2309
Alignment statistics

Expect	Identities	Gaps
0.0	354/358(99%)	0/358 (0%)

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Query  96    CAGTTTGCTGGCTCCGGTGCCGTCGTGCCCTGGATGATCAGCGAGGCTCTTCGCAAAGCT  155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1910  CAGTTTGCTGGCTCCGGTGCCGTCGTGCCCTGGATGATCAGCGAGGCTCTTCGCAAAGCT  1969

Query  156   ATAGGGAAGCGGTGAAGGGGCAGCTGGAAATTTGGACATCGACGGGCATGGAAGGCTTCA  215
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1970  ATAGGGAAGCGGTGAAGGGGCAGCTGGAAATTTGGACATCGACGGGCATGGAAGGCTTCA  2029

Query  216   TGGGATCGAAGCAAAGATGCATTTCTTGTTTCTTTAGATAACAGGCATGAATCGGATCTC  275
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2030  TGGGATCGAAGCAAAGATGCATTTCTTGTTTCTTTAGATAACAGGCATGAATCGGATCTC  2089

Query  276   TATATCAACAATTATATTGGCATGAATACTTAGACTCCCCCCCTTAACACGTAGAAACTC  335
             |||||||||||||||||||||||||||||||||||  ||||||||||||||||||||
Sbjct  2090  TATATCAACAATTATATTGGCATGAATACTTAGACTCCAGCCCTTAACACGTAGAAACTC  2149

Query  336   AAAAAAAGAAAAAAGGAAGCTAAAGACTAAGCGTAAGGTATATTTGGAAGTAAATTATTT  395
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Sbjct  2150  AAAAAAAGAAGAAAGGAAGCTAAAGACTAAGCGTAAGGTATATTTGGAAGTAAATTATTT  2209

Query  396   TTATAGTTTCTAAGCAATCCCATGGTTTATAAAAATACTAGAGTGTTTATGGCATAAG  453
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  2210  TTATAGTTTCTAAGCAATCCCATGGTTTATAGAAATACTAGAGTGTTTATGGCATAAG  2267
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Genetic studies regarding the control of seed pigmentation of an ancient European pointed maize (*Zea mays* L.) rich in phlobaphenes: the ‘‘Nero Spinoso’’ from the Camonica valley

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Abstract

Several preclinical studies have suggested that the regular consumption of flavonoid-rich foods is associated to a reduced risk of chronic diseases. For this reason, in the last years a renewed interest for the ancient landraces rich in flavonoids or other bioactive molecules is growing. Preservation and valorisation of these ancient landraces is very important, not only for economic considerations regarding the farmers within the small rural communities, where the particular maize germplasm has been developed, but also from a scientific point of view. In this work we characterized the ancient cultivar named "Nero Spinoso" from the Camonica valley, the biggest valley in the north-west region of Lombardy (Italy). The peculiarity of this landrace is the colour and the pointed shape of the kernels. We showed after spectrophotometric and TLC analysis that this variety accumulates high amounts of phlobaphenes (320 A_{510}/100 g flour).

Genetic data demonstrate that phlobaphene pigmentation is under the control of a monogenic dominant gene. Further mapping and sequencing data showed that the pigmentation is driven by the presence of a strong allele of *Pericarp color1* (*P1*) gene, a transcription factor belonging to the myb transcription factor gene family. The ''Nero Spinoso'' variety represents an ancient landrace that could be considered a real functional food and a useful tool in future breeding programmes.

Introduction

Domestication of corn (*Zea mays* L.) can be traced back to about 8,700 BP in Mexico and from this center it spread within the Americas (Piperno et al., 2009; Ranere et al., 2009; Van Heerwaarden et al., 2011). From the Americas, three main sources of corn were introduced into Europe: the photoperiod insensitive Cateto types, the Pearl White and the corn lines from the American east coast with high latitude growth adaptation (Brandolini and Brandolini, 2009; Eschholz et al., 2010). The spread of maize in Europe started from Spain and other southern European countries such as Italy in which it had great success thanks to several favourable environmental and social conditions (Anderson and Cutler, 1942; Bianchi et al., 1963; Brandolini and Brandolini, 2009). In Italy, the first reports on the use of corn date from 1600 in the North East where maize was adapted to the climatic zones of cultivation and to local traditions of the people (Brandolini, 1958; Brandolini and Brandolini, 2009). Its spread led to the establishment of many local varieties genetically adapted to environmental conditions.

After World War II the introduction of mechanized farming practices and the utilization of dent hybrids which were much more productive, mainly for use as animal feed, led to the gradual disappearance of local varieties (Brandolini and Brandolini, 2009).

Fortunately, in more recent years many efforts have been made to try to recover and preserve the genotypes of the old varieties: in Italy the main maize collection is preserved ex situ at the CREA-Council for Agricultural Research and Agrarian Economy located at Stezzano (BG).

In this work, we characterize an ancient landrace of colored pointed flint maize used for polenta: the “Nero Spinoso” (Black Pointed), which until now has been cultivated in a small isolated field (about 800 m a.s.l.) in the Annunciata area of Piancogno Municipality near Esine, an Italian town in the Camonica valley, province of Brescia (BS). This maize cultivar has two peculiarities: the pointed shape of the seed and the pigmentation of the kernel. Concerning pigmentation, it is well known that maize is able to synthesize and accumulate two types of pigments: anthocyanins and phlobaphenes, secondary metabolites synthesized through the flavonoids pathway that perform several functions during the growth and development of plants (Grotewold, 2006; Falcone Ferreyra et al., 2012; Casas et al., 2014). Both these types of pigments are responsible for some beneficial effects on human health due to their antioxidant capacity (Grotewold et al.,

2000; West et al., 2002; Rodriguez et al., 2013; Casas et al., 2014; Lago et al., 2014a, Lago et al., 2014b; Petroni et al., 2014). The inheritance of pigmentation depends on the tissue in which the pigment is accumulated. The accumulation of pigments in the seeds may occur in two tissues: in the pericarp, a tissue of maternal origin, or in the aleurone layer that covers the endosperm (Dooner et al., 1991). The anthocyanin pathway in maize is known to be controlled by two classes of regulatory genes: the *r1*/*b1* family, that encodes proteins with sequence homology to the basic helix-loop-helix (*bHLH*) and the *c1*/*pl1* family, that encodes proteins with sequence homology to the DNA-binding domains of the MYB related oncoproteins (Pilu et al., 2003). The interaction of these regulatory genes allows the activation of about 20 structural genes required for anthocyanin pigment production (Dooner et al., 1991).

Phlobaphenes are reddish insoluble pigments; the biosynthetic pathway of these compounds begins with the condensation of three malonyl-CoA molecules with p-coumaroyl-CoA by chalcone synthase (CHS), encoded by the *colorless2 locus* (*c2*), leading to the formation of naringenin chalcone (Styles and Ceska, 1977; Casas et al., 2014). The chalcone isomerase (CHI) enzyme converts naringenin chalcone into the flavanone naringenin that is converted to apiforol and luteoforol by the *A1* locus coding for dihydroflavonol reductase-DFR enzyme and the *Pr1* locus coding for flavanone-3-hydroxylase-F3-H enzyme which are polymerized into phlobaphenes (Winkel-Shirley, 2001; Grotewold, 2006; McMullen et al., 2004; Morohashi et al., 2012; Falcone Ferreyra et al., 2012).

In the maize pericarp layer the accumulations of phlobaphene pigments are under the control of the R2R3-MYB transcription factor *pericarp color1* (*p1*) whereas different *P1* alleles confer different pericarp and cob glume colors (Grotewold et al., 1991; Casas et al., 2014). The presence of *P1-rr* allele determines the coloration of both pericarp and cob glumes, *P1-rw* only the pericarp, *P1-wr* only the cob glumes and *P1-ww* has both the tissues colorless (Anderson, 1924; Chopra et al., 1996; Casas et al., 2014).

For the pointed shape of the seeds, we known that this is an ancient characteristic of wild maize, in fact the maize ancestor was probably both pod corn (tunicate maize) and a popcorn with pointed kernels (Mangelsdorf and Reeves, 1959). In this work we studied from several point of view this rediscovered ancient opv (open pollinated variety), determining which pigments are accumulated in the seeds and the heritability of this character by genetic and molecular analysis.

Materials and methods

Plant and sampling material

The "Nero Spinoso" maize variety (kindly provided by Mr. Saloni of Saloni's farmhouse, Piancogno) was cultivated, during the 2014 season, in different fields situated in the Camonica valley, Italy (the locations were: Esine, Largarolo, Malonno, Plemo, Plerio, Pregasso, Santicolo and Volpera) and in the experimental field of the University of Milan located in Landriano (PV), Italy (45° 18' N, 9° 15' E). The colourless B73 inbred line and the coloured Millo Corvo variety, *R-sc* (self-coloured aleurone) and *P1* homozygous plants which were used as control and for breeding activities came from the collection of germplasm at the Department of Agricultural and Environmental Sciences-Production, Landscapes, Agroenergy at the University of Milan. About 200 seeds, for all genotypes tested, were sown in adjacent rows, under the same agronomic conditions. These plants were selfed and the ears obtained were harvested at the same time at the end of the season. About 80 ears of "Nero Spinoso", cultivated in Landriano, were shelled and the seeds obtained mixed to create a single bulk. The seeds so obtained were used for the determination of anthocyanins, flavonols and phenolic acids.

The same procedures were followed for the Millo Corvo variety and the B73 inbred line used as the colorless control.

Milling

Flour samples were obtained using a ball mill (Retsch MM200, Retsch GmbH Germany), and seeds (cleaned from the glumes) were ground for 5 min at 21 oscillations s^{-1} frequency.

Spectrophotometer determination of anthocyanins, flavonols and phenolic acids

15 mg of flour were first boiled with 100 µL of distilled water for 30 min and then left in an overnight agitation with 1 mL of the extraction buffer (1% HCl, 95% ethanol).

After another agitation time of 2 h with 500 µL of extraction buffer, the supernatants were collected together and centrifuged for 30 min. Their absorbance was determined spectrophotometrically at 530 nm for anthocyanins, at 350 nm for flavonols and at 280

nm for phenolic acids (Pilu et al., 2011). The amounts of anthocyanins were calculated as cyanidin 3-glucoside equivalents (molar extinction coefficient (ε 26,900 L m^{-1} mol^{-1}, M.W. 484.82), flavonols content as quercetin 3-glucoside equivalents (ε 21,877 L m^{-1} mol^{-1}, M. W. 464.38) and the amount of phenolics as ferulic acid equivalents (ε 14,700 L m^{-1} mol^{-1}, M.W. 194.18). The analyses were conducted four times for each genotype, and the confidence interval (C.I.) at 95% was calculated.

Bleaching test

Twenty seeds of coloured "Nero Spinoso" and *R-sc* (self coloured aleurone) seeds, used as a control, were bleached following immersion in 7% sodium hypochlorite for 1 h. After this period the seeds were rinsed with tap water and pericarp tissue decoloration was checked.

Qualitative determination of anthocyanins: TLC (thin layer chromatography)

The fine powder of the pericarp layer of the "Nero Spinoso" and *P1* inbred line kernels (obtained using a manual electric drill) was boiled at 100 °C in 2 mL of 2 N HCl for 40 min. After adding 1 mL of isoamyl alcohol, the upper phase was dried and suspended in EtOH 95% and HCl 1% for the TLC analysis. Cyanidin, pelargonidin and delphinidin standards were loaded together with the extracts on a pre-coated plastic sheet (POLYGRAM CEL 300, MACHEREY–NAGEL) for TLC using formic acid:HCl:water 5:2:3 as solvent. Run TLC plates were dried and the results recorded by a digital camera (A430 Canon) using both white and UV illumination.

Cosegregation analysis

In order to perform cosegregation analysis we used F2 populations, obtained by selfing the progeny of the cross "Nero Spinoso" X B73. A total of 109 F2 plants were screened for the ear color and from every plant a leaf sample was used for DNA extraction (Dellaporta et al., 1983). PCRs were performed using *PHI095*, simple sequence repeat (SSR) marker within the gene *P1* on chromosome 1 (bin1.03) from MaizeGDB (http://www.maizegdb.org).

Polymerase chain reactions were performed in a final volume of 10 µL and the reactions were carried out as follows: 94 °C for 2 min, 35 cycles at 94 °C for 45 s, 67 °C for 1 min, 72 °C for 1 min, and a final step at 72 °C for 5 min. The amplification fragments were resolved on 3% agarose gels. Polymerase chain reactions and gel running conditions were performed as described in the SSR Methods Manual by MaizeGDB (http://www.maizegdb.org/documentation/maizemap/ssr_protocols.php).

Amplification and sequencing

The partial sequencing of the *P1* gene was conducted starting from genomic DNA extracted from leaves.

DNA was amplified by high fidelity PCR (Pfu polymerase; Stratagene, La Jolla, CA, USA) using the specific primers sP1-4F: 5'-ATGGACGCCCTGATGCCTAT-3' and sP1-4R: 5'-CTGTACACACGA GCAACG CC-3'. PCR reaction was performed in a 25 µL volume containing about 50 ng of genomic DNA; 1X polymerase buffer; 2.5 mM $MgCl_2$; 200 µM each of dATP, dCTP, dGTP, and dTTP; 0.1 µM of each primer and 0.25 unit of Taq DNA polymerase.

The reactions were carried out as follows: 94 °C for 2.5 min, 35 cycles at 94 °C for 45 s, 63 °C for 1 min, 72 °C for 1 min, and a final step at 72 °C for 5 min.

Five independent amplicons were sequenced in outsourcing to deduce the consensus DNA sequence by freely available computer software CLUSTALW (http://www.ebi.ac.uk/clustalw/).

We used BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) to study the sequence obtained.

Histological analysis

Coloured "Nero Spinoso", and the controls, coloured Millo Corvo and colourless B73 seeds were imbibed in water overnight and fixed in freshly prepared 4% paraformaldehyde (Sigma P4168) in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM $NaH_2PO_4 \cdot H_2O$) at 4 °C overnight, then rinsed in 0.85% NaCl and transferred in 70% ethanol at 4 °C until processing.

Following successive dehydration in ethanol series and embedding in Paraplast Plus (Sigma P3683), 15 µm-thick sections were cut and serially arranged on microscope slides.

To preserve anthocyanin pigments in situ, sections were mounted on slides using *tert*-butyl alcohol instead of water. To determine the pericarp thickness, images were taken and elaborated using a Zeiss IMAGE R.D1 microscope equipped with an AxioCam MRc1 camera.

Results

Phenotypic characterization of the "Nero Spinoso" landrace

The "Nero Spinoso" open pollinated variety was recovered from a small isolated terraced field owned by the Saloni family located in the Annunciata area of Piancogno municipality (BS) in the Camonica valley (45° 55' N, 10° 13' E), at 680 m a.s.l. (Fig. 1). Starting from a sample of seeds this variety was cultivated and studied at the Experimental Field of University of Milan located at Landriano (PV) where the genetic study was conducted and then in different fields all located in the Camonica valley: Esine, Largarolo, Malonno, Plemo, Plerio, Pregasso, Santicolo and Volpera. As shown in Fig. 1B and Fig. 2 the peculiarities of this cultivar are the color and the pointed shape of the kernel. During the agronomic season 2014, different agronomic traits were measured (Table 1). The plants reached maturity in about 90 days after sowing in these environments. In these fields the plants were, on average, 252.41 ± 4.16 cm in height (plant height was recorded at the level of the flag leaf). The ears were of cylindrical-conical shape with 14.03 ± 0.56 rows, measuring 18.12 ± 1.02 cm in length with an ear diameter of 4.22 ± 0.12 cm and cob diameter of 2.97 ± 0.11 cm (Table 1). The ears height was 106.34 ± 2.74 cm (Table 1). The kernels were pointed flint type and pigmented, with an average weight of 0.26 ± 0.05 g. The weight of seeds carried by a single ear was 120.74 ± 58.7 g for an estimated potential yield of about 7.2–8.4 tonnes per hectare (sowing 6–7 seeds per square meter).

Characterization of seed pigments: anthocyanins, flavonols, phenolic acids and phlobaphenes

The main characteristic of this variety is the color of the seeds as previously described, but other tissues are also pigmented, such as the cob, husks, roots and seedlings (Table 2; Fig. 2).

It is well known that maize plants can accumulate anthocyanins and/or phlobaphenes in different tissues, and with the aim to establish which kind of pigments were accumulated we carried out spectrophotometric analysis. Table 3 shows the results on the amounts of anthocyanins, flavonols, phenolic acids and phlobaphenes present in the seed flour of the "Nero Spinoso" in comparison to the B73 inbred line used as the colorless control and the Millo Corvo variety, used as the colored control which accumulates anthocyanins in the kernel. We found that "Nero Spinoso" is pigmented by the accumulation of phlobaphenes (320.24 A_{510}/100g). To confirm this finding we performed thin layer chromatography (TLC): we loaded as control the three main anthocyanidins accumulated in maize (cyanidin, pelargonidin and delphinidin) and the extract of the *P1* (*pericarp 1*) inbred line, able to accumulate phlobaphenes in the pericarp. As shown in Fig. 3, in the "Nero Spinoso" lane no anthocyanidins were observed whilst two orange spots were present, with the same retention factor (Rf) present in the *P1* lane (although with different relative amounts).

Determination of the pigmented tissues in the seed

In maize seeds two type of tissues can accumulate pigments; the aleurone layer (the outermost triploid tissue) and the pericarp layer (of maternal origin).

To assess which tissue was pigmented in the ''Nero Spinoso'' we treated a seed sample with a strong oxidant, sodium hypochlorite at 7%: a strong oxidant is able to bleach completely the pigment present in the pericarp layer while the pigment present in the aleurone layer remains unoxidized. As shown in Fig. 4, the ''Nero Spinoso'' seeds completely lost their color while the inbred line *R-sc* (accumulating anthocyanin in the aleurone layer) remained unchanged. These data were confirmed by histological analysis of transverse sections of mature seeds, showing the pigmentation only in the pericarp layer (Fig. 5). Furthermore a different structure of the pericarp layer was noticed compared to the B73 colourless line and to the Millo Corvo coloured variety controls. In fact as shown in Table 4 the pericarp thickness of the ''Nero Spinoso'' variety (173.11 ± 12.16 µm) was much greater, compared to B73 and Millo Corvo varieties (respectively 73.5 ± 6.13 µm, 59.16 ± 9.88 µm).

Heritability of the colored seed trait

It is well known that phlobaphenes can be accumulated in the pericarp layer of the kernel, a tissue of maternal origin, by the action of *Pericarp color1* gene (Dooner et al., 1991). Hence, starting from the hypothesis that the "pigmented ear" trait was due to the presence of a dominant allele at the *P1* locus we determined the heritability of this trait in F1 and F2 populations obtained through controlled crosses. As shown in Fig. 6 the coloured "Nero Spinoso" was used as a male line (pollen donor), while the line B73 was used as the colorless female line. The F1 seeds obtained from the cross were all colorless, (indicating that the pigment is present in pericarp maternal tissue) while the F1 generation gave all pigmented ears although their color was less intense compared to the colored parent used for the cross. In the F1 generation we observed a noticeable reduction in the "pointed" characteristics of the kernels (data not shown). The following F2 progeny segregated 3:1 for ear color, confirming that a monogenic dominant character drives the accumulation of phlobaphenes in the pericarp layer (Table 5; Fig. 6).

This evidence led us to hypothesize that the *P1* gene might be responsible for the kernel phlobaphenes biosynthesis. To test this hypothesis we performed a cosegregation analysis using 109 F2 plants phenotyped for the ear color. We extracted genomic DNA from each plant and using *PHI095* SSR marker (inside the *P1* gene), on chromosome 1 (bin1.03) we found a perfect cosegregation between a *PHI095* polymorphism and the trait "pigmented ear", which strengthens the relationship between the presence of a dominant *P1* allele and the ear pigmentation (data not shown). Studying this opv it was possible to observe some variability in the ears' pigmentation, in fact out of 730 ears scored, 686 ears were dark red (93.97%), 23 ears showed different shades of red (3.15%) and 21 ears were completely colorless (2.87%) (Fig. 7). Assuming that the 23 red ear were due to a variable expressivity of *P1* allele present in this population, we used the Hardy–Weinberg principles to calculate the *p1* allelic frequency (colourless ear) and we obtained the value of 0.169 (fr *p1* allele = square root of 21/730) while for *P1* it was 0.831 (fr *P1* = 1 - fr *p1*).

Molecular analysis of the P1 gene

To confirm the presence of a strong *Pericarp 1* allele in this landrace, we sequenced a portion of the P1 gene using specific primers (see "Materials and methods" chapter).

The sequencing of 5 independent amplicons and the following alignment with the CLUSTALW program allowed us to obtain a consensus sequence of 334 nucleotides used for the analysis by the BLASTN program. The results obtained in Fig. 8, show that the best alignment is with the *Zea mays MYB*-like transcription factor *P1* gene, *P1-rw1077* allele (accession number AY702552.1). However we found, respect the *P1-rw1077* allele, two polymorphisms (2 deletions/336 nucleotides) indicating the presence of a new allele needing further investigation.

Discussion

The center of domestication of maize (*Zea mays* L.) is located in south-central Mexico, and from here it spread within the Americas over thousands of years and, successively, to the rest of the world including Europe (Matsuoka et al., 2002; Mir et al., 2013). The spread of maize to a variety of geographical locations has led to its local selection and adaptation to new environments and, consequently, the development of many landraces, or farmer's varieties (Mir et al., 2013). These varieties were characterized by low yields, when compared with modern hybrids, but had considerable phenotypic and genetic variability (Liu et al., 2003; Vigouroux et al., 2008; Warburton et al., 2008; Mir et al., 2013). Recent studies indicate that the spread of maize outside the Americas is complex. In Europe, Asia and Africa the different varieties imported over the centuries from the Americas still coexist (Mir et al., 2013). In Italy the use of corn in agriculture dates back to the second half of the sixteenth century, since then the adaptation to different environments together with human selection led to the diversification of hundreds of landraces (Messedaglia, 1924; Brandolini and Brandolini, 2009). Before these landraces disappeared, being replaced by modern hybrids, hundreds of them were preserved ex situ at the CREA-Council for Agricultural Research and Agrarian Economy located at Stezzano (BG). Analysis of 17 phenological, morphological and geographical characteristics allowed the classification of the accessions of Italian corn into 65 agroecotypes, representing 34 landraces derived from 9 racial complexes (Brandolini and Brandolini, 2009). These nine racial complexes are: Eight row flints (Ottofile) located throughout Italy; Conical flints (Conici) located in Central and Northern Italy; Late south cylindrical flints (Cilindrico tardivo) located in Appenine valleys and Sicily; South cylindrical flints (Cilindrici meridionali di ciclo medio) located in Southern Italy and

Sicily; Early dwarf flints (Nani precoci) located in mountainous areas in North and Central Italy; Microsperma flints (Microsperma) located in Northern and Central Italy; Insubrian flints (Insubri or Padani); Pearl white flints (Bianco perla) and White dents (Dentati bianchi) grown in the Veneto and Friuli regions (Brandolini and Brandolini 2009). In this system of subdivisions, the ‘‘Nero Spinoso’’ (named also “Spinusa” and “Spinato Nero della Valcamonica”) the ancient landrace from the Camonica valley (BS), subject of this study, taking together the characteristics of the ear, seed (Fig. 2) and the data reported in Table 1, can be classified in the Insubrian flints group (Insubri or Padani). The main features of this cultivar are the dark red pigmentation and the shape of the seed that appears pointed (Fig. 1, Fig. 2).

It is well known that maize is able to accumulate pigments in the kernels belonging to two classes of flavonoids, anthocyanins and phlobaphenes (reviewed by Petroni et al., 2014) and with the aim to establish and quantify the pigments present we performed spectrophotometric and TLC analysis. As reported in Table 3, the data obtained revealed that the pigments accumulated in the “Nero Spinoso” variety are phlobaphenes and not anthocyanins as in the case of the Millo Corvo cultivar used as control. We detected also a small amount of anthocyanin (16.66 mg/100g of flours) that, most likely, does not represent actual anthocyanins but rather un-polymerized phlobaphenes (phlobaphenes are complex molecules derived from the polymerization of flavan-4-ols, mainly apiforol and luteoforol) extracted by the anthocyanin extraction buffer (see “Materials and methods” chapter). To strengthen this finding we carried out TLC analysis of the pigments extract (Fig. 3) using as control an extract coming from an inbred line carrying the *P1* gene, and the three main anthocyanidins accumulated in maize: the “Nero Spinoso” pattern observed after chromatographic runs is very similar to one present in the *P1* line that is able to accumulate phlobaphenes in the pericarp layer (Grotewold, 2006; Pilu et al., 2011; Casas et al., 2014). The presence of phlobaphenes in “Nero Spinoso” kernels allows us to consider it as a functional food compared to colorless corn varieties. In fact the beneficial properties derived from the anthocyanins and from other classes of flavonoids on human health have been well studied in recent years (Grotewold et al., 2000; West et al., 2002; Lopez-Martinez et al., 2009; Žilić et al., 2012; Lago et al., 2013; Rodriguez et al., 2013; Casas et al., 2014; Lago et al., 2014a, Lago et al., 2014b; Petroni et al., 2014). Furthermore, phenylpropanoids and in particular phlobaphenes seem to be a resistance factor to kernel infection and fumonisin accumulation by *Fusarium verticillioides*, making it likely that this landrace is more safe

for direct human consumption (Pilu et al., 2011; Sampietro et al., 2013). We also confirmed that the phlobaphenes pigments were accumulated in the pericarp layer, as shown by Fig. 4 and Fig. 5. We also found that the pericarp of "Nero Spinoso" is thicker compared to the two controls B73 and Millo Corvo variety (Table 4; Fig. 5). This characteristic could explain the high amount of phlobaphenes accumulated in this landrace and the consequent dark red/black seed color, compared to other varieties carrying strong *pericarp color1* gene such as *P1-rr* conferring a brick red seed color (Pilu et al., 2011; Petroni et al., 2014). Hence when we crossed "Nero Spinoso" with a colorless line, the F1 obtained always produced seeds which were red and not dark red/black (Fig. 6), probably because of the reduction in pericarp thickness. Of course we cannot also exclude that the specific genetic background of "Nero Spinoso" could also boost the phlobaphenes biosynthesis. The character "pointed kernel" in the F1 produced seeds was less strongly marked compared to the original one and preliminary data suggest that this could be a simple Mendelian character exhibiting incomplete dominance (Fig. 6). However future work will be necessary to study this ancient trait in depth.

The genetic data definitely confirmed that the trait "colored ear" is under control of a dominant monogenic character (Table 5; Fig. 6) driving the accumulation of phlobaphenes in the pericarp layer as expected by the presence of a strong *P1* allele. To support our hypothesis a cosegregation analysis was performed using SSR marker, chosen inside the *P1* gene in an F2 population where the ears were screened for the color. A polymorphism always associated to the trait "colored ear" was found in all 109 individuals analysed, confirming the hypothesis (data not shown).

We also notice that not all the colored ears had the full pigmentation (3.15%) and in some case were colorless (2.87%). Using Hardy–Weinberg principles, as shown in the Results section, we calculated the allelic frequency of this *P1* allele conferring pigmentation, which was found to be 0.831 (Fig. 7). The observation of this variability is not surprising considering that maize is a highly heterogeneous crop where most of the genetic diversity is observed within each population rather than between populations (Warburton et al., 2008; Mir et al., 2013). Finally the presence of a strong *P1* allele has been further confirmed by sequencing and following alignment analysis by BLAST program showing an identity of 99% (334/336 bp) with the *P1-rw1077* allele previously sequenced (Fig. 8). However we think that our allele should belong to the *P1-rr* class of alleles having both pericarp and cob colored as shown in Table 2, as it is different from

a *P1-rw* allele having only the pericarp colored. Further work will be necessary to obtain the complete sequence of this new allele at the *P1* locus.

Although today the "Nero Spinoso" is grown in small plots in the Camonica Valley, in collaboration with the municipalities of Esine and Piancogno (BS), "Nero Spinoso" has been included into the list of "Variety of Conservation" of the National Register of Varieties of Agricultural and Horticultural Species at MIPAAF (Ministry of Agriculture, Food and Forestry) in order to prevent the loss of local traditions as well to preserve the genetic variability (published in the Official Gazette of the Italian Republic n. 9, 13.01.2016). Due its "splendid isolation" (see Fig. 1A) the "Nero Spinoso" went through the centuries unchanged becoming not only a potential functional food but also useful to further clarify the origin and spread of maize as well as to prevent the loss of important sources of genetic variability for further genetic improvement programs.

Acknowledgments

We wish to thank Dr. Davide Reginelli and Dr. Mariarosa Buffoli for their hard work in the field and Dr. Lesley Currah for her editing and suggestions. This study was partly supported by "Accordo di Programma, affermazione in Edolo del Centro di Eccellenza Universita` della Montagna" MIUR-Università degli Studi di Milano, Prot. No. 386 1293-05/08/2011 and by Fondazione della Comunità Bresciana-Onlus.

Figures

Figure 1. Sampling site of the "Nero Spinoso" maize cultivar. (A) Terraced field where this landrace has been cultivated by the Saloni family in the Annunciata area of Piancogno municipality. (B) Harvested ears hung in farmhouse for drying according to tradition.

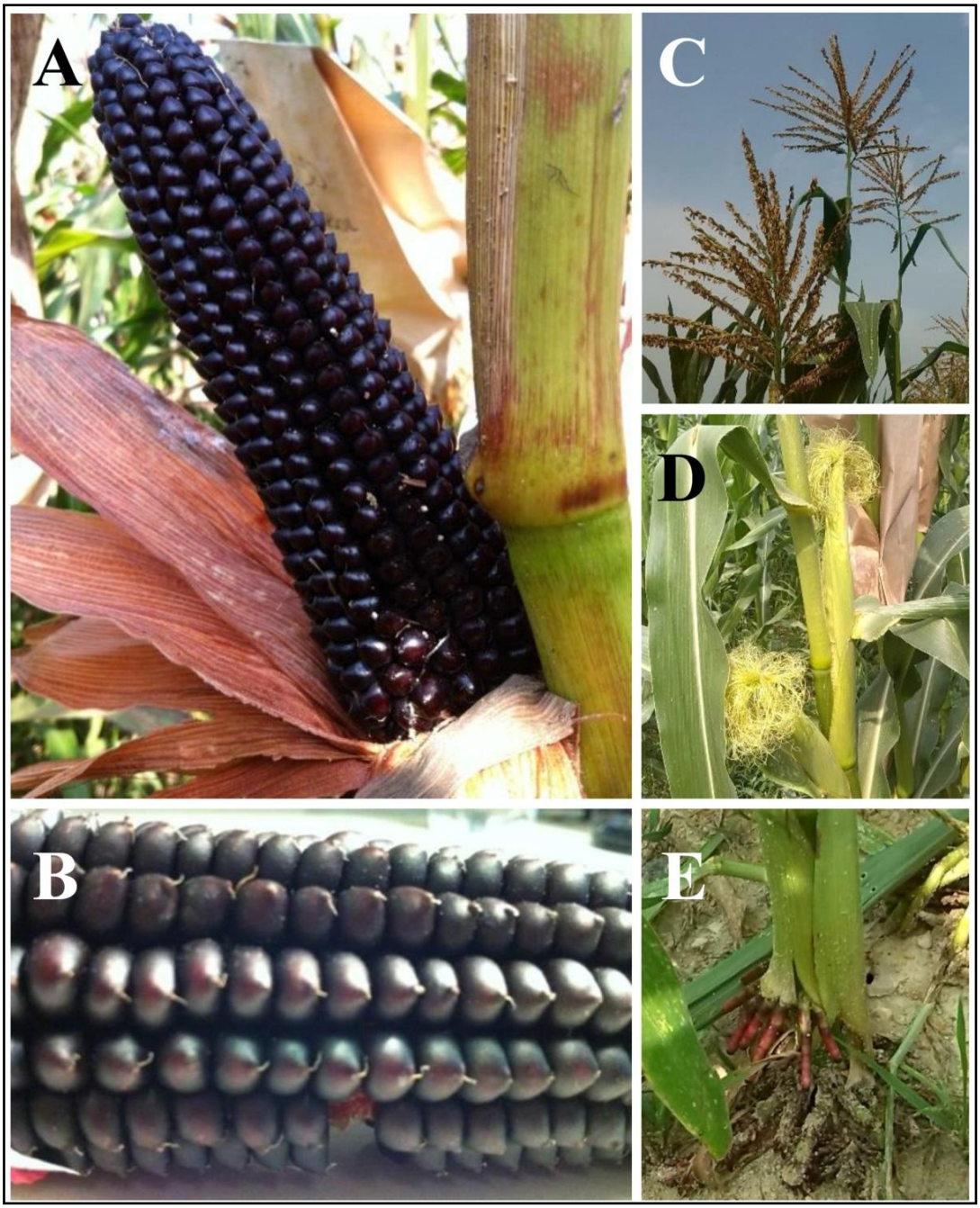


Figure 2. Phenotype of the "Nero Spinoso" maize cultivar. (A) Dark red/black ear at maturity and (B) the characteristic pointed kernels, (C) tassel and (D) immature ears with silk, (E) pigmented roots.

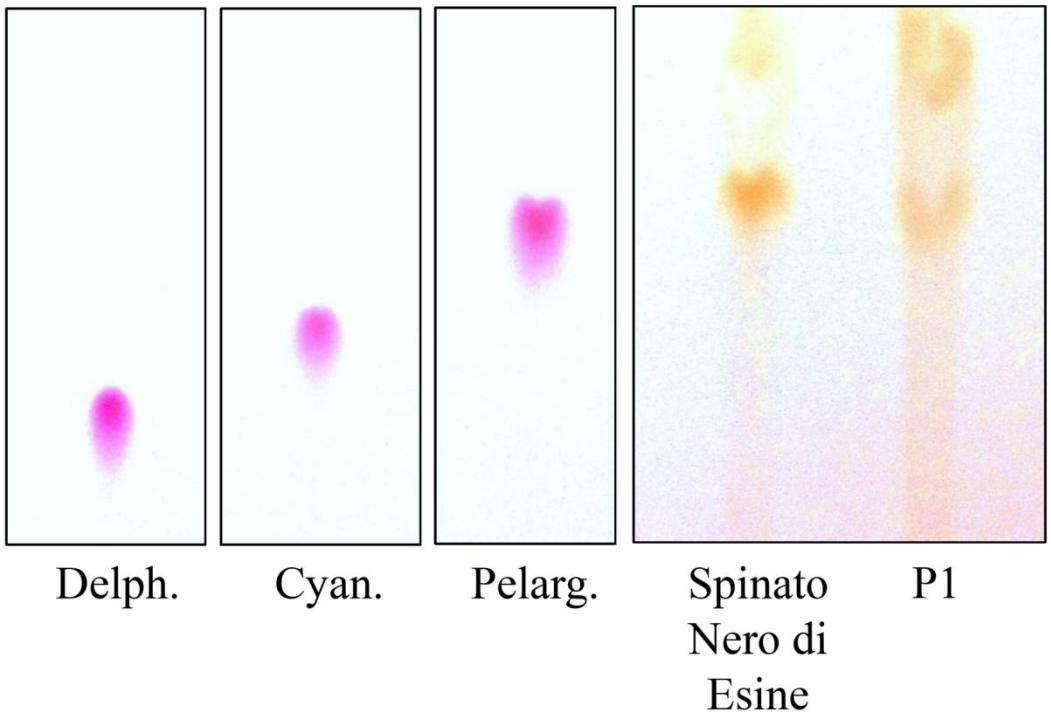


Figure 3. TLC analysis of "Nero Spinoso" compared to a colored *P1* homozygous variety. The standards used for the TLC analysis were: cyanidin (cyan.), delphinidin (delph.) and pelargonidin (pelarg.).

Figure 4. Bleaching test. (A) "Nero Spinoso" seeds before and (B) after bleaching test in which the complete depigmentation of the seed can be seen; (C) *R-sc* seeds (having the aleurone layer pigmented), used as control, before and (D) after bleaching test.

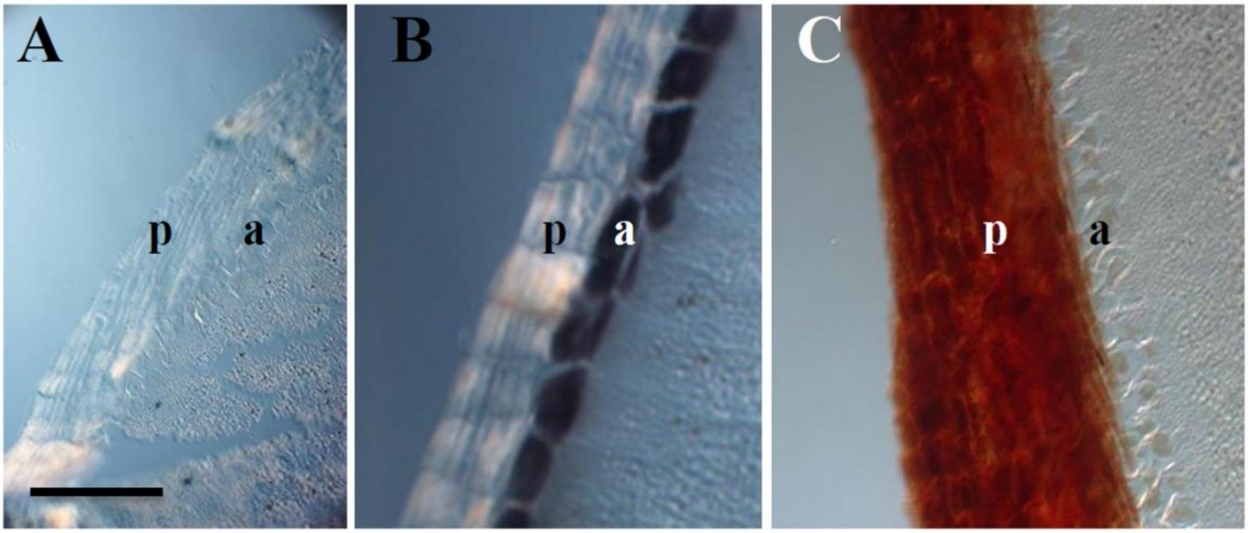


Figure 5. Histological analysis of seeds preserving pigments *in situ*. (A) B73 colourless seed used as control, (B) coloured Millo Corvo seed where the pigments are accumulated in the aleurone layer and (C) "Nero Spinoso" seed where the pigments are accumulated in the pericarp layer. p pericarp layer; a aleurone layer; Bar = 100 µm.

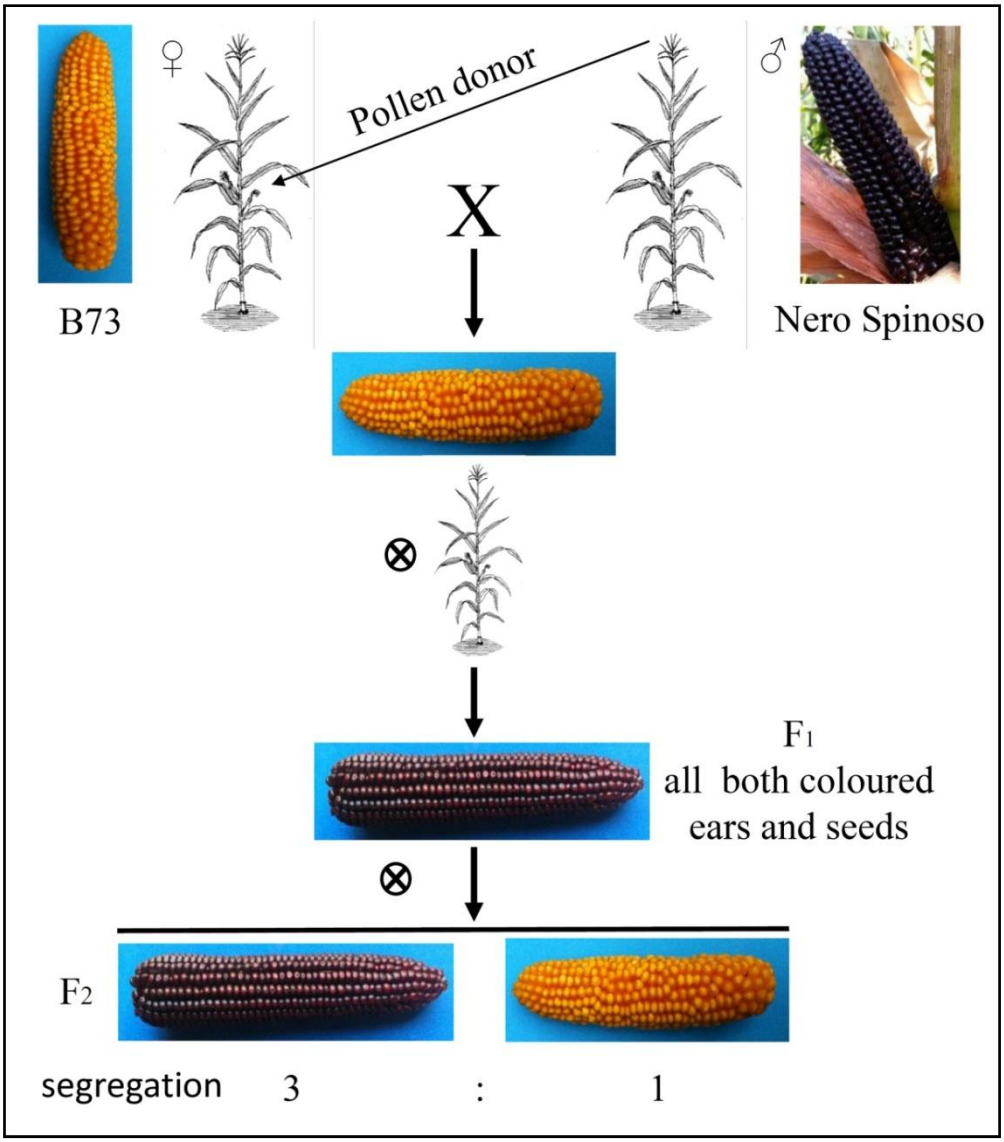


Figure 6. Segregation of the "colored ear" trait observed in the F1 and F2 progenies starting from the cross B73 x "Nero Spinoso" plants. The expected segregation values for "colored ear" was 3:1 in the case of the presence of a single dominant gene driving the pigmentation in the pericarp layer maternal tissue.

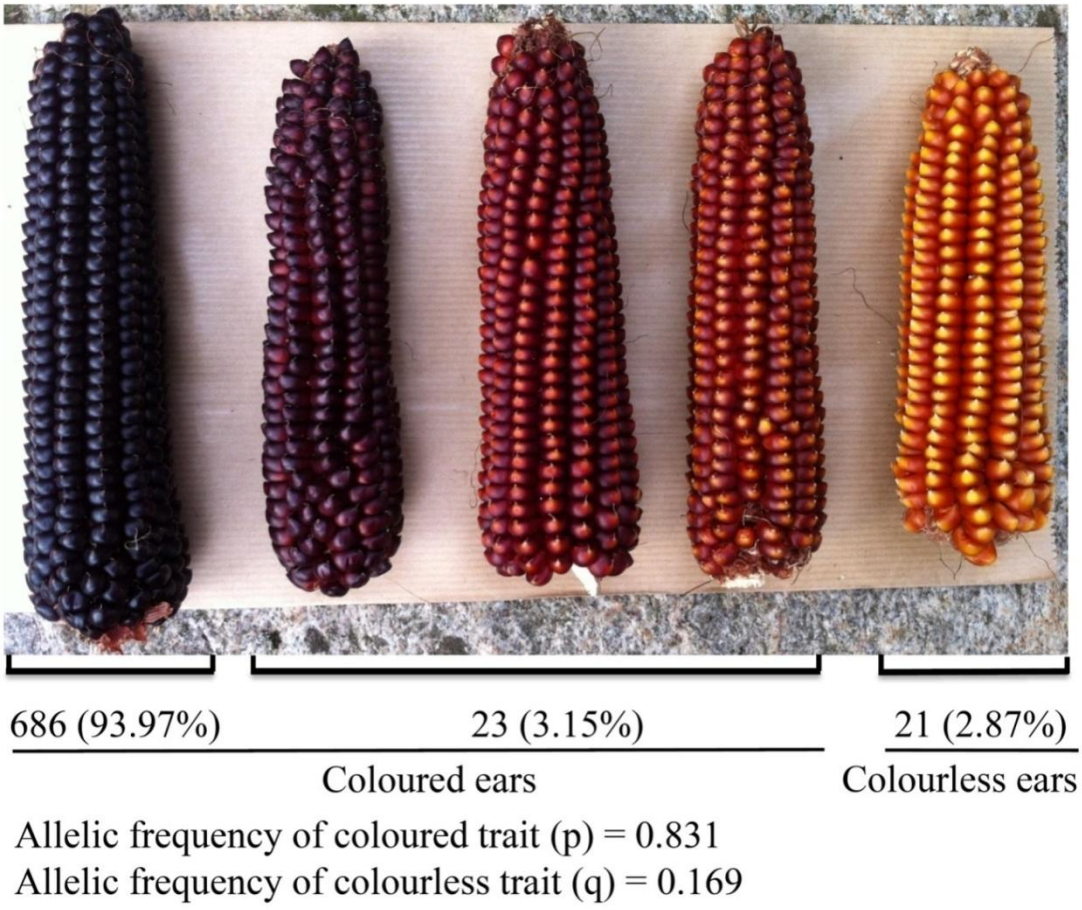
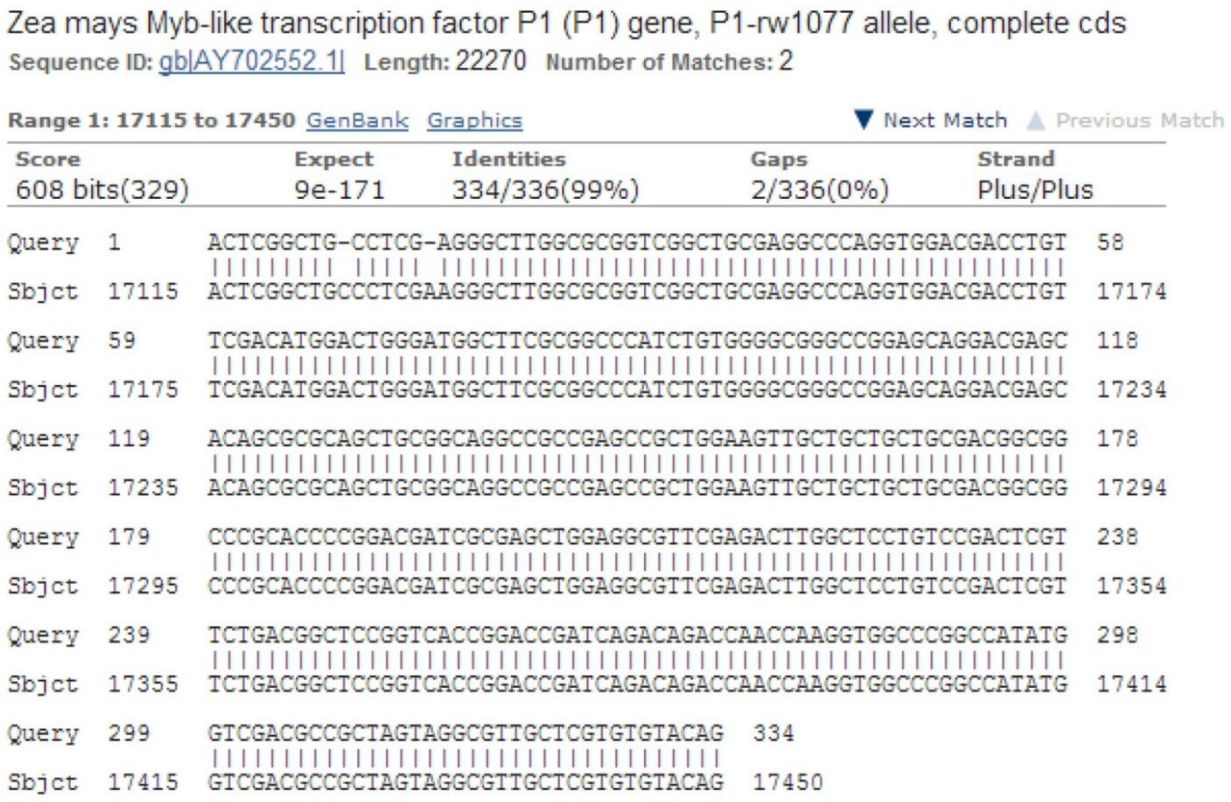


Figure 7. Expressivity and frequency of *P1* allele present in the "Nero Spinoso" opv. Out of 730 ears scored, 686 showed strong pigmentation, 23 showed different red color gradations and 21 appeared completely colorless. Using Hardy-Weimberg principles the allelic frequency of *P1* allele was 0.831 (assuming the presence of *P1* in all the colored individuals) while the allelic frequency of *p1* was 0.169.



Zea mays Myb-like transcription factor P1 (P1) gene, P1-rw1077 allele, complete cds

Sequence ID: gb|AY702552.1| Length: 22270 Number of Matches: 2

Range 1: 17115 to 17450 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
608 bits(329)	9e-171	334/336(99%)	2/336(0%)	Plus/Plus

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Query  1      ACTCGGCTG-CCTCG-AGGGCTTGGCGCGGTCGGCTGCGAGGCCCAGGTGGACGACCTGT  58
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Sbjct  17115  ACTCGGCTGCCCTCGAAGGGCTTGGCGCGGTCGGCTGCGAGGCCCAGGTGGACGACCTGT  17174

Query  59     TCGACATGGACTGGGATGGCTTCGCGGCCCATCTGTGGGGCGGGCCGGAGCAGGACGAGC  118
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Sbjct  17175  TCGACATGGACTGGGATGGCTTCGCGGCCCATCTGTGGGGCGGGCCGGAGCAGGACGAGC  17234

Query  119    ACAGCGCGCAGCTGCGGCAGGCCGCCGAGCCGCTGGAAGTTGCTGCTGCTGCGACGGCGG  178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17235  ACAGCGCGCAGCTGCGGCAGGCCGCCGAGCCGCTGGAAGTTGCTGCTGCTGCGACGGCGG  17294

Query  179    CCCGCACCCCGGACGATCGCGAGCTGGAGGCGTTCGAGACTTGGCTCCTGTCCGACTCGT  238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17295  CCCGCACCCCGGACGATCGCGAGCTGGAGGCGTTCGAGACTTGGCTCCTGTCCGACTCGT  17354

Query  239    TCTGACGGCTCCGGTCACCGGACCGATCAGACAGACCAACCAAGGTGGCCCGGCCATATG  298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17355  TCTGACGGCTCCGGTCACCGGACCGATCAGACAGACCAACCAAGGTGGCCCGGCCATATG  17414

Query  299    GTCGACGCCGCTAGTAGGCGTTGCTCGTGTGTACAG  334
             ||||||||||||||||||||||||||||||||||||
Sbjct  17415  GTCGACGCCGCTAGTAGGCGTTGCTCGTGTGTACAG  17450
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Figure 8. Partial sequencing analysis of *P1* "Nero Spinoso" allele. Alignment obtained by BLASTN program using as query the consensus sequence of 334 nucleotide at the 3' portion of *P1* gene.

Tables

Table 1. Agronomic parameters of "Nero Spinoso" in the 2014 agronomic season. The data were collected from plants grown in different fields located in Camonica valley (BS): Esine (300 m a.s.l.), Largarolo (850 m a.s.l.), Malonno (600 m a.s.l.), Plemo (300 m a.s.l.), Plerio (800 m a.s.l.), Pregasso (350 m a.s.l.), Santicolo (850 m a.s.l.) and Volpera (600 m a.s.l.).

	Esine	Largarolo	Malonno	Plemo	Plerio	Pregasso	Santicolo	Volpera	Average year 2014
Plant height [a] (cm)	294.23±6.47	242.46±10.7	232.45±16.52	274.11±7.63	238.1±10.6	246.42±11.35	214.8±19.64	228.6±14.02	252.41±4.16
Ear height (cm)	127.43±5.23	109.06±6.82	92.75±8	114.97±5.24	88.8±5.67	106.51±4.47	80.8±5.36	105.9±29.44	106.34±2.74
Ear length (cm)	18.87±4.2	19.95±6.65	*n.d.*	19±3.98	17.72±2	17.42±5.71	19.07±1.73	14.77±2.85	18.12±1.02
Ear diameter (cm)	4.2±0.45	4.37±0.5	*n.d.*	4.35±0.23	3.95±0.69	4.32±0.9	4.27±0.48	4.07±0.38	4.22±0.12
Cob diameter (cm)	2.87±0.55	3.02±0.48	*n.d.*	2.9±0.25	3.17±0.87	2.9±0.7	2.9±0.33	3.05±0.24	2.97±0.11
Seed weight [b] (g)	0.262	0.304	*n.d.*	0.305	0.192	0.227	0.321	0.232	0.26±0.05
No. of rows	14±3	13.75±2.31	*n.d.*	14.5±1.84	13.3±3.52	14±2.94	14.75±2.76	13.75±3.14	14.03±0.56

Confidence intervals at 95% are shown, n > 50.

Table 2. Tissues in which pigments are accumulated in "Nero Spinoso" cultivar.

Tissues	Pigmentation
Seedling	+/-
Roots	+
Culm	-
Anthers	-
Silk	-
Husks	+
Cob	+
Seeds	+

The symbol + indicates the presence of pigment and the symbol – its absence.

Table 3. Spectrophotometric quantification of anthocyanins, flavonols, phenolic acids and phlobaphenes in "Nero Spinoso" cultivar. The analyses were conducted four times for each genotype, and the confidence interval at 95% was calculated.

	Anthocyanins (mg/100g)	Flavonols (mg/100g)	Phenolic Acids (mg/100g)	Phlobaphenes (A_{510}/100g)
Nero Spinoso	16.66 ± 2.52	162.13 ± 20.80	108.21 ± 44.06	320.24 ± 104.85
Millo Corvo[a]	83.45 ± 11.44	74.21 ± 17.83	216.63 ± 29.05	2.19 ± 1.78
B73[a]	3 ± 1	66 ± 10	113 ± 0.2	0.8 ± 0.2

[a] Spectrophotometric quantification from Lago et al. (2015).

Table 4. Measurements of pericarp thickness expressed as µm. The confidence interval at 95% was calculated. Mean calculated from >25 measurements.

B73	Millo Corvo	Nero Spinoso
75.5 ± 6.13	59.16 ± 9.88	173.11 ± 12.16

Table 5. Segregation of the trait "coloured ears" in the "Nero Spinoso" cultivar observed in F_2 progeny obtained by selfing F_1 ("Nero Spinoso" x B73) plants. The hypothesis made for the $\chi 2$ test was of 3:1 segregation values for "coloured ears" as expected for a monogenic dominant characters.

Field code	Number of coloured ears	Number of colourless ears	$\chi 2$	P
R4309 selfed	15	3		
R4309 selfed	17	4		
R4230 selfed	12	3		
Total	44	10	1.20	0.8-0.7

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Development and characterization of a coloured sweet corn line as a new functional food.

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Abstract

The standard sugary sweet corn (*Zea mays saccharata* Sturt) is a maize variety grown for the fresh, frozen and canned markets, traditionally appreciated. Its kernels are characterized by the presence of some antioxidant substances suggested to be beneficial for cancer prevention. For this reason an interesting challenge for breeders is the development of sweet corn genotypes with naturally high antioxidant levels, starting from flavonoids. In fact important sources of antioxidants in maize are anthocyanins, considered as nutraceuticals because they have been proven to lower the risk of many chronic diseases.

In this paper we report the development of a new coloured sugary line and the results of some analyses concerning flavonoid content before and after two different cooking treatments are discussed. Attention was mainly focused on the anthocyanins, the molecules suggested as being responsible for the nutraceutical properties of the new coloured sugary line. The results show that the presence of the anthocyanins also pushes up the flavonol and the phenolic acid amounts and gives the new coloured sugary line a higher scavenging power compared to the uncoloured control. The mild cooking seems not to significantly change the metabolites analyzed in the coloured kernels, while the stronger treatment seems to drastically decrease the amounts of pigments, without changing the structure of the leftover molecules. All these findings suggest that the new colored sugary line can be considered a new functional food, able to introduce healthy compounds into the diet of many people.

Introduction

According to the data for the years 2012-2013, the corn global yield overtook 850 million metric tonnes, so that maize can be considered as the most produced cereal in the world (USDA data).

Corn is characterized by a high versatility: it is used for food, forage and for industrial purposes. In USA the amount of corn used as food is about 1.4 billion bushels (35.56 million metric tonnes), to produce high-fructose corn syrup, starch, corn oil and various other food products (Brester, 2012; http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/).

Among the different varieties of maize used for different purposes an important one is sweet corn. Sweet corn (*Zea mays saccharata* Sturt) is a corn type grown for fresh, frozen and canned markets (Bülent Coşkun et al., 2006). In USA the fresh market accounts for nearly 70% of the total production of the sweet corn crop, and it is the second largest processing crop, surpassed only by tomatoes (Hansen R, content specialist, AgMRC, Iowa State University, Sweet corn profile http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/sweet-corn-profile/; Haynes et al, Sweet Corn, Iowa State University Horticulture Guide). It differs from starchy field corn by a single recessive naturally-occurring genetic mutation causing a higher sugar content in the kernels. As a consequence sweet corn is harvested during the milk stage, before physiological maturation, approximately 15 to 23 days after the silks emerge, when it retains the highest amount of sugar and its maximum sweetness (Hansen R, content specialist, AgMRC, Iowa State University, Sweet corn profile http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/sweet-cornprofile/). There are three different mutations resulting in the three most widely diffused genetic varieties of sweet corn: *sugary* (*su*), *sugarenhanced* (*se*), and *shrunken2* (*sh2*): they vary in sweetness, shelf life and cold soil vigour. The most diffused and ancient sweet corn variety is the *sugary*. This variety has a harvest, storage and shelf life slightly shorter than the others, the sugar content is not so high compared to *se* and *sh2*, but it is characterized by a flavour and a texture traditionally appreciated by consumers (Juvik et al., 2003).

The sweet corn kernels are characterized by a high starch and sugar content, important energy sources, by cellulose and ß-glucan which are important dietary fibre for the

enteric flora (Topping and Clifton, 2001; Tokuji et al., 2009) and by the presence of zinc, an essential mineral to assure the functioning of many enzymes and transcription factors (Haase et al., 2008; Tokuji et al., 2009). Some antioxidant substances can also be found in sweet corn kernels, such as the *β*-carotene and the lutein carotenoids (Kurilich and Juvik, 1999; Tokuji et al., 2009) and above all the phenolic compound ferulic acid (Balasubashini et al., 2003; Tokuji et al., 2009). This molecule seems to be very important for health, in fact Tokuji and colleagues collected data indicating that this compound found in dietary sweet corn can be beneficial for cancer prevention (Tokuji et al., 2009).

The antioxidant power seems to be the mechanism through which the molecules carry out their preventive function against human chronic diseases (Virgili and Marino, 2008), and cancer. Therefore vegetable foods containing high levels of antioxidant compounds are now entering the human diet as essential constituents, endowed with the added value of the functional food. So developing sweet corn genotypes with naturally high antioxidant level could be an interesting challenge for breeders. Important sources of antioxidants in maize are the anthocyanins. Anthocyanins are a class of flavonoids: they are water-soluble glycosides of simple or acylated polyhydroxy and polymethoxy derivates of flavylium salts and they are responsible for the red, purple, and blue colours of many fruits, vegetables, and cereal kernels (Giusti and Wrolstad, 2003; Zilić et al., 2012). They are very important for human health because they have been proven in animal system to reduce the risk of death from heart disease (Rissanen et al., 2003; Tsuda., 2012), to be able to lower LDL cholesterol levels (Castilla et al., 2008; Tsuda, 2012) and to fight obesity (Seymour et al., 2009; Titta et al., 2010; Peng et al., 2011; Tsuda, 2012) and diabetes (Prior et al., 2008; Tsuda., 2008; DeFuria et al., 2009; Tsuda, 2012), to improve visual function (Matsumoto et al., 2005; Iwasaki-Kurashige et al., 2006) and to prevent neurodegenerative diseases (Goyarzu et al., 2004; Lau et al., 2007; Shukitt-Hale et al., 2008; Tsuda, 2012).

Corn (*Zea mays* L) presents around 20 structural and regulatory genes that compose the anthocyanin biosynthetic pathway (Chandler et al., 1989; Dooner et al., 1991; Pilu et al., 2003). The regulatory genes concerned belong to two different multigene families: the class of *bHLH* transcription factors among which are the *r1*/*b1* genes, and the class of MYB transcription factors, among which are the *c1*/*pl1*/*p1* genes (Chandler et al., 1989; Dooner et al., 1991; Pilu et al., 2003). A member of each family must be present and active in the dominant form to activate anthocyanin structural genes expression.

According to the combination of these alleles, the pigments will be synthesized in different plant tissues, for example the *B*/*Pl* genes combination confers purple colour to the pericarp (Chandler et al.,1989; Bodeau and Walbot, 1992; Gaut, 2001; Pilu et al., 2003).

In this paper we describe how a new coloured sugary line has been developed and, together with an uncoloured control, was subjected to three different food processing treatments: raw, steam cooked and autoclaved. Some analyses concerning the quantitative and qualitative characterization of the main flavonoid molecules in the uncoloured and coloured samples are presented and the results obtained after the different cooking treatments are discussed. Attention has been focused on the anthocyanins, the molecules that are supposed to be responsible for the nutraceutical properties of the new coloured sugary line, which is therefore proposed as a new functional food.

Materials and methods

Plant material

A backcrossing breeding scheme was used to develop a sugary maize line rich in anthocyanins, in the experimental field of the University of Milan located in Landriano (PV, Italy). The source of the anthocyanin biosynthesis regulatory genes was a tropical maize line carrying the homozygous form of the *Booster1* (*B1*) and *Purple Plant1* (*Pl1*) genes, that determine the pigmentation in the pericarp and in the plant. This line was crossed with a commercial yellow sugary line, used as the recurrent parent for 5 cycles of backcrossing. Then 3 cycles of self pollination were performed, selecting in each cycle, the plants with the highest content of anthocyanins by Marker Assisted Selection (MAS).

Molecular Marker assay

Two SSR molecular markers were used to select the coloured sugary plants: the *nc009* SSR molecular marker (5'CGAAAGTCGATCGAGAGACC3'/5'CCTCTCTTCACCCCTTCCTT3'), that is part of the *pl1* gene located on chromosome 6 and the *bnlg1064* SSR molecular marker (5'CTGGTCCGAGATGATGGC3'/5'TCCATTTCTGCATCTGCAAC3') located next to the *b1* gene on the short arm of chromosome 2 (http://www.maizeg db.org/ssr.php).

After the DNA extraction from the leaves of parental (P1 and P2) and progenies' plants (Dellaporta et al., 1983), the Polymerase Chain Reactions (PCR) and gel running were performed as described in the SSR Methods Manual by MaizeGDB (http://www.maizegdb.org/documentation/maizemap/ssr_protocols.php).

Material sampling

For the genotypes tested (sugary maize line rich in anthocyanins and his colourless control) in the 2012 field season about 300 plants were grown in adjacent rows, under the same agronomic conditions, in the experimental field of the University of Milan, Italy (45°18' N, 9°15' E). These plants were selfed (using paper bags) and then harvested at the same time at the end of the season.

About 50 ears were shelled and the seeds obtained mixed to create a single bulk used for the analyses.

Seed treatments

With the aim to mimic the processing treatments used for the sweet corn commercially available, we decided to test the uncoloured control seeds and the new coloured ones raw and after 2 different cooking treatments (100 seeds each). The steam cooking method involved a mild cooking of 10 minutes during which no contact between the seeds and the boiling water occurred. Other seeds underwent an autoclave cycle, consisting of 20 minutes of a constant pressure of 1 atm and a constant temperature of 120 °C. After these treatments the seeds were analysed as described below.

Metabolite quantification (Anthocyanins, flavonols and phenolic acids quantification)

A pool of 10 seeds per treatment (raw, steam cooked and autoclaved) and per line was used to extract flavonoid metabolites. The seeds were ground in a mortar with the extraction buffer (1% HCl, 95% ethanol) in the presence of quartz sand. A sequence of consecutive washing steps of 30 minutes were performed until the extraction buffer turned out to be transparent. Finally the collected supernatants underwent a centrifugation at 13,000 rpm for 30 minutes, and then were used to determine

anthocyanins using a spectrophotometer at λ = 530 nm, flavonols at λ=350 nm and phenolic acids at λ = 280 nm.

The amount of anthocyanins was calculated as cyanidin 3-glucoside equivalents (molar extinction coefficient (ε) 26,900 L m^{-1} mol^{-1}, MW 449.2), flavonoids as quercitin 3-glucoside equivalents (molar extinction coefficient (ε) 21,877 L m^{-1} mol^{-1}, MW 464.38) and phenolic compounds as ferulic acid equivalents (molar extinction coefficient (ε) 14,700 L m^{-1} mol^{-1}, MW 194.18) for 100 g of seed weight.

The analyses were conducted on four seeds bulk (10 seeds each) randomly selected for each type. The confidence interval (C.I.) at 95% was calculated.

Qualitative determination of anthocyanins: TLC

The pericarp layers of 2 kernels per treatment of coloured and uncoloured lines were excised and boiled at 100°C with 2 mL of 2N HCl for 40 minutes.

After adding 1 mL of isoamyl alcohol, the upper phase was dried and dissolved in EtOH 95% and HCl 1%. The standards of cyanidin, pelargonidin and delphinidin were loaded on a pre-coated TLC (Thin Layer Chromatography) plate (POLYGRAM CEL 300, Macherey-Nagel) together with the samples to be tested. The solvent used for the TLC running was formic acid:HCl:water 5:2:3. The developed plates were photographed with a digital camera (A430 Canon) using both white and UV illumination.

Antiradical ability assay

The free radical-scavenging activity was tested using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Brand-Williams et al., 1995; Leong and Shui, 2002; Cevallos-Casals and Cisneros-Zevallos, 2003; Hu et al., 2004; Yang and Zhai, 2010). Five coloured sugary seeds per each treatment were excised from the pericarp; the same procedure was also followed for the uncoloured untreated seeds. The pool of 5 pericarps for each treatment was ground with liquid nitrogen.

An adequate aliquot was extracted with acetone 70% (acetone:water 70:30 v/v) according to the ratio 1:8 (w/v) for 3 hours. Then the samples were centrifuged for 10 minutes at 13,000 rpm and the colored extracts were equalized with a dilution based on the anthocyanin content of each treatment.

Then a 0.12 mM DPPH ethanolic solution was added to increasing aliquots of each sample extract, conveniently diluted. The final volumes of 2.5 mL of these preparations were

left 1 hour in the dark at room temperature before the discoloration absorbance was spectrophotometrically recorded at 516 nm. The percentage of the scavenged DPPH was calculated as: % DPPH = (Ac−As) × 100 / Ac where Ac is the absorbance of the control, and As is the absorbance of each increasing aliquot of thesample (Leong and Shui, 2002; Hu et al., 2004; Yang and Zhai, 2010). Finally the amounts of the increasing aliquots of each extract were interpolated with the corresponding DPPH scavenged percentage, tracing the reported curve.

Panel test

To test the acceptability of the new coloured sugary line, 12 blinded subjects were randomly recruited and asked to taste 4 kernels of both the uncoloured and coloured line. No cooking treatment, nor salt nor dressing were added to the kernels. Each subject expressed his judgment about his appreciation according to a scale from 1, the worst, to 10, the best. The mean, the median and the mode of the judgments relative to the two different kinds of kernels were calculated.

Results

Development of a coloured sugary line

Sugary corn is a well-established product in the market and a very popular ingredient in the diet, especially in the USA. Some reports showed that dietary consumption of sweet corn seems to be able to inhibit tumour growth in mice (Tokuji et al., 2009), probably because of the presence of phenolic compounds, particularly ferulic acid (Tokuji et al., 2009). The ability of some molecules to prevent several chronic diseases such as cancer is supposed to originate from their antioxidant potential (Virgili and Marino, 2008).

In maize the antioxidant potential could be increased thanks to its capacity to accumulate anthocyanins in the kernels. In fact anthocyanins are antioxidant molecules whose regular consumption is associated with a high number of health benefits.

Therefore improving sweet corn by increasing the anthocyanins content could lead it to being considered as a functional food.

For this purpose a recurrent breeding scheme was planned (Fig. 1A). A tropical black corn plant bearing the *Pl* and *B* regulatory genes, required to activate the anthocyanin accumulation in the seed pericarp, was used as source of the genes for the pigment

biosynthesis, while a commercial sugary yellow line was used as the recurrent parent (Fig. 1A). The selection of the heterozygous plants used for crossing and the homozygous plants during the self-pollination cycles was based on the use of 2 molecular markers, *nc009* and *bnlg1064*, polymorphic for the *Pl* and *B* genes between the uncoloured and coloured parents of the cross (Fig. 2). This breeding scheme allowed us to obtain a sugary plant with a pigmented ear, harvested 21 days after pollination (DAP) (Figure 1B). Simultaneously, in the same field in Landriano (PV, Italy), the yellow commercial sugary line was grown and harvested 22 DAP to be used as the control (Fig. 1B).

The breeding scheme provided good results: both the fresh and dry mean seed weights did not show significant differences on comparing coloured with non-coloured raw seeds (Fig. S1).

The coloured and the uncoloured sweet corn lines are near-isogenic lines, and as a consequence a near-isogenic food, that differs only in the content of specific phytonutrients and thus appears to be an useful tool to reduce the complexity of the studies about the diet-health relationship (Martin et al., 2011).

Then for both the coloured and uncoloured sugary lines the seed anthocyanin, flavonol and phenolic acid compounds were spectrophotometrically quantified (Table 1).

The introgression of the colour genes allowed us to obtain a red sugary line able to accumulate 118.92 ± 14.97 mg 100 g^{-1} of anthocyanins in the fresh kernels (Table 1), while no pigment was detected in control sugary kernels (Table 1). This appeared to be a good amount in comparison with berries that accumulate 25 to 698 mg 100 g^{-1} (Mazza and Miniati, 1993; Wang and Lin, 2000; Wu et al., 2006; Koponen et al., 2007), black rice, that accumulates 10 - 493 mg 100 g^{-1} (Ryu et al., 1998) and coloured popcorn, that accumulates around 36 - 66.44 mg 100 g^{-1} (Lago et al., 2013).

Also for each of the other metabolite classes, the red line showed a significantly higher value compared to the yellow line: 81.04 mg 100 g^{-1} vs 31.23 mg 100 g^{-1} of flavonols and 121.67 mg 100 g^{-1} vs 52.49 mg 100 g^{-1} of phenolic acids. In addition to anthocyanins, sweet corn is also able to synthesize phenolics compounds, particularly ferulic acid (Balasubashini et al., 2003; Tokuji et al., 2009). So we quantified the amount of phenolic acids and flavonols in both coloured and uncoloured sweet corn lines. The results showed a significantly higher amount of both in the new coloured sugary line, than in the control uncoloured one (Table 1): 81.04 mg 100 g^{-1} vs 31.23 mg 100 g^{-1} of flavonols and 121.67 mg 100 g^{-1} vs 52.49 mg 100 g^{-1} of phenolic acids.

This could be expected because these classes of molecules share the first part of the biosynthetic pathway with anthocyanins, so that the active alleles of the anthocyanin regulatory genes could have pushed up the quantities of all the structural genes of the flavonoids biosynthesis. Therefore the presence of the anthocyanin pigments in the new coloured sugary line is a nodal point because they also seem to boost the amounts of other flavonoids and health-promoting compounds too: the anthocyanin presence makes the new sugary coloured line a good candidate as an everyday functional food in the diet of many people.

Effects of the cooking treatments on anthocyanins, flavonols and phenolic acids content

The steam treatment caused a small decrease in the anthocyanins content of the new coloured line: the 118.92 mg 100 g^{-1} amount in the fresh seeds fell to 96.82 mg 100 g^{-1} (Table 1). The effect of the autoclave cycle was dramatic, destroying a large part of the anthocyanins, which reached the final amount of 19.6 mg 100 g^{-1} (Table 1). Strikingly, neither the flavonols nor the phenolic acids were degraded by the steaming procedure, on the contrary this treatment caused a significant increase in the flavonol amounts (Table 1). This increase seems to be higher for the coloured seeds (81.04 mg/100 g before the treatment and 115.28 mg/100 g after) than for the uncoloured ones (31.32 mg/100 g before the treatment and 39.51 mg/100 g after). The same pattern was found for the phenolic acids, with the red line scoring 81.043 mg/100 g before and 156.66 mg/100 g after the treatment and the yellow line 31.23 mg/100 g before and 64.07 mg/100g after the steam treatment (Table 1).

The autoclave cycle led to a marked decrease in flavonols and phenolic acids content in the red line, while this decrease was less evident in the yellow line (Table 1).

Qualitative analysis of anthocyanins

To understand whether the cooking treatments modified the chemical structure of the leftover anthocyanins, a TLC was performed comparing the extracts of raw and treated seeds uncoloured and coloured. The plate in Fig. 3A shows the spots corresponding to the 3 standards delphinidin, cyanidin and pelargonidin (lanes 1-3), then the 3 coloured samples -raw, steam cooked and autoclaved- (lanes 4-6) and finally the 3 uncoloured samples (lanes 7-9).

Cyanidin was the most abundant anthocyanin in the 3 coloured samples, while no spots corresponding to the standards were identified in the uncoloured samples. The extract obtained from the raw seeds (lane 4), also revealed the presence of pelargonidin, less abundant than cyanidin, and another spot, not identified by the standards. The same pattern even if less intense, was shown by the coloured steam cooked sample (lane 5). The spots relative to the coloured autoclaved sample were very weak (lane 6), so that only the cyanidin was visible. The UV picture of the TLC plate revealed another unidentified spot (Fig. 3B), not detected in visible light, and present in both the coloured autoclaved sample (lane 6) and the uncoloured untreated sample (lane 7). This spot was also present, even if weaker, in the uncoloured steam cooked and autoclaved samples too (lanes 8-9).

DPPH Scavenging ability

The diagram representing the DPPH scavenging ability clearly showed that the new coloured sugary line has a much higher antioxidant activity compared to the uncoloured sample (Fig. 4A). After the equalization of the extracts among the three different treatments, through suitable dilutions based on the anthocyanins content, the curves of the raw and the steam cooked coloured samples were characterized by a similar tendency, while the autoclaved coloured extract showed a lower radical scavenging ability (Fig. 4B).

Panel test - consumer test

Twelve blinded subjects, randomly chosen, were asked to express a judgment about the acceptability of the new coloured sugary corn and of the respective control (Table 2). Both lines were tested without cooking, salt and dressing. The acceptability mean scores were 6.75 for both lines, attesting no significant differences between the acceptability for taste alone of the traditionally uncoloured and the new coloured sugary products (Table 2).

Discussion

Sugary corn is a well-established product in the market and a very popular ingredient in the diet especially in the USA. Some reports showed that dietary consumption of sweet corn seems to be able to inhibit tumour growth in mice (Tokuji et al., 2009), probably because of the presence of phenolic compounds, particularly ferulic acid (Tokuji et al., 2009). The ability of some molecules to prevent several chronic diseases such as cancer is supposed to originate from their antioxidant potential (Virgili and Marino, 2008).

In maize the antioxidant potential could be increased thanks to its capacity to accumulate anthocyanins in the kernels. In fact anthocyanins are antioxidant molecules whose regular consumption is associated with a high number of health benefits.

Therefore improving sweet corn by increasing the anthocyanins content could lead it to being considered as a functional food.

For this purpose a recurrent breeding scheme was planned (Fig. 1A). A tropical black corn plant bearing the *Pl* and *B* regulatory genes, required to activate the anthocyanin accumulation in the seed pericarp, was used as source of the genes for the pigment biosynthesis, while a commercial sugary yellow line was used as the recurrent parent (Fig. 1A). The selection procedure was based on the use of 2 molecular markers, *nc009* and *bnlg1064,* polymorphic for the *Pl* and *B* genes between the parents of the cross (Fig. 2). The result of this breeding scheme is a coloured sugary plant, characterized by the genetic background of the commercial uncoloured sugary line with the exception of the presence of the anthocyanin regulatory genes in the dominant form (Fig. 1B). The new coloured sugary line was then analysed using the uncoloured commercial sugary isogenic line as control.

Being the coloured and the uncoloured sweet corn lines near-isogenic, they represent near-isogenic foods, differing only in the content of specific phytonutrients and thus appears to be an useful tool to reduce the complexity of the studies about the diet-health relationship (Martin et al., 2011).

Sweet corn is harvested before the time of field maize physiological maturity, at about 20-21 DAP The fresh and dry seed weight did not show significant differences between the two isogenic lines (Fig. S1), attesting to the good result coming from the breeding work. The introgression of the colour genes allowed us to obtain a red sugary line able

to accumulate 118.92 ± 14.97 mg/100g of anthocyanins in the fresh kernels (Table 1), while no pigment was detected in control sugary kernels (Table 1).

This appeared to be a good amount in comparison with berries that accumulate 25 to 698 mg/100g (Mazza and Miniati, 1993; Wang and Lin, 2000; Wu et al., 2006; Koponen et al., 2007), black rice, that accumulates 10 - 493 mg/100g (Ryu et al., 1998) and coloured popcorn, that accumulates around 36 - 66.44 mg/100g (Lago et al., 2013). In addition to anthocyanins, sweet corn is also able to synthesize phenolics compounds, particularly ferulic acid (Balasubashini et al., 2003; Tokuji et al., 2009). Ferulic acid is synthesized starting from phenylalanine following the phenylpropanoids pathway.

Therefore phenolic acids share a part of the biosynthetic way with anthocyanins and with flavonols, the most abundant group of flavonoids among plants, proven to have many human health beneficial effects (Formica and Regelson, 1995; Duthie et al., 2000). So we quantified the amount of phenolic acids and flavonols in both coloured and uncoloured sweet corn lines. The results showed a significantly higher amount of both in the new coloured sugary line, than in the uncoloured control one (Table 1). This could be expected because these classes of molecules share the first part of the biosynthetic pathway with the anthocyanin pathway, so that the active alleles of the anthocyanin regulatory genes could have pushed up the quantities of all the structural genes of the flavonoids biosynthesis. Therefore the presence of the anthocyanin pigments in the new coloured sugary line is a nodal point because they also seem to boost the amounts of other flavonoids and health-promoting compounds too: the anthocyanin presence makes the new sugary coloured line a good candidate as an everyday functional food in the diet of many people. The DPPH scavenging ability test seems to strengthen this hypothesis (Fig. 4A): the raw uncoloured commercial sugary seed extract showed a much lower antioxidant ability compared to the raw coloured one, attesting the anthocyanins' remarkable power (Fig. 4A). This is in agreement with previously reported data about a coloured popcorn line (Lago et al., 2013), consequently the coloured sweet corn can be considered a new functional food. Although while part of the sweet corn crop is consumed as fresh grains or fresh ears, most of it is consumed as processed canned sweet corn (Dewanto et al., 2002). Some of the thermal procedures required for sweet corn processing are known to lower the nutritional level of grains and vegetables in comparison with the fresh ones (Lathrop et al., 1980; Rao et al., 1981; Burge et al., 1995; Murcia et al., 2000; Dewanto et al., 2002). Therefore it is important to understand

the effect of sweet corn processing on the anthocyanin molecules, at both quantitative and qualitative levels.

Big companies, *e.g.* Bonduelle and Conserve Italia in Italy, and Allens in USA, studied the best methods for thermal processing and conservation of canned food: first of all small amounts of salt water and sugars are added to the kernels inside the can, where the vacuum is imposed. Then the product underwent a steam cooking, but the presence of the vacuum allows a lowering and shortening of the heating procedure, so that the kernels are subjected only to a sterilization and not to a proper cooking. As a consequence the vegetable can keep its flavour and its nutritional properties. The correct balance between vacuum and temperature is often held as a trade secret by the companies (http://www.bonduelle.it/lacottura-al-vapore-secondo-bonduelle/ accessed 26 august 2013). For this reason we decided to subject the two sweet corn lines to different cooking processes: a mild cooking with steam and a severe one with the autoclave. The steam cooking treatment seems to only slightly decrease the anthocyanins amount (Table 1), as already found by Vallejo et al. (Vallejo et al., 2003). The autoclave cooking on the other hand resulted in a more dramatic effect causing the reduction of the anthocyanins level by about 83% in comparison with the untreated kernels. This result was in agreement with previous data reporting that the stability of anthocyanins in cooked foods is dependent on the temperature and on the heating time of the thermal process (Cabrita et al., 2000; Abdel-Aal et al., 2003; Hiemori et al., 2009). The big difference in the degrading ability of the cooking processes used could be explained by the fact that the steam cooking was not only milder but also shorter than the autoclave treatment so that it was able only to inactivate some oxidative enzymes and not to destroy the pigments that are present in the edible part of the vegetable (Howard et al., 1994; Vallejo et al., 2003).

Moreover steam cooking seems to increase flavonols and phenolic acids in both the coloured (+42.25% and +28.76%, respectively) and the uncoloured line (+26.51% and +22.06%, respectively) (Table 1).

The autoclave cooking caused an increase of 9.75% for the flavonols and 7.44% for the phenolic acids in the coloured kernels and of 94.46% and of 71.40% respectively in the uncoloured ones (Table 1). This was in agreement with the results of Dewanto et al. (Dewanto et al., 2002) who found that the free phenolic portion in their sweet corn significantly increased after the thermal process. This can be explained by the fact that

the heating, causing the breakdown of the cellular constituents allowed the release of the bound phenolic acids portion (Dewanto et al., 2002).

At this point it was important to understand whether the cooking was able to change the structure of the pigments and consequently the antioxidant ability of the leftover anthocyanins, not degraded by the heating. With this purpose the DPPH assay was also performed on the extracts coming from the raw, the steam cooked and from the autoclaved coloured kernels, equalized through proper dilutions on the basis of the anthocyanin amount. Anthocyanin amounts being equal among them, the raw and steamed kernels showed the same scavenging ability (Fig. 4B), attesting that no structural changes occurred in the leftover pigment molecules after the steam cooking. On the contrary the extract obtained from the autoclaved kernels had a much lower antioxidant power (Fig. 4B). This could be caused by the strong treatment of the autoclave, in contrast to the lighter one of the steam treatment: probably one hour of heating coupled with the high pressure was able to degradate not only the anthocyanin molecules but also some other antioxidant compounds, such as for example vitamin C (Burge and Fraile, 1995; Murcia et al., 2000; Dewanto et al., 2002) or *β*-carotene and lutein carotenoids (Kurilich and Juvik 1999; Tokuji et al., 2009).

To confirm that no structural or chemical changes in anthocyanin molecules occurred after the thermal processes, Thin Layer Chromatography was performed (Fig. 3). The spots of the coloured samples clearly showed that the anthocyanin aglycons did not change their structure following cooking, only their amount decreased (Fig. 3A). We noticed the presence of a little spot above the pelargonidin one (Fig. 3A): it is not present in other *B*/*Pl* maize genotypes, such as the coloured popcorn (Lago et al., 2013). This could be explained by the fact that sweet corn is a fresh product, composed by developing kernels that are still accumulating pigments in the pericarp; therefore the metabolite profile is not definitive as in the dry maize kernels. Deeper and more precise analyses are needed to finely characterize the metabolite profile of this coloured line. In the meantime the acceptability of the new product in comparison to the uncoloured one was tested on 12 blinded subjects, randomly chosen (Table 2). The kernels were tested with no cooking, no salt and no dressing in order to level the taste. The appreciation scores did not show significant differences between the uncoloured and coloured sugary kernels (Table 2), suggesting that the healthier properties due to the pigment presence could persuade the consumer to prefer the coloured sweet corn to the uncoloured one.

Conclusions

This study suggested that the new coloured sugary line is a good source of anthocyanins, of other beneficial flavonoids and of antioxidant potential, and thus it can be considered a good functional food. Our results also suggest that to preserve the healthy properties of the coloured sweet corn it is better to consume it fresh but if processing is needed it would be better to use a mild process, such as the steam treatment, in order to benefit from the best nutritional composition. It could be likely that consumers will choose this new product for its healthy properties given that no differences in appreciability were scored.

Acknowledgements

We wish to thank Dr Davide Reginelli for his hard work in the field and Dr Lesley Currah for her precious suggestions.

Figures

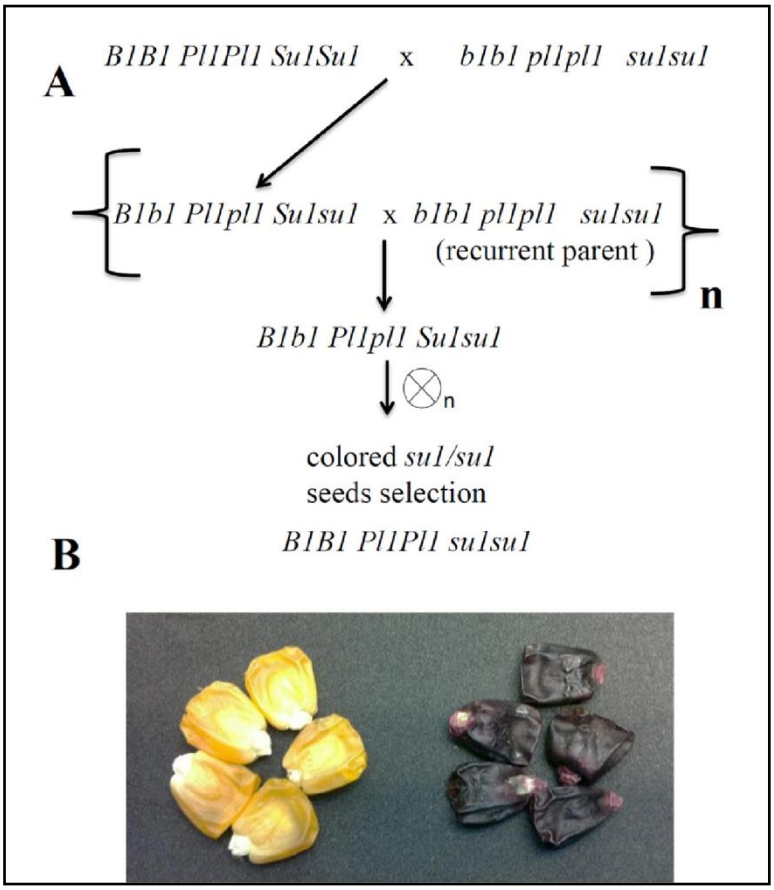


Figure 1. Recurrent Selection Scheme: the cross between the *B1Pl1* line, source of the regulatory biosynthetic genes and the commercial uncoloured sugary corn gave rise to heterozygous plants for the *B*/*Pl* genes. Among them, the highest anthocyanin content plants were selected for the backcrossing with the recurrent parent. Then the best plants underwent some cycles of self-pollination (A). Phenotype of uncoloured (left) and coloured (right) sweet corn kernels (B).

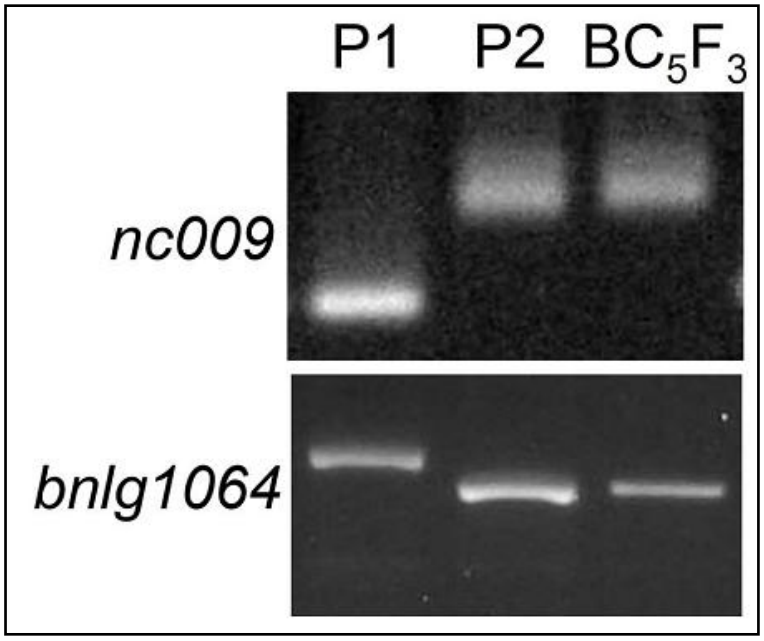


Figure 2. Marked Assisted Selection: the *nc009* SSR, part of the *pl1* gene and the *bnlg1064* SSR, next to the *b1* gene, was found to be polymorphic between the coloured and the colourless parents. The heterozygous individuals were easily detected and selected to carry on the breeding selection scheme. P1 colourless sugary corn line; P2 *B1Pl1* line; BC_5F_3 coloured sugary corn line developed.

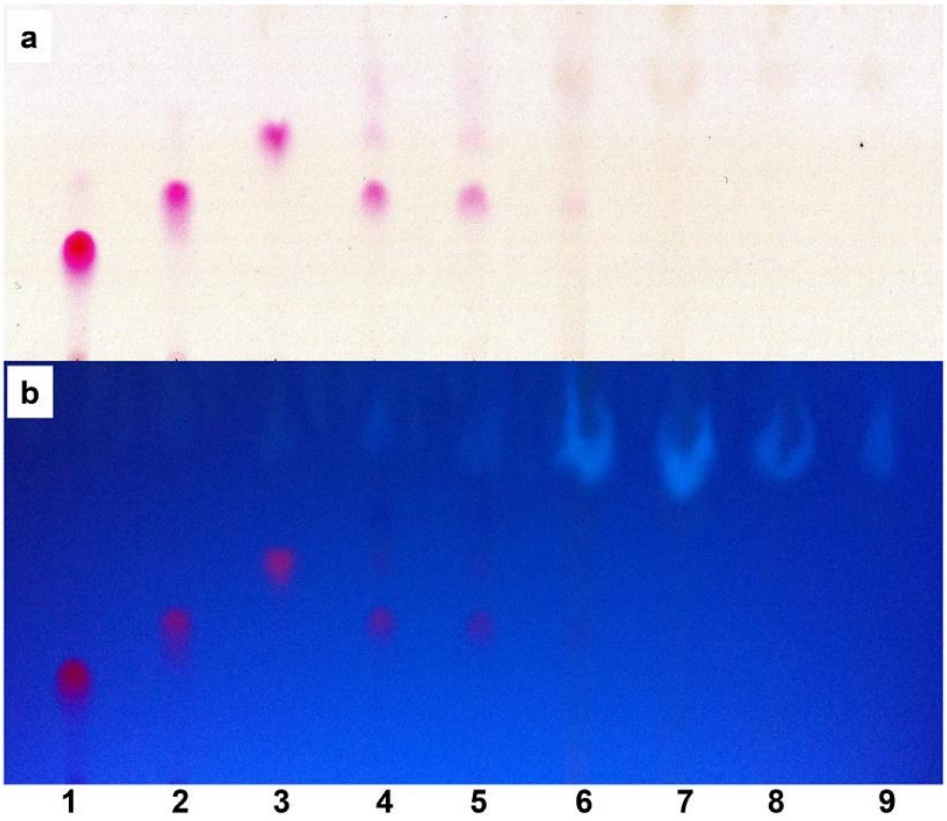


Figure 3. Pictures of the TLC plate taken under visible (a) or UV (b) light. The spots represent: from lane 1 to 3, the delphinidin, cyanidin and pelargonidin standards, from lane 4 to 6 the anthocyanin extracts coming from the coloured raw, steamed cooked and autoclaved kernels, while the respective uncoloured controls are represented in lanes 7 to 9.

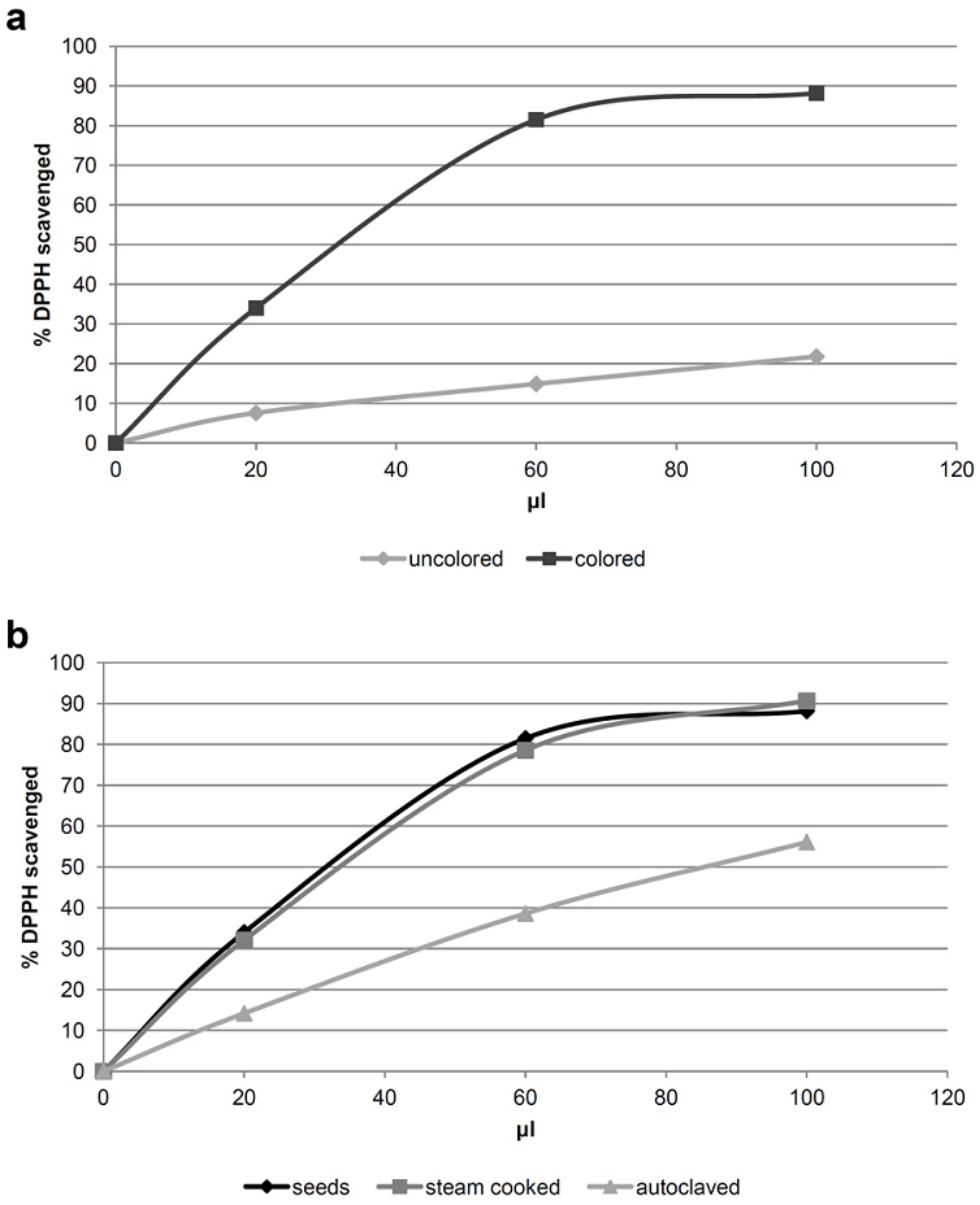


Figure 4. Comparison of the antioxidant ability in the DPPH radical scavenging assay of the coloured raw vs the uncoloured raw seed extracts (a) and of the raw vs steam cooked vs autoclaved coloured seed extracts (b), equalized and diluted according to the anthocyanins concentration.

Tables

Table 1. Spectrophotometric quantification of anthocyanins, flavonols and phenolic acids content

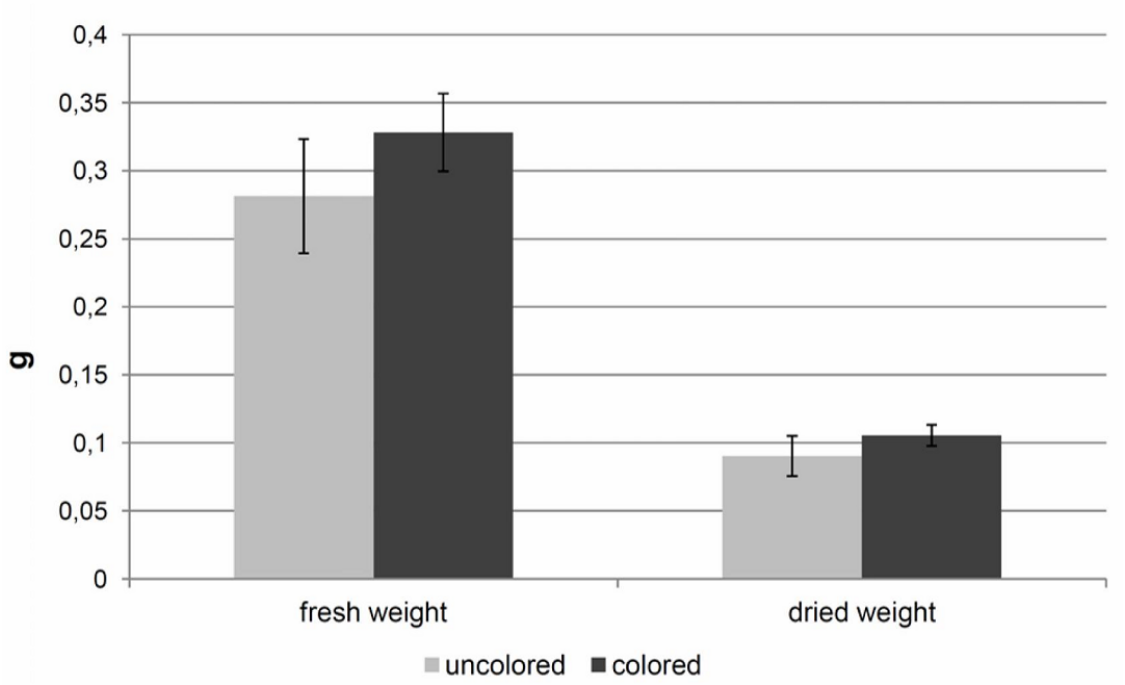
		raw (mg $100g^{-1}$)	Steam cooked (mg $100g^{-1}$)	autoclaved (mg $100g^{-1}$)
anthocyanins[a]	uncoloured	0.23±0.24	0.57±0.70	0.22±0.15
	coloured	118.92±14.97	96.82±2.21	19.6±1.75
flavonols[b]	uncoloured	31.23±5.24	39.51±4.8	60.73±9.25
	coloured	81.04±14.54	115.28±2.61	88.94±9.11
phenolic acids[c]	uncoloured	52.49±5.68	64.07±2.68	89.97±2.63
	coloured	121.67±25.67	156.66±3.34	130.72±3.97

[a] quantified as cyanidin-3-glucoside equivalents; [b] quantified as quercitin 3-glucoside equivalents; [c] ferulic acid equivalents. The confidence intervals at 95% are shown.

Table 2. Comparison of the panel test scores between the new coloured sugary kernels and the commercial uncoloured one

	acceptability degree	
	uncoloured	coloured
mean	6.75	6.75
mode	7	7
median	7	7

Supporting information



SI1. Comparison of the mean seed weight of the fresh and dried kernels of the uncoloured control line with the new coloured sugary line

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Nutritional and phenotypical characterization of two South African maize (*Zea mays* L.) varieties sampled in the Qwa-Qwa region

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Abstract

Zea mays L. represents one of the main source of energy in the diet in many African countries, especially in the sub-Saharan regions. White maize varieties, characterized by the lack of carotenoids, are usually widely preferred in Africa for human consumption, and this contributes to the occurrence of vitamin A deficiency; yellow varieties, often derived from commercial hybrids, are usually destined for animal feeding. In this study we characterized from the phenotypical and nutritional points of view one white and one yellow South African landrace maize cultivar obtained directly from the farmers in the rural region of Qwa-Qwa (Free State Province). Calorific value, oil, protein, starch, minerals, flavonoids and carotenoids content were determined, together with free and phytic P. Both the varieties showed low protein and Fe content in comparison to the ones used as control, and the yellow one also had a low content of Zn. The white variety was characterized by a higher free P content but also by a very low level of carotenoids. Our data show that there are no nutritional reasons to prefer the white variety for human consumption, with the exception of the large size of the seeds, which make them particularly adapted for milling; hence the nutritional value of these varieties, and in particular of the white one, should be improved (protein, Fe and carotenoids), contributing in this way to tackle the problem of malnutrition in South African rural areas.

Introduction

Maize is diffused all over the world where temperatures unable its cultivation. In Africa, 16 of the 22 countries where corn represents the main source of energy in the diet are located (Dowswell et al., 1996; Nuss and Tanumihardjo, 2011).

Maize consumption in the local cuisine is comparable to that of rice in Asia. Its flour is used to produce beverages and porridges (Gouse et al., 2006; Nuss and Tanumihardjo, 2011). In South Africa, where pap (white maize meal porridge) (Fig. 1C) is a staple food for a great part of the population (Oldewage-Theron et al., 2005), corn represents the 30% of the daily energy and protein intake (Doria et al., 2015).

Denutrition and micronutrient malnutrition or deficiency are still relevant public health problems in South Africa (Vorster et al., 1997; Steyn et al., 2006; Acham et al., 2012): more than the 20% of the local population is affected by stunting/underweight (Doria et al., 2015). Iron and zinc intakes are particularly low (Oelofse et al., 2002). More than the 10% of the population is affected by iron and vitamin A deficiency (Doria et al., 2015). Vitamin A deficiency can cause anemia and blindness, reduces resistance to infections and increases the risk of death (Gannon et al., 2014). Zinc intake is inadequate for 45.3% of South African children between 1 and 9 years of age (Samuel et al., 2010). Zinc represents a key component in enzymes which are crucial for metabolism and body functions and is also an anti-inflammatory and antioxidant agent working in cell-mediated immune processes (Prasad, 2007); its deficiency in children can cause adverse effects on both physical growth and cognitive development (Black, 1998; Brown et al., 2001; Gibson, 2006).

Thanks to its wide diffusion, maize can greatly help to improve nutrition in several countries, considering its important role in the diet of many people.

Maize seeds are characterized by a high starch content (about 75-80% of their weight), they contain protein (10-15%) (even though the content of essential aminoacids tryptophan and lysine is low) and lipids (5%) (Panzeri et al., 2011), and they also represent a source of micronutrients and macronutrients (*e.g.* Na, Mg, P, K, Ca, Fe, Zn). Phosphorus availability is a relevant issue for seeds' nutritional value, in fact it is present in the kernel in three fractions: free P, phytic P (as a component of the phytate salts) and cellular P (bound to other cellular compounds).

Phytic acid is accumulated mainly in the scutellum (O'Dell et al., 1972; Raboy, 1990), and is the main form of phosphate present in the seed, representing about 50-80% of the total amount of phosphorus (Doria et al., 2015), as a mixture of phytate salts of several cations, such as potassium, iron, zinc, magnesium (Raboy, 2002). During seeds' germination phytic acid is degraded by phytase, leading to the release of free P, myo-inositol and cations necessary for seedling growth (Badone et al., 2010). Furthermore, phytic acid has a relevant role in protecting the seeds' embryos from ageing-related damage, thanks to its antioxidant activity, avoiding a decrease in their germination capacity (Badone et al., 2010). Despite the potential health benefits due to its antiradical power, phytic acid represents an anti-nutritional factor for monogastric animals (and humans), since it is able to interfere with protein and starch digestion, and to chelate metal cations, reducing their availability in the digestive apparatus (Nuss and Tanumihardjo, 2011), thus contributing to deficiencies of nutrients in the most vulnerable members of the population.

Many phenolic compounds are accumulated in maize seeds; flavonoids and in particular anthocyanins and flavonols are among the main classes. After ingestion, free phenolics are rapidly absorbed by the small intestine and conjugate, leading to a reduced aglycones accumulation in the blood (Scalbert and Williamson, 2000): instead bound phenolics are released only through colonic fermentation (Andreasen et al., 2001; Adom and Liu, 2002). Maize is known to contain a higher amount of phenolics compared to other cereals (Adom and Liu, 2002; Ndolo and Beta, 2014). They are mainly present in the insoluble-bound form, associated with cell wall polysaccharides; the free form represents only a small fraction of the total amount (Lloyd et al., 2000; Bunzel et al., 2001). Phenolics are mainly accumulated in the outermost layers of the grains: Das and Singh (2016) observed that 74-83% of bound phenolics are accumulated in the pericarp, and the remaining fraction is accumulated mainly in the germ (Das and Singh, 2016).

Anthocyanins, flavonols and phenolic acids are able to exert positive effects on human health thanks to their antioxidant activity, contributing to reduce the negative effects of several degenerative and chronic diseases (Lago et al., 2014; Lago et al., 2015). Anthocyanins are water-soluble pigments belonging to the class of flavonoids (Escribano-Bailòn et al., 2004); they confer a purple-blue pigmentation to maize seeds and other plant tissues (Lago et al., 2015), but they are present only in traces in the kernels of yellow and white varieties.

Carotenoids can also be accumulated in maize seeds; they are tetraterpenes, conferring a yellow-orange pigmentation to seeds' endosperm, depending on their concentration. The most abundant carotenoids in maize are the xanthophylls lutein (*β,ε*-carotene-3,3'-diol) and zeaxanthin (*β,β*-carotene-3,3'-diol), that constitute together 90% of the total amount (Doria et al., 2015). Other compounds belonging to this family can be accumulated in the kernel: the xanthophylls *β*-cryptoxanthin (*β,β*-caroten-3-ol), the carotenes, *β*-carotene (*β,β*-carotene) and *α*-carotene (*β,ε*-carotene), and also pro-vitamin A. This class of molecules plays a role in the prevention of several degenerative diseases (*e.g.* cardiovascular diseases, cancer and cataracts), and in particular in the prevention of age-related macular degeneration (AMD), one of the main causes of irreversible blindness (Snodderly, 1995; Faulks and Southon, 2001; Ahmed et al., 2005; Kuhnen et al., 2011). In African countries white maize varieties are usually preferred for human consumption, rather than yellow ones which are often destined for animal feeding. Unfortunately white varieties are unable to accumulate high amounts of carotenoids due to the presence of recessive homozygous mutations belonging to the y_s class (Lago et al., 2015); this also confers on them a lower antioxidant power compared to yellow and pigmented ones (Lago et al., 2015).

In this study we characterized from the phenotypical and nutritional point of view two South African maize landrace cultivars: a white one used for human consumption, and a yellow one used for animal feeding. The seeds were sampled directly from the farmers in the Qwa-Qwa region, a mountainous area in Free State province, not far from the northern Lesotho border. We analysed the seeds to assess their nutritional value for several parameters (calorific value, oil, protein, starch, mineral nutrients, repartition between free and phytic P, flavonoids and carotenoids content).

Our results led us to plan a breeding program aimed to increase the nutraceutical properties of this staple food, contributing in this way to tackle the problem of malnutrition affecting a considerable fraction of the population in South Africa.

Materials and methods

Plant material

The maize varieties studied in this article were sampled in South Africa in the mountainous region of Qwa-Qwa, Thibela, Phomolong (28° 37' 20.81" S, 28° 53' 58.07" E), and cultivated in the experimental field of the University of Milan situated in Landriano (PV), Italy (45°18' N, 9°15' E).

Flour samples used for the analysis were obtained by grinding seeds, cleaned from the glumes, with a Retsch MM200 (Retsch GmbH Germany) ball mill for 3 min at 21 Hz.

Phenotypical characterization

To determine the repartition between germ and endosperm 6 seeds for each variety were imbibed overnight in distilled water and the germ was manually separated from the endosperm using a scalpel. The germ and the endosperm were dried separately at 60°C for 24 hours and weighed again to determine their dry weight.

Seeds of both the varieties (n>50 each) were germinated in the dark after a disinfectant treatment (2% NaClO for 10 min) to determine their germination rate. Plantlets were kept in the dark for 6 days before being exposed to the light, and observed for 15 days to determine the seedlings' tissue-specific pigmentation, both in the dark and in the light.

25 seeds of both the varieties were sown in the same agronomic conditions at 45° of latitude. The plants so obtained were measured after flowering: plants height was measured at the tip of the flag leaf; the height of the ears was measured at their attachment to the stalks.

Bromatological analysis (calorific value, dry matter, crude protein, and ether extract)

Dry seed weight was calculated after weighing in three replicates for each sample. Calorific value measures and chemical analyses were performed using approximately 50 g of seeds for each genotype. Gross energy value was determined using an adiabatic calorimeter (IKA 4000, Staufen, Germany). Chemical analyses were performed according

to AOAC standard methods (AOAC, 2000), milling and analysing the samples for dry matter, crude protein, and ether extract (oil).

Determination of ionomic content (Na, Mg, P, K, Ca, Fe, Zn) in maize flour

For the determination of elements of interest, 0.3 g of maize flour samples were digested by a microwave digestor system (Anton Paar MULTIWAVE-ECO) in Teflon tubes filled with 10 mL of 65% HNO_3 by applying a one-step temperature ramp (at 210°C in 10 min, maintained for 10 min)

After 20 min of cooling time, the mineralized samples were transferred into polypropylene test tubes.

Samples were diluted 1:40 with MILLI-Q water and the concentration of elements was measured by ICP-MS (BRUKER Aurora-M90 ICP-MS). An aliquot of a 2 mg/L of an internal standard solution (^{72}Ge, ^{89}Y, ^{159}Tb) was added both to samples and calibration curve to give a final concentration of 20 µg/L.

Typical polyatomical analysis interferences were removed by using CRI (Collision-Reaction-Interface) with an H_2 flow of 93 mL/min flown through skimmer cone.

Average values regarding Na, Mg, K, Ca, Fe, Zn were expressed as µg/g seed flour; values regarding P were indicated as mg/g seed flour.

Determination of phytic phosphate in seeds

5 mL extraction buffer (0.4 M HCl + 0.7 M Na_2SO_4) were added to 50 mg seed flour (three replicates for each sample); the solutions were vortexed and incubated overnight at room temperature. After centrifugation (13000 rpm for 10 min) 1 mL of a 15 mM $FeCl_3$ 0.2 N HCl solution was added to 1 mL supernatant in plastic screw top 2 mL tubes. The tubes were left in the dry bath at 100°C for 30 min and centrifuged at 13000 rpm for 10 min to obtain the ferric phytate precipitate; the supernatant was removed. 1 mL 0.2 N HCl was added to wash the pellet, and removed after centrifugation. The samples were digested to completion on a hot plate in H_2SO_4 (400 mL), adding H_2O_2 every three hours until the solution remained clear. All the solutions were diluted adding distilled H_2O to reach a final volume of 2 mL. Phytic phosphorus in the digests was determined spectrophotometrically through the colorimetrical Chen assay (Chen et al., 1956).

The reference standard curve was obtained adding 1998 µL, 1996 µL, 1994 µL, 1992 µL, 1990 µL and 1972 µL of a freshly prepared Chen's reagent (distilled H_2O, 6 N H_2SO_4, 10% ascorbic acid and 2.5% ammonium molybdate in the ratio 2:1:1:1 v/v/v/v) to 2 µL, 4 µL, 6 µL, 8 µL 10 µL and 28 µL of a KH_2PO_4 solution (atomic P 1µg/µL) respectively. 2 mL Chen's reagent was used as blank. 1800 µL of Chen's reagent were added to 200 µL of digested solution for each sample. All the solutions were vortexed and incubated at room temperature for 2.5 h before reading the absorbance of the reaction mixture at 650 nm. The concentration of phytic P in the samples was determined considering the measured absorbance, according to the standard curve.

Determination of free phosphorus in seeds

50 mg seed flour were extract with 2 mL 12.5% trichloroacetic acid (TCA) 25 mM $MgCl_2$ solution (three replicates for each sample). The solutions were mixed and kept in agitation for 30 min at room temperature before being incubated overnight at 4°C. Free phosphorus in the extracts was determined spectrophotometrically through the colorimetrical Chen assay (Chen et al., 1956). Four solutions, containing respectively atomic P 0.62, 1.24, 2.48, 3.72 µg/mL were prepared using a 2 mM Na_2HPO_4 solution: 1980 µL, 1960 µL, 1920 µL and 1880 µL of a freshly prepared Chen's reagent (distilled H_2O, 6 N H_2SO_4, 10% ascorbic acid and 2.5% ammonium molybdate in the ratio 2:1:1:1, v/v/v/v) were added to 20 µL, 40 µL, 80 µL and 120 µL of a 2 mM Na_2HPO_4 solution. 2 mL Chen's reagent was also used as the blank and 1800 µL were added to 200 µL of each extract collected after centrifuge, to reach a final volume of 2 mL.

All the solutions were agitated and incubated at 50 °C for 1 h before reading. The absorbance of the reaction mixture was measured at 650 nm.

Free P concentration was calculated according to the standard curve.

Flavonoids quantification

About 15 mg seed flour were weighed and transferred into a 2 mL tube (four replicas for each sample); 200 µL distilled water were added, and the samples were boiled at 100°C for 30 min. 1 mL of extraction buffer was added to each sample (94.8 mL EtOH 95%, 2 mL distilled water and 3.2 mL 37% HCl were mixed to obtain 100 mL extraction buffer). The solutions were vortexed and left overnight in agitation. The samples were centrifuged at 13000 rpm for 15 min and the supernatants were collected. 500 µL

extraction buffer were added to each pellet; the samples were vortexed and left in agitation for two hours. After centrifugation (13000 rpm for 15 min) the supernatant was collected and unified with the first one. The whole amount of supernatant collected from each sample was centrifuged again at 13000 rpm for 30 min before reading. The absorbance was measured spectrophotometrically at 530 nm, at 350 and 280 nm respectively for anthocyanins, flavonols and phenolic acids, using the extraction buffer as blank. The anthocyanin content was calculated as cyanidin 3-glucoside equivalents (molar extinction coefficient (ε) 26900 L m^{-1} mol^{-1}, M.W. 484.82), the amounts of flavonols and phenolic acids were calculated as quercetin 3-glucoside (ε 21877 L m^{-1} mol^{-1}, M.W. 464.38) and ferulic acid (ε 14700 L m^{-1} mol^{-1}, M.W. 194.18) equivalents. The analyses were conducted four times for each genotype, and the confidence interval (C.I.) at 95% was calculated.

Carotenoids extraction and quantification

3 mL of extraction buffer (acetone, methanol, hexane 1:1:1) were added to 0.25 g seed flour in 15 mL tubes (four replicas for each sample). The samples were vortexed and left in agitation in ice for 30 min, vortexing them again every 10 min. 1 mL nanopure water was added to each sample, then the samples were vortexed and kept in agitation 5 min before centrifuge (3000 rpm for 10 min). 1 mL non-polar phase was collected and filtered through a 0.22 µm syringe filter. The extracts were conserved at -20°C in the dark until reading.

1.8 mL extraction buffer (acetone, methanol, hexane 1:1:1) was added to 200 µL extract (dilution 1:10) to obtain a final volume of 2 mL. The extraction buffer was used as blank. The absorbance was measured spectrophotometrically at 450 nm using glass cuvettes. Carotenoids content was calculated according to the standard curve obtained using five lutein solutions (0.25, 0.5, 1, 2, 4 µg/mg). Standard deviation was calculated.

Informatic tools

Microsoft Excel® was used to analyse the collected data.

Results and discussion

In this paper two South African maize varieties (a yellow and a white one), sampled directly from the farmers in the mountain region of Qwa-Qwa (28°37'20.81"S, 28°53'58.07"E) (Fig. 1A, Fig. 1B), were analysed and characterized from the nutritional and phenotypical points of view. The white variety, characterized by very big ears and large flint dent seeds (Fig. 2B, Fig. 2E) is used by the local population for human consumption, and milled to prepare a traditional maize meal porridge called pap (Fig. 1C), similar to the Italian polenta. The yellow one was characterized by smaller flint seeds, with a more pronounced dent shape (Fig. 2A, Fig. 2E); its kernel is manually ground by the local farmers and used as feed for poultry.

Both the varieties were maintained by the local farmers as open pollinated varieties and cultivated in kitchen gardens; unfortunately the two varieties were not always kept in isolation, as demonstrated by the presence of cross contamination.

The average dry weight of the white seeds was 0.655 ± 0.065 g, higher than that of the yellow seeds (0.389 ± 0.06 g) and also, to our knowledge, higher than that of any landrace still cultivated in Europe. Because of their dimensions, white seeds appear particularly adapted for milling, allowing the users to obtain flour with a very fine particle size thanks to the favorable ratio endosperm/pericarp.

The germination rate was higher, but not significantly, for the yellow variety (98.18 ± 3.56%) compared to the white (94.54 ± 6.05%). Despite this, the seedlings of the white variety showed a greater vegetative vigour and a more developed root system. (Fig. 2C, Fig. 2D).

The seedlings of both the varieties were characterized by the absence of tissue pigmentation in the dark; the yellow variety showed very weak seedling pigmentation after light exposure (Fig. 2C). All the observed plantlets of the white variety showed the accumulation of red-purple pigments in both roots and mesocotyl, following light exposure (Fig. 2D) suggesting the presence of a *Sn* dominant allele. *Sn* regulatory gene belongs to the *r1*/*b1* gene family, that together with the *c1*/*pl1* gene family, regulates anthocyanin accumulation in plant tissues. *Sn* locus is situated on chromosome 10 near the *r1* locus, and probably originated from an intrachromosomal duplication (Pilu et al., 2003). Even if cultivars adapted to low latitudes are often unable to reach maturity and set seeds at medium-high latitudes because of the longer photoperiod (Petroni et al.,

2014), the two South African cultivars, sampled at 28° of latitude, and cultivated in open field conditions in Italy at 45° of latitude, were able to reach maturity. Mature plants did not have high amounts of pigments in their tissues. Plants of the white variety reached 276.6 ± 10.1 cm in height (average height of the ear 197.1 ± 8.1 cm): in fact, low latitude origin maize varieties often reach greater heights when grown at higher latitudes. However, the plants of the yellow variety only reached an average height of 162.6 ± 8.3 cm (height of the ear 111.5 ± 6.9 cm); their limited height, despite their subtropical origin, suggests a high level of homozygosity causing inbreeding depression, probably due to the incorrect conservation of this variety (genetic drift) in recent years. It is highly probable that the yellow variety, even if maintained by the local farmers as a population, derives from a commercial dent hybrid that lost its hybrid vigour after many years of cultivation.

However, the white cultivar is probably an ancient landrace and appears more interesting from the scientific point of view because of its higher variability and its characteristically large seeds, so it is a good candidate for future breeding programmes.

The calorific value, indicated as J/g, and the percentage of oil and protein in the two South African varieties was found to be comparable to that shown by colorless modern hybrids (Panzeri et al., 2011) (Table 1), which are known for their low nutritional value in comparison with several ancient landraces. In fact the Scagliolo cultivar (an Italian traditional flint maize) was found to show higher values, in particular for its protein content (Panzeri et al., 2011) (Table 1). Berta et al. (Berta et al., 2014) also reported a higher protein content in the Italian variety Ostiglia (9.5 g/100g), and a starch content of 68.7 g/100g, comparable to that in the yellow South African variety (68.4 g/100g).

The content of micro and macronutrients (Na, Mg, P, K, Ca, Fe and Zn) in the two varieties was quantified by ICP-MS (Table 2, Table 3) using the B73/Mo17 colorless hybrid and the traditional Spanish Millo Corvo pigmented variety as controls. Among the minerals analysed, no significant differences were observed between the two varieties for Na, Mg and K content (Table 2).

The yellow variety was characterized by a higher content of Ca (50.45 ± 3.65 µg/g) compared to the white variety and to the Millo Corvo seeds used as control; even though it was somewhat higher, its Ca content was not significantly higher than that of B73/Mo17 hybrid (Table 2).

Zinc is an essential mineral to assure the functioning of many enzymes and transcription factors, and also an anti-inflammatory and antioxidant agent working in cell-mediated

immune processes (Prasad, 2007; Haase et al., 2008; Tokuji et al., 2009): its deficiency can cause adverse effects on both physical growth and cognitive development (Black, 1998; Brown et al., 2001; Gibson, 2006). Unfortunately 45.3% of South African children have an inadequate zinc intake (Samuel et al., 2010), but our results show that the white South African variety found in Qwa-Qwa, used for human nutrition, has a significantly higher zinc content in the kernel (23.44 ± 6.06 µg/g) compared to the yellow one that is fed to animals (15.28 ± 1.21 µg/g); despite this, Zn content in the white variety was not particularly high as it was similar to that of one of the varieties used as control (Table 2), and lower than that one reported by Berta et al. (Berta et al., 2014) for the variety Ostiglia (33.5 ± 1.1 µg/g).

Iron concentration was low in both the South African varieties, especially in the white one, compared to the ones used as control (Table 2) and to the value reported for the Ostiglia variety: 26.3 ± 1.5 µg/g (Berta et al., 2014); this appears particularly worrying considering that iron deficiency affects more than 10% of the South African population (Doria et al., 2015).

To better characterize the two South African varieties from the nutritional point of view, the total amount of phosphorus and its repartition between free and phytic forms were also quantified (Table 3). The total content of phosphorus quantified through ICP-MS was found to be higher in the white variety (3.48 ± 0.12 mg/g) than that observed in the yellow variety (2.91 ± 0.05 mg/g). Free P reached 0.53 ± 0.07 mg/g in the white cultivar and only 0.32 ± 0.02 mg/g in the yellow, corresponding respectively to 15 and the 11 percent of the total P amount (Table 3). Phytic P content was similar in the two South African varieties: 2.58 ± 0.3 mg/g and 2.39 ± 0.1 mg/g respectively in the white and in the yellow one; the remaining amount of P in the two varieties is represented by the cellular phosphorus. Both the varieties, especially the white one, contained a higher amount of free P and a lower amount of phytic P compared for example, to that measured by Pilu et al. (Pilu et al., 2005) in the B73 colorless inbreed line (0.29 mg/g and 3.52 mg/g). Considering that free and phytic P in the seeds are accumulated mainly in the germ (O'Dell et al., 1972; Raboy, 1990), we initially supposed that the higher content of free P in the white variety could be due to a higher ratio germ/endosperm; instead our results showed that the germ represented only 10.5% of the total weight in the white seeds, and 14.4% in the yellow; hence free P concentration must actually be higher in the white variety.

Many compounds can exert an antioxidant activity in seeds, protecting tissues from oxidative stresses due to biotic or abiotic stress conditions: the presence of high amounts of phenolic compounds and carotenoids in seeds directly contributes to higher antioxidant power (Lopez-Martinez et al., 2009; Žilić et al., 2012; Lago et al., 2015). In this paper we quantified spectrophotometrically the amount of anthocyanins, flavonols and phenolic acids in the South African varieties, using the Millo Corvo pigmented variety, able to accumulate anthocyanins in the seeds' aleurone layer (Lago et al., 2015) as the coloured control, and the B73 inbred line as the colourless control (Table 4). As expected for colourless varieties, both the South African ones showed a very low anthocyanin content in the seed flour, expressed as cyanidin 3-glucoside equivalents, comparable to that of the B73 colourless inbreed line and lower than that measured in the coloured variety Millo Corvo (Table 4). No significant differences were observed between the two SA varieties and the ones used as controls for their flavonols content (indicated as quercetin 3-glucoside equivalents) (Table 4). Among the phenolic compounds, ferulic acid seems to be very important for health, as it can be beneficial for cancer prevention (Virgili and Marino, 2008; Tokuji et al., 2009); both the South African varieties showed a content of phenolic acids, expressed as ferulic acid equivalents, similar to that of the B73 inbreed line (113 ± 0.2 mg/100g): 94.71 ± 21.07 mg/100g for the white variety and 130.54 ± 26.58 mg/100g for the yellow, much lower (by nearly a half) than that observed in Millo Corvo (216.63 ± 29.05 mg/100g ferulic acid equivalents) (Table 4). In fact a higher anthocyanin content, such as the one observed in Millo Corvo, is often related to a higher content of others flavonoids sharing a part of the same biosynthetic pathway (Lago et al., 2014; Lago et al., 2015).

Carotenoids are known to exert antioxidant (Handelman, 2001) and anti-angiogenic (Kuhnen et al., 2009) actions, contributing to the prevention of degenerative diseases, such as cardiovascular diseases, cancer, age-related macular degeneration (AMD) and cataract (Faulks and Southon, 2001; Ahmed et al., 2005; Kuhnen et al., 2011). They are hydrophobic C40 isoprenoids synthesized in amyloplasts conferring a yellow-orange pigmentation to the seeds, depending on their concentration. Those accumulated in maize endosperm are mainly lutein and zeaxanthin (Kirk and Tinley-Basset, 1978; Kurilich and Juvik, 1999; Tokuji et al., 2009; Žilić et al., 2012). More than 30 loci are involved in their biosynthesis and the main class of mutations reducing or depleting carotenoids in maize kernel is y_s (Chander et al., 2008); as a consequence of these mutations, seeds' endosperm appears pale or white (Lago et al., 2015). White maize

varieties are worldwide consumed and appreciated, in particular in many developing countries, even though they are well known to be lacking in vitamin A (derived from carotenoids) which is essential for human health, and thus contributing to the occurrence of vitamin A deficiency (VAD) in those populations (West et al., 2002). A inadequate consumption of carotenoids may cause blindness, growth retardation and anemia, increasing infectious morbidity and mortality (Sommer and Davidson, 2002; Žilić et al., 2012).

Unfortunately the white South African variety that is used for human consumption, showed a low carotenoids content (1.09 ± 0.4 µg/g), as expected, which was found to be similar to the average value (4.95 ± 0.62 µg/g) observed in three flint maize varieties having a white endosperm (the Italian Bianco Perla and Bianco Vitreo, and the Spanish Millo Corvo) (unpublished data of our group), suggesting the presence of a recessive homozygous mutation belonging to the white endosperm class (*y*).

The yellow South African variety contained a higher amount of carotenoids (22.57 ± 2.5 µg/g), corresponding to the average value observed in 12 Italian flint landraces characterized by a yellow endosperm: 21.94 ± 5.74 µg/g (unpublished data of our group). Our results are in agreement with the content of carotenoids (lutein and zeaxantin) in maize seeds reported by Mangels et al. (Mangels et al., 1993), between 0.05 and 23 µg/g.

Finally, a breeding program has been planned to ameliorate the nutritional profile of the two cultivars which are already adapted to South African growing conditions (climate, photoperiod).

Pigmented maize varieties, carrying the dominant alleles of the regulatory genes of the anthocyanins and carotenoids biosynthesis will be used as pollen donors in a breeding programme based on pedigree selection, to obtain enriched varieties, characterized by a higher antioxidant power compared to the original ones and contributing to tackle the VAD problem.

Particular attention will be focused on the white variety which is used for human consumption: plants will be selected with the aim of increasing protein and Fe content, while maintaining the large size of the seeds that makes this variety particularly adapted for milling.

The breeding programme will be also conducted in South Africa, re-distributing the seeds to the local farmers in poorer communities, thus involving them in participatory plant breeding.

Conclusions

In this work we characterized for the first time, from the phenotypical and nutritional points of view two maize varieties cultivated by South African farmers in the rural region of Qwa-Qwa: a white variety, used for human consumption, and a yellow one destined for animal feeding. The yellow variety shows a low variability and is probably derived from a commercial hybrid, sown for many years by the local farmers. Both the varieties showed low oil and protein content compared to the Scagliolo Italian flint variety used as control, and low iron content compared to the B73/Mo17 hybrid and to the Millo Corvo cultivar. The white variety was characterized by a higher Zn content, but also by a lower content of Ca in comparison with the yellow one. The total content of P and free P was found to be higher in the white variety, while their content of flavonols and phenolic acids was similar, and was low compared to the pigmented Millo Corvo variety. As expected, the white variety was also found to lack carotenoids. Despite its low nutritional value, the white variety appears interesting because of the large dimensions of the seeds that makes them particularly well adapted for milling. Protein, carotenoids and Fe content will be increased, together with flavonoids content, through a breeding program aimed to obtain improved varieties that could be considered as everyday functional foods for the local population.

Acknowledgements

We wish to thank Davide Reginelli for his hard work in the field and Giorgio Lucchini for performing ICP-MS analysis.

Figures

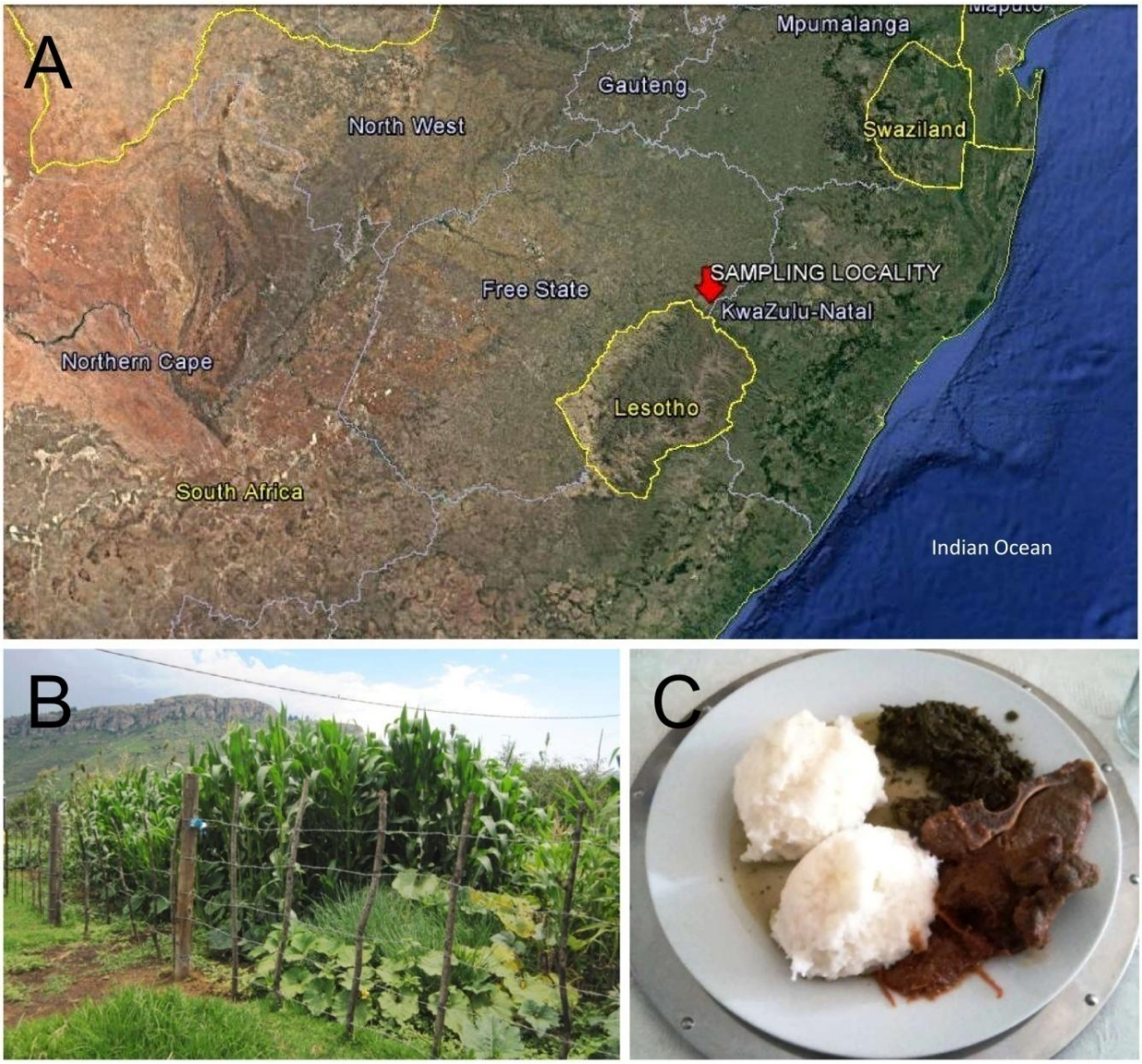

Figure 1. Sampling site of the white and yellow South African maize cultivars in the mountain region of Qwa-Qwa (28° 37’ 20.81” S, 28° 53’ 58.07” E) (A). Vegetable garden where the yellow variety was cultivated (B). South African traditional Maize meal porridge, pap, obtained using white maize flour and water (C).

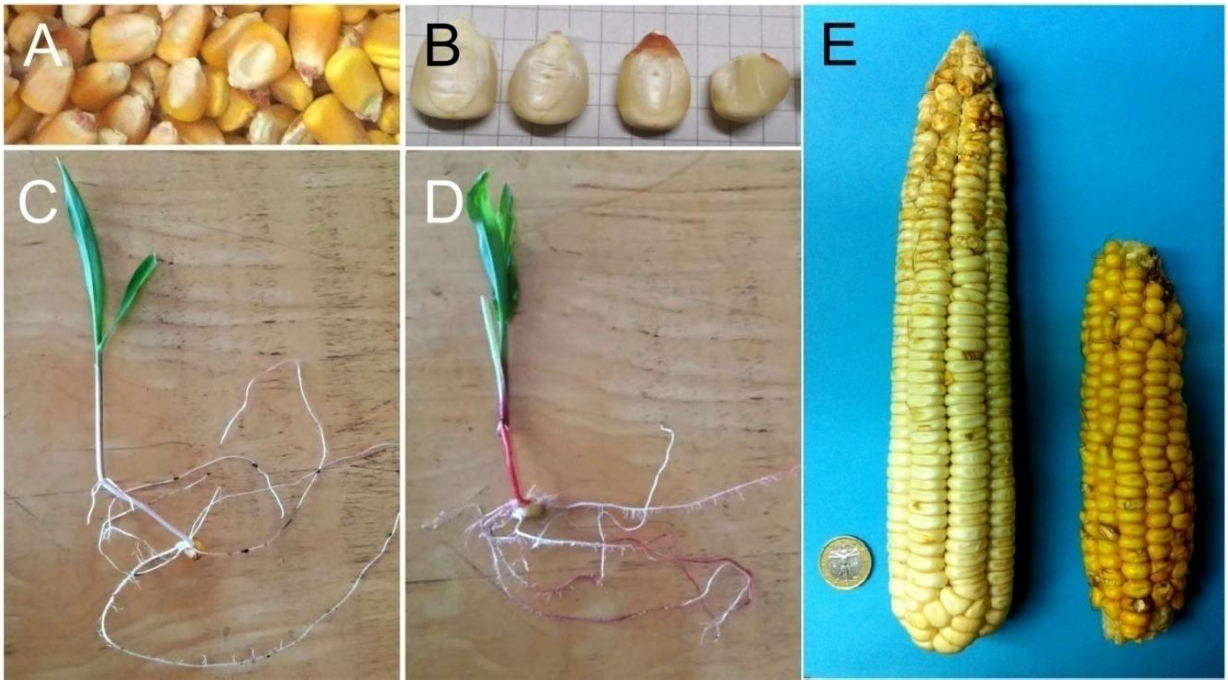

Figure 2. Seeds and seedlings of the two South African varieties. Seeds of the yellow South African variety (A) and of the white one (B). Seedlings of the yellow (C) and white (D) South African varieties after light exposure. Ears of the white (left) and yellow variety (right) (E).

Tables

Table 1. Measures of calorific value, oil, protein and starch in seeds of the genotypes analysed. Mean values and standard errors of the traits are shown. Data regarding calorific value, oil and protein content in the Scagliolo, B73/Mo17, DK 440, PR 33A46, NK HELEN controls varieties are taken from Panzeri *et al.*, 2011.

Variety	Calorific value (J/g)			Oil (%)			Protein (%)			Starch (%)		
S.A. White	18930	±	34.3	4.06	±	0.082	8.78	±	0.165	63.3	±	1.18
S.A. Yellow	18690	±	33.8	5.56	±	0.106	7.44	±	0.140	68.4	±	1.27
Scagliolo	19362	±	31.3	6.02	±	0.015	13.05	±	0.293	62.8	±	1.16
B73/Mo17	18790	±	15.9	4.63	±	0.022	8.54	±	0.290	ND		
DK 440	18723	±	45.5	4.29	±	0.316	8.6	±	0.278	ND		
PR 33A46	18654	±	43.2	3.29	±	0.048	10.71	±	0.067	ND		
NK HELEN	18616	±	33.6	3.47	±	0.008	10.01	±	0.018	ND		

Table 2. Mineral nutrients quantification through ICP-MS. The South African white and yellow varieties are compared to the B73/Mo17 hybrid and the Spanish Millo Corvo traditional variety. Average values are indicated as µg/g. Confidence intervals at 95% are shown.

Elements	S.A. White	S.A. Yellow	B73/Mo17	Millo Corvo
Na	13.31 ± 2.87[a]	11.51 ± 4.22[a]	12.89 ± 0.79[a]	10.51 ± 3.86[a]
Mg	1363.74 ± 149.68[a]	1241.55 ± 69.33[a]	1272.33 ± 42.64[a]	1213.28 ± 105.99[a]
K	3323.11 ± 330.39[ab]	3293.76 ± 152.15[a]	3767.90 ± 134.97[b]	3195.43 ± 294.11[a]
Ca	36.80 ± 4.14[a]	50.45 ± 3.65[b]	41.46 ± 6.61[ab]	33.82 ± 8.03[a]
Fe	15.81 ± 3.10[a]	18.33 ± 3.75[ab]	22.93 ± 1.43[b]	22.07 ± 1.69[b]
Zn	23.44 ± 6.06[a]	15.28 ± 1.21[b]	26.35 ± 1.18[a]	18.94 ± 2.46[ab]

Table 3. Phosphorus quantification in whole seed flour. Total P was quantified through ICP-MS. Free and phytic P repartition was determined. Average values are indicated as mg/g. Standard Deviation is shown.

	SA White	SA Yellow
Total P	3.48 ± 0.12	2.91 ± 0.05
Phytic P	2.58 ± 0.3	2.39 ± 0.1
Free P	0.53 ± 0.07	0.32 ± 0.02

Table 4. Flavonoids spectrophotometrical quantification. Anthocyanins, flavonols and phenolic acids were quantified as mg cyanidin-3-glucoside equivalents, quercetin 3-glucoside equivalents and ferulic acid equivalents respectively per 100 g of dry seed flour. The analyses were conducted four times for each genotype. Data regarding Millo Corvo and B73 controls varieties are taken from Lago *et al.*, 2015. Confidence interval at 95% are shown.

Compound	S.A. White	S.A. Yellow	Millo Corvo	B73
Anthocyanins	6.98 ± 4.46^{ab}	6.25 ± 0.63^{a}	83.45 ± 11.44^{c}	3.00 ± 1.00^{b}
Flavonols	41.72 ± 16.07^{a}	66.19 ± 9.20^{a}	74.21 ± 17.83^{a}	66.00 ± 10.00^{a}
Phenolic acids	94.71 ± 21.07^{a}	130.54 ± 26.58^{a}	216.63 ± 29.05^{b}	113.00 ± 0.20^{a}

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