UNIVERSITÀ DEGLI STUDI DI MILANO

Dottorato di ricerca in

Agricoltura, Ambiente e Bioenergia

XXXI CICLO

PREBREEDING OF MAIZE TRADITIONAL FARMERS' VARIETIES AND THEIR BIOFORTIFICATION FOR FOOD SAFETY AND SECURITY.

Settore disciplinare: AGR/07

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Anno accademico 2018-2019

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General introduction

Maize is the main cereal grain cultivated worldwide (*Zea mays L. ssp. mays*), providing 15% of the protein and 20% of the calories in the human diet supplying an energy density of 365 Kcal/100 g and covering a cultivated area of 184.8 million hectares in 2014 (Faostate 2014, Food and Agriculture Organization of the United Nations, Crops Production 2009, Zeppa et al. 2012).

Maize (*Zea mays L. ssp. mays L.*) was domesticated about the 8,700 BP in Mexico and from there it spreads within the Americas (Piperno et al., 2009; van Heerwaarden et al., 2011). European explorers took maize to Europe since the end of the XV century and only later it was taken to Asia and Africa (Brown and Darrah, 2002; Ranum et al., 2014).

Three main sources of corn were introduced to Europe: the photoperiod insensitive Cateto types, the Pearl White, and the varieties adapted to higher latitudes from the American east coast (Brandolini and Brandolini, 2009; Eschholz et al., 2010). Maize initially was a botanical curiosity, but it became a staple food for its its flexibility of usage and the higher yields.

In Europe, the spread of maize 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; Brandolini and Brandolini 2009).

In Italy, the first reports regarding the utilization of corn date from 1600 in the North East where it was adapted to the climatic zones of cultivation and to local traditions of people (Brandolini 1958; Brandolini and Brandolini 2009).

The process of diversification of hundreds different landraces and traditional farmer's varieties maintained as OPVs (Open Pollination Varieties) was due to the long process of adaptation to different environments, together with human selection (Messedaglia, 1924; Brandolini and Brandolini, 2009; Cassani et al., 2016).Landrace is a population of cultivated plants that has historical origin, distinct identity and lacks formal crop improvement; traditional farmers' varieties were submitted to the farmers crop improvement with massal selection being selected for nutraceuticals and for resistance to biotic and abiotic stresses. These cultivars are locally adapted and associated with traditional farming systems (Newton et al., 2009). An important distinction is possible between landraces and traditional farmers varieties with modern cultivars that are the result of breeding programs aimed to obtain plants genetically uniform. In contrast

landraces and traditional farmers' varieties are genetically heterogeneous: these populations have heterozygous components that lead to a genetic competition among plants.

Traditional varieties and landraces were cultivated until the twentieth century; starting from this period modern cultivars and inbred lines caused their disuse, but in disadvantaged environments they supported subsistence farming all over the world. Moreover these sources of genetic variability has been preserved from germplasm banks and breeders that reduced the loss of genetic variability due to the utilisation of modern hybrids that were more productive. In this way through generations of natural and massal selection for positive genes it was obtained resistance to biotic and abiotic stresses. In fact several important resistance genes were first identified in cereal reducing the pathogen spread, moreover may represent a potential source of varieties adapted to low input cropping systems. The hybridization during cultivation, of these corn sources in different environments, led to the establishment of many local varieties that under different photoperiod, temperature, humidity and altitude allowed the constitution and the differentiation of local European varieties and landraces genetically adapted to specific environmental conditions (Brandolini and Brandolini et al., 2009; Eschholz et al.,2010; Lago et., al 2015).

These important genetic sources with their inter-genotypic balance are well-known to have natural variation in important nutraceuticals, which has been lost in several years during the breeding of modern cultivars having a superior nutritional value (Bouis and Welch, 2010; Newton et al., 2009). Furthermore, in low input system, they present yield stability in response to biotic and a-biotic stresses: in this case the heterogeneity of these populations can react to external stresses giving satisfactory yields. For these reasons the longer-term breeding target, should be based on the utilisation of valuable genes present in landraces increasing productivity in high-stress environments, focusing on adaptation and on the conservation of genetic diversity increasing at the same time the nutritional value. Conventional breeding has obtained good results in optimal growth conditions, but it was less useful in adverse environment. In these cases, participatory breeding can have a significant and positive influence ameliorating some negative consequences of modern agriculture, such as the use of high inputs, the loss of genetic diversity and the decrease of yield in less favourable areas (Newton et al., 2009; Ortiz-Monasterio et al., 2007).

To preserve the genetic diversity of landraces and traditional farmers' varieties and to use these in breeding programmes, will be necessary to involve gene banks and local

farmers, in this way would be possible to study the genetic variability of these cultivars using advances in molecular genotyping and data basing technologies (Newton et al., 2009; Ortiz-Monasterio et al., 2007).

Moreover the characterization of the ancient landraces is very important, for their conscious protection, preservation and valorization (Lago et al., 2015), not only considering the possible economic interests of the farmers and the higher content of nutraceuticals, but also because their study unable to further clarify the origin and spread of maize. Moreover they can be considered a useful tool in future breeding programs thanks to their high genetic variability (Liu et al., 2003; Vigouroux et al., 2008; Warburton et al., 2008; Mir et al., 2013), in particular for what concerns the peculiar traits characterizing some of them, and their adaptation to specific environments, that could unable to identify novel alleles and haplotypes useful in agro-ecologically productive systems characterized by a low level of energy input, in a context of sustainable or subsistence agriculture (Kuhnen et al., 2011; Lago et al., 2015). For poor rural and urban populations living in developing countries, the fundamental source of micronutrients and provitamin A are foods of plants origin (Ortiz-Monasterio et al., 2007). Maize 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); in Africa, in the major part of the countries, corn represents the main source of energy (Dowswell et al., 1996; Nuss and Tanumihardjo, 2011).In some part of Africa and in particular in Sub-Saharian countries, maize is a staple food as rice in Asia (Oldewage-Theron et al., 2005; Gouse et al., 2006; Nuss and Tanumihardjo., 2011) (Table. 1).

In poor regions of the world nutrients deficiency (Zn, Fe and vitamin A) has a relevant role in child and adult mortality. In particular vitamin A deficiency ranks in top positions among factors contributing to blindness, and affect cognitive development in children (Ortiz-Monasterio et al., 2007).

In these areas bio-fortification represents a sustainable strategy to improve human nutrition, where population bases diet principally on cereals and on derived products (Bouis and Welch, 2010; Pfeiffer and McClafferty, 2007): micronutrient malnutrition and other nutritional deficiencies can be reduced by enhancing the nutrient content of staple crops using modern breeding technologies involving at the same time farmers. Bio-fortification is a term that merges the most used endogenous fortification strategies: breeding, mutagenesis, genetic modification, and the use of fertilizers. Bio-fortification

through breeding exploiting the natural genetic variability has been the most used technique, despite it's the slowest. This slowness led to mutagenesis and transgenic techniques; they have given a great knowledge of biosynthetic pathways. Finally fertilization is largely used in particular with the arise of hybrids with high yields requiring the same level of inputs to obtain satisfactory results (Newton et al., 2009). The process of bio-fortification using breeding techniques should be based on four pillars:

1. Identify and study the genetic variability sources,
2. Focus on the introgression of this variability into high yielding and stress tolerant genotypes with an acceptable end-use quality,
3. Test the stability of nutritional elements accumulation across the target environment,
4. Large scale deployment of seeds of these improved cultivars to farmers. They will maintain these new varieties as OPVs to allow self-production of seeds.

Maize is a good candidate for biofortification in fact it can be used in several ways as feed for live-stock, forage, silage and grain, but also for industrial uses. However, human nutrition remains one of the main uses, being a staple food for poor populations and determining the selection of varieties for producing many typical dishes such as polenta in Italy and pap in South Africa (Zeppa et al., 2012). Polenta is a very popular dish in the northern regions of Italy (Zeppa et al., 2012).

Thus, while productivity remains the major target for breeders, focusing on grain quality, could be reduced deficiencies of some minerals and provitamin A increasing the concentration of functional compounds and the nutritional value for poor populations that use maize as staple food (Messias et al., 2015).

The nutritional value can be improved increasing carotenoids and vitamins content, but also the content of minerals such as zinc, iron, phosphorous and calcium, thus obtaining a cheap and easily accessible functional food (Ranum et al., 2014). Moreover the presence of phenolics and in particular phlobaphenes is thought to have an important role in plants' resistance against various pathogens, e.g. by reducing fungal infection, and also having beneficial effects on humans and animal health due to their high antioxidant power (Pilu et al., 2011). Phlobaphenes are reddish insoluble pigments, whose biosynthetic pathway begins with the condensation of malonyl-CoA with p-coumaroyl-CoA, catalysed by the enzyme chalcone synthase (CHS) (encoded by the colorless2 locus, *c2*). The chalcone isomerase (CHI) enzyme converts the resulting naringenin chalcone into the flavanone

naringenin that is converted to apiforol and luteoforol by the enzymes dihydroflavonol reductase-DFR (encoded by the *A1* gene) and the flavanone-3-hydroxylase-F3-H (encoded by the *Pr1* gene). Apiforol and luteoforol are then polymerized into phlobaphenes (Sharma et al., 2012). The accumulation of phlobaphene pigments in the maize pericarp layer is regulated by the R2R3-MYB transcription factor *PERICARP COLOR1* (*P1*), with different *P1* alleles conferring different pericarp and cob glume colors (Grotewold et al., 1991; Casas et al., 2014). Phlobaphenes are accumulated in a limited number of tissues, such as seed pericarp, and cob glumes (Grotewold et al., 1991), conferring them a typical red-brown pigmentation, sometimes very dark. The *P1-rr* allele determines the coloration of both pericarp and cob glumes: with the *P1-rw* allele only the pericarp is colored, with *P1-wr* only the cob glumes, and with *P1-ww* both the tissues are colorless (Casas et al., 2014). Preliminary results indicated that phlobaphenes could be associated with a reduced level of mycotoxin contamination, in particular a reduction of fumonisins accumulation in maize kernel (Pilu et al., 2011). 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).Thanks to their diffusion, consumers appreciation and importance in the diet, staple foods appear particularly interesting for biofortification strategies and are of crucial importance. Bio-fortification activities could be useful for rich countries where in the last years the demand of gluten-free, vegetarian, vegan and high nutritional value food is growing obtaining products with added value; but mainly for poor countries where the main goal is to reduce the target population having low intake of these fundamental nutrients, guarantying them at least the minimum intake needed to improve health, avoiding deficiencies (Bai et al., 2011; Graham and Rosser, 2000; Mellado-Ortega and Hornero-Méndez, 2015).

Summary of the thesis work

In order to explore and identify the genetic variability of Italian and European landraces and traditional farmers' varieties and to use these in breeding programs, we performed a pre-breeding activity that started with "Traditional ancient farmers' varieties: a valuable source of genetic variability for biofortification programs". With the aim to assess the nutritional value of traditional farmers' varieties collaborating with CREA - Council for Agricultural Research and Agrarian Economy located at Stezzano (BG), and the germplasm bank of the University of Milan located in Landriano (PV) (Fig.1) we collected 12 local farmers varieties from CREA in Bergamo and sampled two varieties directly from farmers (Millo Corvo in Spain and Nero Spinoso at Esine) (Fig. 2). At the end of the selection's process, we determined the nutritional value. In this pre-breeding work we demonstrate the nutritional superiority, for the major part of nutraceuticals that we analyzed, of traditional farmers' varieties if compared with modern hybrids. In particular Spinato di Gandino is the best variety for milling properties and for oil, protein, and total phosphorus content; Storo is the best variety for calorific value and for carotenoids and free phosphorus content, Nero Spinoso is an interesting variety for nutrient amount in general and mainly for phlobaphenes presence. Moreover, we found a significant negative relation between embryo's dimensions and carotenoids content ($R = -0.697$; $P < 0.05$) finding that white varieties have bigger embryo than yellow ones, supporting the thesis of a strong selection of white varieties for human consumption. Regarding the mineral concentration in the kernel Nostrano dell'Isola has the highest amount of iron (31.05 μg g^{-1}) and Cinquantone (36.53 μg g^{-1}) gave the highest amounts of zinc being the candidate variety for future breeding programs. Moreover in our study we found a significant relation between iron and zinc in the kernel ($R = 0.581$; $P < 0.05$), we could therefore imagine simultaneous increases of both elements in a breeding activity pointed to increase mineral amount in the grain. From these varieties we started a bio-fortification program aimed to obtain neo-synthesis populations with high nutritional value. The aim of these actions will be oriented to: i) a reduction of nutrient deficiencies in poor countries with a program of participatory plant breeding, and ii) an increase of the added value. In particular after our pre-breeding activity we focused on Nero Spinoso 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 this landrace, that is known for the pointed shape of the seeds and the dark brown-black pigmentation. Our analysis showed that this landrace accumulates very high amounts of

phlobaphenes (320 A_{510}/100 g flour). We demonstrated that also in this landrace phlobaphenes pathway is under the control of a monogenic dominant gene *pericarp colour1* (*P1*). 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 3’ sequence of the *P1* gene and the *P1-rw1077* allele previously sequenced. Thanks to the high phlobaphenes content and the good nutritional value Nero Spinoso could be considered as functional foods, able to increase the amount of antioxidants introduced with the diet in fact their high antioxidant power suggests effects similar to the ones of the anthocyanins. Moreover, these molecules are thought to have an important role in plant’s resistance against different pathogens and are probably involved in flavonoids antifungal activity: phlobaphenes and other flavonoids are thought to harden maize pericarp, acting as a physical barrier against fungal infection and reducing the mycelial progress from a seed to another. With the aim of further dissecting the relationship between phlobaphenes accumulation and resistance to *Fusarium* infection in the paper “Phlobaphenes in maize kernel modify pericarp thickness and fumonisins accumulation” we analyzed maize seeds from 4 different genetic backgrounds characterized by the presence of high levels of phlobaphenes (Syn 1r, Syn 2r) in the pericarp and the corresponding colorless isogenic lines as controls (Syn 1c, Syn 2c). Moreover we used the pigmented variety Spinoso Nero di Esine della Val Camonica which can accumulate in the pericarp layer a high level of phlobaphenes and the weakly pigmented sub-population present at the frequency of 2.87% in this colored variety (Cassani et al., 2017). We named these two sub populations NSr (pigmented ears) and NSw (weakly pigmented ears). The data showed that in the flour of colorless varieties, mycotoxins’ content was higher than in the colored ones (Table 2); Syn1r (*P1*/*P1*) showed an average decrease of fumonisins content of 39.2% compared with the corresponding isogenic colorless population Syn1c (*p1*/*p1*). A similar decrease was observed for Syn2r (*P1*/*P1*) which showed an average decrease of 19% compared with the equivalent isogenic colorless population Syn2c (*p1*/*p1*).To further verify this hypothesis we quantified phlobaphenes, founding that the *P1* allele was responsible for a more than 10 fold increase of these pigments in colored lines (Syn1r, Syn2r and Nsr) in comparison with the colorless controls (Syn1c, Syn2c, Nsw). The histological analysis showed differences in pericarp thickness among colored and colorless lines. In particular the colorless lines (Syn1c = 80μm ±7; Syn2c = 66μm ± 4.18; Nsw= 128 μm ± 9.08) had a thinner pericarp if compared with equivalent isogenic colored lines (Syn1r = 132 μm ± 4.47; Syn2r

= 94 µm ± 9.61; Nsr = 290 µm ± 15.81). Taken together these data highlighted a strong correlation between pericarp thickness and phlobaphenes concentration. Considering the role of the *P1* gene in phlobaphenes accumulation and in pericarp thickness determination, these results suggested 3 different hypotheses: a) *P1* gene has a direct role in regulating pericarp thickness and color; b) pericarp thickness is a consequence of phlobaphenes accumulation driven by *P1* gene; c) there is a linkage drag between *P1* gene, regulating phlobaphenes accumulation, and the gene "X" playing a central role in regulating the pericarp thickness. The histological characterization of the Ac line, seems to suggest a direct involvement of the *P1* gene in both pericarp color and thickness and thus seems to exclude the third hypothesis. Our pre-breeding activity was carried out also in South Africa where maize is a staple food for the population (Table 1) that prefers white maize varieties, characterized by the lack of carotenoids contributing 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 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 of 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. In collaboration with the Vaal University of Technology we started a bio-fortification program using the best European varieties in order to increase the nutritional value of the white variety. We will try to improve protein, Fe and carotenoids amount, contributing in this way to tackle the problem of malnutrition in South African rural areas.

Country	Year	Food supply (kcal/capita/day)
United Rep. of Tanzania	2013	523
Paraguay	2013	551
Togo	2013	572
Burkina Faso	2013	596
Egypt	2013	596
Bosnia and Herzegovina	2013	631
Eswatini	2013	642
Nicaragua	2013	643
El Salvador	2013	659
Kenya	2013	663
Rep. of Moldova	2013	684
Honduras	2013	733
Zimbabwe	2013	743
Guatemala	2013	820
South Africa	2013	858
Mexico	2013	986
Zambia	2013	999
Malawi	2013	1125
Lesotho	2013	1379

Table 1. Food supply (kcal/capita/day) provided from corn in different countries. (FAOSTAT 2013)

Figure 1. Germplasm bank of the University of Milan located in Landriano (PV)

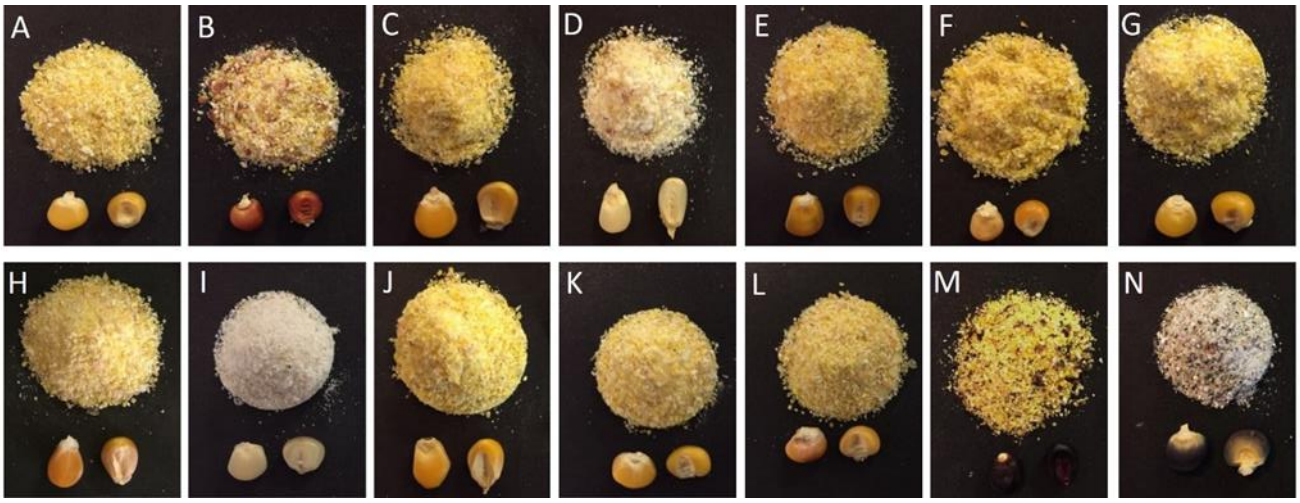

Figure 2. Panel of ancient varieties with seeds and flour (commonly used for the polenta production): Nostrano dell'isola (A), Pignoletto di Tortona (B), Cinquantino (C), Bianco Vitreo (D), Marano (E), Storo (F), Cinquantone (G), Scagliolo (H), Bianco Perla (I), Spinato di Gandino (H), Ottofile Tortonese (K), Ottofile (L), Nero Spinoso (M), Millo Corvo (N).

REFERENCES

Adom K, Liu R (2002). Antioxidant activity of grains. Journal of agricultural and food chemistry, 50(21), 6182-6187.

Anderson E, Cutler H (1942). Races of Zea mays: I. Their recognition and classification. Annals of the Missouri Botanical Garden, 29(2), 69-88.

Avila-Curiel A, Shamah-Levy T, Chávez-Villasana A. (1997). Encuesta Nacional de Alimentación y Nutrición en el Medio Rural, 1996. Resultados por entidad, 1.

Bai C, Twyman R, Farré G, Sanahuja G, Christou P, Capell T, Zhu C (2011). A golden era—pro-vitamin A enhancement in diverse crops. In Vitro Cellular & Developmental Biology-Plant, 47(2), 205-221.

Bouis H, Welch R (2010). Biofortification—a sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. Crop Science, 50(Supplement_1), S-20-S-32.

Brandolini A. (1958). Il germoplasma del mais e la sua conservazione. Maydica, 3, 4-14.

Brandolini A, Brandolini A (2009). Maize introduction, evolution and diffusion in Italy. Maydica, 54(2), 233.

Brown W, Darrah L (2002). Origin, adaptation, and types of corn, national corn handbook. Cooperative Extension Service. Iowa: Iowa State University. NCH-10

Cantaluppi E, Manzi S, Egal A, Puglisi D, Cassani E, Toschi I, Cesari VT, Landoni M, Scapin A, Pilu R (2017). Nutritional and phenotypical characterization of two South African maize (Zea mays L) varieties sampled in the Qwa-Qwa region. Maydica

Cassani E, Puglisi D, Cantaluppi E, Landoni M, Giupponi L, Giorgi A, Pilu R (2016) 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. Genetic Resources and Crop Evolution, 1-13.

Casas MI, Duarte S, Doseff AI, Grotewold E (2014) Flavone-rich maize: an opportunity to improve the nutritional value of an important commodity crop. Front Plant Sci 5:440. doi: 10.3389/fpls.2014.00440.

Das A, Singh V (2015). Antioxidative free and bound phenolic constituents in pericarp, germ and endosperm of Indian dent (Zea mays var. indentata) and flint (Zea mays var. indurata) maize. Journal of Functional Foods, 13, 363-374.

Dowswell C, Paliwal R, Cantrell R (1996). Maize in the third world. Boulder, CO, USA.

Eschholz T, Stamp P, Peter R, Leipner J, Hund A (2010). Genetic structure and history of Swiss maize (Zea mays L. ssp. mays) landraces. Genetic resources and crop evolution, 57(1), 71-84.

Escribano-Bailón M, Santos-Buelga C, Rivas-Gonzalo J (2004). Anthocyanins in cereals. Journal of Chromatography A, 1054(1), 129-141.

Faostat—Food and Agriculture Organization of the United Nations, 2014. http://www.faostat.fao.org. Accessed April 5, 2017.

Food and Agriculture Organization of the United Nations (FAO), 2004. UBET Unified Bioenergy Terminology. Available from http://www.fao.org/docrep/007/j4504e/j4504e00.HTM

Food and Agriculture Organization of the United Nations, 2009: Crops production. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID= 567 (last accessed on April 20, 2011).

Graham R, Rosser J (2000). Carotenoids in staple foods: their potential to improve human nutrition. Food and Nutrition Bulletin, 21(4), 404-409.

Grotewold E, Athma P, Peterson T (1991). Alternatively spliced products of the maize P gene encode proteins with homology to the DNA-binding domain of myb-like transcription factors. Proc. Natl. Acad. Sci. USA 88: 4587–4591.

Grotewold E, Drummond BJ, Bowen B, Peterson T (1994). The myb-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell. 76: 543-53.

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL (2000). Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor R. Proc Natl Acad Sci USA. 97: 3579-84.

Grotewold E (2006). The genetics and biochemistry of floral pigments. Annu. Rev. Plant Biol. 57:761-780

Gouse M, Pray C, Schimmelpfennig D, Kirsten J (2006). Three seasons of subsistence insect-resistant maize in South Africa: have smallholders benefited.

Kuhnen S, Lemos P, Campestrini L, Ogliari J, Dias P, Maraschin M (2009). Antiangiogenic properties of carotenoids: a potential role of maize as functional food. Journal of Functional Foods, 1(3), 284-290.

Kuhnen S, Menel Lemos P, Campestrini L, Ogliari J, Dias P, Maraschin M (2011). Carotenoid and anthocyanin contents of grains of Brazilian maize landraces. Journal of the Science of Food and Agriculture, 91(9), 1548-1553.

Lago C, Landoni M, Cassani E, Cantaluppi E, Doria E, Nielsen E, Giorgi A, Pilu R (2015). Study and characterization of an ancient European flint white maize rich in anthocyanins: Millo Corvo from Galicia. PloS one, 10(5), e0126521.

Liu K, Goodman M, Muse S, Smith J, Buckler E, Doebley J (2003). Genetic structure and diversity among maize inbred lines as inferred from DNA microsatellites. Genetics, 165(4), 2117

Lopez-Martinez L, Oliart-Ros R, Valerio-Alfaro G, Lee C, Parkin K, Garcia H (2009). Antioxidant activity, phenolic compounds and anthocyanins content of eighteen strains of Mexican maize. LWT-Food Science and Technology, 42(6), 1187-1192.

Mellado-Ortega E, Hornero-Méndez D (2015). Carotenoids in cereals: an ancient resource with present and future applications. Phytochemistry Reviews, 14(6), 873-890.

Messedaglia L (1924). Notizie storiche sul mais: Una gloria veneta. Saggio di storia agraria. Quaderno mensile No. 7. Sez. Credito Agrario Istituto Federale Credito del Risorgimento delle Venezie, Verona, Italy. Premiate officine grafiche C. Ferrari-2128.

Messias R, Galli V, Silva S, Schirmer M, Rombaldi C (2015). Micronutrient and functional compounds biofortification of maize grains. Critical reviews in food science and nutrition, 55(1), 123-139.

Mir C, Zerjal T, Combes V, Dumas F, Madur D, Bedoya C, Dreisigacker S, Franco J, Grudloyma P, Hao P, Hearne S, Jampatong C, Laloe D, Muthamia Z, Nguyen T, Prasanna B, Taba S, Xie C, Yunus M, Zhang S, Warburton M, Charcosset A (2013). Out of America: tracing the genetic footprints of the global diffusion of maize. Theoretical and applied genetics, 126(11), 2671-2682.

Nuss E, Tanumihardjo S (2011). Quality protein maize for Africa: closing the protein inadequacy gap in vulnerable populations. Advances in Nutrition: An International Review Journal, 2(3), 217-224.

Oldewage-Theron W, Dicks E, Napier C, Rutengwe R (2005). Situation analysis of an informal settlement in the Vaal Triangle. Development Southern Africa, 22(1), 13-26.

Ortiz-Monasterio J, Palacios-Rojas N, Meng E, Pixley K, Trethowan R, Pena R (2007). Enhancing the mineral and vitamin content of wheat and maize through plant breeding. Journal of Cereal Science, 46(3), 293-307.

Panzeri D, Cesari V, Toschi I, Pilu R (2011). Seed calorific value in different maize genotypes. Energy Sources Part A-Recovery 33(18): 1700-1705

Petroni K, Pilu R, Tonelli C (2014). Anthocyanins in corn: a wealth of genes for human health. Planta, 240(5), 901-911.

Pfeiffer W, McClafferty B (2007). HarvestPlus: breeding crops for better nutrition. Crop Science, 47(Supplement_3), S-88.

Pilu R, Cassani E, Sirizzotti A, Petroni K, Tonelli C (2011). Effect of flavonoid pigments on the accumulation of fumonisin B1 in the maize kernel. J. Appl. Genetics 52:145-52.

Piperno D, Ranere A, Holst I, Iriarte J, Dickau R (2009). Starch grain and phytolith evidence for early ninth millennium BP maize from the Central Balsas River Valley, Mexico. Proceedings of the National Academy of Sciences, 106(13), 5019-5024.

Ranum P, Peña-Rosas J, Garcia-Casal M (2014). Global maize production, utilization, and consumption. Annals of the New York Academy of Sciences, 1312(1), 105-112.

Rodriguez-Amaya D, Kimura M, Godoy H, Amaya-Farfan J (2008). Updated Brazilian database on food carotenoids: Factors affecting carotenoid composition. Journal of Food Composition and Analysis, 21(6), 445-463.

Sampietro D, Fauguel C, Vattuone M, Presello D, Catalán C (2013). Phenylpropanoids from maize pericarp: Resistance factors to kernel infection and fumonisin accumulation by *Fusarium verticillioides*. Eur. J. Plant Pathol. 135:105-113.

Sharma M, Chai C, Morohashi K, Grotewold E, Snook ME, Chopra S (2012). Expression of flavonoid 3′-hydroxylase is controlled by *P1*, the regulator of 3-deoxyflavonoid biosynthesis in maize. BMC Plant Biol. 12, 196.

Treutter D (2006). Significance of Flavonoids in Plant Resistance A Review. Environmental Chemistry Letters, 4, 147-157.

Van Heerwaarden J, Doebley J, Briggs W, Glaubitz J, Goodman M, Gonzalez J, Ross-Ibarra J (2011). Genetic signals of origin, spread, and introgression in a large sample of maize landraces. Proceedings of the National Academy of Sciences, 108(3), 1088-1092.

Venturini G, Babazadeh L, Casati P, Pilu R, Salomoni D, Toffolatti S (2016). Assessing pigmented pericarp of maize kernels as possible source of resistance to fusarium ear rot, Fusarium spp. infection and fumonisin accumulation. International Journal of Food Microbiology, 227, 56-62.

Vigouroux Y, Glaubitz J, Matsuoka Y, Goodman M, Sánchez J, Doebley J (2008). Population structure and genetic diversity of New World maize races assessed by DNA microsatellites. American Journal of Botany, 95(10), 1240-1253.

Villalpando S (2004). Tortilla fortification working group meeting. El problema de la biodisponibildad de hierro en harina de maiz nixtamalizada. National Institute of Public Health: Mexico City.

Warburton M, Reif J, Frisch M, Bohn M, Bedoya C, Xia X, Crossa J, Franco J, Hoisington D, Pixley K, Taba S, Melchinger A (2008). Genetic diversity in CIMMYT non-temperate maize germplasm: landraces, open pollinated varieties, and inbred lines. Crop Science, 48(2), 617-624.

Zeppa G, Bertolino M, and L Rolle (2012). Quantitative descriptive analysis of Italian polenta produced with different corn cultivars. J. Sci. Food Agr. 92, 412—417.

Traditional farmers' varieties: a valuable source of genetic variability for biofortification programs

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This is a pre-copy-editing, author-produced of an article submitted for publication in *Maydica*.

Maydica

Accepted: July 2018

ISSN: 2279-8013

ABSTRACT

Several studies underlined the superiority from a nutritional point of view of ancient varieties. In the last years the interest in landraces has been growing, for this reason preservation and valorisation of these genetic sources is very important. In particular these varieties are source of precious genetic variability interesting from a scientific point of view to preserve biodiversity but also for biofortification programs aimed to support small rural communities, where the particular maize germplasm has been developed. In this work we characterized from the nutritional point of view 13 ancient Italian varieties and one coming from Spain (Millo Corvo). In this pre-breeding work we demonstrate the nutritional superiority of ancient varieties if paragoned with modern hybrids. In particular Spinato di Gandino is the best variety for milling properties and for oil, protein, and total phosphorus content; Storo is the best variety for calorific value and for carotenoids and free phosphorus content. Using these varieties in the next future we will start a bio-fortification program aimed to obtain new populations with improved yields and high nutritional value.

INTRODUCTION

Maize (*Zea mays* L. ssp. *mays*) is the main cereal grain cultivated worldwide. It is responsible for providing 15% of the protein and 20% of the calories in the human diet, supplying an energy density of 365 Kcal/100 g and covering a cultivated area of 184.8 million hectares in 2014 (Faostate 2014, Food and Agriculture Organization of the United Nations, Crops Production 2009, Zeppa et al., 2012). Corn can be used in several ways as feed for live-stock, forage, silage and grain, but also for industrial uses. However, human nutrition remains one of the main uses, determining the selection of varieties for producing many typical dishes such as polenta in Italy and pap in South Africa (Zeppa et al., 2012; Cantaluppi et al., 2017).

Maize has a matrix rich in organic compounds and minerals with potential benefits to health. These compounds may in fact act as antioxidants (carotenoids and phenolic compounds), as cofactors for antioxidant enzymes (selenium, zinc) or as indirect antioxidants (betaine, choline, and folate) complementing those found in fruits and vegetables (Hansch and Mendel 2009; Liu- 2007). Moreover, consumption of whole grains is preventative against cardiovascular disease, some types of cancer, type 2 diabetes, and obesity (Messias et al., 2015).

Thus, while productivity remains the major target for breeders, focusing on grain quality could reduce deficiencies of some minerals and pro-vitamin A increasing the concentration of functional compounds and the nutritional value, and this will be of particular importance especially for poor populations that use maize as staple food (Messias et al., 2015).

Maize was domesticated about 8,700 BP in Mexico and from there it spread within the Americas (Piperno et al., 2009; van Heerwaarden et al. 2011).

After the discovery of the Americas by Europeans, three main maize sources (corn from the American east coast with higher latitude adaptation, the photoperiod insensitive CATETO types and the Pearl White) played an essential role for the adaptation of maize to Europe (Brandolini and Brandolini et al., 2009; Eschholz et al., 2010).

In Europe, the spread of maize 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; Brandolini and Brandolini 2009).

In Italy, the first reports regarding the utilization of corn date from 1600 in the North East where it was adapted to the climatic zones of cultivation (Brandolini 1958; Brandolini and

Brandolini 2009). The hybridization during cultivation of these corn sources in different environments, led to the establishment of many local varieties that under different photoperiod, temperature, humidity and altitude allowed the constitution and the differentiation of local European varieties and landraces (Brandolini and Brandolini et al., 2009; Eschholz et al., 2010). During this process the work of selection was kept by farmers, in fact they maintained 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 alleles with high adaptation to the local environments (Lago et., al 2015).

After World War II the introduction of mechanized farming practices and the utilization of dent hybrids which were much more productive, mainly for animal feed, led to the gradual disappearance of local varieties and thus to the loss of the valuable alleles they contained (Brandolini and Brandolini 2009). Fortunately, in more recent years many efforts have been made to recover and preserve the genotypes of the old varieties: in Italy the main maize collections are preserved *ex situ* at CREA Research Centre for Cereal and Industrial Crops located in Bergamo, and in the germplasm bank of the University of Milan located in Landriano (PV).

In this work, after a pre-breeding activity, we studied the nutritional value of fourteen traditional varieties typically used for the production of polenta, comparing these varieties with modern hybrids to find those with valuable properties that could be part of future bio-fortification breeding programs. In particular we assessed their nutritional value for several parameters (calorific value, oil, protein, mineral nutrients and carotenoids content and the repartition between free and total P).

Our results led us to plan a breeding program aimed to obtain neo-synthesis populations with increased nutritional properties for the production of polenta and others typical dishes.

MATERIALS AND METHODS

Plant Material

We collected 14 ancient varieties: Nostrano dell'Isola, Pignoletto di Tortona, Cinquantino, Bianco Vitreo, Marano, Storo, Cinquantone, Scagliolo, Bianco Perla, Spinato di Gandino, Ottofile Tortonese and Ottofile, maintained in the gene bank of the

University of Milan located in Landriano (PV), Italy (N 45°180, E 9°150), Nero Spinoso and Millo Corvo sampled directly from the farmers, in Spain and at Esine (Italy) respectively (Fig. 1).

For all the genotypes tested we performed three cycles of massal selection (seasons 2014-2015 and 2016): about 200 seeds were sown in adjacent rows, under the same agronomic conditions, with B73/MO17 as control. The analyses were performed on the materials harvested on 2017: about 70 ears of each variety were shelled and the seeds obtained mixed to create a single bulk used for the determination of the nutritional value.

Conservation and propagation of local varieties in open field

To preserve local varieties, we performed controlled pollination to maintain the mechanic isolation of these varieties. Using paper bags, we performed sib pool pollination taking pollen from several plants and bulking it (Fig. 2). Then we put the bulked pollen on the ears of different plants of the same population

Milling

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

Dry seed weight

Dry seed weight was calculated weighing 50 seeds per genotype in three replicates, after drying for 48 h at 70 °C.

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

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 free phosphorus in seeds

50 mg seed flour were extracted 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 0.62, 1.24, 2.48, 3.72 µg/mL atomic P 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.

Determination of ionomic content (P tot, 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 (72Ge, 89Y, 159Tb) 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 Ca, Fe, Zn were expressed as µg/g seed flour; values regarding P were indicated as mg/g seed flour.

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 collect data, SPSS® was used to perform one-way ANOVA on sampled data and SIGMA PLOT® was used to obtain graphs.

RESULTS AND DISCUSSION

In Italy the use of corn in agriculture dates back to the second half of the sixteenth century, since when the adaptation to different environments together with human selection led to the diversification of hundreds of landraces (Messedaglia 1924; Brandolini and Brandolini 2009).

These varieties were characterized by low yields, when compared with modern hybrids, but had considerable phenotypic and genetic variability that led to yield stability (Liu et al. 2003; Vigoroux et al. 2008; Warburton et al. 2008; Mir et al. 2013). At the end of the II World War these farmer's varieties were replaced by modern hybrids. Nowadays a renewed interest for these traditional varieties is growing, determining the selection of varieties for producing many typical dishes such as polenta in Italy and pap in South Africa (Zeppa et al. 2012, Cantaluppi et al. 2017). Moreover, maize is really important in our diet, in particular for the health benefits due to the ingestion of a matrix rich in organic compounds and minerals (Messias et al., 2015; Hansch and Mendel 2009, Liu 2007). Thus, while productivity remains the major target for breeders, in the future it will be important to focus on grain quality. (Messias et al., 2015). For these reasons the study of landraces genetic diversity is a prerequisite for efficient conservation and management and

effective use of landraces in breeding programmes (Newell-Mcgloughlin et al., 2008; Cakmak, 2008; Newton et al.,2009).

To pursue this aim we collected 12 local farmers varieties from CREA in Bergamo and sampled two varieties directly from farmers (Millo Corvo in Spain and Nero Spinoso at Esine, Italy) (Fig. 1). With the aim to preserve and to standardize, from the genetic point of view, the material stored in the gene bank of the University of Milan we performed three cycles of massal selection (2014, 2015 and 2016). We sowed 200 plants for each genotype and among them in every agronomic season we selected the best 15 phenotypes. The varieties were maintained in isolation by using paper bags and performing sib pool pollination to avoid cross contamination (Fig. 2).

During the harvest season we selected the 40 best ears both for yield and phenotype and bulked their grains, storing all these varieties in the germplasm bank at 4 °C and 30% of moisture.

At the end of the selection's process, in 2017, the seeds obtained from about 70 ears of each variety were shelled and mixed to create a single bulk which was used for the determination of nutritional value.

The protein quantification indicated that the protein amount in traditional varieties is higher (10.65 % ± 1.18) than in modern hybrids (9.48 % ± 0.93) with the highest value in Spinato di Gandino (12.98 % ± 0.19), Nostrano dell'Isola (12.35% $\pm$ 0.19) and Cinquantone (12.35 % ± 0.27) (Fig. 3A). These results confirm the data from Berta et al (2014) that reported a higher protein content in the Italian variety Ostiglia (9.5 g 100 g^{-1}). Calorific values for different substances and biomasses are well known. For example, wood and cereal straw range from 17 to 19 KJ/g and wheat grain 17 KJ/g (FAO, 2004; Panzeri et al., 2011). Panzeri et al. (2011) found that among the genotypes expressing normal seed phenotypes, the highest calorific values was in 'Scagliolo' that is a traditional population selected for human nutritional purposes. Analysing our data we can confirm this thesis, as we found the highest values in ancient varieties (19052 J/g ± 156.6) with the best performances of Storo (19334 J/g ± 31.51) and Cinquantino (19326 J/g ± 22.47) (Fig. 3B).

Oil is confined for the major part in the germ, about 85% of the total kernel oil; the rest is contained in the endosperm. Normal maize provides around 2-6% oil, but some high-oil maize contains more than 6% of oil providing a valuable product because of its low levels of saturated fatty acid (i.e. 11% palmitic acid and 2% stearic acid) (Lambert et al., 1997). Corn oil is also recognized as an excellent source of tocopherols that are important antioxidants and a source of Vitamin E (Dormann 2003). Screening our traditional varieties

we saw that also for this parameter the average value (5.81% ±0.98) is higher than in B73/MO17 hybrids (Fig. 3C). Also in this case Spinato di Gandino gave the best performances (7.22 % ± 0.14). These results are in agreement with those found from Panzeri et al. (2011), proving that traditional varieties could be a valuable source of genetic variation to increase oil in the kernel and that Spinato di Gandino could be considered as a high oil variety. It's well known that also seed weight is an important parameter for the milling industry; in fact maize is dry milled to obtain grit, meal and flour and wet milled to produce starch and other valuable by-products such as gluten, germ and bran. From this point of view the best varieties are: Bianco Perla (0.36 g ± 0.05), Spinato di Gandino (0.33 g ± 0.02) and Ottofile Tortonese (0.33g± 0.04) (Fig. 3D). Spinato di Gandino is among the best varieties also for the total phosphorus (4.58 mg g^{-1}±0.13) (Fig. 4A). In poor countries free phosphorus deficiencies are a relevant topic for this reason a geneticist's goal is the reduction in phytic acid content in seeds and the corresponding increase in the level of free phosphorus (Cerino et al., 2010). We analysed the traditional varieties for the free phosphorus, looking for varieties that naturally accumulate high amounts of this element, finding the highest concentration in Storo (0.28 mg g^{-1} ±0.01) (Fig. 4B). Storo proves to be a good variety also for a bio-fortification purpose regarding carotenoids amount in the kernel (33.95 µg g^{-1}) (Fig. 4C) , in fact it is known that globally one third of preschool-age children and 15% of pregnant women are estimated to be vitamin A deficient (Sommer 1982; WHO 2009). Maize presents a broad natural variation for kernel carotenoids, with the best genotypes accumulating 66.0 µg g^{-1} (Harjes et al., 2008). Moreover, we found a significant negative relation between embryo's dimensions and carotenoids content ($R = -0.697$; $P < 0.05$) (Fig. 5B) finding that white varieties have bigger embryo than yellow ones, supporting the thesis of a strong selection of white varieties for human consumption (Cantaluppi et al. 2017). Several cycles of selection of white varieties, for high oil and calorific value, provoked an increase of embryo's dimensions. In developing countries mineral malnutrition is an important topic in particular iron deficiency is the most common and widespread nutritional disorder in the world. Iron deficiency can retard mental development and learning capacity and impair physical growth (Bouis 2002). Among our varieties Nostrano dell'Isola has the highest amount of iron (31.05 µg g^{-1}) (Fig. 4E); even overtaking the common hybrid B73/Mo17 (22.93 µg g^{-1} ±0.57) and showing to be a valuable source for a breeding program pointed towards an iron increase. Deficiency of zinc, likewise to iron one, lowers the intestinal absorption of fat and fat-soluble vitamins including retinol (Ahn and Koo 1995). Maize

contains an average of 20 μg g^{-1} zinc, corresponding to approximately 40% of the requirement for non-pregnant women and infants after breast feeding (Cakmak, 2008). Among the varieties in our study Cinquantone (36.53 μg g^{-1}) (Fig. 4F) gave the highest amounts of zinc being the candidate variety for future breeding programs. Moreover in our study we found a significant correlation between iron and zinc content in the kernel ($R = 0.581$; $P < 0.05$) (Fig. 5A), we could therefore imagine simultaneous increases of both elements in a breeding activity pointed to increase mineral amount in the grain. This result is in agreement with Welch and Graham 2002 who found a tight correlation between iron and zinc concentration in wheat. Calcium, like zinc, is an important cofactor and molecular signal and is essential in the blood coagulation cascade (Relea et al., 1995). Cinquantone (53.98 μg g^{-1}±1.73), Bianco Vitreo (56.23 μg g^{-1}±1.15) and Marano (58.65 μg g^{-1}±2.81) respectively gave the best results for what concern this element exceeding B73/Mo17 (41.46 μg g^{-1} ±2.66) (Fig.4D).

In conclusion, we demonstrated, from a nutritional point of view, that traditional varieties could be used in breeding programs aimed to bio-fortify maize for different important nutritional elements. From these varieties it could be possible to start a bio-fortification program aimed to obtain new synthesis populations with high nutritional value. The aim of these actions will be oriented to: i) a reduction of nutrient deficiencies in poor countries with a program of participatory plant breeding, and ii) an increase of the added value of the final products by increasing their nutritional characteristics.

FIGURES

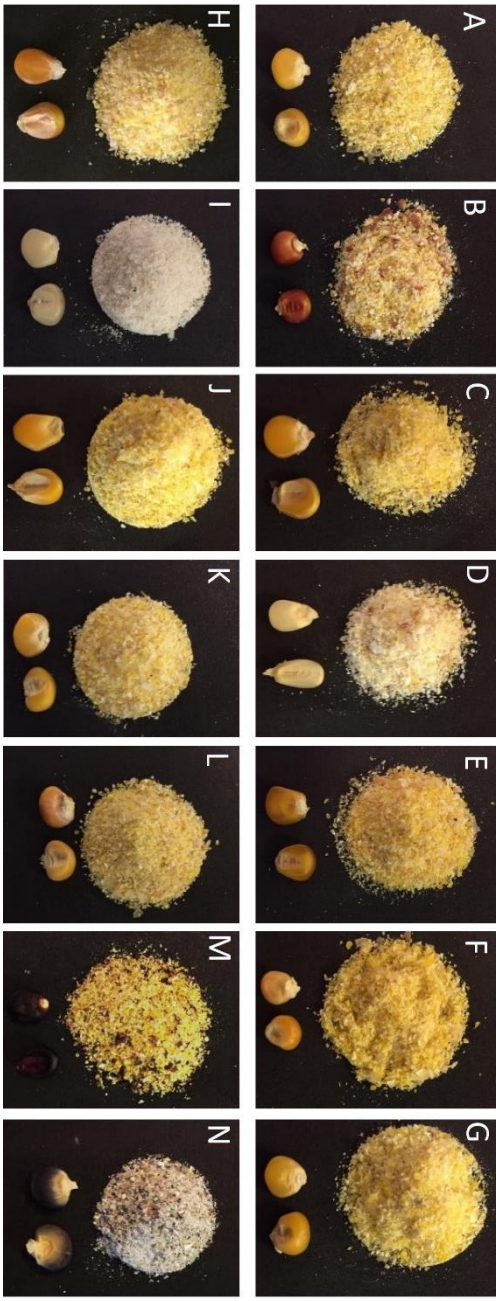

Figure 1. Panel of ancient varieties with seeds and flour (commonly used for the polenta production): Nostrano dell'isola (A), Pignoletto di Tortona (B), Cinquantino (C), Bianco Vitreo (D), Marano (E), Storo (F), Cinquantone (G), Scagliolo (H), Bianco Perla (I), Spinato di Gandino (H), Ottofile Tortonese (K), Ottofile (L), Nero Spinoso (M), Millo Corvo (N).

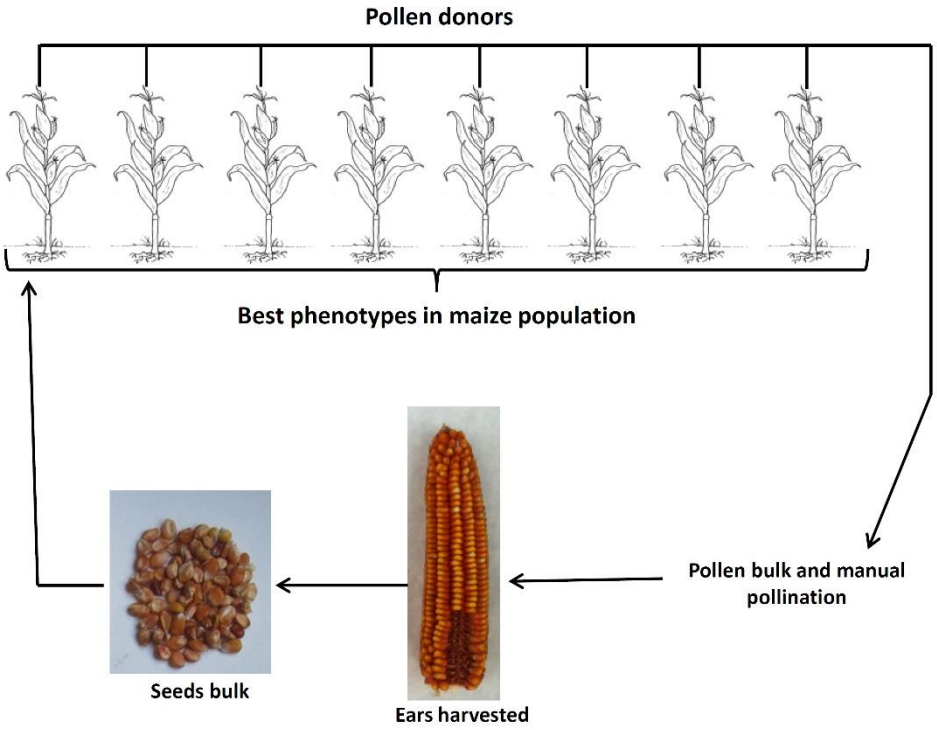

Figure 2. Scheme of the sib pool pollination of maize ancient varieties used for the conservation and preservation of fourteen ancient varieties

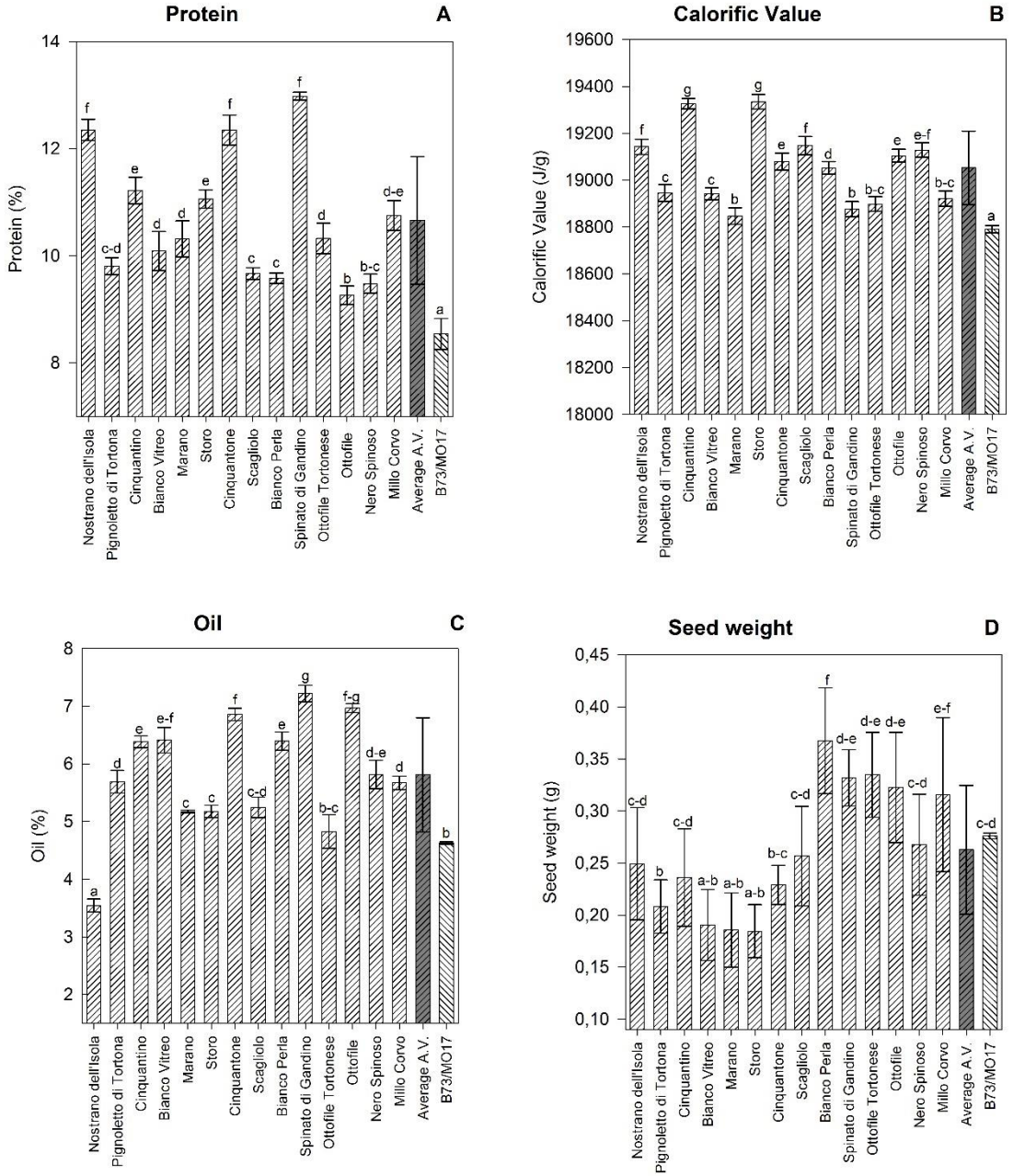

Figure 3. Main results of bromatological analysis performed on maize flour: Protein (A); Calorific Value (B); Oil (C) and Dry Seed Weight (D). Division in homogenous groups is the result of the Tuckey test, performed as a post hoc test after one way Anova (p = 0.05).

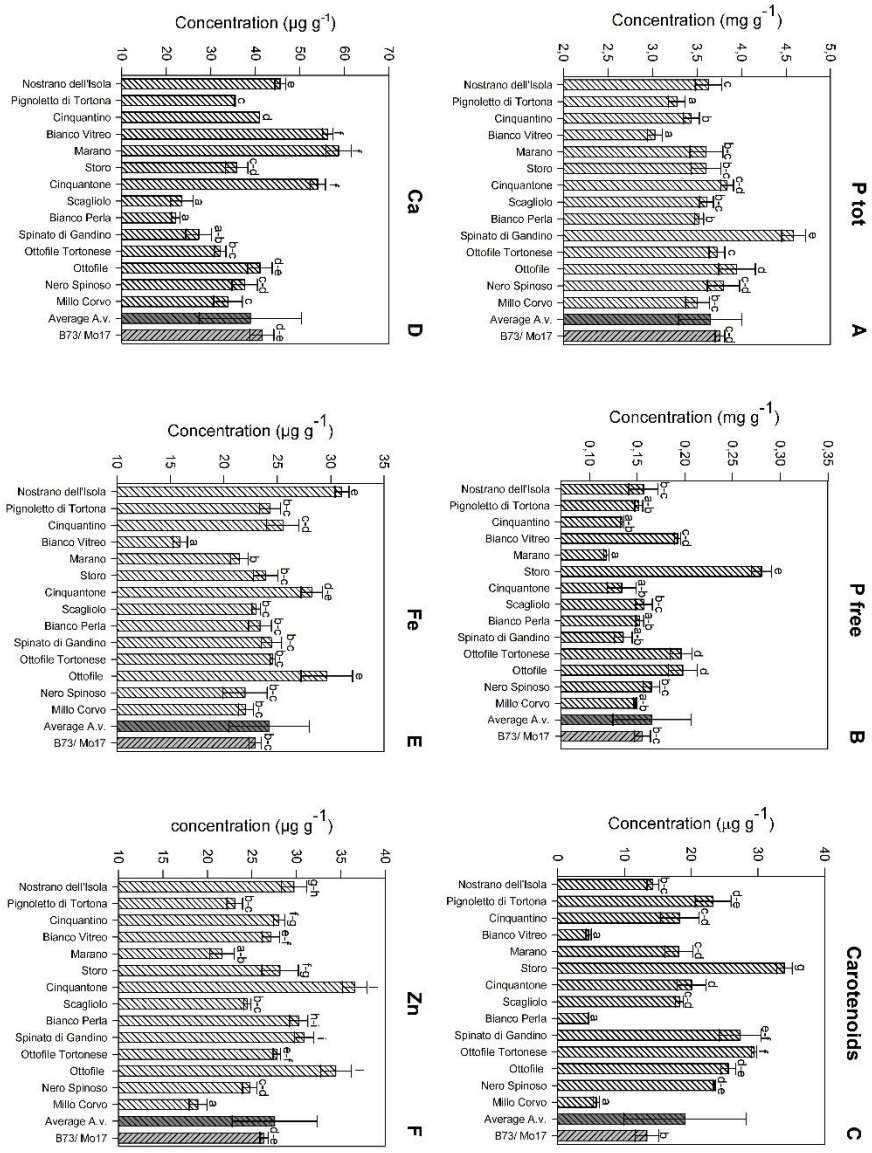

Figure 4. Mineral Nutrient and the Trace Element Content Determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS): P tot (A); Carotenoids (C); Ca (D); Fe (E) and Zn (F). Analysis performed on Maize flour. P free (B) was determined using Chen assay. Division in homogenous groups is the result of the Tuckey test, performed as a post hoc test after one-way Anova (p = 0.05).

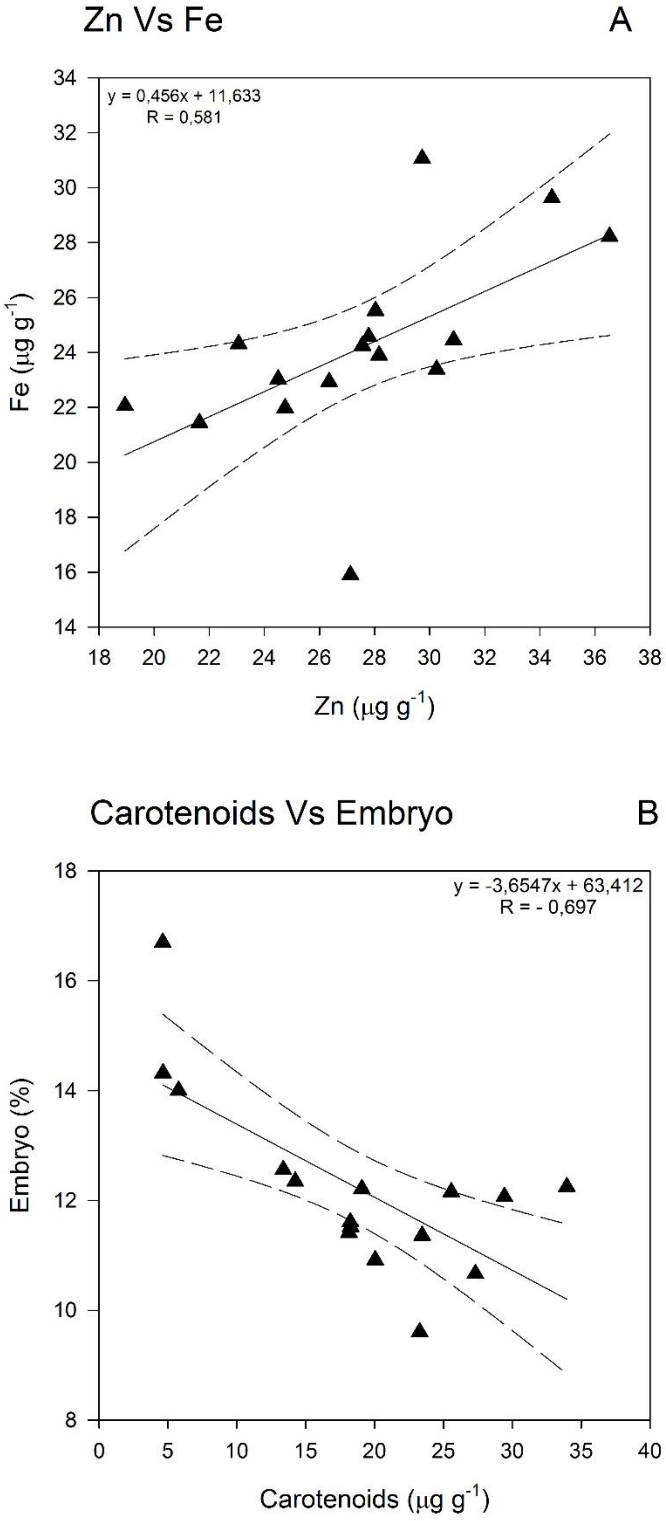

Figure 5. Significant correlation among nutrient in ancient Maize varieties: Correlation Zn vs Fe (A), Correlation Carotenoids Vs Embryo (B). The r showed in both graphs is the Pearson coefficient.

REFERENCES

Ahn J, Koo SI, (1995). Effects of zinc, and essential fatty acid deficiencies on the lymphatic absorption of Vitamin A and secretion of phospholipids. J Nutr Biochem 6:595-603

Anderson E. and Cutler HC, (1942). Races of *Zea mays*: I. Their recognition and classification. Ann. Mo. Bot. Gard. 21:69–88.

Brandolini A, (1958). Il germoplasma del mais e la sua conservazione. Maydica, 3, 4-14

Brandolini A, Brandolini A (2009). Maize introduction, evolution and diffusion in Italy. Maydica 54: 233–242.

Bouis EH, (2002). Plant breeding: a new tool for fighting micronutrient malnutrition. J Nutr 132:491S-494S

Cantaluppi E, Manzi S, Egal A, Puglisi D, Cassani E, Toschi I, Cesari VT, Landoni M, Scapin A, Pilu R, (2017). Nutritional and phenotypical characterization of two South African maize (Zea mays L) varieties sampled in the Qwa-Qwa region. Maydica

Cakmak, I, (2008). Enrichment of cereal grains with zinc: Agronomic or genetic biofortification Plant Soil. 302:1–17.

Cerino F, Cassani E, Landoni M, Doria E, Panzeri D, Lago C, Mesiti F, Nielsen E, Pilu R, (2010). The low phytic acid1-241 (lpa1-241) maize mutation alters the accumulation of anthocyanin pigment in the kernel. Planta 231:1189–1199

Dormann P, (2003). Corn with enhanced anti-oxidant potential Nat Biotechnol 21:1015-1016

Eschholz TW, Stamp P, Peter R, Leipner J, Hund A, (2010). Genetic structure and history of Swiss maize (*Zea mays* L. ssp. *mays*) landraces. Genet Resour Crop Evol 57: 71–84.

Faostat—Food And Agriculture Organization of the United Nations, (2014). http://www.faostat.fao.org. Accessed April 5, 2017.

Food and Agriculture Organization of the United Nations (FAO), (2004). UBET Unified Bioenergy Terminology. Available from http://www.fao.org/docrep/007/j4504e/j4504e00.HTM

Food and Agriculture Organization of the United Nations, (2009): Crops production. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID= 567 (last accessed on April 20, 2011).

Hänsch R and Mendel RR, (2009). Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Curr. Opin. Plant. Biol. 12:259–266.

Harjes CE, Rocheford TR, Bai L, Brutnell TP, Kandianis CB, Sowinski SG, Stapleton AE, Vallabhaneni R, Williams M, Wurtzel ET, Yan JB, Buckler ES, (2008). Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. Science 319:300-333

Lago C, Landoni M, Cassani E, Cantaluppi E, Doria E, Nielsen E, Giorgi A, Pilu R, (2015). Study and characterization of an ancient European flint white maize rich in anthocyanins: Millo Corvo from Galicia. PloS One 10(5): e0126521 DOI: 10.1371/journal.pone.0126521.

Lambert RJ, Alexander DE, Mollring EL, Wiggens B, (1997). Selection for increased oil concentration in maize kernels and associated changes in several kernel traits. Maydica 42:39–43.

Liu K, Goodman M, Muse S, Smith JS, Buckler E, Doebley J, (2003). Genetic structure and diversity among maize inbred lines as inferred from DNA microsatellites. Genetics 165:2117-2128.

Liu RH, (2007). Whole grain phytochemicals and health. J. Cereal Sci. 46:207–219.

Messedaglia L, (1924). Notizie storiche sul mais. Quaderno mensile N°7. Sez. Credito Agrario Istituto Federale Credito del Risorgimento delle Venezie, Verona, Italy.

Messias RDS, Galli V, Silva SDDAE, Schirmer MA, Rombaldi CV, (2015). Micronutrient and functional compounds biofortification of maize grains. Crit. Rev. Food Sci. Nutr. 55, 123–39. doi:10.1080/10408398.2011.649314

Mir C, Zerjal T, Combes V, Dumas F, Madur D, Bedoya C, Dreisigacker S, Franco J, Grudloyma P, Hao PX, Hearne S, Jampatong C, Laloë D, Muthamia Z, Nguyen T, Prasanna BM, Taba S, Xie CX, Yunus M, Zhang S, Warburton ML, Charcosset A, (2013). Out of America: tracing the genetic footprints of the global diffusion of maize. Theor Appl Genet 126:2671-2682.

Newton AC, Akar T, Baresel JP, Bebeli PJ, Bettencourt E, Bladenopoulos KV, Czembor JH, Fasoula DA, Katsiotis A, Koutis K, Koutsika-Sotiriou M, Kovacs G, Larsson H, De Carvalho MAAP, Rubiales D, Russell J, Santos TMM, Patto MCV, (2009). Cereal landraces for sustainable agriculture. Sustain. Agric. 2, 147–186. doi:10.1007/978-94-007-0394-0_10

Panzeri D, Cesari V, Toschi I, Pilu R, (2011). Seed calorific value in different maize genotypes. Energy Sources Part A-Recovery 33(18): 1700-1705

Piperno DR, Ranere AJ, Holst I, Iriarte J, Dickau R, (2009). Starch grain and phytolith evidence for early ninth millennium B.P. maize from the Central Balsas River Valley, Mexico. Proc. Natl. Acad. Sci. U. S. A. 106: 5019-5024. Doi: 10.1073/pnas.0812525106 PMID: 19307570.

Relea P, Revilla M, Ripoll E, Arribas I, Villa LF, Rico H, (1995). Zinc, biochemical markers of nutrition, and type I osteoporosis. Age Ageing. 24:303–307.

Sommer A, (1982). Nutritional blindness: xerophthalmia and keratomalacia. Oxford University Press, New York

Van Heerwaarden J, Doebley J, Briggs W, Glaubitz CJ, Goodman M, Gonzalez JDS, Ross-Ibarra J, (2011). Genetic signals of origin, spread, and introgression in a large sample of a maize landraces. Proc. Natl. Acad. Sci. U. S. A. 108: 1088-1092. Doi: 10.1073/pnas.1013011108 PMID: 21189301.

Warburton ML, Reif JC, Frisch M, Bohn M., Bedoya C, Xia XC, Crossa J, Franco J, Hoisington D, Pixley K, Taba S, Melchinger AE, (2008). Genetic diversity in CIMMYT non temperate maize germplasm: landraces, open pollinated varieties, and inbred lines. Crop Sci 48(2):617-624 doi:10.2135/cropsci2007.02.0103.

Welch RM, Graham RD, (2002). Breeding crops for enhanced mineral content. Plant and Soil 245: 205-214.

WHO, (2009). Global prevalence of vitamin A deficiency in populations at risk 1995-2005. In: WHO global database on vitamin A deficiency, World Health Organization, Geneva, pp 1-55

Zeppa G, Bertolino M, and L Rolle, (2012). Quantitative descriptive analysis of Italian polenta produced with different corn cultivars. J. Sci. Food Agr. 92, 412—41

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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Genetic Resources and Crop Evolution

Available online: April 2016

DOI 10.1007/s10722-016-0399-7

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 programs.

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 points 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 ears 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 safer 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.

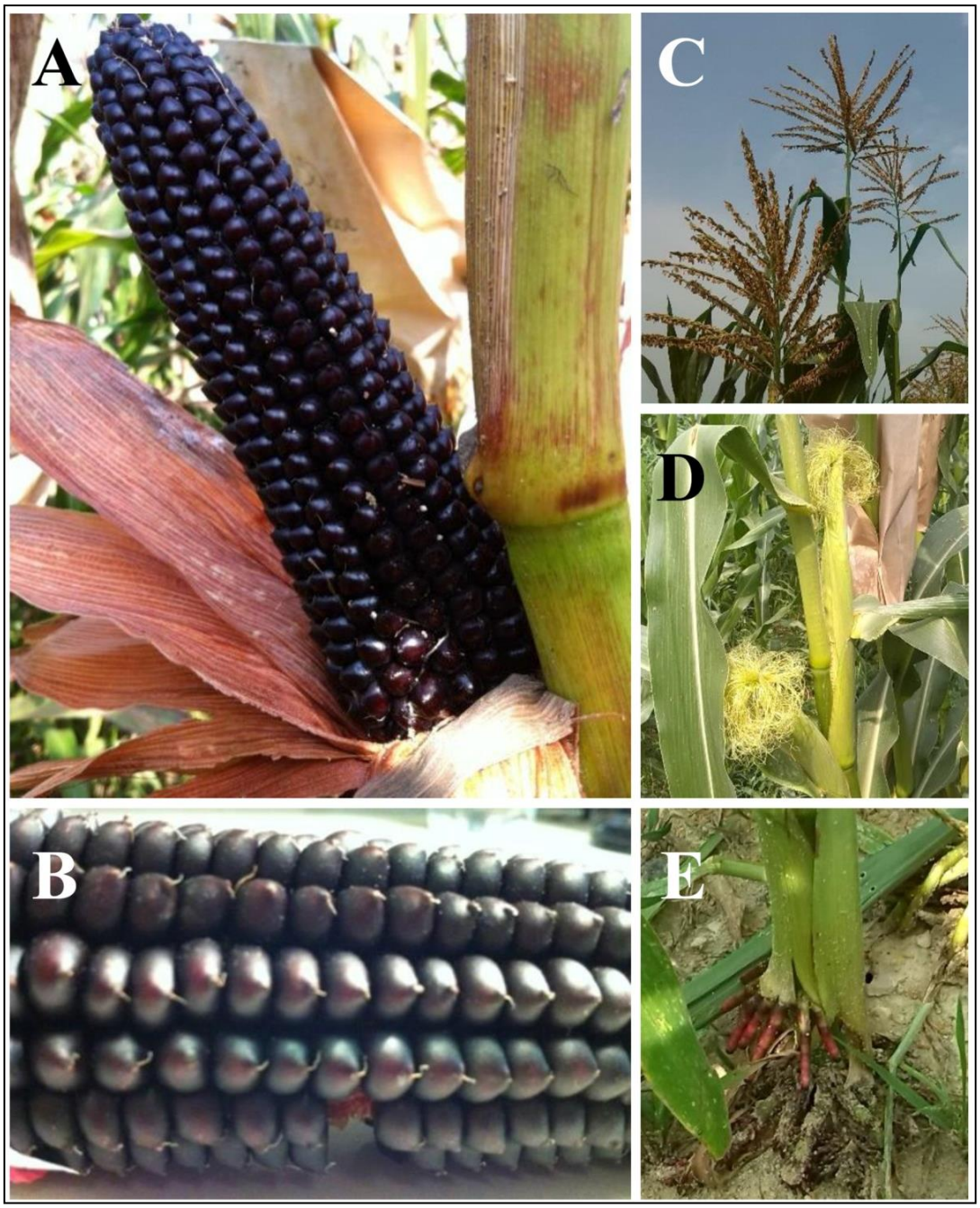

Figure 2. Phenotype of the "Nero Spinoso" maize cultivar. (A) Dark red/black ear at maturity and (B) the characteristic pointed kernels, (C) tassel and (D) immature ears with silk, (E) pigmented roots.

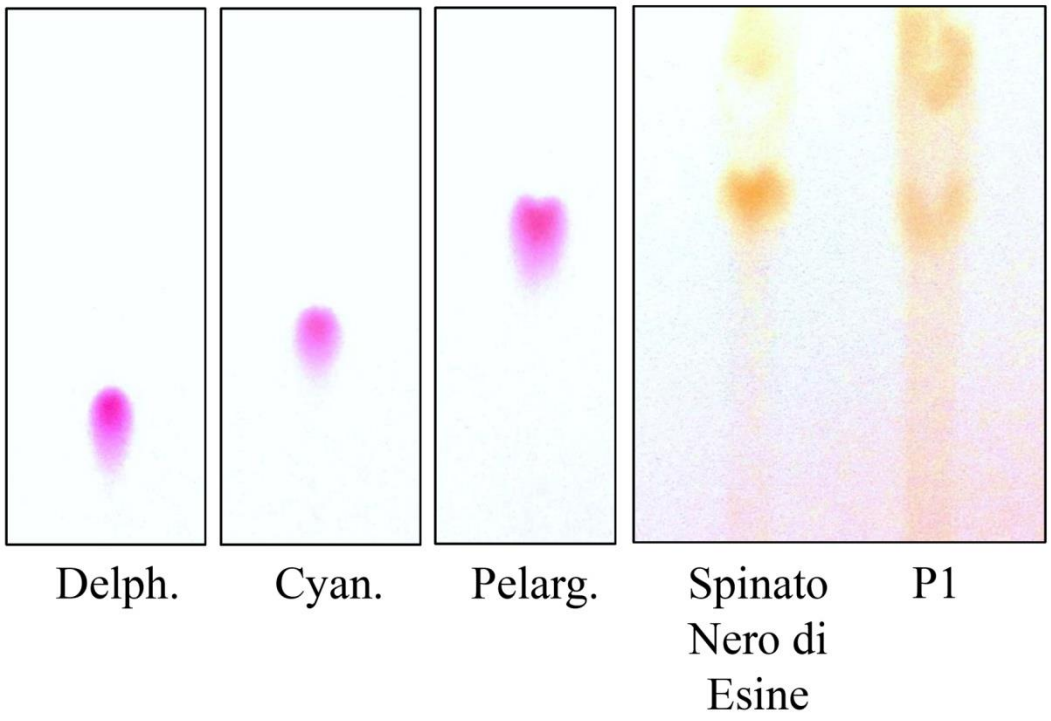

Figure 3. TLC analysis of "Nero Spinoso" compared to a colored *P1* homozygous variety. The standards used for the TLC analysis were: cyanidin (cyan.), delphinidin (delph.) and pelargonidin (pelarg.).

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.

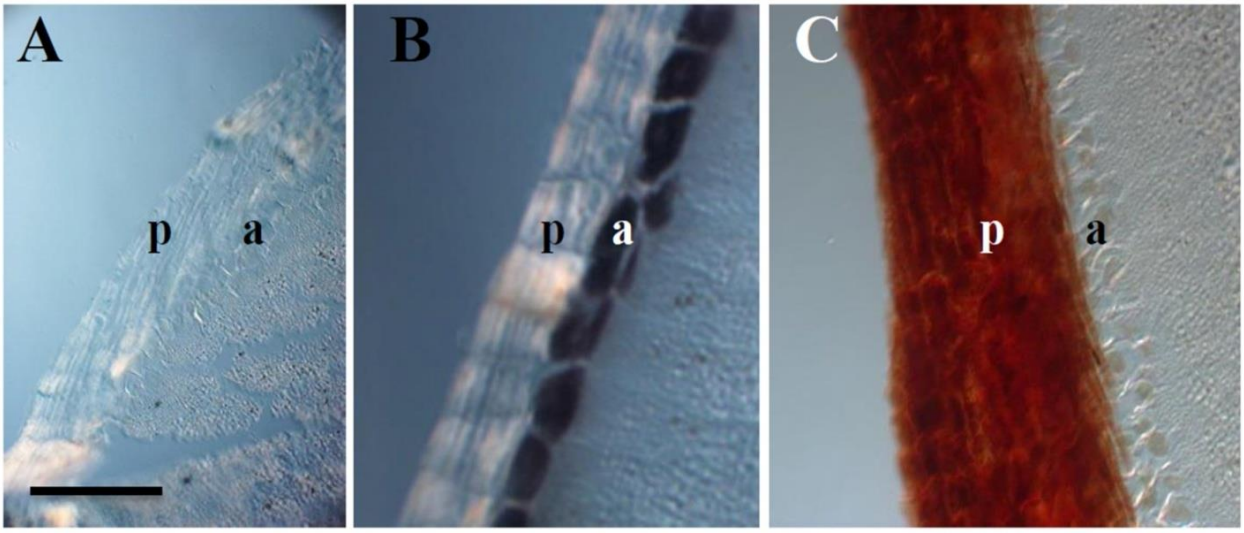

Figure 5. Histological analysis of seeds preserving pigments *in situ*. (A) B73 colourless seed used as control, (B) coloured Millo Corvo seed where the pigments are accumulated in the aleurone layer and (C) "Nero Spinoso" seed where the pigments are accumulated in the pericarp layer. p pericarp layer;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

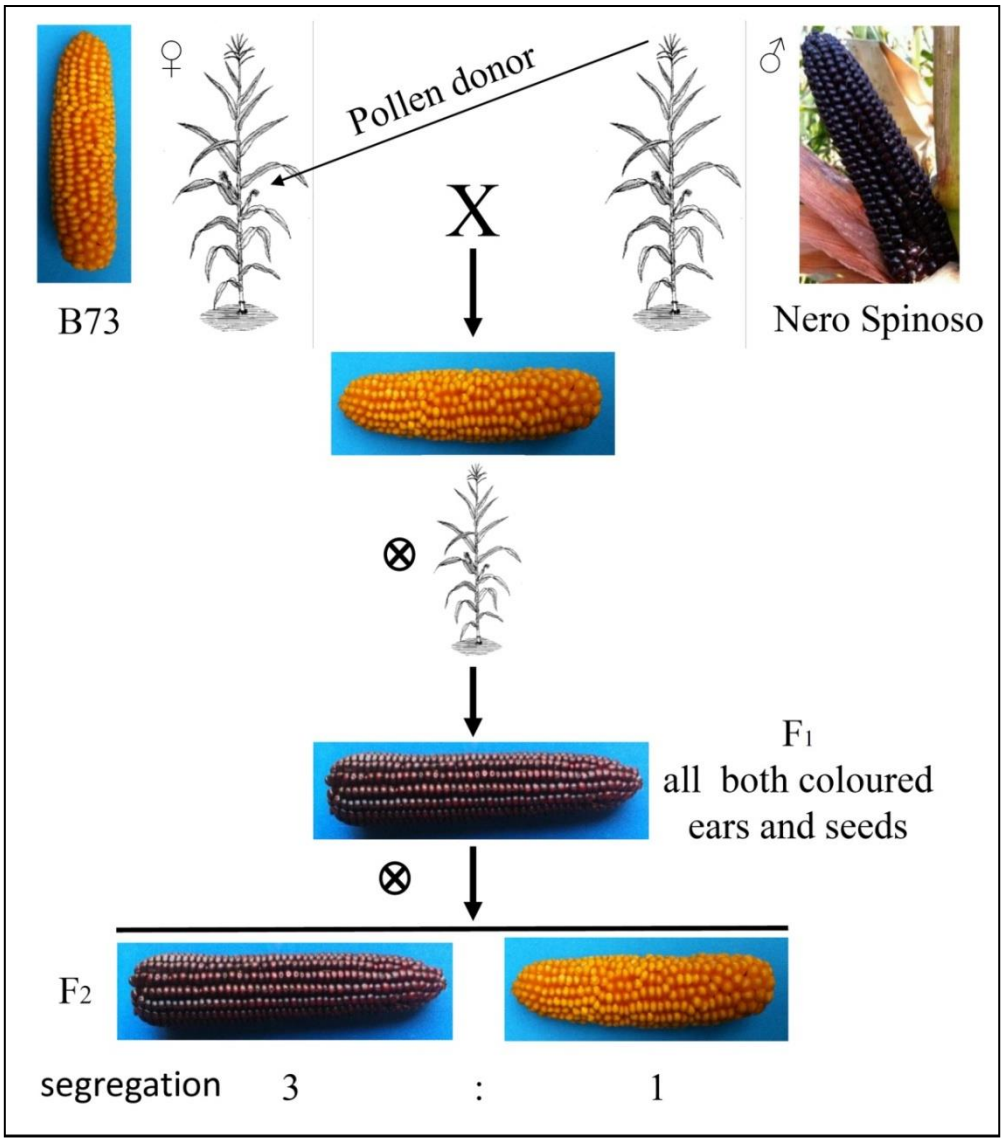

Figure 6. Segregation of the "colored ear" trait observed in the F1 and F2 progenies starting from the cross B73 x "Nero Spinoso" plants. The expected segregation values for "colored ear" was 3:1 in the case of the presence of a single dominant gene driving the pigmentation in the pericarp layer maternal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

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

REFERENCES

Anderson EG, (1924). Pericarp studies in maize II. The allelomorphism of a series of factors for pericarp color. Genetics 9:442–453

Anderson E, Cutler H, (1942). Races of maize: their recognition and classification. Ann Mo Bot Gard 29:69–88

Bianchi A, Ghatnekar MV, Ghidoni A, (1963). Knobs in Italian maize. Chromosoma 14:601-617

Brandolini A, (1958). Il germoplasma del mais e la sua conservazione. Maydica 3:4–14

Brandolini A, Brandolini A, (2009). Maize introduction, evolution and diffusion in Italy. Maydica 54:233–242

Casas MI, Duarte S, Doseff AI, Grotewold E, (2014). Flavonerich maize: an opportunity to improve the nutritional value of an important commodity crop. Front Plant Sci 5:440. doi:10.3389/fpls.2014.00440

Chopra S, Athma P, Peterson T, (1996). Alleles of the maize P gene with distinct tissue specificities encode Myb-homologous proteins with C-terminal replacements. Plant Cell 8:1149–1158. doi:10.1105/tpc.8.7.1149

Dellaporta SL, Wood J, Hicks JB, (1983). A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Dooner HK, Robbins TP, Jorgensen RA, (1991). Genetic and developmental control of anthocyanin biosynthesis. Ann Rev Gen 25:173–199. doi:10.1146/annurev.ge.25.120191.001133

Eschholz TW, Stamp P, Peter R, Leipner J, Hund A, (2010). Genetic structure and history of Swiss maize (Zea mays L. ssp. mays) landraces. Genet Resour Crop Evol 57:71–84

Falcone Ferreyra ML, Rodriguez E, Casas MI, Labadie G, Grotewold E, Casati P, (2012). Identification of a bifunctional maize C- and O-glucosyltransferase. J Biol Chem 288:31678–31688

Grotewold E, (2006). The genetics and biochemistry of floral pigments. Annu Rev Plant Biol 57:761–780. doi:10.1146/annurev.arplant.57.032905.105248

Grotewold E, Athma P, Peterson T, (1991). Alternatively spliced products of the maize P gene encode proteins with homology to the DNA-binding domain of myb-like transcription factors. Proc Natl Acad Sci USA 88(11): 4587–4591

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL, (2000).Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor. Proc Natl Acad Sci USA 97:13579–13584

Lago C, Landoni M, Cassani E, Doria E, Nielsen E, Pilu R, (2013). Study and characterization of a novel functional food: purple popcorn. Mol Breed 31:575–585

Lago C, Cassani E, Zanzi C, Landoni M, Trovato R, Pilu R, (2014a). Development and study of a maize cultivar rich in anthocyanins: coloured polenta, a new functional food. Plant Breed. 133:210–217

Lago C, Landoni M, Cassani E, Attanasiu S, Cantaluppi E, Pilu R, (2014b). Development and characterization of a coloured sweet corn line as a new functional food. Maydica 59:191–200

Lago C, Landoni M, Cassani E, Cantaluppi E, Doria E, Nielsen E, Giorgi A, Pilu R, (2015).Study and characterization of an ancient European flint white maize rich in anthocyanins: Millo Corvo from Galicia. PLoS ONE 10(5):e0126521. doi:10.1371/journal.pone.0126521

Liu K, Goodman M, Muse S, Smith JS, Buckler E, Doebley J, (2003). Genetic structure and diversity among maize inbred lines as inferred from DNA microsatellites. Genetics 165:2117–2128

Lopez-Martinez LX, Oliart-Ros RM, Valerio-Alfaro G, Lee CH, Parkin KL, Garcia HS, (2009). Antioxidant activity, phenolic compounds and anthocyanins content of eighteen strains of Mexican maize. LWT-Food Sci Technol 42:1187–1192

Mangelsdorf PC, Reeves RG, (1959). The origin of com. III. Modern races, the product of teosinte introgression. Bot Mus LeaH Harvard Univ 18:389–411

Matsuoka Y, Vigouroux Y, Goodman MM, Sanchez GJ, Buckler E, Doebley J, (2002). A single domestication for maize shown by multilocus microsatellite genotyping. Proc Natl Acad Sci USA 99:6080–6084

McMullen MD, Kross H, Snook ME, Corte ´s-Cruz M, Houchins KE, Musket TA, Coe EH Jr, (2004). Salmon silk genes contribute to the elucidation of the flavone pathway in maize (Zea mays L.). J Hered 95(3):225–233. doi:10.1093/jhered/esh042

Messedaglia L, (1924). Notizie storiche sul mais. Quaderno mensile No. 7. Sez. Credito Agrario Istituto Federale Credito del Risorgimento delle Venezie, Verona, Italy

Mir C, Zerjal T, Combes V, Dumas F, Madur D, Bedoya C, Dreisigacker S, Franco J, Grudloyma P, Hao PX, Hearne S, Jampatong C, Laloe ¨ D, Muthamia Z, Nguyen T, Prasanna BM, Taba S, Xie CX, Yunus M, Zhang S, Warburton ML, Charcosset A, (2013). Out of America: tracing the genetic footprints of the global diffusion of maize. Theor Appl Genet 126:2671–2682

Morohashi K, Casas MI, Falcone Ferreyra ML, Mejı´a-Guerra MK, Pourcel L, Yilmaz A, Feller A, Carvalho B, Emiliani J, Rodriguez E, Pellegrinet S, McMullen M, Casati P, Grotewold E, (2012). A genome-wide regulatory framework identifies maize Pericarp Color1 controlled genes. Plant Cell 24(7):2745–2764

Petroni K, Pilu R, Tonelli C, (2014). Anthocyanins in corn: a wealth of genes for human health. Planta 240:901–911

Pilu R, Piazza P, Petroni K, Ronchi A, Martin C, Tonelli C, (2003). pl-bol3, a complex allele of the anthocyanin regulatory pl1 locus that arose in a naturally occurring maize population. Plant J 36:510–521

Pilu R, Cassani E, Sirizzotti A, Petroni K, Tonelli C, (2011). Effect of flavonoid pigments on the accumulation of fumonisin B1 in the maize kernel. J Appl Genet 52(2): 145–152

Piperno DR, Ranere AJ, Holst I, Iriarte J, Dickau R, (2009). Starch grain and phytolith evidence for early ninth millennium B.P. maize from the Central Balsas River Valley, Mexico. Proc Natl Acad Sci USA 106:5019–5024. doi:10. 1073/pnas.0812525106

Ranere AJ, Piperno DR, Holst I, Dickau R, Iriarte J, (2009). Preceramic human occupation of the Central Balsas Valley, Mexico: cultural context of early domesticated maize and squash. Proc Natl Acad Sci USA 106:5014–5018

Rodriguez VM, Soengas P, Landa A, Ordas A, Revilla P, (2013). Effects of selection for color intensity on antioxidant capacity in maize (Zea mays L.). Euphytica 193:339–345

Sampietro DA, Fauguel CM, Vattuone MA, Presello DA, Catala ´n CAN, (2013). Phenylpropanoids from maize pericarp: resistance factors to kernel infection and fumonisin accumulation by Fusarium verticillioides. Eur J Plant Pathol 135:105–113

Styles ED, Ceska O, (1977). The genetic control of flavonoid synthesis in maize. Can J Genet Cytol 19:289–302

Van Heerwaarden J, Doebley J, Briggs W, Glaubitz CJ, Goodman M, Gonzalez JDS, Ross-Ibarra J, (2011). Genetic signals of origin, spread, and introgression in a large sample of a maize landraces. Proc Natl Acad Sci USA 108:1088–1092. doi:10.1073/pnas.1013011108

Vigouroux Y, Glaubitz JC, Matsuoka Y, Goodman MM, Sanchez GJ, Doebley J, (2008). Population structure and genetic diversity of New World maize races assessed by microsatellites. Am J Bot 95:1240–1253

Warburton ML, Reif JC, Frisch M, Bohn M, Bedoya C, Xia XC, Crossa J, Franco J, Hoisington D, Pixley K, Taba S, Melchinger AE, (2008). Genetic diversity in CIMMYT nontemperate maize germplasm: landraces, open pollinated varieties, and inbred lines. Crop Sci 48(2):617–624. doi:10.2135/cropsci2007.02.0103

West CE, Eilander A, van Lieshout M, (2002). Consequences of revised estimates of carotenoid bioefficacy for dietary control of vitamin A deficiency in developing countries. J Nutr 132:2920S–2926S

Winkel-Shirley B, (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 126(2):485–493. doi:10. 1104/pp.126.2.485

Zîlić S, Serpen A, Akıllıoglu G, Vural Gokmen V, Vancetovic J, (2012). Phenolic compounds, carotenoids, anthocyanins, and antioxidant capacity of colored maize (Zea mays L.) kernels. J Agric Food Chem 60:1224–1231

Phlobaphenes in maize kernel modify pericarp thickness and fumonisins accumulation.

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This is a pre-copy-editing, author-produced of an article submitted for publication in *Genetic Resources and Crop Evolution following peer review.*

Submitted to:
Genetic Resources and Crop Evolution December 2018

ABSTRACT

Phlobaphenes are insoluble phenolic compounds which are accumulated in a limited number of tissues such as seed pericarp and cob glumes, conferring on them a typical red-brown pigmentation. These secondary metabolites, derived from 3-deoxy flavonoids, are thought to have an important role in plants' resistance against various pathogens, e.g. by reducing fungal infection, and also to have beneficial effects on human and animal health due to their high antioxidant power. The aim of this work was to determine the role of phlobaphenes in reducing mycotoxin contamination on maize kernels. We analysed the effect of the *P1* gene on phlobaphenes accumulation, pericarp thickness and fumonisins accumulation. Analysing fumonisins accumulation in different genetic backgrounds through three seasons, we found a clear decrease of these toxins (ranging from 45 to 15%) in coloured lines compared with the isogenic non-coloured ones. This decrease was linked to a corresponding increase in pericarp thickness (about 60-70%) associated with the presence of the *P1* allele and to the corresponding increases in amounts of phlobaphenes (about 10-14-fold).

Taken together these results suggest that the *P1* gene plays a central role in regulating phlobaphenes accumulation in maize kernels, and indirectly, also tackles mycotoxins accumulation.

The development and cultivation of corn varieties rich in phlobaphenes could be a powerful tool to reduce the loss of both quality and yield due to mycotoxin contamination, increasing the safety and the quality of the maize product.

INTRODUCTION

The growing interest in foods rich in flavonoids and other bioactive molecules, regular consumption of which is associated with a reduced risk of chronic diseases (Rodriguez et al., 2013; Lago et al., 2014; Petroni et al., 2014; Casas et al., 2014), has brought about a rediscovery of the importance of pigmented maize varieties. In particular the geneticist's attention was focused on ancient varieties and landraces to find valuable genes for developing functional foods with high amounts of antioxidant compounds. It's known that ancient farmer's varieties and landraces possess a broad natural variation in valuable nutraceuticals, but this has been impoverished during the breeding process which has led to modern cultivar development (Newton et al., 2010; Monasterio and Graham, 2000).

Phenolic compounds, and in particular anthocyanins and phlobaphenes exhibit a noticeable antioxidant activity and confer a red, blue or black pigmentation to the plant tissues (Ranum et al., 2014; Das and Singh, 2016).

Flavonoid biosynthesis is a complex pathway made up of more than 20 structural genes and it is regulated by two classes of transcription factors, the bHLH genes *(r1*/*b1*) and the *c1*/*pl1*/*p1* Myb genes families. This pathway is divided into two branches: one, regulated by the MYB genes *b1*, *c1* and *pl1* leads to flavonols and anthocyanins synthesis and the other, regulated by the *p1* gene leads to phlobaphenes and maysin production.

Phlobaphenes are reddish insoluble pigments, whose biosynthetic pathway begins with the condensation of malonyl-CoA with p-coumaroyl-CoA, catalysed by the enzyme chalcone synthase (CHS) (encoded by the colorless2 locus, *c2*). The chalcone isomerase (CHI) enzyme converts the resulting naringenin chalcone into the flavanone naringenin that is converted to apiforol and luteoforol by the enzymes dihydroflavonol reductase-DFR (encoded by the *A1* gene) and the flavanone-3-hydroxylase-F3-H (encoded by the *Pr1* gene). Apiforol and luteoforol are then polymerized into phlobaphenes (Sharma et al., 2012; Winkel-Shirley 2001; Grotewold 2006; Casas et al., 2014).

The accumulation of phlobaphene pigments in the maize pericarp layer is regulated by the R2R3-MYB transcription factor PERICARP COLOR1 (*P1*), with different *P1* alleles conferring different pericarp and cob glume colors (Grotewold et al., 1991; Casas et al., 2014). The *P1-rr* allele determines the coloration of both pericarp and cob glumes: with the P*1-rw* allele only the pericarp is colored , with *P1-wr* only the cob glumes, and with *P1-ww* both the tissues are colorless (Anderson et al., 1924; Chopra et al., 1996; Casas et al., 2014).

Preliminary results indicated that phlobaphene pigments could be associated with a reduced level of mycotoxin contamination, in particular a reduction in fumonisins in maize kernels, as reported in our previous paper (Pilu et al., 2011).

The contamination of maize kernels by mycotoxin produced by *Fusarium*, in particular *F. verticillioides* and *F. proliferatum*, is implicated in different serious animal diseases and seems to be the cause of some types of human cancer (Ross et al., 1992). Crop growing techniques can reduce the risk of fusarium infection but there are no definitive strategies to prevent fungal infection and fumonisin accumulation in maize kernels. In 2016 in Italy about the 27% of the sample analysed showed a level of fumonisin contamination higher than the legal limit (http://mangimiealimenti.it/articoli/2331-micotossine-in-mais-campagna-2017).

The selection of varieties rich in phlobaphenes and other flavonoids sharing with them a part of the biosynthetic pathway, could thus represent an interesting opportunity, not only because of their beneficial effects on human health due to their antioxidant activity, but also because of their role in protecting maize plants from fungal infections, reducing mycotoxin contamination, thus increasing the quality of the kernel and its healthiness.

The maize pericarp has been shown to play an important role in resistance against Fusarium attacks, as demonstrated by the inverse correlation between pericarp thickness and Fusarium attacks susceptibility (Hoenisch and Davis 1994), and the identification of the pericarp and its wax layer as resistance factors to fumonisins accumulation (Sampietro et al., 2013).

In this context the ancient cultivar Nero Spinoso from the Camonica Valley (Italy) appears very interesting. It is characterized by a very high level of phlobaphenes and a pericarp layer thickness which was significantly higher than that in the colourless control (Cassani et al., 2017).

The aim of this work was to better clarify previous preliminary data regarding the relationship between the presence of phlobaphenes in maize pericarp and mycotoxin content, using new genetic materials characterized by the presence of high levels of phlobaphenes, together with the isogenic colourless lines.

MATERIALS AND METHODS

Plant Material

In this work we used 4 synthetic populations, one Italian landrace, and one inbred line carrying the Ac transposon in *P1* gene (PVV) obtained as described below.

The 4 synthetic populations were obtained by crossing each of the two commercial hybrids PR 33A46 and DK 6530 with an inbred line carrying the *P1* gene which leads to phlobaphenes accumulation in the pericarp (*P1* line throughout the text).

The two F1s obtained were selfed, about 1000 seeds were sown in the following season and about 200 plants were selfed. The *p1*/*p1* control synthetic population was obtained by bulking the seeds coming from the colorless ears (segregation ratio 3/4 colored: 1/4 colorless).

The F3 colored ears (*P1*/*P1* and *P1*/*p1*) were selected for *P1*/*P1* genotype by testing the segregation of colored ears obtained from a sample of 20 seeds/ear. In this way we selected about 50 *P1*/*P1* ears that were shelled and the seeds obtained bulked to obtain the colored population. We named the synthetic populations coming from the PR33A46 parental Syn1r (red seeds) and Syn1c (colorless seeds), and the synthetic populations coming from the DK6530 Syn2r (red seeds) and Syn2c (colorless seeds).

We used the pigmented variety Spinoso Nero di Esine della Val Camonica which can accumulate in the pericarp layer a high level of phlobaphenes and the weakly pigmented sub-population present at the frequency of 2.87% in this colored variety (Cassani et al., 2017). We named these two sub populations NSr (pigmented ears) and NSw (weakly pigmented ears) throughout the text.

Finally we used an inbred line carrying the *Ac* autonomous component of the *Ac*/*Ds* system in the *P* locus, conferring a variegated pericarp due to transposon excision sectors (PVV system) (Pilu et al., 2002).

The 4 synthetic populations used for the determination of fumonisin amounts were tested in 3 field seasons (2015, 2016, 2017). For each population 1200 seeds were sown in adjacent separated plots of 140 square meters, under the same agronomic conditions, in the experimental field of the University of Milano located in Landriano (PV).

Sample preparation and milling

About 70 ears of each variety/year were shelled and the seeds obtained mixed to create a single bulk used to perform various analyses. Flour samples were obtained using a ball mill (Retsch MM200, Retsch GmbH Germany), and seeds were ground for 5 min at 21 oscillations s^{-1} frequency.

Spectrophotometer determination of flavonols and phenolic acids

Fifteen mg of flour were boiled with 100 µl of distilled water for 30 minutes and then left in an overnight agitator with 1 ml of extraction buffer (1% HCl, 95% ethanol). After a further agitation 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 (Pilu et al., 2011).
The amounts of flavonols were calculated as quercetin 3-glucoside equivalents (ε 21877 Lm-1 mol-1, M. W. 464.38) and the amounts of phenolics as ferulic acid equivalents (ε 14700 Lm-1 mol-1, M.W. 194.18). The analyses were conducted three times for each genotype.

Spectrophotometer determination of phlobaphenes

Phlobaphenes were extracted from individual seeds with 1 volume of concentrated HCl and 4 volumes of dimethyl sulfoxide (DMSO) added sequentially with vigorous vortexing after each addition, essentially as described by Das et al. (1994). Extracts were then centrifuged and cleared supernatants were diluted with methanol (20% final concentration). Phlobaphenes concentration was expressed as absorbance value at their λmax (510 nm) per g of dry weight. The analyses were conducted three times for each genotype.

Enzyme immunoassay for the detection of fumonisins

For the determination of fumonisins concentration we used the kit "I' screen FUMO" (Tecna®) according to manufacturer's instructions.

Briefly: for each sample, 50 g of flour were extracted with methanol 70%, a dilution 1:20 of the extracts was loaded in the reaction plate and then the enzyme conjugate and the fumonisins antibody were added to each sample. After 4 washes the samples were incubated for 30 min with the developing solution and after the addition of the stop solution the absorbance was measured at 450 nm. The analyses were conducted three times for each genotype.

Histological analysis

Seeds of Syn1c, Syn1r, Syn2c, Syn2r, Nsw, Nsr and *Ac* lines were imbibed in water overnight and longitudinally cut into halves with a scalpel.
To determine the pericarp thickness, images were taken and elaborated using a Leica MZ6 Stereoscope equipped with the application software LAS V3.8. Statistical analysis was performed on at least 15 measurements of pericarp thickness for each of the 7 genotypes studied.

Informatic tools

Microsoft Excel® was used to collect data, PAST program (Paleontological Statistics, version 3.12) was used to perform statistical analysis.

RESULTS

Constitution of the genetic material and flavonoid analysis

With the aim of studying the relationship between the presence of the *P1* gene and the mycotoxin contamination on maize seeds, we produced as described in the Materials and Methods section, 4 synthetic populations, with Syn1r and Syn2r characterized by the presence of the *P1* gene, and Syn1c and Syn2c the colorless *p1* controls. We also used two sub populations of "Spinoso Nero di Esine della Val Camonica" characterized by a high level of phlobaphenes in the pericarp layer: NSr (fully pigmented ears) and NSw (weakly pigmented ears) (Fig. 1).

We cultivated these genetic materials for three years (2015-2016-2017) at the experimental field of the Faculty of Agricultural and Food Sciences of Milan. The materials were analyzed every year for the phlobaphenes, flavonols and phenolic acids amounts.
Higher amounts of phenolic compounds were found in *P1*/*P1* varieties compared with the *p1*/*p1* controls (Table 1). In particular, our analysis showed that the *P1* allele conferred a tenfold higher concentration of phlobaphenes in comparison with the colorless controls. In fact Syn1c (*p1*/*p1*) showed a phlobaphenes concentration of 13.44 ± 2.69 (A510/100g) and the isogenic colored Syn1r (*P1*/*P1*) had a concentration of 183.516 ± 20.96 (A510/100g). The phlobaphenes content in Syn2c (*p1*/*p1*) was 9.28 ± 1.29 (A510/100g) and in Syn2r (*P1*/*P1*) was 105.03 ± 9. 93 (A510/100g). Similarly Nsw *(p1*/*p1)* had a phlobaphenes concentration of 46.43 ± 9.31 (A510/100g) and Nsr (*P1*/*P1*) of 517.86 ± 38.52 (A510/100g). The trend was maintained similarly for flavonols content, the colorless varieties Syn1c and Syn2c (*p1*/*p1*) showing an amount of 84.18±6.26 mg/100g and 72.59 ±5.43 mg/100g respectively, while in the colored isogenic hybrids Syn1r and Syn2r the flavonols content was respectively of 91.79 ± 7 mg/100g and 86.01 ± 5.75 mg/100g. Similar results were also obtained for phenolic acids content, colorless varieties Syn1c and Syn2c showed a concentration of 1.46 ± 0.11 mg/100g and 1.35 ± 0.1 mg/100g respectively while colored varieties, Syn1r and Syn2r had amounts of 1.65± 0.2 mg/100g and 1.58± 0.08 mg/100g. We also observed that the synthetic populations derived from the commercial hybrid PR 33A46, the colored Syn1r and the colorless Syn1c, had a higher amount of all phenolic compounds tested compared respectively with the colored Syn2r and the colorless Syn2c lines derived from commercial hybrid DK 6530 (Table 1). We noticed small differences between the same genotypes in the different seasons (data not shown), confirming the role of the *P1* gene as the main regulatory gene of the phlobaphenes pathway, and a small environmental effect as expected for a qualitative trait.

Fumonisins quantification

In order to establish a possible correlation between pigmentation and fumonisins contamination in maize seeds we cultivated in adjacent separated plots of 140 square meters, under the same agronomic conditions, the 4 synthetic populations (Syn1c/r and Syn2c/r) for 3 years (seasons 2015, 2016, 2017). We determined the total fumonisins accumulation by an immune-enzymatic assay for the quantitative analysis of fumonisins

(B1 and B2 fumonisins). Data shown in Table 2 indicate that in each sample analyzed the amount of mycotoxins was higher in the colorless flour in comparison with the colored one. In particular Syn1r (*P1*/*P1*) compared with the equivalent isogenic colorless variety Syn1c (*p1*/*p1*) showed an average fumonisins decrease over the three years of trials of 39.2%. A decrease of 19% was also present in Syn2r (*P1*/*P1*) compared with the equivalent isogenic colorless Syn2c (*p1*/*p1*). Using a non-parametric Wilcoxon rank sum test to analyze these data we found statistically significant differences ($p<0.05$) for fumonisins content in colored and colorless materials (Table 2).

Morphological analysis of the seed: pericarp thickness

In order to study whether different levels of phlobaphenes were associated with differences in seed morphology we focused our attention on the pericarp, where these pigments are accumulated. The histological analysis showed differences in pericarp thickness among the different varieties, in agreement with the data concerning phlobaphenes quantification. In fact, the colorless varieties (Syn1c = 80µm ±7; Syn2c = 66µm ± 4.18; Nsw = 128 µm ± 9.08) had a thinner pericarp if compared with equivalent isogenic colored varieties (Syn1r = 132 µm ± 4.47; Syn2r = 94 µm ± 9.61; Nsr = 290 µm ± 15.81) (Fig. 2 and Tab. 3). A strong correlation ($R= 0.9318$, $p= 0.00679$) was found between phlobaphenes content and pericarp thickness by analyzing the data collected (Fig. 3).

P1 gene is responsible for increasing pericarp thickness

With the aim of strengthening the idea that the *P1* gene is able in a specific way to modify the pericarp thickness, we measured colorless and colored sectors in PVV seeds. We used an inbred line characterized by a variegated pericarp color due to the excision of an Ac element in the *P1* locus (PVV system). Hence the only difference present in these sectors was due to the presence of the pigments/activity of *P1* gene, all the other genes are the same. As shown in Fig. 4, in the *p1* colorless sectors the pericarp thickness was of 99.34 ± 6.8 µm whilst in the *P1* colored sectors it was 138.95 ± 8.5 µm, i.e. the higher level of phlobaphenes is associated with higher pericarp thickness.

DISCUSSION

Phlobaphenes are reddish insoluble pigments derived from 3-deoxy flavonoids 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). The biosynthetic pathway of phlobaphenes is regulated by the *P1* gene (Dooner et al., 1991), a transcription factor member of the MYB gene family. In maize they are accumulated in a limited number of tissues, such as seed pericarp, a tissue of maternal origin corresponding to the ovary wall, and cob glumes (Grotewold et al., 1994; Falcone Ferreyra et al., 2010; Cassani et al., 2017), which confers on them a typical red-brown pigmentation which is sometimes very dark, as in the Italian Nero Spinoso variety traditionally cultivated in the Camonica Valley (Cassani et al., 2017).

Preliminary data had shown the role of phlobaphenes and other flavonoids accumulated in maize pericarp on the reduction of fungal ear rot and fumonisins accumulation (Pilu et al., 2011; Bernardi et al., 2018). The phlobaphenes and other secondary metabolites are accumulated in many ancient landraces traditionally cultivated in the Padana Plane and in mountainous regions of Northern Italy (Cassani et al., 2017; Bernardi et al., 2018).

Fumonisins are mycotoxins produced by different species of *Fusarium* able to infect maize, especially *F. verticillioides* and *F. proliferatum*, and are associated with the development of various serious diseases in both humans and animals (Ross et al., 1992). Correct cultural practices can reduce the spread of *Fusarium* infection but the most effective strategies rely on genetic improvement of maize. In particular, due to the multigene nature of *Fusarium* resistance, a promising approach seems to be the development of maize lines with high phenolic compounds accumulated in the pericarp, which have proven to be more resistant to *Fusarium* infection and to have a reduced content of mycotoxins. The phenolic compounds have been reported to be involved in the reduction of the susceptibility of maize plants to *Fusarium* attack, acting as a physical barrier by hardening the maize pericarp (Hoenisch and Davis 1994; Pilu et al., 2011), or inactivating fungal proteins by the formation of irreversible complexes (Treutter 2006), or targeting the fungal antioxidative stress response (Kim et al., 2006).

With the aim of further dissecting the relationship between phlobaphenes accumulation and resistance to *Fusarium* infection we analyzed maize seeds from 4 different genetic

backgrounds characterized by the presence of high levels of phlobaphenes in the pericarp and the corresponding colorless isogenic lines as controls. In particular we studied 2 synthetic populations obtained by crossing two commercial hybrids with a line source of the *P1* gene, with colorless controls (Syn1r, Syn1c, Syn2r and Syn2c), a landrace, the Spinoso Nero di Esine della Val Camonica, characterized by a high level of phlobaphenes accumulated in the pericarp and a weakly colored subpopulation as control, and a line with the *Ac* element inserted in the *P1* gene, conferring a variegated pericarp.

The data showed that in the flour of colorless varieties, mycotoxins' content was higher than in the colored ones (Table 2). In particular Syn1r (*P1*/*P1*) showed an average decrease of fumonisins content of 39.2% compared with the corresponding isogenic colorless population Syn1c (*p1*/*p1*). A similar decrease was observed for Syn2r (*P1*/*P1*) which showed an average decrease of 19% compared with the equivalent isogenic colorless population Syn2c (*p1*/*p1*). Our results are in agreement with previously reported data suggesting that phlobaphenes and other flavonoids are implicated in kernel resistance against fungal infection. In particular the decrease of fumonisins amounts in colored lines could be due to a direct effect of phlobaphenes that could form irreversible complexes with fungal proteins leading to their inactivation (Treutter 2006) or to the barrier effect due to the increased pericarp thickness in colored lines in comparison to the colorless ones (Venturini et al., 2016). To check these hypotheses we quantified the phlobaphenes, flavonol and phenolic compound content and the pericarp thickness in each variety. The amount of phenolic compounds, despite some differences that can be explained by the variation of climatic conditions in the different agronomic seasons, was similar in every agronomic season analyzed, with an higher amount of phenolic compounds in colored lines (*P1*/*P1*) compared with the corresponding isogenic colorless (*p1*/*p1*) (Table 1). Focusing on phlobaphenes, we found that the *P1* allele was responsible for a more than 10 fold increase of these pigments in colored lines (Syn1r, Syn2r and Nsr) in comparison with the colorless controls (Syn1c, Syn2c, Nsw) (Table 1). In particular the population Nsr showed the highest level of pigments, responsible for the dark pigmentation of the seeds.

The histological analysis showed differences in pericarp thickness among colored and colorless lines. In particular the colorless lines (Syn1c = 80µm ±7; Syn2c = 66µm ± 4.18; Nsw= 128 µm ± 9.08) had a thinner pericarp if compared with equivalent isogenic colored lines (Syn1r = 132 µm ± 4.47; Syn2r = 94 µm ± 9.61; Nsr = 290 µm ± 15.81) (Tab. 3, Fig. 2). Taken together these data highlighted a strong correlation between pericarp thickness and phlobaphenes concentration (Fig. 3). The association between the phlobaphenes

accumulation, driven by the *P1* gene, and the increase of pericarp thickness is also supported by the histological analysis of the *Ac* line. In this line in fact, the Ac element inserted in *P1* gene confers variegated color and thickness to the pericarp. In these seeds, in the colorless *p1*/*p1* sectors the pericarp thickness is 99.34 ± 6.8 μm while in colored *P1*/*P1* sectors the thickness is 138.95 ± 8.5 μm (Fig. 4).

Considering the role of the *P1* gene in phlobaphenes accumulation and in pericarp thickness determination, these results suggested 3 different hypotheses: a) *P1* gene has a direct role in regulating pericarp thickness and color; b) pericarp thickness is a consequence of phlobaphenes accumulation driven by *P1* gene; c) there is a linkage drag between *P1* gene, regulating phlobaphenes accumulation, and the gene "X" playing a central role in regulating the pericarp thickness. The histological characterization of the Ac line, seems to suggest a direct involvement of the *P1* gene in both pericarp color and thickness and thus seems to exclude the third hypothesis. The analysis of new genetic material will be necessary to collect evidence supporting one of the two alternative remaining hypotheses.

Our findings therefore suggest that the cultivation of maize varieties rich in phlobaphenes would enhance crop quantity and quality in areas which are characterized by a humid and rainy climate particularly favorable to fungal development. In these varieties in fact the presence of phlobaphenes will ensure high resistance to fungal infection, and therefore a low level of mycotoxin contamination, together with the health promoting effect associated with this class of flavonoids. The beneficial properties derived from flavonoids have been well studied in recent years (Grotewold et al., 2000; Lago et al., 2013; Petroni et al., 2014; Casas et al., 2014) and although the effects of phlobaphenes on human and animal health are not yet completely known, their high antioxidant power suggests effects similar to those of the anthocyanins (Rodriguez et al., 2013).

Our work therefore highlighted the importance of the rediscovery of ancient pigmented varieties for use in breeding programs aiming to obtain a functional and safe food for human nutrition and animal feeding.

FIGURES

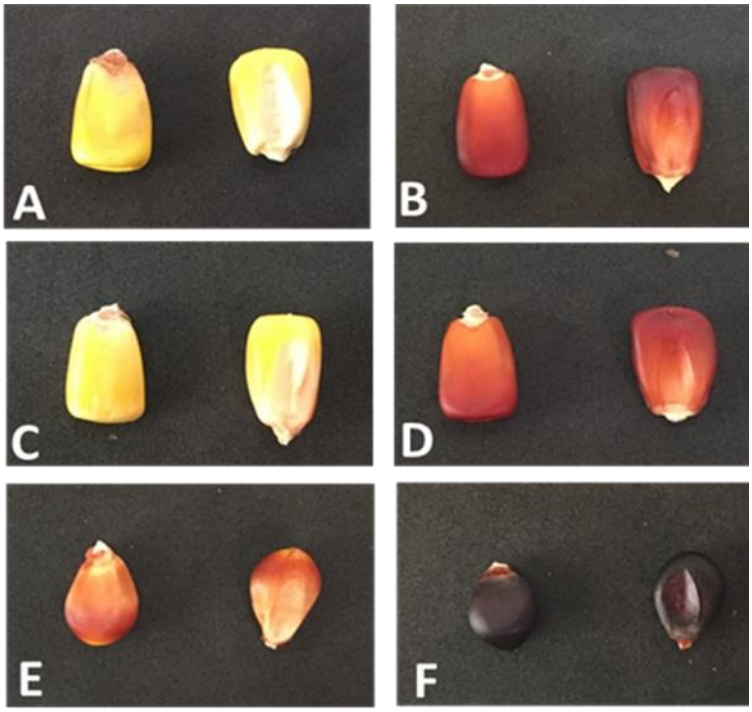

Figure 1. Mature seeds of the maize lines used in this work: Syn1c (A) , Syn1r (B) Syn2c (C), Syn2r (D), Nsw (E), Nsr (F).

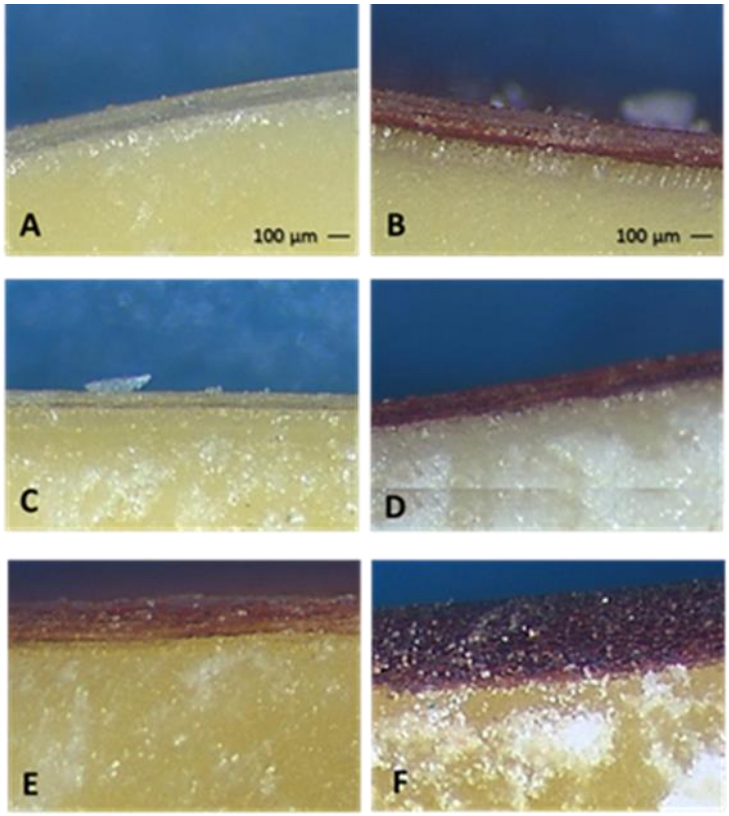

Figure 2. Histological analysis of mature seeds pericarp thickness: Syn1c (A), Syn1r (B), Syn2c (C), Syn2r (D), Nsw (E) and Nsr (F).

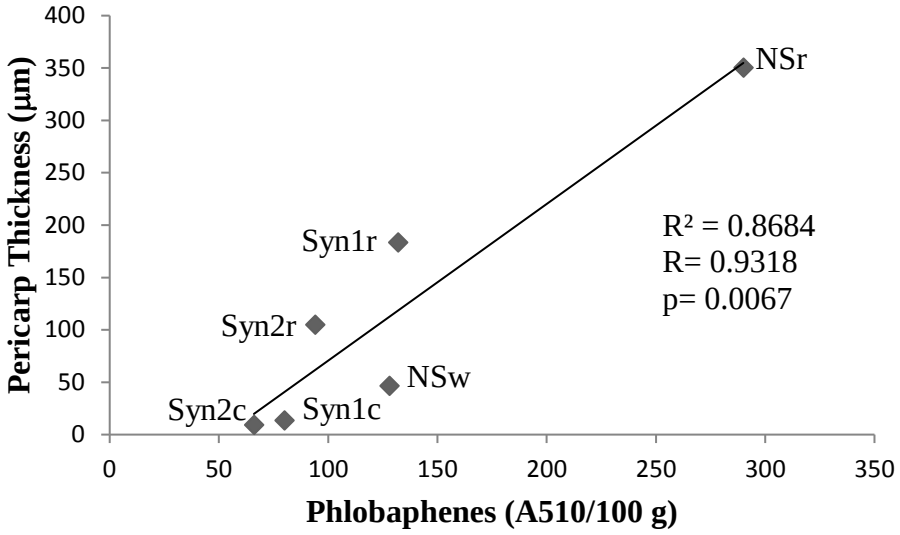

Figure 3. Correlation analysis between phlobaphenes amount and pericarp thickness of the seed in Syn1c, Syn1r, Syn2 c, Syn2r, Nsw and Nsr.

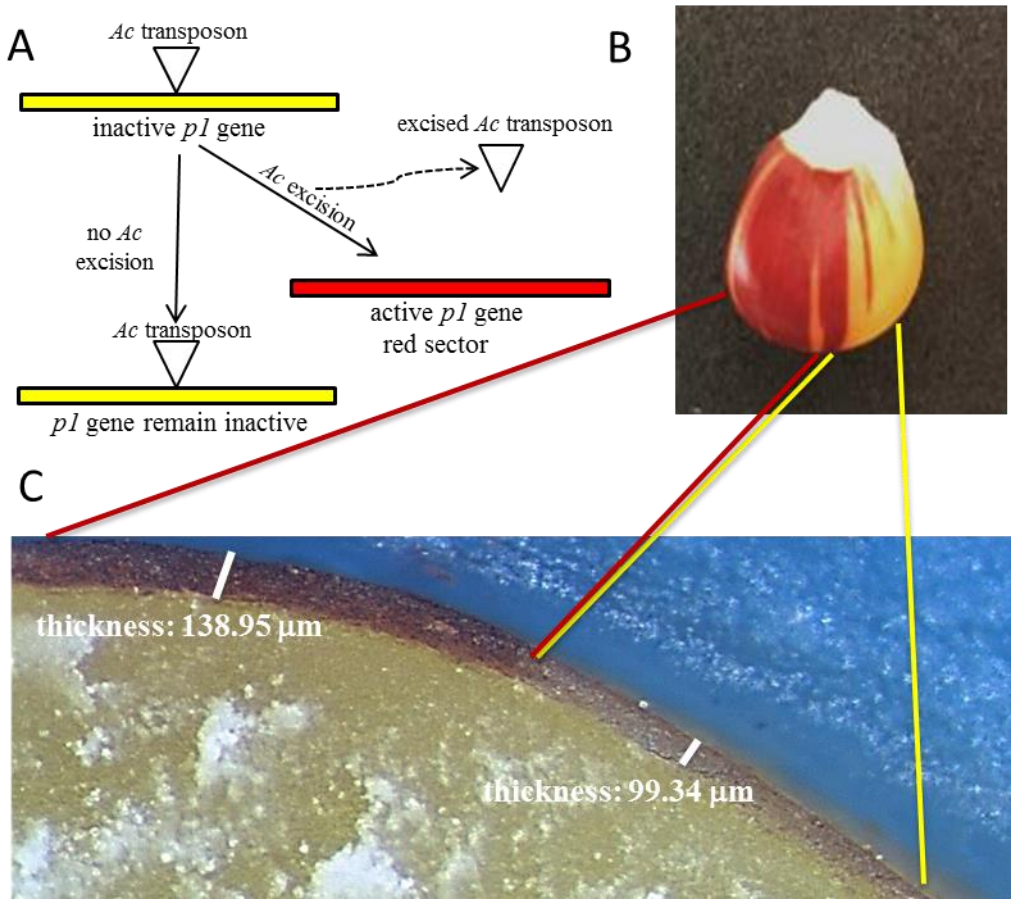

Figure 4. Scheme explaining how the Ac excision from *P1* gene produced somatic sectors due to the reactivation of phlobaphenes pathway (A). Seed sectors caused by *Ac* excision from the locus *p1* (*PVV* unstable allele). (C) Histological analysis of mature PVV seeds and pericarp thickness of colorless and red sectors.

TABLES

Table 1. Spectrophotometric analysis of phlobaphenes, flavonols and phenolic acids quantified respectively as absorbance at 510 nm, mg quercetin 3-glucoside equivalents and ferulic acid equivalents respectively per 100 g of dry seed flour. SD are shown (n=3). The analyses were carried out in the 2015 season.

Code	**Genotype**	**Phlobaphenes** (A510/100g)	**Flavonols** (mg/100g)	**Phenolic acids** (mg/100g)
Syn1c	*p1*	13.44 ±2.69	84.18 ±6.26	146 ±11
Syn1r	*P1*	183.516 ±20.96	91.79 ±7.0	165 ±23
Syn2c	*p1*	9.28 ±1.29	72.59 ±5.43	135 ±16
Syn2r	*P1*	105.03 ±9.93	86.01 ±5.75	158 ±18
NSw	*p1*	46.43 ±9.31	101± 17	146± 22
NSr	*P1*	350.24 ±38.52	152±34	170±14

Table 2. Determination of fumonisins concentration in maize flour (ppb) using ELISA'S test. SD are shown (n=3).

	Genotype		**Decrease** (%)	**Genotype**		**Decrease** (%)
	Syn1c (*p1*)	**Syn1r (*P1*)**		**Syn2c (*p1*)**	**Syn2r (*P1*)**	
1° year	128.4± 13	69.6± 9	45.8	349.2± 36	294.5±42	15.7
2° year	4520.4± 312	2888.8± 218	36.1	22176.8± 553	17436.3± 498	21.4
3° year	2171.9± 210	1398.3± 139	35.6	10654.4± 374	8543.9± 242	19.8
Average decrease (%)			39.2			19

The non-parametric Wilcoxon rank sum test revealed statistically significant differences ($p < 0.05$) between colored and colorless materials.

Table 3. Measurements of mature seeds pericarp thickness expressed as µm. The analyses were carried out in the 2015 season. SD are shown (n>15).

Code	**Genotype**	**Pericarp thickness** (µm)
Syn1c	*p1*	80 ±7.07 [ab]
Syn1r	*P1*	132 ±4.47 [c]
Syn2c	*p1*	66 ±4.18 [a]
Syn2r	*P1*	94 ±9.61 [b]
NSw	*p1*	128 ±9.08 [c]
NSr	*P1*	290 ±15.81 [d]

Means followed by the same letter are not significantly different (Tukey test , $p < 0.05$)

REFERENCES

Anderson EG, (1924). Pericarp studies in maize II. The allelomorphism of a series of factors for pericarp color. Genetics 9: 442-453.

Bernardi J, Stagnati L, Lucini L, Rocchetti G, Lanubile A, Cortellini C, De Poli G, Busconi M, Marocco A, (2018). Phenolic profile and susceptibility to *Fusarium* infection of pigmented maize cultivars. Front Plant Sci 9:1189 https://doi.org/10.3389/fpls.2018.01189

Casas MI, Duarte S, Doseff AI, Grotewold E, (2014). Flavone-rich maize: an opportunity to improve the nutritional value of an important commodity crop. Front Plant Sci 5:440. doi: 10.3389/fpls.2014.00440.

Cassani E, Puglisi D, Cantaluppi E, Landoni M, Giupponi L, Giorgi A, Pilu R, (2017). 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. Genetic Resources and Crop Evolution 64:761-773.

Chopra S, Athma P, Peterson T, (1996). Alleles of the maize P gene with distinct tissue specificities encode Myb-homologous proteins with C-terminal replacements. Plant Cell 8:1149–1158.

Das OP, Morales M, Messing J, (1994). Quantitative extraction of pericarp pigments. Maize Genet Crop Newsl 68:79-83.

Das AK, and Singh V, (2016). Antioxidative free and bound phenolic constituents in botanical fractions of Indian specialty maize (Zea mays L.) genotypes. Food Chemistry 201, 298-306.

Dooner HK, Robbins TP, Jorgensen RA, (1991). Genetic and developmental control of anthocyanin biosynthesis. Ann Rev Gen 25: 173-199

Falcone Ferreyra ML, Rius S, Emiliani J, Pourcel L, Feller A, Morohashi K, Casati P, Grotewold E, (2010). Cloning and characterization of a UV-B-inducible maize flavonol synthase. Plant J. 62: 77–91.

Grotewold E, Athma P, Peterson T, (1991). Alternatively spliced products of the maize P gene encode proteins with homology to the DNA-binding domain of myb-like transcription factors. Proc. Natl. Acad. Sci. USA 88: 4587–4591.

Grotewold E, Drummond BJ, Bowen B, Peterson T, (1994). The myb-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell. 76: 543-53.

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL, (2000).Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor R. Proc Natl Acad Sci USA. 97: 3579-84.

Grotewold E, (2006). The genetics and biochemistry of floral pigments. Annu. Rev. Plant Biol. 57:761-780

Hoenisch RW, and R M Davis, (1994). Relationship between kernel pericarp thickness and susceptibility to Fusarium ear rot in field corn. Plant Disease 78:517-519.

Kim JH, Mahoney N, Chan KL, Molyneux RJ, Campbell BC, (2006). Controlling food-contaminating fungi by targeting antioxidant stress-response system with natural phenolic compounds. Appl. Microbiol. Biotechnol. 70: 735–739

Lago C, Landoni M, Cassani E, Doria E, Nielsen E, Pilu R, (2013). Study and characterization of a novel functional food: purple popcorn. Mol Breed 31: 575-585.

Lago C, Cassani E, Zanzi C, Landoni M, Trovato R, Pilu R, (2014). Development and study of a maize cultivar rich in anthocyanins: coloured polenta, a new functional food. Plant Breed. 133: 210–217.

Monasterio JI, Graham RD, (2000). Breeding for trace minerals in wheat. Food Nutr. Bull.

21: 393–396.

Newton AC, Akar T, Baresel JP, Bebeli PJ, Bettencourt E, Bladenopoulos, K V, Czembor JH, Fasoula DA, Katsiotis A, Koutis K, Koutsika-Sotiriou M, Kovacs G, Larsson H, Pinheiro De Carvalho MAA, Rubiales D, Russell J, Dos Santos TMM, Vaz Patto MC, (2010). Cereal landraces for sustainable agriculture. A review. Agronomy for Sustainable Development 30: 237-269

Pilu R, Consonni G, Busti E, MacCabe AP, Giulini A, Dolfini S, Gavazzi G, (2002). Mutations in two independent genes lead to suppression of the shoot apical meristem in maize. Plant Physiol 128: 502-11

Pilu R, Cassani E, Sirizzotti A, Petroni K, Tonelli C, (2011). Effect of flavonoid pigments on the accumulation of fumonisin B1 in the maize kernel. J. Appl. Genetics 52:145-52.

Petroni K, Pilu R, Tonelli C, (2014). Anthocyanins in corn: a wealth of genes for human health. Planta 240:901-911.

Ranum P, Peña-Rosas JP, Garcia-Casal MN, (2014). Global maize production, utilization, and consumption. Annals of the New York Academy of Sciences, 1312: 105-112.

Rodriguez VM, Soengas P, Landa A, Ordas A, Revilla P, (2013). Effects of selection for color intensity on antioxidant capacity in maize (*Zea mays* L.). Euphytica 193: 339-345.

Ross PF, Rice LG, Osweiler GD, Nelson PE, Richard JL, Wilson TM, (1992). A review and update of animal toxicoses associated with fumonisin-contaminated feeds and production of fumonisins by Fusarium isolates. Mycopathologia. 117:109-14

Sampietro DA, Fauguel CM, Vattuone MA, Presello DA, Catalán CAN, (2013). Phenylpropanoids from maize pericarp: Resistance factors to kernel infection and fumonisin accumulation by *Fusarium verticillioides*. Eur. J. Plant Pathol. 135:105-113.

Sharma M, Chai C, Morohashi K, Grotewold E, Snook ME, Chopra S, (2012). Expression of flavonoid 3′-hydroxylase is controlled by *P1*, the regulator of 3-deoxyflavonoid biosynthesis in maize. BMC Plant Biol. 12, 196.

Treutter D, (2006). Significance of Flavonoids in Plant Resistance A Review. Environmental Chemistry Letters, 4, 147-157.

Venturini G, Babazadeh L, Casati P, Pilu R, Salomoni D, Toffolatti S L, (2016). Assessing pigmented pericarp of maize kernels as possible source of resistance to fusarium ear rot, Fusarium spp. infection and fumonisin accumulation. International Journal of Food Microbiology, 227, 56-62.

Winkel-Shirley B, (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126(2):485-493 doi: http://dx.doi.org/10.1104/pp.126.2.485.

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

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This is a pre-copy-editing, author-produced of an article submitted for publication in *Maydica*.

Maydica

Accepted: November 2016

ISSN: 2279-8013

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_5 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

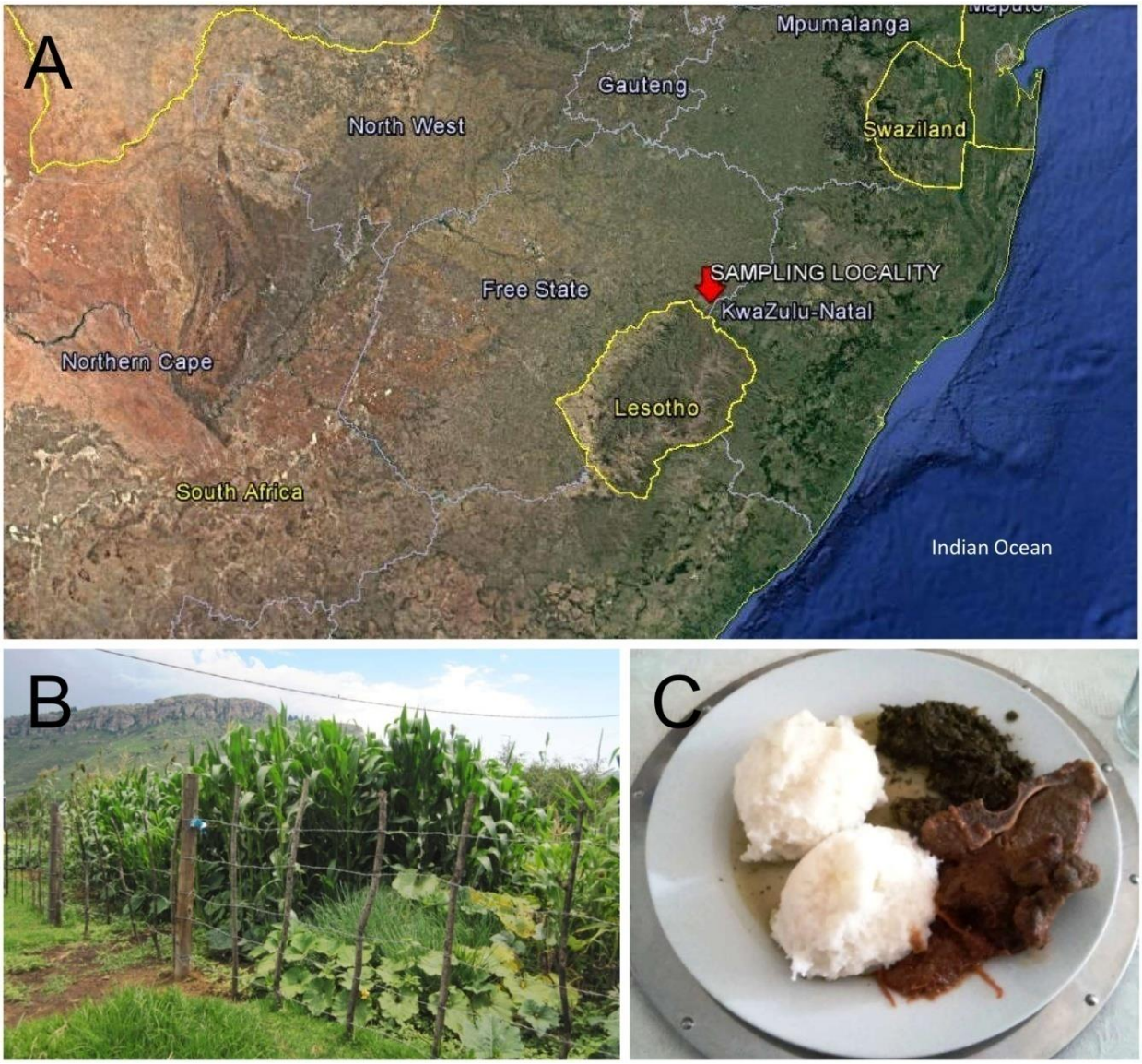

Figure 1. Sampling site of the white and yellow South African maize cultivars in the mountain region of Qwa-Qwa (28° 37' 20.81" S, 28° 53' 58.07" E) (A). Vegetable garden where the yellow variety was cultivated (B). South African traditional Maize meal porridge, pap, obtained using white maize flour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

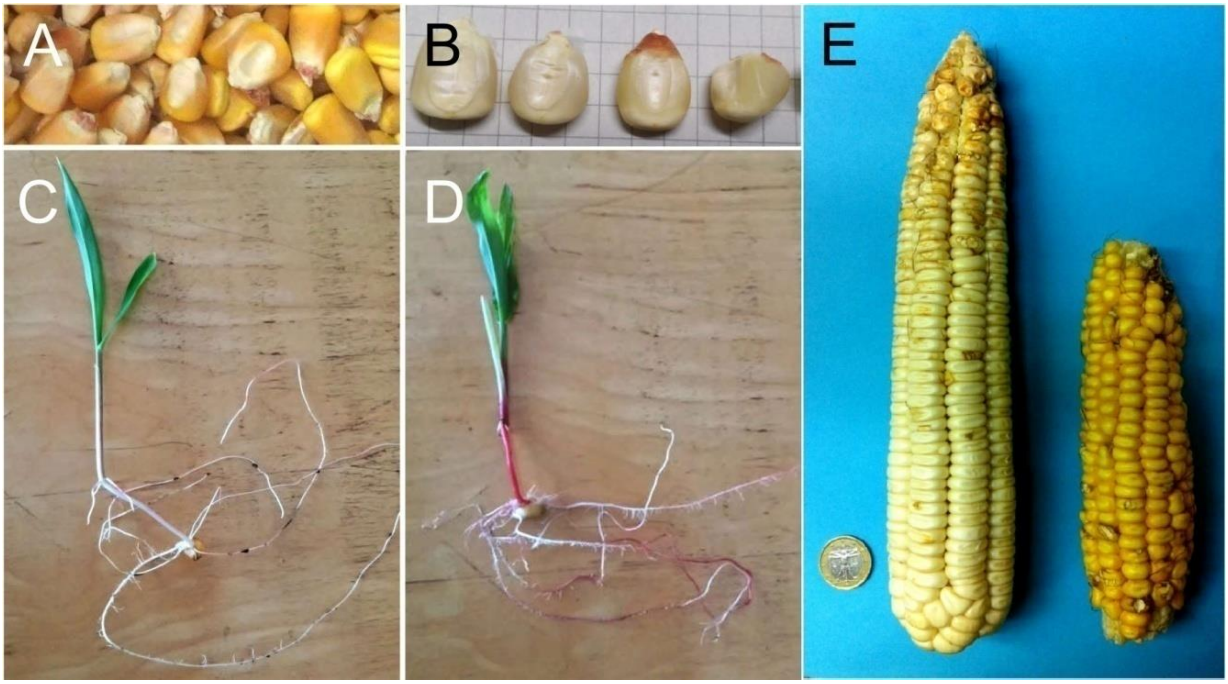

Figure 2. Seeds and seedlings of the two South African varieties. Seeds of the yellow South African variety (A) and of the white one (B). Seedlings of the yellow (C) and white (D) South African varieties after light exposure. Ears of the white (left) and yellow variety (right) (E).

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}

REFERENCES

Acham H, Egal AA, Oldewage-Theron WH, (2012). Household Asset Index and Total Iron Intake, but not education, best predict iron status in a black population sample in Gauteng, South Africa. Sci. Res. Essays, 7(9), 1035-1050.

Adom KK, Liu RH, (2002). Antioxidant activity of grains. J Agr Food Chem, 50(21), 6182-6187.

Ahmed SS, Lott MN, Marcus DM, (2005). The macular xanthophylls. Surv Ophthalmol, 50(2), 183-193.

Andreasen MF, Kroon PA, Williamson G, Garcia-Conesa MT, (2001). Intestinal release and uptake of phenolic antioxidant diferulic acids. Free Radical Bio Med, 31(3), 304-314.

Badone FC, Cassani E, Landoni M, Doria E, Panzeri D, Lago C, Mesiti F, Nielsen E, Pilu R, (2010). The low phytic acid1-241 (lpa1-241) maize mutation alters the accumulation of anthocyanin pigment in the kernel. Planta, 231(5), 1189-1199.

Berta G, Copetta A, Gamalero E, Bona E, Cesaro P, Scarafoni A, D'Agostino G, (2014). Maize development and grain quality are differentially affected by mycorrhizal fungi and a growth-promoting pseudomonad in the field. Mycorrhiza, 24(3), 161-170.

Black MM, (1998). Zinc deficiency and child development. Am J Clin Nutr, 68(2), 464S-469S.

Brown KH, Wuehler SE, Peerson JM, (2001). The importance of zinc in human nutrition and estimation of the global prevalence of zinc deficiency. Food Nutr. Bull., 22(2), 113-125.

Bunzel M, Ralph J, Marita JM, Hatfield RD, Steinhart H, (2001). Diferulates as structural components in soluble and insoluble cereal dietary fibre. J Sci Food Agr, 81(7), 653-660.

Chander S, Guo YQ, Yang XH, Zhang J, Lu XQ, Yan JB, Song TM, Rocheford TR, Li JS, (2008). Using molecular markers to identify two major loci controlling carotenoid contents in maize grain. Theor Appl Genet, 116(2), 223-233.

Chen Jr PS, Toribara TT, Warner H, (1956). Microdetermination of phosphorus. Anal Chem, 28(11), 1756-1758.

Das AK, Singh V, (2016). Antioxidative free and bound phenolic constituents in botanical fractions of Indian specialty maize (*Zea mays* L.) genotypes. Food Chem, 201, 298-306.

Doria E, Daoudou B, Egal AK, Oldewage-Theron WH, Pilu R, (2015). Preliminary analysis and biochemical characterization related to health implications for African populations in some maize cultivars. A special look at the South African environment. J Food Sci Nutr, 1(005).

Dowswell CR, Paliwal RL, Cantrell RP, (1996). Maize in the third world. Westview Press, Boulder, Colorado, USA. p. 8.

Escribano-Bailón MT, Santos-Buelga C, Rivas-Gonzalo JC, (2004). Anthocyanins in cereals. J Chromatog A, 1054(1), 129-141.

Faulks RM, Southon S, (2001). Carotenoids, metabolism and disease. Handbook of Nutraceuticals and Functional Foods, CRC Press pp. 143-156.

Gannon B, Kaliwile C, Arscott SA, Schmaelzle S, Chileshe J, Kalungwana N, Mosonda M, Pixley K, Masi C, Tanumihardjo SA, (2014). Biofortified orange maize is efficacious as a vitamin A supplement in Zambian children even in the presence of high liver reserves of vitamin A: a community-based, randomized placebo-controlled trial. Am J Clin Nutr, 100(6), 1541-1550.

Gibson RS, (2006). Zinc: the missing link in combating micronutrient malnutrition in developing countries. P Nutr Soc, 65(01), 51-60.

Gouse M, Piesse J, Thirtle C, (2006). Output and labour effects of GM maize and minimum tillage in a communal area of KwaZulu Natal. J Dev Perspect, 2(2), 192-207.

Haase H, Overbeck S, Rink L, (2008). Zinc supplementation for the treatment or prevention of disease: current status and future perspectives. Exp Gerontol, 43(5), 394-408.

Handelman GJ, (2001). The evolving role of carotenoids in human biochemistry. Nutrition, 17(10), 818-822.

Kirk JTO, Tilney-Bassett RAE, (1978). Proplastids, etioplasts, amyloplasts, chromoplasts and other plastids. The Plastids: Their Chemistry, Structure, Growth and Inheritance, eds J.T.O. Kirk and R.A.E. Tinley-Bassett. Amsterdam: Elsevier/North Holland Biomedical Press, pp. 217-239.

Kurilich AC, Juvik JA, (1999). Quantification of carotenoid and tocopherol antioxidants in Zea mays. J Agr Food Chem, 47(5), 1948-1955.

Kuhnen S, Lemos PMM, Campestrini LH, Ogliari JB, Dias PF, Maraschin M, (2009). Antiangiogenic properties of carotenoids: a potential role of maize as functional food. J Funct Food, 1(3), 284-290.

Kuhnen S, Menel Lemos PM, Campestrini LH, Ogliari JB, Dias PF, Maraschin M, (2011). Carotenoid and anthocyanin contents of grains of Brazilian maize landraces. J Sci Food Agr, 91(9), 1548-1553.

Lago C, Landoni M, Cassani E, Atanassiu S, Cantaluppi E, Pilu R, (2014). Development and characterization of a coloured sweet corn line as a new functional food. Maydica, 59(3), 191-200.

Lago C, Landoni M, Cassani E, Cantaluppi E, Doria E, Nielsen E, Giorgi A, Pilu R, (2015). Study and characterization of an ancient European flint white maize rich in anthocyanins: Millo Corvo from Galicia. PloS one, 10(5): e0126521. doi:10.1371/journal.pone.0126521

Lloyd BJ, Siebenmorgen TJ, Beers KW, (2000). Effects of commercial processing on antioxidants in rice bran 1. Cereal Chem, 77(5), 551-555.

Lopez-Martinez LX, Oliart-Ros RM, Valerio-Alfaro G, Lee CH, Parkin KL, Garcia HS, (2009). Antioxidant activity, phenolic compounds and anthocyanins content of eighteen strains of Mexican maize. LWT-Food Sci. Technol., 42(6), 1187-1192.

Mangels AR, Holden JM, Beecher GR, Forman MR, Lanza E, (1993). Carotenoid content of fruits and vegetables: an evaluation of analytic data. J Am Diet Assoc, 93(3), 284-296.

Ndolo VU, Beta T, (2014). Comparative studies on composition and distribution of phenolic acids in cereal grain botanical fractions. Cereal Chem, 91(5), 522-530.

Nuss ET, Tanumihardjo SA, (2011). Quality protein maize for Africa: closing the protein inadequacy gap in vulnerable populations. Adv. Nutr., 2(3), 217-224.

O'Dell BL, De Boland AR, Koirtyohann SR, (1972). Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. J Agric Food Chem, 20(3), 718-723.

Oelofse A, Van Raaij JM, Benadé AJ, Dhansay MA, Tolboom JJ, Hautvast JG, 2002. Disadvantaged black and coloured infants in two urban communities in the Western Cape, South Africa differ in micronutrient status. Public Health Nutr, 5(2), 289-294.

Oldewage-Theron WH, Dicks EG, Napier CE, Rutengwe R, (2005). Situation analysis of an informal settlement in the Vaal Triangle. Dev. South. Afr., 22(1), 13-26.

Panzeri D, Cesari V, Toschi I, Pilu R, (2011). Seed calorific value in different maize genotypes. Energy Sources Part A-Recovery, 33(18), 1700-1705.

Petroni K, Pilu R, Tonelli C, (2014). Anthocyanins in corn: a wealth of genes for human health. Planta, 240(5), 901-911.

Pilu R, Piazza P, Petroni K, Ronchi A, Martin C, Tonelli C (2003) plbol3, a complex allele of the anthocyanin regulatory pl1 locus that arose in a naturally occurring maize population. Plant J 36:510–521

Pilu R, Landoni M, Cassani E, Doria E, Nielsen E, (2005). The Maize *lpa241* mutation causes a remarkable variability of expression and some pleiotropic effects. Crop Sci, 45(5), 2096-2105.

Prasad AS, (2007). Zinc: mechanisms of host defense. J Nutr, 137(5), 1345-1349.

Raboy V,(1990). Biochemistry and genetics of phytic acid synthesis. Plant biol (USA).

Raboy V, (2002). Progress in breeding low phytate crops. J Nutr, 132(3), 503S-505S.

Samuel FO, Egal AA, Oldewage-Theron WH, Napier CE, Venter CS, (2010). Prevalence of zinc deficiency among primary school children in a poor peri-urban informal settlement in South Africa. Health SA Gesondheid (Online), 15(1), 1-6.

Scalbert A, Williamson, G, (2000). Dietary intake and bioavailability of polyphenols. J Nutr, 130(8), 2073S-2085S.

Snodderly DM, (1995). Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. Am J Clin Nutr, 62(6), 1448S-1461S.

Sommer A, Davidson, FR, (2002). Assessment and control of vitamin A deficiency: the Annecy Accords. J Nutr, 132(9), 2845S-2850S.

Steyn NP, Maunder EMW, Labadarios D, Nel JH, (2006). Foods and beverages that make significant contributions to macro-and micronutrient intakes of children in South Africa–do they meet the food-based dietary guidelines?. S Afr J Clin Nutr, 19(2), 66-76.

Tokuji Y, Akiyama K, Yunoki K, Kinoshita M, Sasaki K, Kobayashi H, Wada M, Ohnishi M, (2009). Screening for beneficial effects of oral intake of sweet corn by DNA microarray analysis. J Food Sci, 74(7), H197-H203.

Virgili F, Marino M, (2008). Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity. Free Radic. Biol. Med., 45(9), 1205-1216.

Vorster HH, Oosthuizen W, Jerling JC, Veldman FJ, Burger HM, McLachlan M, (1997). The nutritional status of South Africans: a review of the literature from 1975-1996. Durban Health Syst Trust, 1: 1-22; 2, 1-22.

West CE, Eilander A, Van Lieshout M, (2002). Consequences of revised estimates of carotenoid bioefficacy for dietary control of vitamin A deficiency in developing countries. J Nutr, 132(9), 2920S-2926S.

Žilić S, Serpen A, Akıllıoglu G, Gokmen V, Vancetović J, (2012). Phenolic compounds, carotenoids, anthocyanins, and antioxidant capacity of colored maize (*Zea mays* L.) kernels. J Agric Food Chem, 60(5), 1224-1231