Title: CORN HAVING HIGH PIGMENT AND REDUCED MYCOTOXIN CONTENT, PREPARATION AND USE THEREOF

Abstract: Corn plant characterised by low susceptibility to micotoxin contamination and by high anthocyanin accumulation in tissues, and use thereof as food and dietary supplement, for either humans or animals, or as the starting material for the extraction of antioxidant pigments.
CORN HAVING HIGH PIGMENT AND REDUCED MYCOTOXIN CONTENT, PREPARATION AND USE THEREOF

The present invention provides a corn plant characterized by a high content of flavonoid pigment and lower susceptibility to micotoxin contamination. The corn plant of the invention, or a part thereof, can be used as food and dietary supplement, for either humans or animals, or as the starting material for the extraction of antioxidant pigments.

BACKGROUND OF THE INVENTION

Corn is one of the most important crop in the world and is the primary source of food for pigs, poultry and, to a lesser extent, for ruminants.

From sixties to nineties, in Italy the production has increased from 50 to more than 100 quintals per hectare, allowing a large development of the zootechnical section, in particular in the northern regions, where corn represent the most important source of calories for the animal feeding as kernels or silage.

In Italy 82% of maize is used in animal food industry, 12% in starch industry, 4% for direct human consumption and 2% for other industrial applications, such as the production of paper, biodegradable plastics, solvents and biofuels.

The corn, in form of kernels and silage, represents one of the raw material more used for animal feeding. It is, in fact, the main component of pig and poultry diet and is used also for ruminant feeding. The high availability of maize flour has promoted in the northern regions of Italy the development of breeding that, for technical level and product quality is at the first places in Europe. Thus, a large amount of maize enters directly or indirectly the human food chain.

One of the major problems for maize cultivation is the occurrence of
fungal infections, which causes loss of yield and contamination of grains with mycotoxins, in particular aflatoxins and fumonisins. Mycotoxins are secondary metabolites potentially dangerous for animals and human health.

Among the different fungal infections, *Fusarium verticilloides* and *F. proliferatum* are the most abundant in several wet zones such as northern Italy, causing contamination of the kernels with fumonisin B2, B3 and most frequently B1 (1, 2, 3). These toxins are involved in a number of serious animal diseases like porcine pulmonary edema (4), neural tube birth defects (5), equine leukoencephalomalacia (6), and in general in loss of weight in farm animals (1, 2). Even human cancer has been reported as a possible consequence of ingesting these toxins (6). For this problem several official agencies such as the FAO/WHO Expert Committee on Food Additives, U.S. Food and Drug Administration and the European Union, established the threshold of fumonisin content in maize as 2-4 ppm in non-processed maize.

So far, no definitive control strategies are available to prevent fumonisin accumulation in kernels. However, it is known that factors favouring or affecting crop growth such as planting date, irrigation, nitrogen fertilization, insects and storage can contribute to fungal proliferation on kernels (3, 4, 7-10).

Another major risk factor is the attack of insects, especially corn borer, which vehiculate fungal infection into seeds, leading to 15-80% loss of yield depending on the season. Bt transgenic maize, which is resistant to corn borer, can be a solution to lower fumonisins in maize, but legal restrictions do not allow cultivation of transgenic maize. In addition, Bt maize does not guarantee that the fumonisin level is kept to a concentration not dangerous for human consumption, if the season is favourable to the invasion of pests (11, 12).

Among maize genotypes, differences in fumonisin accumulation have
been found to correlate with several genetic traits, such as late-maturing (13), thickness of pericarp (14), shape of husks (15). However, due to the complexity of this character many questions are still open. Unquestionably, fusarium infection and consequently mycotoxin contamination are associated with several quantitative trait loci (QTLs) and in particular with two QTLs located on chromosome 5 (10, 16).

Flavonoids and anthocyanins are two of the most important classes of bioactives, providing the red and blue pigments widely present in fruits and vegetables. The antioxidants present in vegetables species used in the human diet are subdivided in nutrient (C vitamin and carotenoid) and not nutrient (flavonoid and phenolyc substance) compounds. They are able to prevent cellular damages caused by active oxygen species and by free radicals formed during aerobic metabolism and by exogenous stresses. In particular, the flavonoids are compounds which provide some of the pigments presents in vegetable kingdom. They are water soluble molecules present in glycosilated form inside the vacuole, and on the basis of their molecular structures they are grouped in several classes: flavanones, flavonols, iso-flavonoids and anthocyanins. These compounds present a base structure constituted by a 15 carbon atoms ring derived by a condensation reaction involving acetate and phenylalanine.

The anthocyanins, which represent a class of secondary metabolites synthesised exclusively in plants having red coloured tissues or parts, carry out many other physiological functions (5). Several studies indicate the importance of anthocyanins also as antioxidant compounds and as protective compounds against age-related diseases, such as cardiovascular disease and cancer. (6-10, 17, 18). In particular experiments performed on rats fed with coloured corn have shown that these animals are more protected against obesity, hyperglycemia (9) and ischemia (19).
Furthermore it has been showed that anthocyanins are also antimicrobial agents able to inhibit the mycotoxin synthesis by mould in vitro (20).

Although several maize cultivars are able to accumulate anthocyanins, the hybrid used so far are colourless.

Flavonoids are a class of plant molecules synthesized in maize by a complex pathway made up of more than 20 genes and regulated by two classes of transcription factors: r1/b1 bHLH genes and c1/pl1/p1 MYB gene families (21). This metabolic pathway is branched into two sections: one leads to the synthesis of flavonols and anthocyanins (responsible for the colour of aleurone and pericarp), and is regulated by the coordinated action of b1 or c1 with c1 or pl1, the second section of the pathway leads to the synthesis of C-glycosyl flavones like phlobaphenes (causing red pericarp pigments) and maysin and is regulated by the p1 gene (11-16, 21-24). Due to large genetic variability present in corn, several alleles of regulatory genes of the anthocyanins pathways have been characterized. They are able to confer a strong pigmentation in different plant tissues: B1 (booster), Pl1 (purple plant), R1 (red color), P1 (pericarp color) (11, 16, 25, 26). In particular the alleles B1 e Pl1, when present in the same genotype induce a big accumulation of anthocyanins in roots, stalk, anther, cob, pericarp and partially in the leaves; R1 induces accumulation of anthocyanins in the aleurone and P1 induces accumulation of phlobaphenes in the kernel and cob. All these alleles act as dominant genes, useful for the production of F1 hybrid seeds.

There is a considerable demand for food colorants from natural sources as an alternative to synthetic colorants, since 25% of consumers perceive foods without artificial ingredients as desirable. Anthocyanin-enriched extracts are currently obtained from grape, specifically
from the skin wastes of wine production. Other plant species potentially supplying anthocyanins are blueberry (900-4500 mg/Kg), blackberry and blackcurrant (600-3500 mg/Kg), but industrial extraction from these fruits is expensive due to a low yield of cultivation.

There is growing evidence that bioactive substances present in the diet may promote health. Flavonoids and related phenols are examples of bioactives from plants that have beneficial effects on a number of important risk factors associated with cardiovascular disease, cancer and age-related degenerative diseases. For this reasons there is a growing interest to find a cheaper vegetable source of pigment easily extractable to supplement human and animal diet (6-10, 17, 18, 26, 27).

**DESCRIPTION OF THE INVENTION**

It has surprisingly been found that a maize plant carrying a genotype which is selected from:

a) \( B1-Pl1-R1-P1-C1- \);
b) \( B1-Pl-C1- \);
c) \( R-P1-C1 \);
d) \( Sn1-Pl1-R1-P1-C1- \);
e) \( Sn1-Pl-C1- \),

wherein \( B1, Pl1, P1, R1, C1, Sn1 \) identify the dominant alleles of the following genes:

\( B1 = \) booster 1gene, mapping on chromosome 2 (bin 2.03); SEQ ID NO:1;

\( Pl1 = \) purple plant 1 gene, mapping on chromosome 6, bin 6.04; SEQ ID NO:2;

\( P1 = \) pericarp color 1 gene, mapping on chromosome 1, bin 1.03; SEQ ID NO:3;

\( R1 = \) red color 1 gene, mapping on chromosome 10, bin 10.06; SEQ ID NO:4;
NO:4;

\[ C1 = \text{colored aleurone 1 gene, mapping on chromosome 9, bin 9.01}; \]

SEQ ID NO:5;

\[ Sn1 = \text{scutellar node color 1 gene, mapping on chromosome 10, bin 10.06, SEQ ID NO:6}; \]

and wherein the hyphen attached to each gene identifier indicates either a dominant or a recessive allele of the same gene, is significantly less susceptible to mycotoxin contamination and contain increased amounts of antioxidant pigments in plant tissues, particularly anthocyanins and phlobaphenes, compared to control genotypes (colourless).

Therefore, in a first embodiment the invention provides a maize plant, or a part thereof including kernels, said maize plant being characterized by a genotype which is selected from those mentioned above.

The \( B1 \) (booster 1), \( Pl1 \) (purple plant 1) and, to a lesser extent, \( Sn1 \) (scutellar node color) genes determine anthocyanin pigment accumulation in the pericarp; the \( R1 \) (red color 1) and \( C1 \) (colored aleurone1) genes determine anthocyanin pigment accumulation in the aleurone while the \( P1 \) (pericarp color 1) gene increases the phlobaphene level in the pericarp (Fig. 1). The hybrids \( B1-Pl1-R1-P1-C1 \), obtained by crossing the constituted, inbred lines \( B1B1 P1P1 C1C1 \) and \( R1R1 P1P1 C1C1 \), with the equivalent \( (Sn1-Pl1-R1-P1-C1-) \), are able to accumulate pigments in all plant tissues and are therefore particularly preferred.

The genes \( B1 \) and \( Pl1 \) or \( Sn1 \) and \( Pl1 \), when present in the same genotype, produce anthocyanin accumulation in kernels and all plant tissues (Fig. 2). The analysis of the anthocyanin content in kernels of \( R1 \) and \( B1 Pl1 \) genotypes showed an approximately 33- and 55-fold increase compared to the control genotype carrying the \( r1 \) gene, respectively (Table 1). The analysis of the phlobaphene content of kernels of \( P1 \) and \( p1 \) genotypes
showed an approximately 14-fold increase compared to the control genotype carrying \( p1 \) gene (Table 1). In open-field cultures, the presence of this pigment in kernels was found to partially reduce the accumulation of mycotoxin. In fact the analysis of kernels mycotoxin content showed a lower \( B1 \) fumonisine accumulation in genotypes able to accumulate pigment compared to the control genotype. In particular, a lower content of mycotoxin has been observed in the genotypes \( P1 \) and \( B1 \ PI1 \), which are able to accumulate pigment in the pericarp, compared to the isogenic colourless control (Figure 3 and Table 2). The aflatoxins B1, B2, G1 and G2 have been quantified below the 0.01 ppb threshold in all the samples analysed. Furthermore the data collected over the last three seasons as regards fumonisin B1 accumulation, moldy kernels and damaged kernels (mainly due to corn borer) on all harvested materials, showed a significant correlation between the percentage of moldy kernels and the content of fumonisin B1 (\( r = 0.729 \)), between the % of damaged kernels and the content of fumonisin B1 (\( r = 0.693 \)) and between the percentage of damaged kernels and the percentage of moldy kernels (\( r = 0.628 \)) (Table 3).

The maize plants having the desired genotype combination according to the invention can be obtained by suitable crossing of the selected inbred lines using known breeding techniques. Figure 4 reports a pattern of crosses used to combine several genes in a single genotype. The crosses have been carried out using paper bags to cover the ear, to prevent pollen cross contamination and to collect the pollen from the tassels.

In another embodiment the invention provides the use of a plant as herein described, or a part thereof, as food for human or animal consumption. The higher level of antioxidant pigment, together with the lower mycotoxin contamination, make the use of maize plants according to the invention particularly advantageous. In a preferred embodiment, the plant is
used to produce seeds for human consumption and silage for animal feeding.

In a further embodiment, the invention provides the use of the maize plant, or a part thereof, as starting material for the extraction of antioxidant pigments, in particular anthocyanines. The extractive processes are known to the skilled person and include extraction with solvents, such as methanol and acetone, and supercritical CO₂ extraction. A technique used for maize plant pigments extraction is described in EP1191071.

TABLES

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Anthocyanins</th>
<th>Flavonols</th>
<th>Phlobaphenes</th>
<th>Phenolic acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>r1/r1</td>
<td>0.01±0.01</td>
<td>0.72±0.09</td>
<td>0.7±0.1</td>
<td>1.20±0.07</td>
</tr>
<tr>
<td>R1/R1</td>
<td>0.66±0.21</td>
<td>0.57±0.05</td>
<td>n.d.</td>
<td>1.20±0.20</td>
</tr>
<tr>
<td>b1/b1 pl1/pl1</td>
<td>0.03±0.01</td>
<td>0.66±0.10</td>
<td>0.8±0.2</td>
<td>1.13±0.20</td>
</tr>
<tr>
<td>B1/- Pl1/-</td>
<td>1.64±0.22</td>
<td>0.63±0.08</td>
<td>n.d.</td>
<td>1.18±0.11</td>
</tr>
<tr>
<td>p1/p1</td>
<td>0.02±0.01</td>
<td>n.d.</td>
<td>1.89±0.30</td>
<td>3.48±0.71</td>
</tr>
<tr>
<td>P1/P1</td>
<td>0.02±0.01</td>
<td>n.d.</td>
<td>27.53±5.80</td>
<td>4.51±0.90</td>
</tr>
</tbody>
</table>

\(^1\) n.d. not determined.
Table 2. Concentration of mycotoxins and frequency of occurrence of moldy kernels and damaged kernels in colored and colorless control populations over 3 years.

<table>
<thead>
<tr>
<th>Field season</th>
<th>Genotype</th>
<th>Ears color</th>
<th>% Moldy(^1)</th>
<th>% Damaged(^1)</th>
<th>Aflatoxin B1, B2, G1, G2</th>
<th>Fumonisin B1 (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>r1/r1</td>
<td>-</td>
<td>14.8</td>
<td>0.4</td>
<td>&lt; 0.01</td>
<td>6.6</td>
</tr>
<tr>
<td>2007</td>
<td>R1/R1</td>
<td>+</td>
<td>11.4</td>
<td>0.8</td>
<td>&lt; 0.01</td>
<td>6.5</td>
</tr>
<tr>
<td>2007</td>
<td>b1/b1 pl1/pl1</td>
<td>-</td>
<td>6.1</td>
<td>0.6</td>
<td>&lt; 0.01</td>
<td>3.2</td>
</tr>
<tr>
<td>2007</td>
<td>B1/- Pl1/-</td>
<td>+</td>
<td>3.4</td>
<td>0.5</td>
<td>&lt; 0.01</td>
<td>2.9</td>
</tr>
<tr>
<td>2007</td>
<td>p1/p1</td>
<td>-</td>
<td>10.2</td>
<td>1.1</td>
<td>&lt; 0.01</td>
<td>5.7</td>
</tr>
<tr>
<td>2007</td>
<td>P1/P1</td>
<td>+</td>
<td>3.2</td>
<td>0.2</td>
<td>&lt; 0.01</td>
<td>2.5</td>
</tr>
<tr>
<td>2006</td>
<td>r1/r1</td>
<td>-</td>
<td>33.2</td>
<td>1.8</td>
<td>&lt; 0.01</td>
<td>12</td>
</tr>
<tr>
<td>2006</td>
<td>R1/R1</td>
<td>+</td>
<td>20.4</td>
<td>0.8</td>
<td>&lt; 0.01</td>
<td>10</td>
</tr>
<tr>
<td>2006</td>
<td>b1/b1 pl1/pl1</td>
<td>-</td>
<td>n.d.(^2)</td>
<td>n.d.</td>
<td>&lt; 0.01</td>
<td>11</td>
</tr>
<tr>
<td>2006</td>
<td>B1/- Pl1/-</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&lt; 0.01</td>
<td>3.6</td>
</tr>
<tr>
<td>2006</td>
<td>p1/p1</td>
<td>-</td>
<td>13.8</td>
<td>1.2</td>
<td>&lt; 0.01</td>
<td>9.5</td>
</tr>
<tr>
<td>2005</td>
<td>P1/P1</td>
<td>+</td>
<td>7.6</td>
<td>0.4</td>
<td>&lt; 0.04</td>
<td>1.2</td>
</tr>
<tr>
<td>2005</td>
<td>r1/r1</td>
<td>-</td>
<td>18.2</td>
<td>0.4</td>
<td>&lt; 0.01</td>
<td>2.9</td>
</tr>
<tr>
<td>2005</td>
<td>R1/R1</td>
<td>+</td>
<td>16.6</td>
<td>0.5</td>
<td>&lt; 0.01</td>
<td>2.5</td>
</tr>
<tr>
<td>2005</td>
<td>p1/p1</td>
<td>-</td>
<td>8.2</td>
<td>1.2</td>
<td>&lt; 0.01</td>
<td>1.1</td>
</tr>
<tr>
<td>2005</td>
<td>P1/P1</td>
<td>+</td>
<td>7.6</td>
<td>0.4</td>
<td>&lt; 0.01</td>
<td>0.7</td>
</tr>
</tbody>
</table>

\(^1\) analyzed by visual inspection of at least 500 seeds

\(^2\) not determined.

Table 3

Table 3. Correlation coefficients between % moldy kernels and fumonisin B1; % damaged kernels and fumonisin B1, % damaged Kernels and % moldy kernels in 2005-07 seasons.

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>n(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% moldy kernels - fumonisin B1</td>
<td>0.729*</td>
<td>17</td>
</tr>
<tr>
<td>% damaged kernels - fumonisin B1</td>
<td>0.693*</td>
<td>17</td>
</tr>
<tr>
<td>% damaged kernels - % moldy kernels</td>
<td>0.628</td>
<td>17</td>
</tr>
</tbody>
</table>

\(^1\) Number of observations

* p < 0.01
DESCRIPTION OF THE FIGURES

FIG. 1. Comparison of kernel pigment accumulation in different genotype combinations obtained by specific breeding program: B1 (booster 1), P11 (purple plant 1), R1 (red color 1), C1 (colored aleurone 1), P1 (pericarp color 1) and respective recessive alleles.

FIG. 2. Comparison of anthocyanine accumulation in mature plants, silage and kernels from two hybrids differing in the genetic constitution only for the presence of B1 and P11 genes. The study was carried out in 2005, 2006 and 2007 field seasons.

FIG. 3. Analysis of mycotoxins content in the kernels of genotype R1, P1, B1 P11 and in the corresponding isogenic control r1, p1 e b1 p1. Analysis carried out in 2005, 2006 and 2007 field seasons.

FIG. 4. Scheme of the crosses made to generate the two inbred lines carrying B1B1 P11P11 and R1R1 P1P1, which can be directly used as parental lines for the hybrid constitution B1b1 P11p11 and R1r1 P1p1 or crossed each other to obtain the hybrid B1b1 P11p1 R1r1 P1p1.

- R1, B1, P11 and P1 are dominant on r1, b1, pl1 e p1 alleles
- The pericarp is a seed tissue of maternal origin
- B1, P11 and P1 have maternal effect expressing in the pericarp
- All plants are homozygous for C1 and all structural genes involved in the anthocyanins and phlobaphenes biosynthesis pathway are dominant
- In this schema the crosses with B1 could be substituted by the Sn1 gene.
EXPERIMENTAL

The inbred lines and hybrids have been developed by artificial pollination using paper bags to avoid cross contamination.

Spectrophotometric analysis of flavonoids

Anthocyanins, flavonols and phenolic acids were extracted from individual seeds with 1% HCl in 95% ethanol. The extracts were centrifuged twice and their absorption determined spectrophotometrically at 530 nm for anthocyanins, at 350 nm for flavonols and at 280 nm for phenolic acids. The amount of anthocyanins was calculated as cyanidin 3-glucoside equivalents (molar extinction coefficient (ε) 26900 L m⁻¹mol⁻¹, M.W. 484.82), flavonols content as quercetin 3-glucoside equivalents (ε 21877 L m⁻¹mol⁻¹, M.W. 464.38) and the amount of phenolics as ferulic acid equivalents (ε 14700 L m⁻¹mol⁻¹, M.W. 194.18). Mean values represent seven independent replicates ±S.D. Phlobaphenes were extracted from individual seeds with 1 volume of concentrated HCl and 4 volumes of dimethylsulfoxide (DMSO) sequentially with vigorous vortexing after each addition, essentially as described by Das et al. (28). 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 ±S.D.

Mycotoxin analysis

The seeds harvested were dried to 12-13% moisture content and about 500 grams of milled seeds were used for mycotoxin analysis. Total B1, B2, G1, G2 aflatoxin and B1 fumonisin quantification was made by the technical service of the Grain Consortium of Milano, using immunoaffinity column HPLC for aflatoxin and fluorimetric methods for the B1 fumonisin (AOAC Official Method 991.31 - “Aflatoxins in corn, Raw peanuts and Peanut butter - Immunoaffinity column Method”).
BIBLIOGRAPHY


23. Guerra M.C.; Galvano F.; Bonsi L. et al. (2005) Cyanidin-3-O- beta-glucopyranoside, a natural free-radical scavenger against aflatoxin B1- and ochratoxin A-induced cell damage in a human hepatoma cell line (Hep G2) and a human colonic adenocarcinoma cell line (CaCo-2). British Journal of Nutrition 94 (2): 211-220.


CLAIMS

1. A maize plant characterized by a genotype which is selected from the
   group consisting of:

5 a) \((B1\cdot PI1\cdot R1\cdot P1\cdot C1)\);
   b) \((B1\cdot PI\cdot C1)\);
   c) \((R\cdot P1\cdot C1)\);
   d) \((Sn1\cdot PI1\cdot R1\cdot P1\cdot C1)\);
   e) \((Sn1\cdot PI\cdot C1)\),

10 wherein \(B1, PI1, P1, R1, C1, Sn1\) identify the dominant alleles of the
   following genes:

\(B1 = \text{booster 1gene, mapping on chromosome 2 (bin 2.03}; \text{ SEQ ID NO:1;})\)
\(PI1 = \text{purple plant 1 gene, mapping on chromosome 6, bin 6.04; SEQ ID NO:2;})\)
\(P1 = \text{pericarp color 1 gene, mapping on chromosome 1, bin 1.03; SEQ ID NO:3;})\)
\(R1 = \text{red color 1 gene, mapping on chromosome 10, bin 10.06; SEQ ID NO:4;})\)
\(C1 = \text{colored aleurone 1 gene, mapping on chromosome 9, bin 9.01; SEQ ID NO:5;})\)
\(Sn1 = \text{scutellar node color 1 gene, mapping on chromosome 10, bin 10.06,}
\text{ SEQ ID NO:6},\)
   and wherein the hyphen attached to each gene identifier indicates either a
   dominant or a recessive allele of the same gene.

25 2. A maize plant according to claim 1, wherein said genotype is selected
   from \((B1\cdot PI1\cdot R1\cdot P1\cdot C1)\) and \((Sn1\cdot PI1\cdot R1\cdot P1\cdot C1)\).
3. A seed of a maize plant according to claims 1 and 2.
4. The use of a maize plant according to claims 1-2, or a part thereof, for
the preparation of foodstuff for human or animal consumption.

5. The use according to claim 4, wherein the maize plant or a part thereof are in form of kernels or silage.

6. The use of a maize plant according to claims 1-2, or a part thereof, as starting raw material for the extraction of antioxidant pigments.

7. The use according to claim 6, for the extraction of anthocyanines for human or animal consumption.

8. Foodstuff for human or animal consumption containing a maize plant or a part thereof according to claims 1 and 2.
Figure 2

Mature plants

Silage

Seeds