INDUCTION AND STUDY OF TETRAPLOID *OPAQUE-2* MAIZE

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Abstract

In this paper we present the results on induction and study of tetraploid maize forms containing *opaque-2* gene which determines a high content of lysine in grain protein, in order to exploit the biochemical effect of this mutation at the tetraploid level. By applying colchicine of 0,15% concentration on plants of hybrid Chişiniovschi 307 PL at 3-4 leaf stage, tetraploid forms were obtained, characterized by vigorous stems, but shorter size and internodes, leaves with darker green hues, poorly branched panicle, but with thicker branches, especially the central one. Study of leaf epidermis revealed larger stomata but fewer per unit area. Diploid pollen of tetraploid forms was larger compared to the haploid, but the degree of fertility was lower in tetraploid forms. Analysis of the phenotypic expression of *opaque-2* mutation of maize tetraploid forms showed that this gene inherits at random chromosomal segregation in a phenotypic ratio of 35:1, which is confirmed by χ^2 test application. With ploidy level increased the amount of grain protein and fat content was reduced. Lysine content in grain protein containing *opaque-2* gene increased in proportion to the dose level in triploid and, partly, in the hexaploid endosperm.

Key words: Zea mays L., Diploids, Tetraploids, Opaque-2, Lysine.

Poliploids are organisms that contain multiple sets of chromosomes or genomes (3x, 4x, 5x, etc) (Tate et al., 2005). These forms may arise spontaneously due to abnormalities in the cell division or can be induced experimentally by the use of the specific agents (Palii A., 1998). In the experimental polyploid series of maize, the only forms of practical importance are tetraploids (4x = 40). The first tetraploids of maize were obtained by L. F. Randolph in 1932 by applying the temperature shock. Comparing to diploids, tetraploids plants of maize have both more valuable morphological, physiological and biochemical traits and some negative features as: reduced fertility, slower growth, longer growing season, low productivity, others that make tetraploids uncompetitive with commercial diploids (Мирюта Ю. П., 1966; Коварский А. Е., Обершт В. М., Чалык Т. С., 1970; Хаджинов М. И., Щербак В. С., 1974; Sockness BA, Dudley JW, 1989a, 1989b). However, tetraploid forms have found use in various scientific studies on genetic variability (Riddle N.C. et al., 2006, 2008), inbreeding and heterosis (Wang Ze-li et al., 2005), gene expression and dosage effects (Guo, M., Davis D., Birchler J. A., 1996; Riddle C. N. et al., 2010; Yao H. et al., 2011), etc.. At the State Agrarian University of Moldova are carried out experiments with the aim of using polyploidy in improving the quality of maize grain. In this paper we present the results on the induction and study of tetraploid forms of maize which contain the *opaque-2* gene (o_2) incorporated into the genotype, that determines a high content of lysine in the protein of the grain, in order to exploit the biochemical effect of this mutation on tetraploid level.

MATERIAL AND METHOD

The research was conducted at the Department of Plant Biology of the State Agrarian University of Moldova and the Institute of Plant breeding "Porumbeni" in 2010-2013. As biological material served a single-cross maize hybrid with increased protein and lysine content Chişiniovschi 307PL with o_2 gene in the endosperm, single-cross hybrid population Porumbeni 331Mrf, and a tetraploid synthetic B with normal endosperm. In order to induce tetraploidy we used 0,15% concentration colchicine ($C_{22}H_{25}NO_6$) (Sigma) with dimethyl sulfoxide as additive. Treatment was made by injections of the colchicine solution in the apex of plants at 3-4 leaf stage in the morning. During vegetation period the possible influence of ploidy level on the following characters was studied: plant height, leaf length and width, height of ear insertion, internode length, number of tassel branches and the length of the central branch. Pollen was studied on B490A microscope with digital camera MD400. The fertility was assessed by staining with iodine solution.

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Colored grains were considered fertile, while the uncoloured - sterile. Anatomical fractions of the grains (endosperm, embryo, pericarp + tip cap) were evaluated after harvest in several tetraploid o_2 families (compared to the original diploid form), diploid single-cross hybrid Porumbeni 331Mrf and in tetraploid synthetic B. Analysis of the anatomy of the grains was performed by separating and weighing each fraction with the torsion balance.

Biochemical analysis was performed by infrared spectroscopy. Protein amino acid content was determined by ion exchange chromatography on an automatic aminoacid analyser T339M.

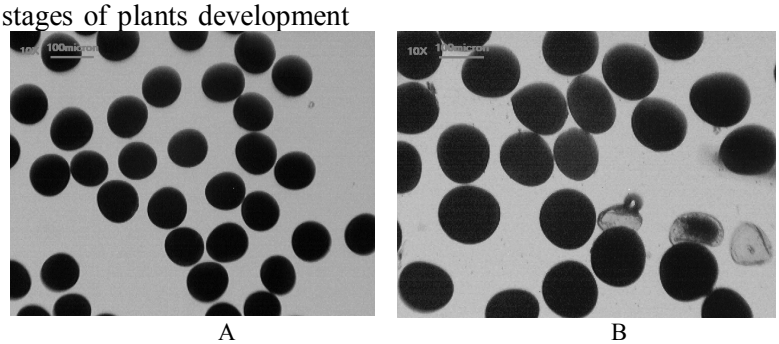
The data were statistically processed using StatGraphics Centurion version XV.

RESULTS AND DISCUSSIONS

Due to biological peculiarities of the corn plants, application of the colchicine at the first stage of the research allowed to obtain chimeric plants, with tissues containing different ploidy level, characterized by different degrees of morphosis. An important criterion in selecting the valuable plants was the size of produced heterogeneous pollen, some grains being about 50% higher than others. Self-pollination of these plants allowed to obtain chimera ears of various classes of kernels, distinguished by size and extent of development of the endosperm. Using these criteria we have selected tetraploid grains that differed by size, color and weight, which was 30% higher than diploid grains. After selecting tetraploid grains, next step was the verification of chromosomes number to avoid the cases with diploid or aneuploidy grains. In the initial stages of plants development, there were no significant differences between tetraploid forms (4x) obtained from the hybrid Chişiniovschi PL 307 and diploid plants (2x). Most essential differences were observed in the later stages of plants development expressed in their vigor (height), stem thickness, leaf appearance and tasseling, these difference became more well pronounced at plants maturity thus, during flowering, tetraploid plants had a different habitus compared to that of diploid plants.

Biometric measurements performed after pollination revealed significant differences in: plant's height, internode length, number of tassel branches, and length of the central branch of the tassel. Some other insignificant differences have been observed in the leaf length and width, height of ear insertion and tassel length. Our results show that the studied traits are differently influenced by ploidy level, some being more affected than others, as noted in other studies [Riddle N. C., Kato A., Birchler J. A., 2006]. The morphology studies of lower epidermis of diploid and tetraploid maize o_2 lamina revealed that stomata size of tetraploids are higher compared to those of diploid forms and kept in offspring. Tetraploid forms exceeded those of diploids after this character on average by 20-25%. It was also noted that the variability coefficient of stomata size in tetraploid forms was higher, indicating the variable nature of the character. Density of stomata per unit of leaf area of tetraploid leaves was lower than that of diploid leaves.

Study of pollen grains showed that the average size of diploid pollen grains of tetraploid o_2 forms were within the limits of 101-115µm , while those of haploid grains of the diploid form ranged between 74 - 88µm, the difference varying 15 to 55% (*Fig. 1*). The data show that in the tetraploid families with the same origin, the variability exists in the average size of the pollen grains due, perhaps, to complex interaction between the ploidy level, genotype and the environment.



A B

Figure 1 **The polen grains of diploid (A) and tetraploid (B) plants of Chişiniovschi PL 307 hybrid**

After calculating the percentage of pollen fertility, we identified that tetraploid forms have had a high degree of pollen sterility comparing to diploid forms, the degree of fertility varying within 76-96% to 95-98%, depending on years of study. In our experiments we noticed some pollen grains with irregular or abnormal shape (dumbbell, pear, strangulations), the frequency of which, on average, was 0.4%. Some researchers believe that abnormalities of this type are caused by the disturbances in microsporogenesis (Lobanova L. P., Kolesov A. Ju., 2010).

Tetraploid plants have produced ears with a thicker cob than the diploids and kernels were larger and distributed in chaotic rows (*Fig. 2*). The degree of filling of the tetraploid ears was mostly low, typical to neopolyploid forms. The quantitative analysis showed that the average grain weight of tetraploids was higher than that of diploids however, it varied considerably. Tetraploid o_2 grains exceeded the diploid grains maximum by 18%, but there were families with 7% lower mass, which in statistical terms was considered as insignificant. During the unfavorable conditions of 2012 year, the average mass of a grain decreased significantly ($P \leq 0.05$). The grain weight of diploid hybrid Chişiniovschi PL 307 was reduced by 50%, while for some families it decreased only by 10%. This highlights the valuable features of these families, which maintain the filling of grains in various environmental conditions. As a result of experiments, we found some structural-anatomic changes of maize embryos. The analysis showed that embryos increased in mass differently depending on form and, similar to whole grain, some forms tend to have a substantial advantage over the mass of diploid grains during the year of 2012. In analysis of tetraploid grains the most obvious differences occurred in the characteristics of the pericarp, which turned out to be thicker and harder, so that all tetraploid grains significantly exceeded the diploid ones by this indicator. An important feature is the share in the whole grain. The results showed that in some tetraploid ears the embryos had higher mass (≈ 14%), while others maintain the diploid level (≈ 12%), or below them (9-11%). The share of pericarp was higher in tetraploid grains (7-12%) than in diploids (6%). One of the objectives of the research was to analyze o_2 gene heredity in tetraploid forms to establish the model of segregation: random chromosomal or chromatid. O_2 gene is a recessive gene, located on chromosome 7 in locus 16 near the centromere and in diploid forms is inherited according to Mendel laws of heredity in a classic mendelian ratio of 3:1 (Палий А. Ф., 1989; Vasal S. K., 2001). The analysis of tetraploid cobs obtained in the second generation (F_2) by self-pollination of a duplex $O_2O_2o_2o_2$ established an empirical 34,33:1 segregation, which is close to the theoretical 35:1. Application of the χ^2-test generated a value $\chi^2 = 0.055$, which showed that the o_2 gene is transmitted in tetraploid maize at random chromosomal segregation model. In our research the final objective was to evaluate the biochemical expression of the o_2 gene in interaction with ploidy level in grain endosperm. In the biochemical experiments as control was used the diploid hybrid Chişiniovschi 307PL, a comercial diploid hybrid Porumbeni 331 MRf (with vitreous endosperm) and a synthetic tetraploid B (with vitreous endosperm). Biochemical analyzes performed on the material under study revealed some essential differences between diploid and tetraploid forms.

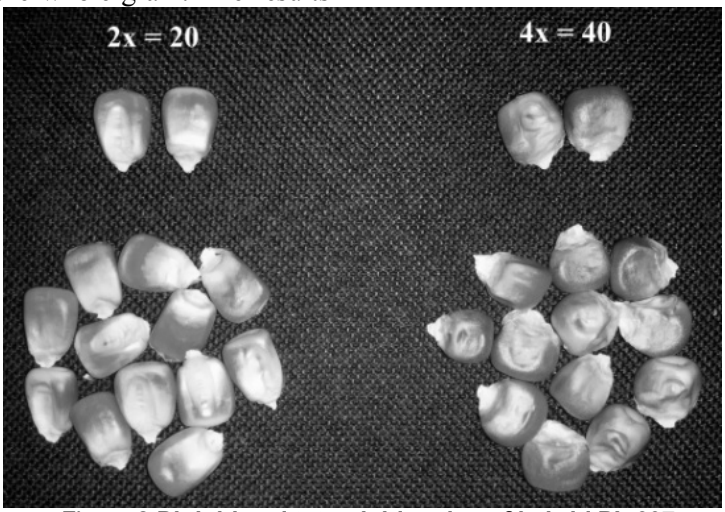


Figure 2 **Diploid and tetraploid grains of hybrid PL 307 Chişiniovschi**

Increasing the level of ploidy, regardless of the presence or absence of o_2 gene in the genotype contributed to a greater amount of protein in the grain. Protein level in tetraploid o_2 grain was on average 17% higher than in the diploid mutant form Chişiniovschi 307 PL and 30% higher than in the diploid hybrid Porumbeni 331 MRf. No differences were found between o_2 tetraploids and the tetraploid synthetic B. The content of lipids in tetraploid mutant grains was lower than that of diploid mutant form, practically at the level of normal diploid grains, but no essential differences were noticed as regard to cellulose content. The analysis of amino acids content in the protein of diploid and tetraploid grains containing o_2 gene, has revealed that with increase in ploidy level there was a tendency to increase the content of aspartic acid, threonine, serine, glutamic acid,

tyrosine, alanine, reducing content of proline, glycine, valine, cysteine, leucine, isoleucine, histidine, arginine. As to the content of leucine, lysine, methionine and ammonia, a clear trend was not observed.

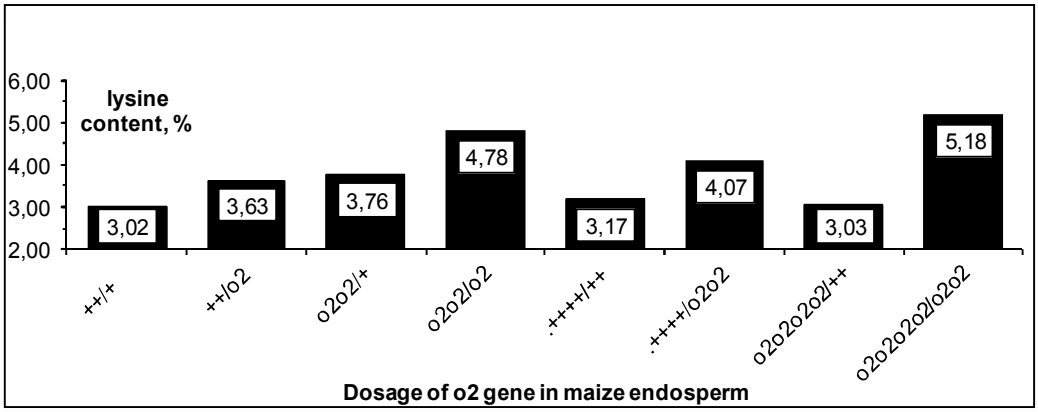


Figure 3 **Dosage effect of opaque-2 gene on lysine content in the protein content of diploid and tetraploid grains.**

Diploid grains have triploid endosperm (two maternal and one paternal genomes) and the tetraploid - hexaploid (four maternal and two paternal genomes). This allows to obtain at the diploid level of the plants endosperm genotypes containing 0, 1, 2, 3 alleles (dose) of a gene and at the tetraploid level – forms containing 0, 2, 4, 6 doses of the gene. So far, in the special literature there is no information on induction and study of biochemical effects of different doses of o_2 gene in tetraploid maize grains. In connection with the above, one of the objectives of the research was to perform a thorough study of the influence of o_2 mutation on the content of amino acids in tetraploid maize forms depending on the number of applied doses. Of particular interest was the content of lysine in the protein (*Figure 3*). From the data obtained, we found that the lysine content increased in proportion to dose only at the diploid level. At the tetraploid level the lysine content varied with an unclear trend of reduction in 4 doses out of 6 possible. However, in the endosperm containing 6 o_2 recessive alleles the lysine content was higher than all other doses of diploid and tetraploid levels. It is worth mentioning the pleiotropic effect of gene dosage on other amino acids. According to the number of o_2 doses was observed an increase in the content of arginine, effect found both at the diploid and tetraploid levels. The content of glycine tended to increase in diploid form and decreased in the tetraploids.

CONCLUSIONS

Application of the 0.15% solution of colchicine with the addition of dimethyl sulfoxide, by injection method at the stage of 3-4 leaves has been shown to be effective in order to obtain tetraploid maize forms.

Transfer of maize from the diploid (2x = 20) in the tetraploid (4x = 40) changes essentially some morphological characters of the plants. Tetraploid plants are usually shorter than the original diploid forms, have a thicker stem, shorter internodes, fewer panicle branches with central branch thicker and longer.

Stomata sizes of in tetraploid forms are on average 20-25% higher than in the diploid but, less per unit of surface. Pollen from tetraploid forms is larger, but with a low degree of fertility. Tetraploid grain mass is greater than the diploid, but varies widely.

The o_2 recessive gene in the genome of tetraploid forms, just as in the diploid, is inherited monogenic according to Mendelian laws of heredity in a F_2 ratio of 35:1.

Doubling the number of chromosomes in diploid maize was accompanied by an increase of the quantity of grain protein and a reduction in the content of lipids. Lysine content in grain protein containing the o_2 gene increases with number of doses in endosperm at diploid level, and partially, at tetraploid.

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