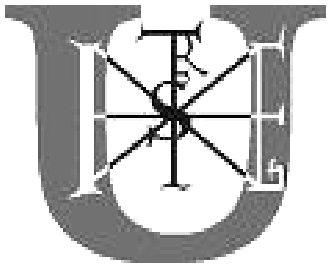

SZENT ISTVÁN UNIVERSITY

IN VITRO ANDROGENEZIS INDUCTION IN WHEAT (*TRITICUM AESTIVUM* L.), TRITICALE (X *TRITICOSECALE* WITTMACK), SPICE PEPPER (*CAPSICUM ANNUUM* L.) AND INTEGRATION OF THE RESULTS INTO BREEDING

Thesis of the Ph.D dissertation

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1. ANTECEDENTS AND AIMS

The doubled haploid (DH) plant production has a history of several decades and has had a consistent connection with the breeding and research since then. The DH plants can be produced by androgenesis (anther culture, isolated microspore culture) and ginogenesis (ovary culture, ovulum culture, oocyta culture) based on the type of the targeted cells.

The first successes are followed by the wide-crossing method (using maize pollen) in wheat breeding; however, this method is not integrated into triticale breeding. The ginogenesis has become a part of basic research (study of ginogenesis, *in vitro* fertilization and microinjection).

The DH plant production methods based on androgenesis are applied to studying microspore embryogenesis, producing mapping populations, genetic transformation, in vitro selection or mutant selection. Wheat anther culture is integrated into breeding programs of some Hungarian and foreign companies. The efficiency of DH plant production is lower in triticale than in wheat; however, anther culture derived triticale instances exist. In pepper, DH plant production methods are improved because of breeding.

Microspore culture is an alternative method for doubled haploid plant production, and has some advantages compared with anther culture: (1) Androgenesis is induced without somatic cell and tissue in isolated microspore culture. (2) Process of microspore culture (microspore embryogenesis) can be observed in a simpler way in isolated microspore culture than in anther culture. (3) Monitoring of the microspore culture can give more information to improve this method.

The first isolated microspore culture derived **wheat** plants were published simultaneously by two scientific groups (Mejza et al 1993, Tuvesson and Öhlund 1993). In most cases of the results published, stressor was applied in order to induce the androgenesis (Hu and Kasha 1999). Ovary co-culture was applied first in 1990 in wheat microspore culture (Mejza et al 1993). Co-culture increased the number of embryoids in microspore cultures, and improved the efficiency of plant regeneration. Letarte et al. (2006) arabinogalactans (Larcoll) and arabinogalactan proteins (gum arabic). In wheat microspore culture the rate of regeneration of embryoids is acceptable but the regenerated plantlets are dominantly albinos. The application of this method in breeding is limited by this phenomenon. The first isolated microspore culture derived **triticale** plants were published by our laboratory (Pauk et al. 2000). Oleszczuk et al. (2004) reported an efficiency microspore culture method for ‘Bogo’ variety. Eudes and Amundsen (2005) increased the number of embryoids and the efficiency of plant regeneration using Ficoll® (osmotic agent) in triticale microspore culture. Zur et al. (2008) studied the changing of ABA and concentration of antioxidants using genotypes

with different responsivity. Improvement of plant regeneration is essential for routinish application of triticale microspore culture.

The first anther culture derived **pepper** plants were published by three different research groups (George and Narayanaswamy 1973, Kuo et al 1973, Wang et al 1973b). Since then anther culture is applied not only to breed new varieties and hybrids but also to produce mapping populations. Despite of first success some factors (genotype effect, regeneration rate and the amount of manual work) restrict the widespread application of it in breeding.

Supena et al. (2006) published the shed microspore culture method in pepper and mentioned the successful application of isolated microspore culture without giving experimental details. Kim et al. (2008) reported a detailed isolated microspore culture method for 'Milyang-jare' genotype. Responsivity of other genotypes was not published by them (Kim et al 2008).

Research programs have started to focus on carrying out of isolated cell cultures worldwide. In our experiments, we aimed the improvement of producing isolated microspore culture of three different species (wheat, triticale and pepper):

I. **Improvement on the induction of *in vitro* androgenesis of crop plants – wheat, triticale and pepper – to produce new methods and genotypes for breeding and research of tested species.**
II. **Improvement on wheat microspore culture; studying basic media and effect of genotype.**
III. **Setting up a methodology for producing triticale microspore culture by analyzing ovary co-culture and genotype effect; integration of regenerated DH lines into breeding.**
IV. **Induction of microspore embryogenesis in isolated microspore culture of pepper. Studying the effect of developmental stage, co-culture, exogenous growth regulators and genotype on the process of androgenesis.**

2. MATERIALS AND METHODS

2. 1. Plant materials

Donor tillers of one spring (’CY-45’) and nine Hungarian winter wheat genotype (’GK Mini Manó’, ’GK Garaboly’, ’GK Hargita’, ’GK Csongrád’, ’GK Délibáb’, ’GK Élet’, ’GK Kata’, ’GK Bán’, and ’Mv Palotás’) were used in experiments. Six spring triticale genotypes (’Kargo’, ’GK Gabo’, ’Rex’, ’AT314’, ’AT322’, and ’EMBR17⁄S2001’) were used in triticale microspore culture. Three Hungarian ‘Szegedi 80’, ‘Szegedi 178’, and ‘Remény’, three Spanish ‘Jeromin’, ‘Jariza’, and ‘Jaranda’ genotypes were used for improving, and setting up a methodology for isolated microspore culture of pepper.

2. 2. Growing conditions and collection of donor plant material

Donor plants were forced in greenhouse except for winter wheat genotypes, which were collected from the nursery of Cereal Research Non-profit Company. Donor plants were grown with natural photoperiod. In the greenhouse, the temperature was 20-28 °C (day) and 15-19 °C (night) for pepper, while the temperature was under 20 °C (day) and 16 °C (night) for wheat and triticale. The donor plants were fertilized by Volldünger® every two weeks.

Donor tillers of wheat and triticale were collected when microspores were in mid and late uninucleate stages. These tillers were cold-treated at 3-4 °C for two weeks. Grouping of pepper flower buds was based on developmental stage in order to test the effect of microspore developmental stage on induction of androgenesis.

2. 4. Pre-treatment of anthers and isolation of microspores

The isolated anthers were pre-treated at 32 °C in 0.3 M mannit solution. This pre-treatment lasted 3 days for wheat and triticale, while it lasted 7 days for pepper.

Microspores were isolated using the method of maceration; the suspension of microspores was fined down by carbohydrate gradient centrifugation. The significant difference between the isolation methods of 3 tested species was that in the case of pepper 21% maltose (Sigma) solution was used instead of 30% maltose solution. The density of microspore suspension was set to 30,000-35,000 microspores/ml.

2. 5. Isolated microspore culture

The effect of two media (A2 and CHB) applied to microspore cultures and two modified media (W14-mi and P4-m) applied to anther cultures were compared in isolated microspore culture of wheat. The wheat microspores were co-cultured with ovaries at 28 °C (10 ovaries/Petri dish).

The effect of ovary co-culture was tested using six spring triticale genotypes - 7 ovaries were put in each Petri dish.

Pepper microspores were cultured in W14mi medium. 7 pepper or 7 wheat ('CY-45') ovaries were put in each Petri dish to test the effect of ovary co-culture and foreign species ovary co-culture (FSOC method).

2. 6. Plant regeneration

The wheat and triticale microspore culture-derived structures were transferred to 190-2Cu regeneration medium. The regenerated green plantlets were placed into glass tubes containing the regeneration medium. The well-rooted plantlets were planted in the greenhouse to acclimatise.

The pepper microspore culture-derived embryoids were transferred to R1 regeneration medium. The regenerated plantlets were placed into glass tubes containing MS medium without growth regulators. The ploidy level of well-rooted plantlets was determined by using flow citometry. The haploid plantlets were colchicine treated. The diploid plants and the colchicin treated plants were planted in the greenhouse.

The regeneration of microspore culture derived structures was carried out in growth cabinet (16 h light, 50 $\mu mol \times m^{-2} \times s^{-1}$ light intensity and 24 ºC temperature).

2. 7. Cytological and histological analysis

The viability of isolated microspores was checked using fluorescein diacetate (FDA) staining in Institute of Plant Biology of Biological Research Center (HAS, Szeged) by Dr. Attila Fehér and Dr. Krisztina Ötvös. The microspore-derived embryoids were studied histological in Department of Botany, Eötvös Lóránd University (Budapest) by Dr. Kristóf Zoltán and Dr. Vági Pál.

2. 8. Analysis of variability of triticale DH lines

8 lines of each 5 spring triticale genotypes were studied in the nursery based on six traits (plant height, length of spikes, thousand kernel weight, hardness, protein content and ash content).

2. 9. Statistical analysis

The experiments were carried out in at least 3 replications. The statistical analysis (two-sample t-probe, ANOVA) was prepared using Microsoft® Excel 2002 software.

3. RESULTS

3. 1. 1. Wheat microspore culture

The critical steps of wheat microspore culture were tracked by culturing of 'CY-45' genotype. The first essential step towards androgenesis induction is the ideal developmental stage of the microspores. The donor tillers contained mid and late uninucleated microspores in the middle part of the donor spikes. Cold treatment, osmotic stress and starvation increased the number of viable isolated microspores. After the isolation, the microspores were in late uninucleated and early binucleated developmental stages on the first day of culturing.

First cell divisions were observed on the 3rd and 4th days of culture. Ovary co-culture was essential in wheat microspore culture, since co-cultivation protected the development of structures. Following one-month, co-cultivation microspore-derived embryoids were placed to solid regeneration medium, where green and albino plantlets were regenerated from these structures within two weeks. The well-rooted plantlets were planted in greenhouse and were grown until harvesting.

3. 1. 2. Effect of medium on embryoid production and plant regeneration in wheat microspore culture

Four different media (A2, CHB3, W14mi and P4-m) were compared in microspore culture of 'CY-45' genotype. Significant difference was noticed between the effect of media in terms of the number of embryoids, green and albino plantlets.

The best results were achieved with W14mi and A2 media based on number of embryoids and green plantlets. Significant difference was not detected between the two media. W14mi medium was applied during the further experiments, which produced the most embryoids and green plantlets in this experiment.

The well-rooted plantlets were planted in greenhouse to acclimatise. Colchicine treatment was not applied; the rate of spontaneous rediploidisation was controlled by harvesting. 78.3% of the 120 planted plants (94 DH lines) were harvested.

3. 1. 3. Isolated microspore culture using Hungarian wheat genotypes

The efficiency of microspore culture was checked by ten wheat genotypes. In the case of all genotypes, androgenesis was induced in wheat microspore culture with ovaries. The development of microspores was intensive and the number of embryoids was varying together with the different genotypes (31.75 – 413.5 embryoids/Petri dishes). The best results were achieved by 'CY-45'

(413.5 embryoids/Petri dish), 'GK Élet' (289.25 embryoids/Petri dish), 'GK Csongrád' (224 embryoids/Petri dish) and 'GK Mini Manó' (176.75 embryoids/Petri dish) genotypes.

Green plantlets were regenerated from structures of 7 genotypes (0.25 – 17.75 green plantlets/Petri dish), while 3 varieties ('GK Kata', 'GK Bán', 'Mv Palotás') produced albino plantlets only. The 'CY-45' (17.75 green plantlets/Petri dish) and 'GK Délibáb' (12 green plantlets/Petri dish) genotypes produced the highest number of green plantlets per Petri dish.

In the case of anther culture–derived 'GK Délibáb' and 'GK Bán' varieties, the number of embryoids was significantly lower than in the case of 'CY-45' or other varieties ('GK Élet', 'GK Csongrád' and 'GK Mini Manó'). Green plant regeneration was relatively high in the case of 'GK Délibáb' variety, while 'GK Bán' produced albino plantlets only. The responsivity of anther culture derived genotypes was significantly different, and was not higher than other varieties bred by classical breeding method.

3. 2. 1. Effect of ovary co-culture in triticale isolated microspore culture

The effect of ovary co-culture was studied in isolated microspore culture of six spring genotypes. Following isolation, microspores were late uninucleated and early binucleated stages. The first cell divisions were noticed on 4^{th} – 5^{th} day of culturing in the case of all genotypes.

Ovary co-culture increased the quality of microspore-derived embryoids and the number of embryoids in the cultures. The highest number of embryoids were produced by 'AT314' (347 embryoids/Petri dish) and 'AT322' (344 embryoids/Petri dish) genotypes. 224.7 embryoids/Petri dishes were the average of the tested genotypes.

The best plant regeneration rate was achieved by 'AT314' (14 green plantlets/Petri dish) genotype, while 0.7 green plantlets/Petri dish were regenerated from structures of 'Rex' genotype.

The well-rooted plantlets were placed in greenhouse to acclimatise. The spontaneous rediploidisation rate was different depending on the genotype (41.46% - 82.14%). Altogether 103 isolated microspore culture-derived DH lines were grown. The 8 most kernel-producing DH lines/genotypes were chosen from each of the DH lines to test six traits of them in the nursery.

3. 2. 2. Analysis of variability of triticale DH lines

Six traits (plant height, length of spike, thousand kernel weight, hardness, protein content and ash content) were controlled in the nursery by 8 DH lines each of two spring triticale varieties ('GK Gabo', 'Kargo') and three population ('AT314', 'AT322', 'EMBR17/S2001') and their controls (super elit). Significant differences were observed among the genotypes and the DH lines.

3. 3. 1. Effect of microspore developmental stage on efficiency of spice pepper microspore culture

Donor buds of two spice pepper varieties ('Remény' and 'Szegedi 80') collected from greenhouse were grouped into 4 different groups on the basis of development. The size of the buds, the size, and colour of anthers are good markers for differentiating between the developmental stages of microspores. Group 1: Yellow anthers of donor buds contained tetrads. Group 2: In this group, the microspores were in late uninucleated stage, which is optimal for anther culture. Less than ¼ of the anthers were purplish. Group 3: The anthers were purplish to the size of 2/3 – 4/5 inside the collected buds. 80% of the microspores were in late uninucleated and 20 % of them were in early binucleated stages. Group 4: Anthers were fully purplish, and contained pollen grains.

Following the pre-treatment of anthers, viable microspores could be isolated from the anthers of second and third groups. After the isolation, the developmental stages of microspores were checked by FDA staining. 90 % of the microspores were in late uninucleated and 10% of them were in early binucleated stage in the case of the second group. In the third group, the amount of uninucleated and binucleated microspores was equal.

The isolated microspores were co-cultured with wheat ovaries in W14mi medium. The rate of cell divisions and the number of embryoids was higher in the cultures of third group than they were in the cultures of second groups.

3. 3. 2. Effect of ovaries of different plant species on the androgenesis of spice pepper in isolated microspore culture

Three different treatments were applied to test the effect of ovary co-culture: (1) without ovaries, (2) with pepper ovaries and (3) with wheat ovaries. Two spice pepper varieties ('Remény' and 'Szegedi 80') were applied to test the effect of ovaries of pepper and wheat. The first cell divisions were detected at the end on the first week in the case of all three treatments. The dividing microspores developed multicellular structures on the second week. These structures got free from the wall of microspores in presence of ovaries, but the development was stopped due to the absence of ovaries. In the presence of pepper ovaries the development continued until the end of third week, proembryoids were detected in the cultures, however embryoids were not. The development of pepper microspore-derived structures could be sustainable using wheat ovaries as nurse agent, and the microspore culture-derived structures were noticed by naked eye on the 5^{th} and 6^{th} week of culture.

Androgenesis was induced in the cultures of all the six tested genotypes. Embryoids developed in the cultures (depending on genotype 3.75 – 65.7 embryoids/Petri dish) in the presence

of ovaries. Significant differences were detected among genotypes in terms of the number of embryoids.

3. 3. 3. Histological study of microspore-derived embryoids and plant regeneration of pepper microspore derived embryoids

The microspore-derived *in vitro* embryoids were transferred to R1 regeneration medium. The longitudinal section of the slightly elongated embryoids showed two distinct poles. The root meristem, central structures, and shoot meristem were detected on the structure. The opposite pole of the embryoid formed two equally developed primordial cotyledons. The embryoids were completely covered by hairy epidermis. Plant regeneration of embryos was a critical step in isolated microspore culture of spice pepper. Some structures produced normal *in vitro* shoots, while most of them were distorted with leaf rosettes and abnormal green leaves, or there was even no shoot response on the regeneration medium. The ploidy level of plantlets with 3-4 leaves was checked. The ploidy level of haploid plantlets was doubled with colchicine treatment. The spontaneous diploid plantlets and the colchicine treated haploid plantlets were planted in the greenhouse. Three ‘Jaranda’, three ‘Jariza’ and a single ‘Szegedi 80’ diploid plant acclimatised, and produced fruits with seeds.

The DH lines were integrated into Hungarian spice pepper program. These lines were used to breed the ’Délibáb’ spice pepper hybrid.

4. DISCUSSION (CONCLUSIONS AND SUGGESTIONS)

4. 1. Wheat isolated microspore culture

The characteristic steps of isolated microspore culture of wheat were studied with model 'CY-45' genotype. Our results were harmonising with the data published earlier (Mejza et al. 1993, Hu and Kasha 1999). The stress-treatment of the donor material and optimal developmental stage were important for androgenesis induction (Mejza et al. 1993, Hu and Kasha 1999). Ovary co-culture was essential to produce a high number of embryoids (Mejza et al. 1993). On the 5th week many embryoids (413.5 embryoids/Petri dishes) developed into microspore cultures, however the regeneration rate of green pants (17.75 green plantlets/Petri dishes) was low.

Four different liquid media were compared in wheat isolated microspore culture of 'CY-45' genotype. The CHB (Chu et al. 1990) and A2 (Indrianto et al. 1999) applied in isolated microspore culture and two modified media (W14mi, P4-m) were tested, W14 (Ouyang et al. 1989) and P4 (Ouyang et al. 1983) media are well-known in anther culture. The significantly best results were achieved by A2 and W14mi media in terms of the number of embryoids and the number of green plantlets. In further experiments W14mi medium was applied, which produced the highest number of embryoids and green plantlets.

Androgenesis was induced in isolated microspore culture of the tested genotypes. The genotype significantly influenced the number of embryoids and plant regeneration (Agache et al. 1988). The responsivity of genotypes from anther culture ('GK Bán' and 'GK Délibáb') was not higher than that of other genotypes. This phenomenon could be caused by the additive gene effect of the responsivity.

Most of the regenerated plantlets of wheat microspore culture were albino depending on genotype (60.33% - 100%); albinism is well known in microspore culture cereal species (Trop and Andersen 2009). Many factors influence the rate of albinism, thus further experiments should decrease the rate of albino plantlets among the regenerants.

The efficiency of green plant regeneration should be increased to produce a number of green plants similarly to anther culture. Following the optimalisation, isolated microspore culture might become an efficient method for breeders.

4. 2. Triticale isolated microspore culture

Stress treatments played a very important role in androgenesis induction of triticale microspore culture (Pauk et al. 2000, Oleszczuk et al. 2004, Eudes and Amundsen 2005, Zur et al. 2008). In our experiments, cold pre-treatment of donor tillers was combined with a 3-day starvation (0.3 M mannit solution, 32 °C) to support the isolation of viable microspores during maceration.

Eudes and Amundsen (2005) were the first who applied the ovary co-culture in triticale, which increased the quantity and quality of microspore-derived embryoids. Likewise, ovary co-culture increased the efficiency of androgenesis in our experiments. Further on, ovary co-culture had been applied to test the responsivity of genotypes.

Genotype proved to be an important factor, which influenced the efficiency of androgenesis in anther culture (Balatero et al. 1995) as well as in isolated microspore culture (Eudes and Amundsen 2005). The amount of embryoids and green plantlets changed depending on the genotype. Genotype was one of the most important factors, which influenced the efficiency.

The average number of tested genotypes was 224.7 embryoids / Petri dish out of which 7.79 green plantlets were regenerated on the average. Some of the regenerated plantlets were albino similarly to wheat (Trop and Andersen 2009). Compared with previously published results (Pauk et al. 2000, Eudes and Amundsen 2005, Zur et al. 2008) more embryoids were produced, and the ability of regeneration of the green plants was increased. The exception was the result of the experiment carried out with the 'Bogo' variety having good responsivity (Oleszczuk et al. 2004). The regenerated plantlets acclimatised to greenhouse conditions properly.

DH lines generally had wider ranges for dry matter yield, grain yield and total biomass than the lines derived from field selections had (Arzani and Darvey 2002). In our experiments, significant differences were observed among genotypes and DH lines in terms of the six tested traits.

As a summary, isolated microspore culture like other methods of DH line production was suitable to eliminate the genetic inhomogenity from the genotypes and to balance them. The triticale microspore culture might become an alternative method to produce homogenous genotypes not only from early generation (F_1 and F_2) but also from further generations (F_5).

4. 3. Pepper microspore culture

The developmental stage of microspores has a significant effect on androgenesis induction and on the efficiency of androgenesis. Study of developmental stage was targeted because of the different published results in pepper microspore culture. In our experiments, the best results were achieved by donor material containing 80% late uninucleated microspores and 20% early binucleated microspores, which were in later developmental stage at the time of collection than that in the case of anther culture.

One-week pre-treatment (0.3 M mannit solution) was important to increase the number of isolated microspores (Supena et al. 2006). The viable microspores could be isolated from the debris of cells and tissues by gradient centrifugation. Mannit/maltose gradient centrifugation was chosen for gradient centrifugation, a modification of which let the isolation be successful.

The isolated microspore culture was improved by foreign species ovary co-culture method (FSOC method). Effect of ovaries from different species (pepper and wheat) was checked in pepper microspore culture. In the absence of ovaries, the division of microspores stopped on the second week of culturing. In the presence of pepper ovaries some proembryoids were induced in the cultures, however, these structures were not able to continue their development further. Well-developed embryoids could be observed by naked eye in the pepper microspore co-culture with foreign ovaries (wheat ovaries).

Ovary co-culture is a well-known method in anther and microspore culture of monocots (Mejza et al. 1993, Eudes and Amundsen 2005) nevertheless, it has not been applied in cell and tissue culture of dicots. FSOC method was applied in pepper microspore culture for the first time in our experiments. This method was tested on three Hungarian and three Spanish genotypes. In the case of all genotypes, microspore derived embryoids were produced, and the genotypic dependence of isolated pepper microspore cultures was demonstrated for the first time.

Plant regeneration is a critical step in spice pepper microspore culture (Supena et al. 2006). In terms of the number of embryoids, the isolated pepper microspore culture is competitive with other DH plant production methods, however by further experiments it is necessary to decrease the genotype effect and increase the efficiency of plant regeneration.

Microspore-derived fertile plants were regenerated from three different varieties (‘Jariza’, ‘Jaranda’ and ‘Szegedi 80’). These plants have been integrated into Hungarian hybrid spice pepper breeding programs. These spice pepper DH lines were used for breeding the ‘Délibáb’ spice pepper hybrid.

NEW SCIENTIFIC RESULTS

In our experiments, isolated microspore cultures of three different crop plants (wheat, triticale and spice pepper) were studied. Several factors, which influence the efficiency of haploid induction, are to be paid attention to during studying the process of androgenesis. In the case of the three species, microspore developmental stage played an important role by influencing the efficiency of androgenesis induction. Ovary co-culture had a positive effect on androgenesis of the three tested species, as observed. The regenerated DH plants have been integrated into breeding programs. The following new results were achieved by our experiments:

1. Genotype of donor materials influenced the efficiency of microspore culture vitally. The responsivity of one spring wheat and nine winter wheat genotypes was compared in isolated microspore culture. In terms of breeding in the case of triticale, valuable breeding materials were used for experiments. A wider genotype background (Hungarian and Spanish genotypes) was tested in pepper microspore culture, so the genotype effect in pepper microspore culture was published for the first time.

2. Microspore developmental stage influenced the androgenesis induction vitally. In the case of pepper, the effect of different developmental stages on induction was tested. Taking the international scientific literature and our experiments into consideration, in the case of the three tested species later developmental stages are suggested to be chosen for microspore culture compared with the ones in anther culture.

3. Ovary co-culture improved the efficiency of microspore culture in the cases of the three species. In wheat and triticale microspore culture ovary co-culture increased the number of embryoids and quality of embryoids being essential for regeneration. Ovary co-culture was applied for the first time in spice pepper microspore culture, namely the FSOC method, which improved the induction of microspore-derived embryoids in the case of all genotypes.

4. Valuable DH lines were produced from triticale and pepper microspore culture. Some of the DH lines have been integrated into our breeding programs and our breeding partners have already used some DH pepper lines for breeding of spice pepper hybrid (’Délibáb’).

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„Én mindent készen kaptam
Áldott elődi kézből,
Éppen csak a szivárvány
Hiányzott még az égről.
Vén vasoszlopokra
Szivárványként feszültem,
Egemet ők tartották
Mohosan és derülten:
Nincs semmi érdemem.”

Citation from the poem of Sándor Reményik (1936)
Title: „Elődeim emberségéből”