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Genetic stability of somatic hybrids between *Solanum tuberosum* cv. Delikat+*Solanum bulbocastanum* and their response to different stress factors

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I. Theoretical overview

I.1. Economical importance and the major pathogens of potato

Potato (*Solanum tuberosum* L.) is one of the most essential cultivated plant of the world, it ranks the third place after rice and wheat as a food crops. It's importance is expected to increase due to the effect of climate change and increased consumption (Obidiegwu *et al.,* 2015).

The origin of potato is in the Andes Mountain in the Peruvian region of South America (CIP). The domestication process started 7000 years ago, but was introduces in Europe only in the 1570's (Bradshaw *et al.,* 2006). In Europe, Romania occupies the third place in terms of area for potato cultivation and the sixth place if the production is chosen as a criterion. The Romanian annual potato consumption shows a decreased tendency, in 2012 it was 98.3 kg/capita, which is 6.4% less than the value recorded in 2006 (Vlad and Done, 2014). The big importance of potato utilization in Romanian gastronomy is reflected in the use of the "second bread" denomination (Baciu *et al.,* 2009).

The domestication process caused shorter stolons, larger tubers, reduction of glycoalkaloid content and changed fruit shape and colour (Chaudhary, 2013). All these changes represent an evolutionary response to human selection.

Comparing the potato with the wild species reveals that the cultivars in use have bigger yield production without toxic content but are more sensitive to herbivores and diseases. This feature of cultivars underlines the primary aims of domestication (Heřmanová *et al.,* 2007).

Potatoes due to these changes in time and sensibility to diseases, became the most chemically protected crop. In every year more and more millions of dollars are spent for treatments against fungi and herbivores. For example, the late blight disease, caused 3 billion dollars loss in every year, which represent the money for disease control and the price of yield loss (Fry, 2008).

The most destructive biotic stressors of potato, which causes substantially yield reduction have some common features which ensure its success, even though they are members of different taxonomically distant groups.

The late blight of potato caused by the oomycete *Phytophthora infestans* (Mont.) de Bary (1876) are responsible for the most of people's deaths caused by phytopathogen. The way in which this pathogen shows the above-mentioned characteristics and the adaptation of the plants immune system in overcoming them, is presented in the next chapters.

I.1.1. Late Blight of potato

The *Phytophthora infestans* is hemibiotrophic oomycete, this means that in the early stage of infection a living tissue is required, and the duration of this period could be of several days (Termorshuizen, 2007).

This pathogen has left its mark in human history due to the big importance of the potato, as part of the basic nutrition. The well-known case is the great Irish Potato Famine in the 19th century, the huge loss of potato crop yield provoking the death and emigration of more than two million of people from Ireland (Goss *et al.*, 2014).

This oomycete has the capacity to develop resistance to modern fungicides; it have a great genetic variability for virulence and is able to overcome the resistance in previously resistant potato cultivars (Jo *et al.*, 2011).

P. infestans is a heterothallic oomycete with two mating types A1 and A2, therefore, they have the possibility to reproduce by both asexual and sexual. Oospores resulted from sexual reproduction are more resistant to abiotic stress, remaining infectious during four years (Turkensteen *et al.*, 2000).

I.2. Plant-pathogen coevolution

The interaction between oomycete and host plant is a very complex process, each part of this pathosystem develops its "attacker arm" and its defensive system. Plant pathogens use diverse strategies to enter in the plant via water pores, intercellular spaces or through wound, accordingly the host plants have developed sophisticated systems to detect the presence of attackers.

The oomycete haustoria intrudes into the plasma membrane by invagination, in this way, establish an interface for the next interaction steps. Similarly with other pathogens, at the beginning of the interaction, it confronts with the basal immunity, which represents the first layer of the plant defence. Transmembrane pattern recognition receptors (PRRs) of the plant detects the highly conserved microbial molecules called PAMP which can be peptides derived from bacteria or polysaccharides, for example chitin or beta-glucans in the case of fungi or oomycete. The confrontation lead to the switching on the PAMP-triggered immunity (PTI) (Rouxel and Balesdent, 2010).

Successful pathogens have a well-developed ability to suppress the PTI by effector molecules, products of oomycete avirulence (*Avr*) genes. These effectors manipulate the host cell structure and functioning in this way facilitates the infection and triggers another defence response of plant (Kamoun, 2006).

At this level of infection through the MAP kinase signalling pathway effectors activate the pathogen-responsive genes transcription, which are responsible for the reactive oxygen species (ROS) production and cell wall reinforcement (Chisholm *et al.,* 2006).

If one effector is recognized by host cells resistance proteins (R), it will activate the effector-triggered response (ETI), which is more rapid and vigorous than PTI (Jiang and Tyler, 2012). This type of interaction complies with the gene for gene theory and lead to the hypersensitive response (HR) and programmed cell death (PCD), at the site of infection. The collapse of the infected tissue creates a physical barrier, in this way prevent the proliferation and spread of the pathogen (Dénes *et al.,* 2015).

There are two types of interactions, based on the infections outcome, the compatible and the incompatible. In the case of compatible interaction the pathogen succefull inhibits the first and second immune layer of plant and the infection became systematically. Incompatible interaction is present when the pathogen is recognized, by resistance genes, which activate the ETI in this way the infection remains localized (Gyetvai *et al.,* 2012).

Role of Reactive Oxygen Species in plant defence response

The promptness of the plant defence system activation in the moment of infection has a major impact on the interaction outcome. The oxidative burst represents the most rapid response of the basic immune system to pathogen attacks, which manifest in the rapid accumulation of reactive oxygen species (ROS) (Wojtaszek, 1997; Torres, 2010). These molecules have a dual role, indirectly reacting with the pathogen or indirectly acting as a second messenger in signalling (Torres *et al.,* 2006).

The rapid accumulation of ROS molecule is ensured by three different synthesis sites, NADPH oxidases in the plasma membrane, peroxidases in the cell wall and amino oxidases localized in the apoplastic space (Grant and Loake, 2000). The primary synthesized singlet oxygen molecules will be transformed by reduction into a superoxide, hydrogen peroxide and hydroxyl radical. During the oxidative burst hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are produced in the highest proportion. Depending on the type of interaction, the hydrogen peroxide synthesis shows different synthesis timing, duration, and intensity level. In the case of the compatible interaction there is one rapid accumulation of hydrogen peroxide, but in the incompatible interaction the first peak is followed by a second, more extended (Apel and Hirt, 2004).

The ROS molecules are cytotoxic, they interact not only with the pathogen, therefore, can disturb the normal functioning of the host cell, leads to unchangeable modifications and finally to the cell death.

I.3. Somatic hybridization and genetic stability of the somatic hybrids

Somatic hybridization represents an attractive alternative for crossing species which cannot be crossed by natural methods. By protoplast isolation and fusion it becomes possible to obtain intraspecific hybrids in a short time, without bridge crosses.

Instead of the isolation and cloning of one or two genes, with this method all genome of one species can be transferred. It is proved to be a good solution especially in the cases where the interested trait of the plant is determined by multiple genes (Glimelius *et al.,* 1991; Grosser *et al.,* 2000; Waara and Glimelius, 1995).

The obtaining of the somatic hybrids is a time consuming process and the precision of work is mandatory, this part of the experiment representing just the beginning of the research and always remains the need to investigate the presence of the desired traits in the regenerated plants.

The analysis of fusion product begins with the determination of the hybrid status and ploidy level. There are many direct or indirect methods for ploidy determination. Between the indirect methods, counting the chloroplast in the guard cells is the most simple and

inexpensive way, established in the 1930's (Kostoff, 1938) and requires only a light microscope and the preparation of epidermal peel.

During the regeneration, the somatic hybrids can lose their chromosomes, this asymmetrization is spontaneous and unpredictable, and can be a consequence of the somatic incompatibility. The mechanisms which are implicated in this phenomenon is not well understood , some observation suggesting that the phylogenetic relation between species being the major determining factor (Glimelius *et al.,* 1991, Rákosy-Tican *et al.,* 2005).

The somatic incompatibility is an intracellular phenomenon resulted from the forced coexistence of two nuclei and cytoplasm, originated from different cells which in natural way cannot form a single cell (Rákosy-Tican *et al.,* 2005).

Modern cytogenetic approaches give the possibility to analyse the chromosomes morphology, to karyotype the genomes and to reveal the origin of alien chromosomes (Gavrilenko, 2007, Benavente *et al.,* 2008). Before starting a cytogenetic analysis is necessary to study genome type and to clarify the relationship between the studied species.

In the case of a closely relationship between species, the cross-hybridization phenomena could appear which are attributed to the high similarity of genomes. Another factor which makes difficult to study the genome of *Solanum* species is represented by the small size and the poorly differentiation of the chromosomes (Iovene *et al.,* 2007). For this reason the well-known classical banding techniques for identification of individual chromosomes are not reliable (Pendinen *et al.,* 2012).

The cultivated potato, member of this section, is an autotetraploid plant with four identical chromosome sets, basic haploid chromosome number x=24 (Gavrilenko, 2007). As were mentioned, potato is an autotetraploid plant characterized by an A genome type, therefore, the genome formula is AAA^tA^t. The wild species, *Solanum bulbocastanum* is a diploid with a haploid chromosome number n =12 and therefore have the genome formula A^bA^b.

Genomic *in situ* hybridization (GISH) is the most used method in case of somatic hybrids when the aim of the study is to distinguish the parental chromosomes and to detect the interogressed sequences resulted from recombination (Brammer *et al.,* 2013). This method is a particular type of fluorescence *in situ* hybridization, because as probe the total genomic DNA form one of the parental species is used.

The goal of biotechnological tools applied in breeding programs is to transfer the desired gene or gene clusters into the crops and by this *in situ* technique is possible to determine if the transfer was achieved and where the gene(s) is located on chromosomes (Devi *et al.,* 2005).

I.4. Drought stress

Drought stress is one of the most limiting abiotic stress of plant growth and development. Water deficit occurs, when the transpiration rate exceeds the uptake rate, to prevent this, the plant reacts in multiple ways (Fleisher *et al.,* 2015).

Potato is a drought sensible plant, moderately water deficit can have a major impact to physiological processes, growth and yield production (Fleisher *et al.,* 2015). The cause of their sensibility is the shallow root system because 85 % of the roots is concentrated in the upper phase of soil (0.3 m) (Costa *et al.,* 2007; Sprenger *et al.,* 2015).

Screening numerous genotypes for drought tolerance require time, space and equipment. At the last stage of the screening, plants have to be tested in the field, but the main disadvantageous of this stage represents the uncontrollable factors effect which cannot be measured. Taking in consideration these reasons a big number of pre-screening were performed *in vitro* conditions. The most used is polyethylene glycol (PEG) which has a series of advantageous characteristics. It is recommended the utilization of PEG with a molecular weight greater than 6000 (Mw≥ 6000), because do not penetrate into plant cell, is non-ionic and mimic dry soil conditions (Gopal and Iwama, 2007). After the in vitro screening of drought tolerant plants, the next step represents the stress-selection in green house condition.

The phenotyping process means the recording of quantitative and qualitative traits of plants (Granier and Vile, 2014). Nowadays modern phenotyping techniques use high-throughput phenotyping platforms [2], which are equipped with different cameras, to capture the traits changing over time. Automatic and semi-automatic systems allow examination of a high number of plants, are non-invasive and non-laborious. Image analysis and computer processing of the obtained data is more time consuming than manually performed measurements and represent the basic methods of phenotyping (Fehér-Juhász *et al.,* 2014).

Drought, salt and heat stresses are between the major limiting factors for photosynthesis, the limitation could be performed by stomatal or non-stomatal pathways (Ashraf and Harris, 2013). The chlorophyll fluorescence intensity shows characteristic kinetic which are named after Kautsky effect or fluorescence induction curve. The light harvesting being the most vulnerable part of the photosynthesis by measuring the fluorescence induction

we get information about the photosynthetic performance of plant (Maxwell and Johnson, 2000). Photosynthesis due to the complexity are very sensitive to the stress factors, assessing the difference between normal and disturbed process can help in the detection of early response to stress, before visual sign on the plant.

The obtained results from these investigations, helps to understand the stress physiology of plants and to choose the best candidates for pre-breeding programs (Fehér-Juhász *et al.,* 2014).

I.5. Somatic hybrids between *Solanum tuberosum* and *Solanumbulbocastanum* used in the experiment

Traditional breeding of plants takes a long time, transferring the resistance genes into a cultivar plant could be a solution, but currently the cultivation of Genetically Modified Organism (GMO) for human consumption in the European Union is not allowed [3].

To accelerate the breeding process Rákosy-Tican *et al.*, (2015) performed somatic hybridization of cultivated potato Delikat cultivar with *S.bulbocastanum* (GLKS-31741, blb41), which cannot be crossed in a natural way.

The main aim of their research was to obtain in a shorter time valuable plant material resistant to late blight, which are not considered GMO. Parts of these somatic hybrids and back-cross progenies represent the plant material used in our study.

The used accession of wild species carries two broad spectrum resistance genes *Rpi-blb1* and *Rpi-blb3,* which provide resistance to foliar late blight. The cultivated potato, Delikat cultivar, is popular due to the tuber production qualities.

Somatic hybridization were performed via protoplast electrofusion, in total 235 hybrids were regenerated. The majority of them were validated for the hybrid nature and ploidy, by using the SSR molecular markers and flow cytometry. In the first selection step at shoot level the hexaploid plants were kept for further researches (Rákosy-Tican *et al.,* 2015).

The experiments with somatic hybrids are continued in order to get information about the presence of resistance genes, the level of resistance to late blight, and to describe the produced tuber quality and quantity (Rákosy-Tican*et al., manuscript*). Resistance tests were performed under greenhouse and field conditions. The presence of resistance genes was

checked with specific primers. In some cases results indicate the segregation of resistance genes in a back-cross generation.

Some somatic hybrids showed resistance to late blight during the field test, but the presence of resistance genes cannot be detected by the used primers. These unexpected, but good results suggested, that probably another resistance gene or QTL's is present in these hybrids.

Tuber production of somatic hybrids showed similar results to Delikat cultivar, but the shape of tubers need to be improved in the future, because it is not suitable for actual market requirements.

II. The objectives of the thesis

Coevolution of potato-*Phytophthora infestans* interaction is still continuing, making it difficult to find a durable protection. Similarly, the increasing water deficit will cause a substantially decrease of the potato yield. The biologists and breeders were always trying to find solutions for these problems performing researches and studies.

Rákosy and collaborators (2015) performed a somatic hybridization, to transfer the valuable characteristics from the *Solanum bulbocastanum* wild species into the cultivated potato. The preliminary characterization of somatic hybrids (presented in previous I.5) demonstrated the fact that the obtained somatic hybrids carry the valuable traits, therefore they have the potential to become good candidates for breeding programs.

The main goals of my PhD thesis was to bring novel information concerning the genetic stability and drought tolerance of the potato somatic hybrids. The specific objectives of this thesis were as follows:

- To investigate the genetic stability of the somatic hybrids;
- To reveal the presence of *S. bulbocastanum* chromosomes in the backcross progenies in order to prove the efficiency of traits transfer from wild species;
- To reveal the role of oxidative burst pattern in somatic hybrids with different genomic composition and to find the resistance gene role in this process;
- To investigate the somatic hybrids drought tolerance, starting from the *in vitro* stress selection of hybrids, which will be analysed in a newest phenotyping platform.

III. Material and methods

III.1. Maintaining and multiplication of plant material

The object of this research is represented by the characterization of the somatic hybrids produced via protoplast electrofusion of cultivated potato and *Solanum bulbocastanum* (Rákosy-Tican *et al.*, 2015). The backcross generations were obtained by crossing the hybrids with other cultivars of cultivated potato (Table). All plant material were maintained *in vitro* in Julius Kühn Institute, Germany.

Table 1. Somatic hybrids and backcross progenies used in the research

Name		**Name**	
Solanum bulbocastanum	parent	**2295/1**	hybrid
***Solanum tuberosum* cv. Delikat**	parent	**2295/1/7**	BC1
2299/2	SH	**2295/1/4/11**	BC2
2284/5	SH	**2295/1/4/59**	BC2
2284/4	SH	**2282/4**	SH
2283/9	SH	**2282/4/4**	BC1
2283/9/3	BC1	**2282/4/68**	BC1
2283/9/27	BC1	**2282/4/68/22**	BC2
2283/9/63	BC1	**2294/5**	SH
2282/9/64	BC1	**2294/5/5**	BC1

III.2. Ploidy determination of somatic hybrids by direct and indirect methods

III.2.1. Ploidy stability determination by flow cytometry

Flow cytometry analysis was performed to characterize the ploidy level of somatic hybrids. The first leaf pairs of 8 weeks old *in vitro* plants were chopped with a razor blade into small pieces adding 800 µL of LB01 buffer.

The measurements were performed after 15 minute sample incubation on ice, using Becton Dickinson FacScan flow cytometer, and the obtained data was analysed using Cell Quest

software version 4.1, in Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary.

III.2.2. Determination of ploidy level by counting chloroplast in guard cells

The first three fully expanded leaflets from the top of the well-developed plants (three plants per genotype) were used to determine the number of chloroplast per guard cell (three pairs of guard cell per leaflets). The abaxial epidermis was peeled and placed onto a glass microscope slide. The observation was made under UV fluorescent microscope (Olympus BX 60), and captured by CCD camera.

III.2.3. Determination of ploidy level and genome composition by traditional cytology

About 1-2 cm long root tips were harvested from young plants grown under *in vitro* conditions and afterwards they were fixed in ethanol/acetic acid (3:1), and stored in 70 % ethanol at 4º C Firstly, the root tips were washed twice in distilled water (10 min each), digested with a 2 % cellulase-20 % pectinase solution (30 min), and squashed in 45 % acetic acid Chromosomes were stained by 4’6 diamidino-2-phenilyndole (DAPI), afterwards being washed with 2 x SSC and distilled water. To preserve the staining, the slides were mounted in antifading solution (Vectashield, Vector Laboratories). For chromosome count, cells with metaphase chromosomes were used. For each hybrid and progenies at least 5 cells were analysed and photographed under UV fluorescent microscope (Olympus BX 60) using the 100X objective.

III.3. Cytogenetic characterization of plant genome composition

III.3.1. Determination of genome composition and chromosome constitution by genomic *in situ* hybridization technique

The genomic *in situ* hybridization technique makes it possible to analyse the chromosome constitution of somatic hybrids and the detection of recombinant sequences. For GISH, a good quality of the chromosome spread and of the genomic DNA is necessary, the parental species DNA being used as a probe in the hybridization step.

1. Preparation of the chromosome spreads

To obtain young root tips, nodal fragments of *in vitro* grown plants were put into Petri dishes containing MSmedium (Duchefa, ph 5.8) supplemented with 0.0 5mg/l 1-Naphthaleneacetic acid (ANA). After two weeks, 1-2 cm long root tips were harvested and pre-treated with 8-hydroxyquinoline. Root tips were fixed in ethanol/acetic acid (3:1). The root tips were digested with 0.4 % pectolyase, 0.4 % cytohelicase 1 % cellulose in a citrate buffer (25 min) and squashed in 70 % acetic acid.

2. Extraction of genomic DNA

DNA extraction was performed by the CTAB method.

3. Labelling the obtained genomic DNA

Probe labelling was performed using the Nick Translation Mix (Roche) protocol, involving the simultaneous activity of two enzymes, DNase I, and DNA Pol I. The labelled DNA size was checked in a 1% (w/v) agarose gel. In order to purify and concentrate the labelled product, the ethanol precipitation method was used.

4. *In situ* hybridization

Before the *in situ* hybridization, the slides with chromosome spreads were pre-treated to remove the cytoplasm and RNA which can cause false signals.

McGISH were performed by using two protocols:

Protocol 1 (modified after Kruppa *et al.*, 2013)

Protocol 2 (modified after Jang and Weiss-Schneeweiss, 2015)

5. Image capturing and analysis

III.4. Indirect methods to visualize first defence responses

Acclimatization of the plant material and agroinfiltration

Plants were grown in a jar on RMB5 media for 10 days and transferred afterwards in plastic pots (10x10x12 cm), being kept in plant growing chambers (Binder KBWF 720 E5.2). The following growing conditions were applied: 16h/8h day/night regime, 21 ºC and 70 % humidity. In order to test the resistance gene functionality and to follow the ROS synthesis, somatic hybrids were classified in different groups based on the presence of the resistance gene and the field test results

1. Preparation of bacterium cultures before agroinfiltration

2. Agroinfiltration

Seven week old plants were used for the experiment. The infection of plants was performed by agroinfiltration with *Agrobacterium tumefaciens* strain AGL1+ pVirg, transformed with pK7WG2 plasmid containing the *Avr* genes from *P. infestans* isolate T30-4, PTIG_22870 clone containing *Avr2* gene and PITG_21388 clone containing *Avrblb1* effector gene.

3. Visualization of oxidative burst

The presence of hydrogen peroxide (H_2O_2) was detected with 3, 3-diaminobenzidine (DAB), (Alfa Aesar) dye. Detection of the superoxide radical was performed with Nitroblue-tetrazolium (NBT) (Alfa Aesar) dye, leaves were placed in 0.05 % solution before sampling time point.

4. Image analysis

After staining and fixation, leaves were captured with Olympus digital camera 5060, the images being edited with the Adobe Photoshop CS6 software.

III.5. Stress selection and phenotyping of somatic hybrids

III.5.1. Pre-selection of somatic hybrids

The somatic hybrids tested for resistance to late blight were used for *in vitro* drought stress-selection. To simulate a drought stress, the culture media (MS) was supplemented with polyethylene glycol (PEG) (Pino *et al.,* 2013), which changes the osmotic potential and causes a water deficit to the plant. The drought stress *in vitro* simulation was performed in two steps: first the culture media was supplemented with 5 % PEG (M_w=6000) and in a second step with 15 % PEG (M_w=6000). The duration of the treatments was three weeks. In each experiment step two groups of plants were used: the control group and a treated group (repeated five times).

In the end of each experiment, the morphological traits of the plants were measured: number and length of roots and stems and the proline concentration. The proline concentration was quantified from plant leaves (Chinnusamy *et al.,* 2005), using the protocol developed by Bates (1973).

III.5.2.Biomass accumulation of somatic hybrids under drought stress

All measurements and investigations were executed on the Phenotyping Platform HAS-RSDS of Biological Research Centre Szeged, Hungary. In this experiment, three pots for each

genotype were exposed to water deficit and the other treated as the control. The soil water capacity was determined and pots were watered to 20 % (water limitation) and 60 % (control, well-watered) of the 100 % soil water capacity. Watering was done automatically by a plant moving system including balance, in a connection with a computer-mediated peristaltic pump. Each pot was equipped with a radio-frequency identifier (RI). The growth of plants was monitored by digital photography, each plant being photographed by an Olympus C-7070WZ (Olympus Ltd., UK) digital camera from eleven different sideway positions, produced by 32-33 step rotation of the pot.

III.5.3. Effect of drought stress on the photosynthesis

Chlorophyll fluorescence emission from the upper leaf surface was measured with a pulse amplitude modulation fluorometer (PAM-2000 Heinz Walz Gmbh) and pocket a Plant Efficiency Analyzer (PEA) chlorophyll fluorimeter (Hansatech Instruments).

IV. Results and discussion

IV.1. Ploidy determination of somatic hybrids by direct and indirect methods

IV.1.1.Ploidy stability determination by flow cytometry

Direct and indirect methods were performed to determine the ploidy level of somatic hybrids, to investigate the methods efficiency and to test the indirect methods predictability in order to determine the real chromosome number of the somatic hybrids. First, the chromosome number was estimated by flow cytometric means, the results being compared with previous examination results (Rákosy-Tican *et al.,* 2015). Six somatic hybrids (SH), eight hybrids from the first backcross event (BC1) and three from the second backcross event (BC2) were analysed. Parental lines, the diploid *S. bulbocastanum* and the tetraploid cultivated potato were used for internal standardization. Taking into consideration that after somatic hybridization only the hexaploid plants were selected for *in vitro* culture (Rákosy-Tican *et al.,* 2015), current findings reveal that somatic hybrids lose chromosomes during the regeneration stage.

IV.1.2. Determination of the ploidy level by counting chloroplast in guard cells

To obtain reliable results, the ploidy level was determined using 9 leaves from each genotype. Thereby, chloroplasts present in 54 guard cells were counted for each genotype.

IV.1.3.Determination of ploidy level and genome composition by classical cytological methods

In order to confirm the utility of the indirect methods, further analysis of the ploidy level based on chromosome counts was undertaken.

The chromosome number for each somatic hybrid was determined from root tip cells. In this study, the chromosome number was determined for seven hybrids (SH) ten genotypes from BC1 and three genotypes from BC2 progeny. Based on our findings, we can state that the backcross progenies ploidy level decreases in time to tetraploid or pentaploid level.

Hybrid plants chromosome number reveals that four somatic hybrids (2299/2, 2284/5, 2283/9, 2294/5) have the expected chromosome number 2n=72. Three hybrids (2284/4, 2295/1, 2282/4) are characterized by a smaller chromosome number that the expected one 2n<72. In the case of the 2282/4 hybrid, it was not possible to estimate the exact chromosome number, results being based only on the flow cytometry measurement and the chloroplast counting.

Similar results were obtained in the BC1 progeny. From the ten investigated genotypes, two have the expected chromosome number 2n=60 (2283/9/63; 2295/1/3), while the eight remaining genotypes are characterized by fewer chromosomes than expected 2n<60.

For the BC2 progeny, surprising result were obtained. Among the three studied genotypes, two have the expected chromosome number 2n=60, but the third genotype (2282/4/68/22) has more chromosomes that expected. This phenomenon might be explained and might suggest the existence of a problem with chromosome separation during meiosis.

Based on these results it becomes conspicuous that the somatic hybrids between *S. bulbocastanum* and cultivated potato are stabilized at tetraploid level. Chromosome elimination in case of somatic hybrids is a well-known phenomenon (Harms, 1983; Menke *et al.,* 1996; Pijnacker *et al.,* 1989; Gavrilenko *et al.,* 2002; 2003).

IV.1.4. Correlation of direct and indirect assays to reveal the ploidy of somatic hybrids

The chromosome counting results were used to statistically analyse which indirect method predicts better the real chromosome number.

Before performing the chromosome number predictability determination based on indirect methods, we statistically confirmed that both flow cytometry and chloroplast number significantly correlate with the chromosome number (Pearson correlation, $p<0.05$). These findings are not surprising taking into consideration that these indirect methods were extensively used before, in several ploidy determination assays (Fahleson *et al.*, 1988; Mattheij *et al.*, 1992; Menke *et al.*, 1996; Maciejevska *et al.*, 1999; Horsman *et al.*, 2001).

The selection of the parental lines as a standard was appropriate for predicting chromosome count, based on the correlation between the flow cytometry results (M1) and the chromosome count data($R^2=0.97$, $p<0.05$).

The second stage of the modelling was the calculation of the chromosome count based on the linear equation and testing if the data obtained correlates with the real chromosome number. Results reveal that based on flow cytometry analysis ($R^2= 0.78$, $p<0.05$) and chloroplast number assay ($R^2=0.85$, $p<0.05$) the chromosome number can be predicted with high accuracy (Figure 1).

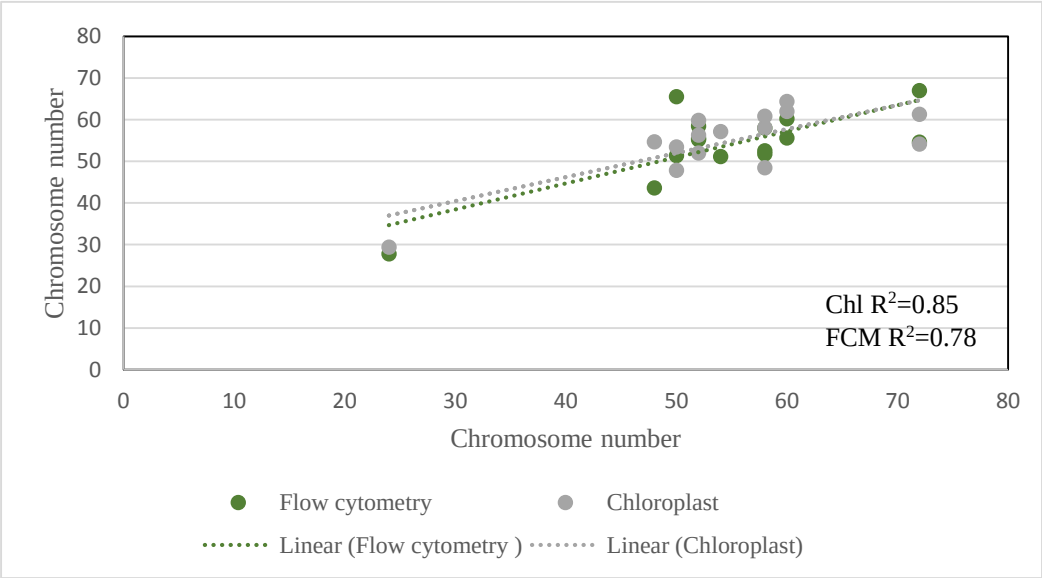

Figure 1. Correlation between real chromosome number and chromosome number determined through chloroplast number assay (Chl) (grey points) and flow cytometry analysis (FCM) (green points)

IV.2. Cytogenetic characterization of plant genome composition

IV.2.1. Genome composition and chromosome constitution of the somatic hybrids

Ploidy analysis of somatic hybrids and backcross progenies reveal the presence of somatic incompatibility between parental species, therefore, chromosome losses during plant regeneration is attributed to this phenomenon (Orczyk *et al.*, 2003).

In order to explore the genome composition and chromosome constitution of somatic hybrids, mcGISH was performed. As mentioned before, after the somatic hybridization, only the hexaploid plants were selected for *in vitro* maintaining (Rákosy-Tican *et al.*, 2015).In this study we analysed five hybrids, five genotypes from BC1 progeny and two genotypes from BC2 progeny. In these plants belong to the series containing hybrid-BC1 and BC2 progeny in one case. It is possible to follow the source of the lost chromosome through the series.

The first studied series proved that the hexaploid hybrid (2295/1) became aneuploid during the *in vitro* maintenance stage, with a total of 66 chromosomes, 46 from *S. tuberosum* and 20 from *S. bulbocastanum* (Figure 2 A). In the studied BC1 progeny (Figure 2 B) we found fewer chromosomes than expected because it has 58 chromosomes overall, 48 from the cultivated potato and 10 from the wild species. These findings demonstrate that the chromosomes from the wild species are lost in bigger proportion.

The members of the BC2 series have the same chromosome number 50, but mcGISH reveals that the chromosome constitution of these genotypes is different. More specifically, one of the genotypes lost more chromosomes from. *S.bulbocastanum* than the other BC2 genotype (Figure 2 C and D).

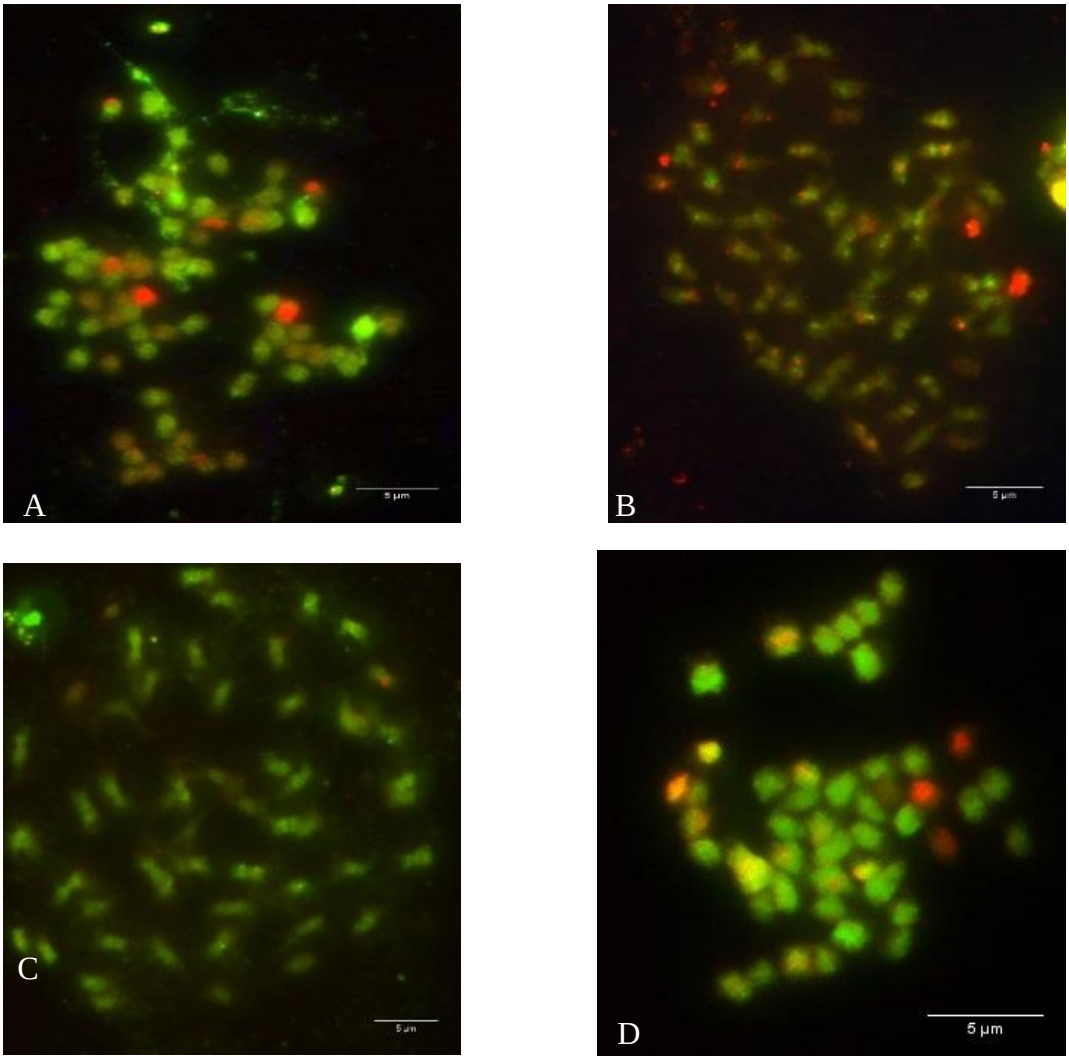

Figure 2. McGISH visualization of mitotic metaphase chromosomes of hybrid-BC1-BC2 series

A-2295/1 with 66 chromosome 46 tbr (green) and 20 blb (red);

B-2295/1/7 with 58 chromosome 48 tbr (green) and 10 blb (red);

C-2295/1/4/11 with 50 chromosome 48 tbr (green) and 2 blb (red);

D-2295/1/4/59 with 50 chromosome 42 tbr (green) and 8 blb (red)

Three genotypes from BC1 progeny (2283/9/3; 2283/9/27; 2283/9/63) produced by backcrossing a hexaploid hybrid (2283/9) with the tetraploid potato, showed different chromosome numbers, demonstrating that the chromosome elimination is a random process. Just one from three BC1 genotypes has the expected chromosome number (60 chromosomes), the rest of the genotypes being aneuploids (Figure 3).

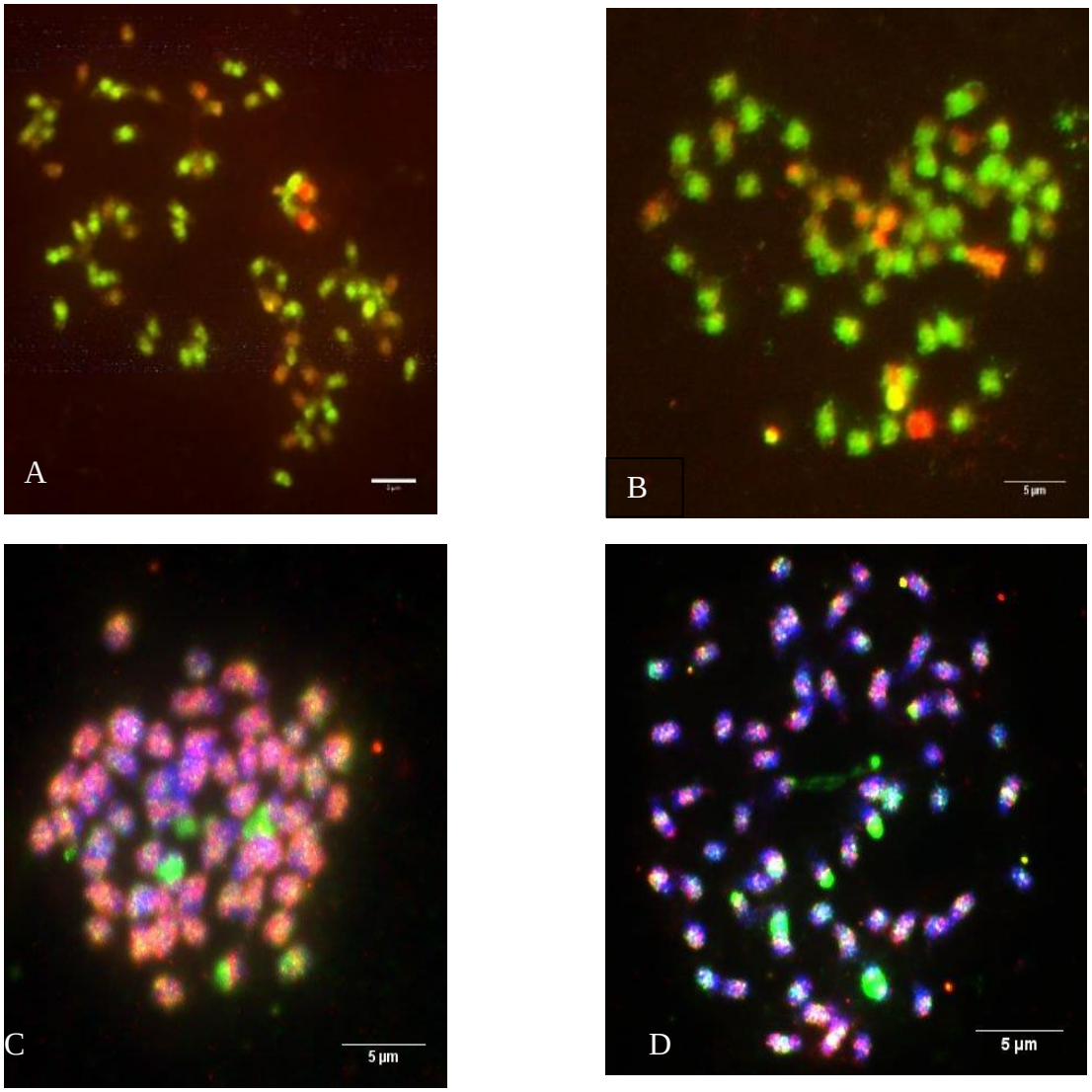

Figure 3. McGISH visualization mitotic metaphase chromosomes of hybrid-BC1 series

A-2283/9 hexaploid genotype with 48 tbr (green) and 24 blb (red);

B-2283/9/3 with 52chromosome 40 tbr (green) and 12 blb (red);

C-2283/9/27 with 52 chromosome 42 tbr (red) and 8 blb (green)

D-2283/9/63 with 60 chromosome 44 tbr (red) andb16 blb (green);

Analysis of the backcross progenies chromosome constitution reveals that even if the real chromosome number is equal with the expected one, the numbers of chromosomes from different parental lines are not the expected ones. A good example is the 2283/9/63 genotype with 16 blb chromosomes, which are more than the expected ones (Figure 3 D).

Assessing numerous somatic hybrids outlined that the ploidy level of hybrid plants seems to become stable at tetraploid level.

Recombination events are present in 2294/5/5 BC1 plant (Figure 4), this progeny being originated from the crossing of the hexaploid hybrid (2294/5) and the cultivated potato.

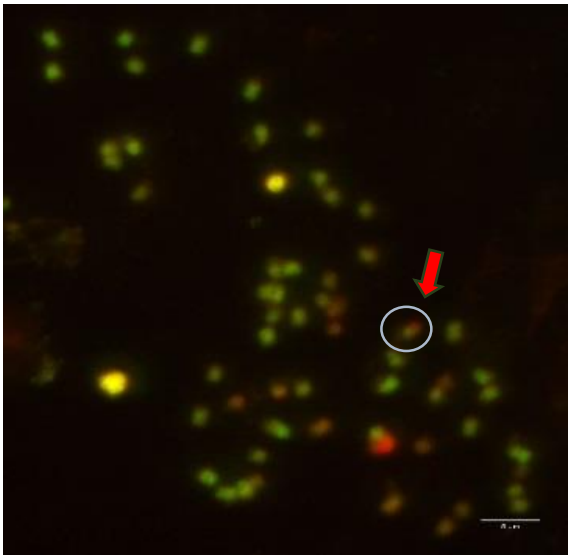

Figure 4. McGISH visualization of mitotic metaphase chromosomes of 2294/5/5-BC1a somatic hybrid (2294/5/5).The recombination site is indicated with an arrow. Chromosome constitution: 46 chromosomes (green) from cultivated potato and 12 chromosomes (red) from *S. bulbocastanum*.

Preferential elimination of chromosomes originating from the wild species were reported in several studies, where the fusion partners were *S. pinnatisectum* (Menke *et al.*, 1996), *S. phureja* (Pijnacker *et al.*,1987, 1989) *S. brevidens* (Gavrilenko *et al.*, 2002) or *S. etuberosum* (Gavrilenko *et al.*, 2003).

Hybrids were crossed with different tetraploid potato cultivars, reason why in the backcross progenies the reduction of the ploidy level of the plants is expected. As previously demonstrated, *S. bulbocastanum* cannot be sexually crossed with potato cultivars because of the differences in the endosperm balance number (EBN) 2:1 (*S. tuberosum***:***S. bulbocastanum*) (Masuelli and Camadro, 1997; Rákosy-Tican *et al.*, 2005).

Two pairs of *S. bulbocastanum* chromosomes in all of the hybridization reactions gave a stronger hybridization signal, which is located in the centromere region. Stupar *et al.*, (2002) found that a highly repetitive 2D8 sequence is responsible for the intense labelling of these chromosomes.

The observed structural chromosome changes indicate the presence of a recombination event between the parental chromosomes (Figure 11). Due to the very small sizes of the chromosomes, only the big recombinant segments can be detected, small segments being undetectable with mcGISH (Gavrilenko *et al.*, 2002).

Successful recombination is essential in a project which aims to transfer the desired traits from wild species into the cultivated potato. Based on the mcGISH results, we can state

that the backcross progeny between cultivated potato and *S. bulbocastanum* is a promising pre-breeding material, the chromosomes from the wild species being present in the hybrids genetic makeup.

IV.3. Oxidative burst, the first defence responses

Histochemical methods allowed the detection and localization of the ROS molecules synthesis site (hydrogen peroxide and superoxide) and the monitoring the evolution of the interaction (Kužniak *et al.*, 2014).

IV.3.1. Detection of hydrogen peroxide

Using special staining, the synthesis site in each plant group was successfully localized. After the infiltration, the water control did not show any specific reaction.

Parental species were used as model plants, representing compatible, respective incompatible interactions, *S.tuberosum* cv. Delikat being susceptible while *S. bulbocastanum* being resistant to late blight.

Hydrogen peroxide synthesis patterns in somatic hybrids were compared with the ones in parental lines and each comparison was statistically validated using a linear model (LM), taking into account the plant group, the time after infection and the expansion of the synthesis site.

Statistical analysis of the results revealed that the synthesis pattern significantly differs in compatible and incompatible interaction. The area of hydrogen peroxide synthesis showed remarkable differences at different times after infiltration in the case of incompatible interaction (LM, $p<0.01$).

In the incompatible interaction the first oxidative burst appeared three hours after infiltration and the second maximum appears 24 hours after infiltration, while in the compatible interaction there is only one maximum (peak), which appears 8 hours after infiltration.

Our results are in accordance with the literature (Apel and Hirt, 2004, Kobayashi *et al.,* 2007; Wi *et al.,* 2012, Han *et al.,* 2013) in which is presented that in incompatible interactions, after the first peak of hydrogen peroxide synthesis, there appears a second, more extended one. The second peak is also called “recognition response” because it occurs when

the pathogens effector molecules are recognized by the matching resistance proteins. This recognition represents the basic condition of the incompatible interaction.

The investigation was started with the BC1 hybrid 2282/4/77, which carries both resistance genes, but proved to be susceptible to late blight, after field tests. This comparison with *S. bulbocastanum* revealed that both first and second maximum hydrogen peroxide synthesis appear later, but the values are not significantly less (t-test, $p>0.05$) for the hybrid (Figure 5). Knowing that the timing and amplitude of the oxidative burst determine the interactions outcome, the delay of the first oxidative burst can have a major impact. It is well-known that the delay of the oxidative burst is characteristic for susceptible plants (Wi *et al.*, 2012).

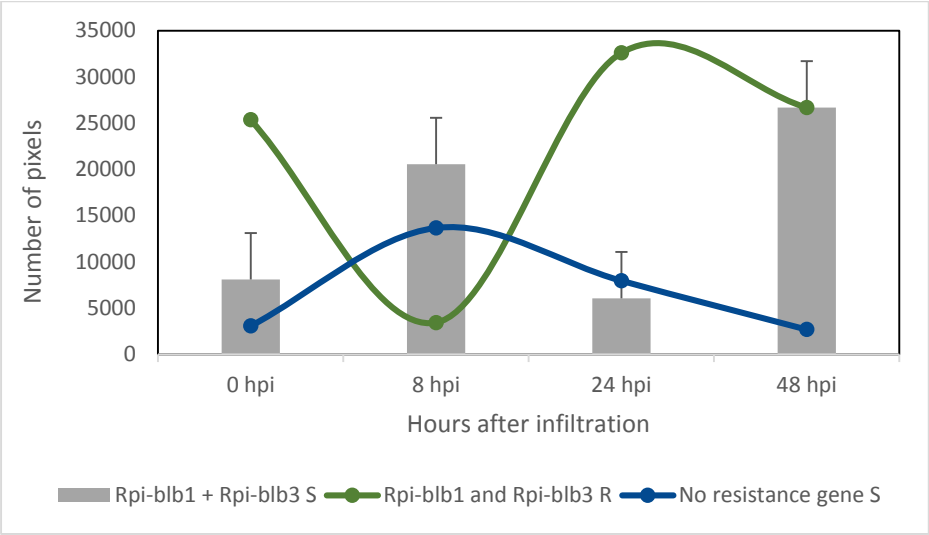

Figure 5. Hydrogen peroxide synthesis in susceptible somatic hybrid carrying two resistance genes (grey column) in comparison with synthesis pattern in resistant *S. bulbocastanum* (green line) and susceptible plants *S. tuberosum* (blue line);

The second stage of the experiment is represented by the analysis of the somatic hybrids group with the *Rpi-blb1* gene: the susceptible (2283/9/27) and the resistant genotypes (2283/9/3). Measurement results showed that the susceptible plant group has similar hydrogen peroxide synthesis pattern to *S. tuberosum* (LM, $p>0.05$), the maximum of synthesis appears 8 hours after infiltration and then decreases sharply until 24 hours after infiltration. The pattern of hydrogen peroxide synthesis in the resistant plant carrying *Rpi-blb1*gene showed just partial similarities with resistant *S. bulbocastanum*. The biphasic synthesis pattern is present, but the timing is different. In this case, the first rapid oxidative burst increased more than two fold, when compared to the one characteristic for the susceptible plant with *Rpi-blb1* gene, while the second burst appeared just 48 hours after infiltration, later than in the case of the control, *S. bulbocastanum*.

The last investigated group was represented by the somatic hybrids with *Rpi-blb3* gene, both resistant (2283/9/63) and susceptible (2283/9/64) genotypes, which showed different levels of hydrogen peroxide synthesis than the other investigated genotypes. First of all, the synthesis area is bigger than in the case of other somatic hybrids or the control groups. In the case of the sensitive genotype, the quantity of hydrogen peroxide starts at very high level, after words stabilizing at a low level.

In the resistant plants, the synthesis rate is constantly growing until 8 hours after infiltration. The second peak of the synthesis appears 48 hours after infiltration.

IV.3.2. Detection of superoxide radical

Superoxide is another main component of the hypersensitive response in plants. Coloration was performed successfully, our results outlining the characteristic pattern for superoxide production in susceptible and resistant genotypes to late blight. In plants resistant to *P. infestans* the superoxide is synthesized in the first 8 hours after infiltration, while in the case of the sensitive genotypes the superoxide appears right after infiltration, being present in the plant tissues for 48 hours after infiltration (Figure 6).

	Sensitive		**Resistant**	
	0 hpi	**48 hpi**	**0 hpi**	**48 hpi**
1				

Figure 6. Superoxide synthesis pattern in plant groups after infiltration

1. Comparison of *Solanum tuberosum*, sensitive genotype to *Solanum bulbocastanum* resistant genotypes;

IV.4. Stress selection and phenotyping of somatic hybrids

Stress selection of drought tolerant somatic hybrids is part of the pre-breeding programs because the presence of different combinations of valuable traits in a single genotype is essential, not only for the breeders.

IV.4.1. *In vitro* stress-selection of the drought tolerant somatic hybrids

Drought stress was induced by supplementing the culture media with PEG, in different concentrations, such that both mild and severe drought stress were simulated.

Based on the morphological trait development (stem and root), genotypes were classified in different groups. Overall we can conclude that the drought stress induced by PEG has a negative effect (t.test, $p<0.01$) on the somatic hybrids development.

The following enumeration contains the morphological characteristics of each class and the members of the groups.

Group 1: well-developed plant with several roots, developed stem with green leaves, development of lateral stems and/or presence of mini-tubers in some hybrids;

Group 2: weakly developed stems, no presence of mini tubers or root;

Group 3: root developed, without stem development;

Group 4: dried plant;

Potato is a drought sensitive plant, this fact being supported by our results which reveal that mild drought stress negatively affects the somatic hybrids development. As mentioned above, drought stressed plants development show signs of stress, when compared to the control plants.

In the previous chapter, we demonstrated that somatic hybrids are genetically different and for this reason it is important to analyse the responses to drought stress in a genotype-dependent manner. Classification of genotypes based on morphological traits helped us understand the genotype resources allocation.

Parental lines used for somatic hybridization are members of group 3 because they are characterized only by root development. This aspect of the plants morphology is not negligible because well-developed roots ensure rapid water uptake during the drought and rapid recovery after the drought stress period.

In case of severe drought stress (15 % PEG) the control plants development was normal, but the drought stressed plants showed poor development. Using the same classification criteria like in mild stress, in this case, no genotypes can be framed in group 1. There are a

few genotypes in group 2, which show 1-2 cm growth of the stem or abaxial stem, while members of group 3 have a slightly developed root system.

Proline content

Results reveal that the proline content in the drought stressed group is significantly increased, when compared to the control plants, in mild drought stress (t.test, $p<0.01$) and also in severe drought stress (t.test, $p<0.01$).

IV.4.2. Biomass accumulation of somatic hybrids under drought stress

Phenotyping platforms provide a good opportunity to assess somatic hybrids morphological and physiological changes over time and under drought stress (Wegener *et al.*, 2015).

In our research, the somatic hybrids response to drought stress was investigated, by evaluating the green biomass and the tubers yield. Determination of biomass accumulation is based on RGB images, in which the counted green pixels represent the stem surface area. After the experiment, the total weight of tubers was measured in drought stressed and in control plants. Observations on their shape were also done.

Drought stress significantly decreased the biomass accumulation of the cultivated potato(ANOVA, $p<0.05$) (Figure 21 A), while in case of *S. bulbocastanum* and the somatic hybrids there were no significant differences observed between control and drought stressed plant development (ANOVA, $p>0.05$). In the vast majority of the studied somatic hybrids, it was remarkable how the biomass accumulation of drought stressed plants ran parallel with the one for the control plants. The negative effect of water deficit is observable in the first two weeks, but from the beginning of the third week the biomass of treated plants begun to grow at the same rate as the one of a control plant (Figure 7 A and B).

The drought tolerance of the somatic hybrids proves the fact that these plants rapidly adapt to new environmental conditions and at the end of the experiment they are capable of catching up to control plant. The biomass accumulation of the drought stressed 2283/9 somatic hybrid is remarkable, because the stressed plants growth precedes the control after the third week of the experiment (Figure 7B)

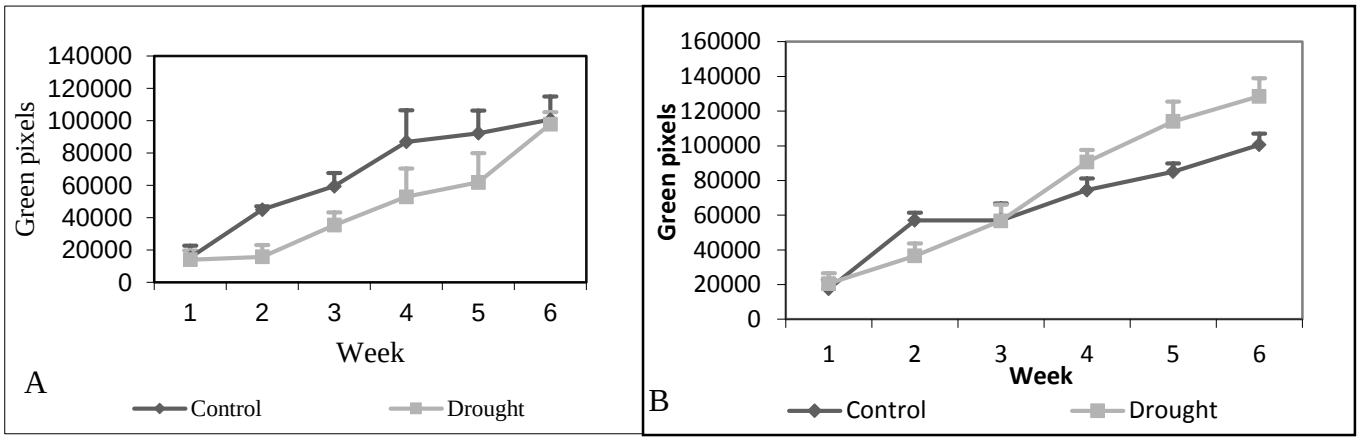

Figure 7. Biomass accumulation of the 2282/4 (A) and 2283/9 (B) somatic hybrids

Quantity and quality of produced tubers are the most followed trait in potato breeding programs. In our experiment the somatic hybrids tuber yield and the shape were assessed. Big difference was observable between somatic hybrids yield, some of them did not had tubers, another presents only 24% reduction. These results was not surprising due to the fact that these hybrids are genetically different, having different chromosome number and genome constitution. Tubers morphology of somatic hybrids both in control and the stressed group is similar to cultivated potato, witch's main feature are yellow skin, smooth surface and round, oval shape. In our somatic hybrids there is no detected tuber defect due to the drought stress (Figure 8).

Figure 8. Tubers from SH 2283-9 control (A) and drought stressed plant (B)

IV.4.3. Drought stress effect on the photosynthesis

Photosynthesis is a very sensitive process, the presence of abiotic or biotic stressor leading to several deviation from normal progress therefore photosynthesis can be used as an indicator of overall plant fitness.

Through the measured and calculated parameters we followed the drought stress effect on the protein complexes which have role in light capturing and transformation into biochemical energy. Firstly we analysed parameters, which reflect the all systems functionality and after that the activity of the individual complexes.

The maximum quantum yield of plants (Fv/Fm), was assessed firstly, because decrease of this ratio reflect the presence of serious disorder in PSII functioning. Both control and drought stressed plants maximal quantum efficiency fell into the optimal interval, characteristic of unstressed plants, 0.70-0.84 (Jefferies 1992; Baker and Rosenqvist, 2004; Rolfe and Scholes, 2010). In several studies was reported the significantly decrease of this ratio, due to drought stress, but in our case, we observed a slightly increased values in the stressed plants.

In the next step were assessed the effective quantum yield of photosynthesis, because this ratio reflects the efficiency of the electron transport and the conversation efficiency of the light energy into the chemical energy (Maxwell and Johnson, 2000; Brestic and Zivcak, 2015). In accordance to biomass assessment, the effective quantum yield of plants was negatively affected in the beginning of the experiment, but at the second measurement there was no difference between control and stressed plants. As Flagella et al. (1998) mentioned, mild and moderate drought stress doesn't decrease significantly, the quantum yield of PSII in this way the Calvin cycle remains just slightly affected (Brestic and Zivcak, 2015).

These results are supported by the individual activity of protein complexes. Drought stress didn't affect the integrity of the system which directs the light energy from the antenna to PSII. The secondly assessed, water splitting complex activity (Fv/Fo), was negatively affected in the beginning of the experiment, but second measurement did not find any difference between stressed and control plants, these findings suggest that somatic hybrids have the capacity to adapt to water scarcity (t.test $p>0.05$).

In the next steps were assessed the plastoquinone pool fluorescence and size by calculating the Fv/2 ratio and area under fluorescence induction curve. We got the expected results, there was no significant decrease in their fluorescence and the size of the plastoquinone pool, controversy in the second measurement the both mentioned parameter

showed significantly increased values in the drought stressed plants. Knowing that, the Fv/2 ratio positively correlates with the pigment number associated to the reaction centre, we demonstrated that in somatic hybrids under drought stress the photosynthetic pigment are reorganized (Fodorpataki, 2004). Moreover we found the increased density of the active reaction centre in drought stressed plant, in the second measurement.

Another important parameter, the performance index (PI) were measured, because reflects the activity of both PSII and PSI, all of the photosynthesis process mechanisms. Obtained results showed, that in the case of the first measurement there was no difference in the density of reaction centres in the control and the drought stressed groups, while at the end of the experiment, the number of the active centre increased in the stressed plant group (t.test, $p<0.05$).

The part of the energy which is dissipated in form of heat or re-emited as a fluorescence, were quantified by calculating the Non-photochemical quenching (NPQ) (Demming and Adams, 1996; Maxwell and Johnson, 2000). The measurement results, showed that the excess energy dissipated by a non-photochemical process in drought stressed group significantly decreased from the first measurement to the second measurement event.

Electron transport rate (ETR) showed differences between the control and the stressed plants, in all genotypes. In the control plants, the ETR became saturated at lower Photosynthetic Photon Flux Density (PPFD) than in the drought stressed plants.

Li et al., (2015) appointed that the high electron transport capacity is a characteristic of drought resistant species. Tubers represent the non-photosynthetic storage, thus their formation in our plants diminished the negative effects of drought stress on the photosynthesis Basu *et al.* (1999) demonstrated that in plants that lack tubers, the photosynthesis is severely affected in comparison with plants with tubers.

Somatic hybrids have the capability to adapt to water limitations, while at the beginning of the experiment drought stress effect is more accentuated, in the end of the experiment the photosynthesis was stabilized.

Stress selection of somatic hybrids has to be continued with field experiment, in order to find the best candidate for breeding programs, which combine late blight resistance and drought stress tolerance.

V. General conclusions

The main goal of my researches was to obtain a more complex characterization of some somatic hybrids between the cultivated potato and *S. bulbocastanum*, complementing previous results. My research was focused on plant resistance to late blight and also on bringing new insights into the oxidative burst and drought tolerance in somatic hybrids.

Therefore

1. We investigated the genetic stability of somatic hybrids and backcross progenies. From preliminary results (Rákosy *et al.*, 2015), only the hexaploid hybrids were kept for *in vitro* culture, after somatic hybridization. Our results demonstrate that somatic hybrids are not genetically stable, chromosomes being lost during the regeneration stage. The different chromosome number of BC1 genotypes, originating from the same crossing event, demonstrates that the number of lost chromosomes is unpredictable. The indirect methods proved to be reliable because they allow us to predict the real chromosome number of somatic hybrids with high accuracy. The chromosome elimination might be explained by the somatic incompatibility between the parental species.

2. The mcGISH method was used to analyse the genome composition and the chromosome constitution of our somatic hybrids. Based on our results we recommend to perform the pre-treatment of root tips with cellulase and pectinase enzymes, because after using this treatment we obtained clearer chromosome preparations. Our findings demonstrate that the somatic hybrids lost chromosomes from both parental species, but in a higher proportion from *S. bulbocastanum.* We also proved that the same chromosome number of two somatic hybrid doesn't mean same chromosome constitution. Our results reveal the presence of recombination between parental species genome. Localization of the resistance genes it was not possible, we suggest to use high-resolution method, to localize similar, single copy resistance genes.

3. We investigated the resistance gene functionality by using the agroinfiltration method. This method proved to be efficient; we successfully identified the resistant genotypes to late blight. Based on our results, we highlighted the importance of the oxidative

burst timing in the plant-pathogen interaction outcome. Our findings revealed the presence of biphasic oxidative burst in resistant somatic hybrids, but the pattern of the maximum synthesis is genotype dependent.

Our results showed a significantly increased hydrogen peroxide level in plants with *Rpi-blb1*gene in comparison with plants containing *Rpi-blb3*gene.

4. In chapter 4.4 we proved that the *in vitro* stress selection is an effective tool to find the drought tolerant somatic hybrids. Based on the assessed morphological traits and on proline content of plants, we were able to make a distinction between drought tolerant and sensitive genotypes. The phenotyping of somatic hybrids revealed that the drought stress didn't decrease significantly the green biomass of plants, but declined substantially the potato yield. Among the parental lines, *S. bulbocastanum* proved to be drought tolerant. These results are confirmed by the assessment of drought stress effects on photosynthesis.

   Our results revealed that drought stress does not affect the maximum quantum yield of photosynthesis, suggesting that the PSII is not damaged. The results of repeated effective quantum yield measurement showed that the carbon fixation is negatively affected only in the beginning of the drought stress. In the case of the first photosynthesis analysis, we observed an enhanced photosynthesis in drought stressed plants, fact that justifies the green biomass accumulation, probably more energy being allocated in the osmotic adjustment.

Summarizing the results of our research, we can state that a combination of drought tolerance and late blight resistance is present in some of our somatic hybrids, thus the traits from the wild species were successfully transferred and maintained in backcross progenies.

Finally we can conclude that our somatic hybrids between cultivated potato and *Solanum bulbocastanum* represent an unique, valuable material not only for pre-breeding programs, but also for biological research, if we take into account that by studying these unique genotypes we can better understand the intercellular mechanisms occurring in incompatible crossings of different plant species.

The originality of my PhD thesis is represented by the following aspects:

- Determination of the ploidy level and genome composition of the somatic hybrids between *Solanum tuberosum cv. Delikat+ Solanum bulbocastanum*;

- First time was used the agroinfiltration for histochemical detection of the reactive oxygen molecules, in plants agroinfiltrated with the corespondent types of effectors, depending on the presence of the resistance genes *Rpi-blb1* and/or *Rpi-blb3;*
- Stress-selection of tolerant somatic hybrids to drought stress, by using the phenotyping platform tools HAS RDS Szeged, Hungary.

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