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The complex analysis of the resistance traits and genomic composition in the somatic hybrids between *Solanum tuberosum* and *Solanum chacoense*, with or without deficiency in DNA mismatch repair system

PhD thesis summary

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Cluj Napoca

2017

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KEYWORDS: Colorado potato beetle, cultivated potato, drought stress, GISH, glycoalkaloids, MMR deficiency, *Solanum chacoense*, somatic hybrids

I. THEORETICAL OVERVIEW

Potato (*Solanum tuberosum* L.) is considered an especially valuable crop when Earth's nutritional problems are increasing. Nowadays potato is considered the fourth most important crop in the world, ranking first in yield among the non-grain food commodity, with total world production being more than 360 million tons per year (FAOSTAT 2014). Romania is the sixth largest harvested area potato grower in Europe, in 2014 more than 3.5 million tons of potato was produced (FAOSTAT 2014). Potatoes are grown in 160 countries and more than 4000 cultivars are known (Camire *et al.* 2009).

Due to potato domestication processes, which were carried out in order to increase the potato production and tubers quality, *via* the selection against high levels of secondary metabolites in tubers (Hermanova *et al.* 2007), the genetic base of the potato became narrow. As a result of genetic background weakening, the susceptibility of potato increased. This crop has been affected not only by large scale of abiotic stresses as cold, frost, and drought, but also countless biotic factor decrease the yield (Hirsch *et al.* 2013).

Nowadays potato crop production is reduced by approximately 160 diseases and several pests: 50 are caused by fungal and fungus-like pathogens, 10 by bacteria, 40 by viruses and the rest by leaf- or tuber-damaging pests.

Developing host plant resistance could be the single long-term solution for disease and pests control, but unfortunately it is difficult to achieve, because modern potato cultivars possess poor resistance gene background, a fact that does not allow to select resistant varieties.

Most of the wild relatives of the cultivated potato provide host-plant resistance to different pests and diseases. These wild species represent a rich and diverse source of resistant genes (Hawkes 1990) which could be useful for potato improvement. In the last decades, potato breeding was based on diversification of the cultivated potato's genetic background, by incorporating the desirable traits from wild tuber-bearing species (Ross 1986). This new genetic resource proved to be useful in disease resistance and also in tolerance of environmental changes. An unpleasant disadvantage of classical breeding process is that these methods are time-consuming. Several

hundreds of selection cycle is necessary to obtain usable varieties, which requires a minimum of 10 year. In several cases, more than 30 years were necessary before releasing a new variety (Gebhardt 2013, Haverkort *et al.* 2009). Somatic hybridization realized by protoplast electrofusion is considered a faster alternative method to transfer resistance genes from wild *Solanum* species into potato crop (Thieme *et al.* 2010). Furthermore, this method made it possible to overcome the maternal inheritance of the cytoplasm, which prevents the mixing of plastids and mitochondria from both parents (Birky 1995). As a result of interspecific hybridization, the obtained somatic hybrids may contain the target beneficial traits from wild species, but also some undesired properties could appear, like high glycoalkaloid concentration in tubers, decreased tuber quality or yield. Therefore, in the case of somatic hybrids produced in this way, a profound characterization is necessary before they are integrated into breeding programs.

Solanum chacoense is a diploid (2n=2x=24), self-incompatible, tuber-bearing *Solanum* species. *S. chacoense* attracted the attention of potato breeders because of its broad resistance against different pathogens. *S. chacoense* is highly resistant against Colorado potato beetle (CPB) (Sinden *et al.* 1986). The insect resistance is attributed to their specific glycoalkaloids: leptines, which are acetylated forms of common α-solanine and α-chaconine. Leptines are synthetized only in the aerial tissues of plants, which is an advantage for breeders, because introgression of leptine synthetizing genes into cultivated potato should confer resistance against several diseases, but does not increase the tubers glycoalkaloid level.

1.1. Somatic hybrids between *Solanum chacoense* and cultivated potato

In order to introgress valuable traits from *S. chacoense*, Thieme *et al.* (unpublished data) and Rakosy *et al.* (2004) produced somatic hybrids and backcross progenies between cultivated potato cv. Delikat and Desiree and *S. chacoense* with or without deficiency in DNA repair system (MMR). In both cases somatic hybridization was performed using the protoplast electrofusion technique.

For somatic hybrid production, Thieme *et al.* (unpublished data) used mesophyll cells of *S. tuberosum* cv Delikat and *S. chacoense* GLKS 30138 (S. chc 138) from Gross Lüsewitz Potato Collections, IPK Satellite Collections North Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Genebank, (Germany). BC_1 plants were obtained after sexual backcrossing of SH

1552/1 with *S. tuberosum* cv. Sonate. BC_1 1552/1/7 plants were sexual backrossed with *S. tuberosum* cv. Romanze to obtain BC_2 progenies.

Rakosy *et al.* (2004; 2015) used as parents in somatic hybridization, Delikat and Desiree cultivars and the highest leptine producing *S. chacoense* accession (PI 458310) (S.chc HL) from NPGS Sturgeon Bay, USA.

MMR deficient *S. chacoense* production was performed using *Agrobacterium*-mediated transformation. For genetic transformation two types of construct were used. The AS construct contained the 1 kb fragment of the *AtMSH2* cDNA in antisense orientation. The DN construct contained the *AtMSH2* coding sequence with a mutation converting a strongly conserved Glycine codon at position 697 to an Aspartic acid codon (Ispas 2004).

The putative transgenic AS and DN lines were tested both phenotypically and by RT-PCR to prove the presence of the target gene. One transgenic line for AS and two for DN, and *S. tuberosum* cv. Delikat (Dk) and Desiree (De) were used in protoplast electrofusion to produce MMR deficient somatic hybrids. The hybridity nature of regenerated plants was validated using SSR and RAPD molecular markers (Unpublished data). MMR deficient somatic hybrids were obtained between these parent lines, in order to study the role of DNA repair system in homeologous recombination and also to increase the possibility to introgress *S. chacoense*'s genetic material into cultivated potato germplasm.

II. THE OBJECTIVES OF THESIS

Potato genotypes produced *via* somatic hybridization technique need to be characterized in order to select SHs with resistance and also with valuable properties from cultivated potato, but without undesired traits. Later, these selected genotypes can be introduced in pre-breeding programs.

The main goal of my PhD thesis was to deeply characterize the potato somatic hybrids between *Solanum tuberosum* and *S. chacoense* (Rakosy-Tican *et al.* 2004; 2015). This characterization was necessary to establish the genetic composition of somatic hybrids, to select leptine producer plants and to determine Colorado potato beetle resistant and also drought tolerant potato genotypes.

The specific aims of the thesis were as follows:

- To investigate the genetic stability and constitution of the wild type and MMR deficient somatic hybrids and backcross progenies.
- To evaluate the precision of flow cytometry method in ploidy level determination
- To determine leptine producer *Solanum* genotypes using RAPD markers described by Bouarte-Medina *et al.* (2002) and Ronning *et al.* (1999)
- To develop SCAR markers, which are effective in determining leptine producer SHs
- To differentiate leptine producer *Solanum* genotypes using different biochemical analysis
- To investigate the correlation between chemical composition and Colorado potato beetle (CPB) resistance in *Solanum* genotypes
- To determine CPB resistant genotypes by evaluating both antibiosis and antixenosis properties of SHs and derivates
- To reveal the reactive oxygen species and anthocyanin role in *Solanum* plants's defense response to mechanical injury
- To determine drought resistant *Solanum* genotypes using *in vitro* and *ex vitro* stress-selection procedures
- To investigate the drought condition effects on *Solanum* plants's development, tuber yield and photosynthesis performance

III. GENETIC CONSTITUTION DETERMINATION OF *SOLANUM* SOMATIC HYBRIDS USING CLASSICAL AND MODERN CYTOGENETIC METHODS

The main disadvantage of somatic hybridization is the cytogenetic instability of the resulted somatic hybrids (Wolters *et al.* 1994). As a result of protoplast fusion near symmetric hybrids with the whole chromosome sets from both parent species, and asymmetric somatic hybrids formation, which lost a part of one parent's genetic material, can be observed. Chromosome losses can be attributed to the differences in the ploidy level of the combined parental nuclei, or even asymmetric hybrids could be the result of the differences in dividing activity of the tissue where parental protoplasts originated from. Besides protoplast fusion processes, *in vitro* culture of regenerates could also provoke chromosome elimination in regenerated somatic hybrid plants (Glimelius *et al.* 1991). Because of the excessive changes within the fused cells, all of the regenerated plants should be considered genetically unique organisms, therefore they must be characterized separately.

The first major step of their characterization is the ploidy level determination. Flow cytometry proved to be an efficient indirect method to determine with high reproducibility the relative nuclear DNA content and ploidy level of different species (Ochatt 2006). The real chromosome numbers were highly correlated with the results obtained after flow cytometry analysis (Pearson correlation coefficient R^2: 0.935, $p < 0.05$). Based on the linear regression analysis results, the chromosome number can be predicted with a ± 4 accuracy to the flow cytometry measurements. The majority of SHs were tetraploid, which could be explained with the fact that SHs eliminated chromosomes after hybridization process. By analyzing the SHs in particular groups, like wild type SHs and MMR deficient SHs, we observed that in case of SHs without MMR deficiency, the most of them belonged to pentaploid plants group, while MMR deficient SHs plants which contained dominant negative *AtMSH2* gene (SH-DN) were mostly hexaploids but numerous tetraploid plants were also observed. SHs with *AtMSH2* gene in antisense orientation (SH-AS) were dominantly tetraploids. This observation suggests that SHs with dominant negative *AtMSH2* gene were the most stable fusion products, they eliminated the least amount of chromosomes, while SH-ASs were more sensitive (**Fig. 1**).

The value of somatic hybrids has not been diminished by losing chromosomes, because the aim of SH production was to introduce resistant genes from wild species into the genetic pool of cultivated potato. For this, it is sufficient to integrate the target DNA sequence *via* insertion or translocation processes into the genetic material of *S. tuberosum*, while the other chromosomes from wild species can be ejected without disturbing the plant. The elimination of chromosomes can facilitate the work of plant breeders, because the undesired traits can easily and quickly be rid of, without backcrossing the obtained hybrids with cultivated plants several times, which is time- and energy-consuming.

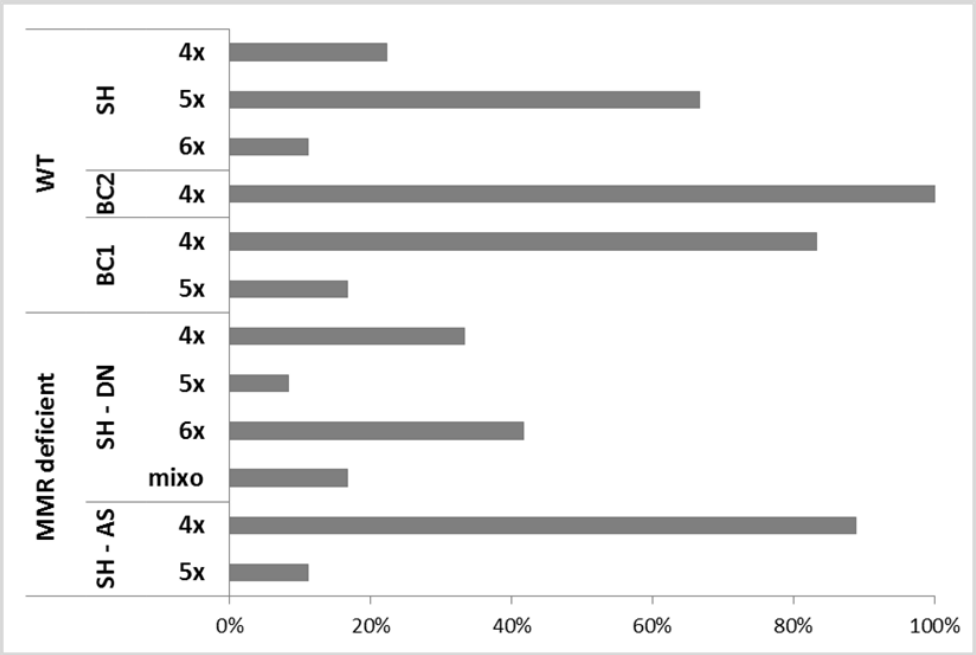

Fig. 1 Percentage distribution of ploidy level of different somatic hybrids group (WT–Somatic hybrids between *S. tuberosum* and *S. chacoense*, BC_1- Backcross progenies between WT SH + *S. tuberosum*, BC_2 – Backcross between BC_1 plants and cultivated potato, MMR deficient somatic hybrids between *S. tuberosum* and MMR deficient *S. chacoense*: SH-DN – Somatic hybrids which contain dominant negative *AtMSH2* gene, SH-AS – Somatic hybrids with antisense orientation of *AtMSH2* gene)

In the case of somatic hybrids between *S. tuberosum* and *S. chacoense*, the parental genome differentiation was really difficult, due to the very close phylogenetic relationship of the parental species. These species belong to the same *Solanum* section (*Petota*) and also to the same 4th Clade class, which was determined by single copy nuclear Granule-Bound Starch Synthase I (Spooner *et al.* 2008) and the nitrate reductase sequence analysis (Rodriguez and Spooner 2009).

Genomic composition of SHs were determined using a modified multicolor genomic *in situ* hybridization procedure described by Jang and Weiss-Schneeweiss (2015), with powerful post-hybridisation washing steps. The increased stringent washing conditions helped to reduce the cross-hybridization phenomenon, because the surplus of hybridised DNA was washed away. The stringent washing steps also contributed to reduced background signals of chromosome preparations, which provided a better visualisation of the lower-intensity signals on the chromosomes. To check the effectiveness of the applied GISH technique, somatic hybrids with full sets of parental chromosomes (72 chromosomes) were used. Using Jang and Weiss-Schneeweiss (2015) modified protocol, we can confirm that this mcGISH technique worked well and therefore we were able to determine the genomic composition of SHs.

Based on our results, we can conclude that the optimised stringency conditions were effective in determining the somatic hybrids's genomic composition with high accuracy, but in the case of some chromosomes cross-hybridization phenomenon can also be observed. The optimization of the used method is necessary in order to entirely eliminate the cross-hybridisation phenomenon. Unfortunately it is almost impossible due to the high degree of genomic similarity of parental lines.

IV. DETERMINATION OF SOMATIC HYBRIDS WHICH POSSESS LEPTINE GLYCOALKALOID ENCODING GENES BY USING MOLECULAR MARKERS

RAPD procedure is simpler and faster than other marker based analysis (RFLP), and several polymorphic loci can be identified in a single PCR amplification.

The main disadvantage of RAPD markers is that they operates like dominant alleles, therefore separation of heterozygous (1 copy) loci is not possible. RAPD technique is sensitive to quality and concentration of the used genomic DNA, to concentration changes of PCR components or PCR cycling conditions. Any kind of changes highly influence the repeatability of the obtained results.

To improve the reproducibility of RAPD primers, Paran and Michelmore (1993) developed a new molecular marker (SCAR), which was based on polymorphic RAPD products.

IV.1. RAPD analysis

RAPD markers used in this study were assorted in order to distinguish the leptine producer somatic hybrids based on the studies of Bouarte-Medina *et al.* (2002) and Ronning *et al.* (1999). Primers selected by Bouarte-Medina *et al.* (2002) recognized specific DNA sequences which were linked to leptine trait, while Ronning *et al.*'s (1999) primer (UBC-370) amplified a specific DNA sequence which was not synthetized by leptine producer plants.

Unfortunately, UBC-370 marker was not effective in our case, because this marker amplified a DNA sequence with 1500 bp length at both parent lines (*S. chacoense*, *S. tuberosum*), which could only able to be recognized in the case of leptine non-producer plants such as the cultivated potato.

Among specific markers of Bouarte-Medina *et al.* (2002), only OPT-20 showed differences between the parent lines. By using OPT-20 RAPD marker, a specific DNA sequence with 250 bp length was amplified in the case of *S. chacoense* HL and *S. chacoense* 138, which are known to produce leptine glycoalkaloids, as opposed to this, in the case of *S. tuberosum* this specific band was not present on agarose gel. Bouarte-Medina *et al.* (2002) found the same sized polymorphic product after bulk segregant analysis.

Among SHs without MMR deficiency, the OPT-20 marker amplified the specific 250 bp length sequence only in the case of SH 1552/1. This hybrid was also used in backcross procedure with the cultivated potato, which led to 1:3 segregation of leptine synthesis ability in descendants based on RAPD- PCR analysis (**Fig. 2**).

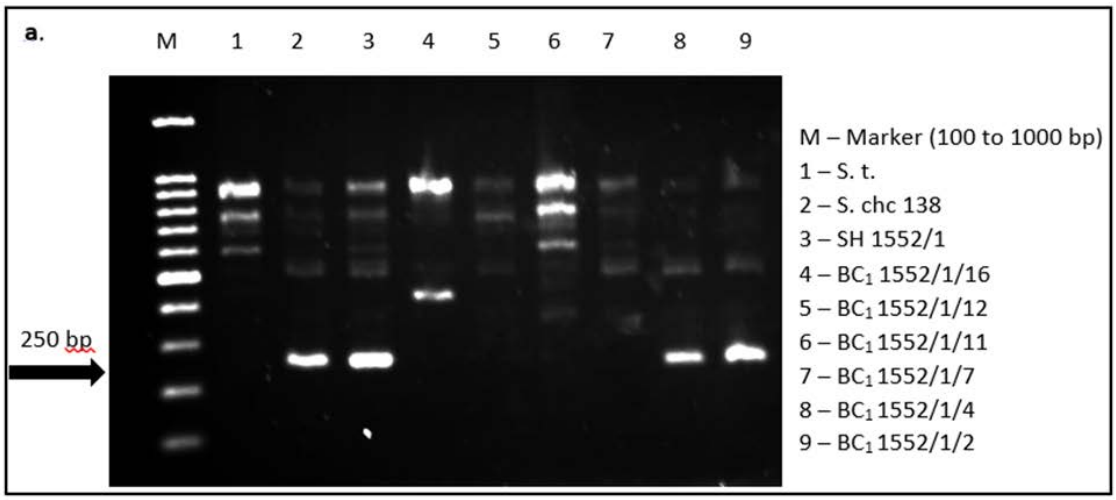

Fig. 2 Selection of leptine producing SHs and BC_1 clones based on OPT-20 RAPD marker linked to leptine biosynthesis

BC_1 1552/1/7 was backcrossed with cultivated potato, which resulted in two BC_2 progenies. This BC_1 genome did not contain the specific DNA sequence, and according to expectations in the case of BC_2 plants, the OPT-20 marker did not amplify the 250 bp sequences.

Somatic hybrids with MMR deficiency performed better in this experiment. Thirteen MMR deficient SH contained the specific DNA sequence, which was amplified by OPT-20 RAPD marker (**Fig. 3**).

Based on the obtained result, we can conclude that deficiency in MMR system increases the possibility of homeologous recombination during hybridization procedure of closely related species, which lead to increased gene transfer from wild parent line. Somatic hybrids carrying *AtMSH2* gene in antisense orientation (AS) possessed the specific DNA sequence with higher proportion (54.54%), which was associated with leptine synthesis, than SHs with dominant negative mutant *AtMSH2* gene (40%).

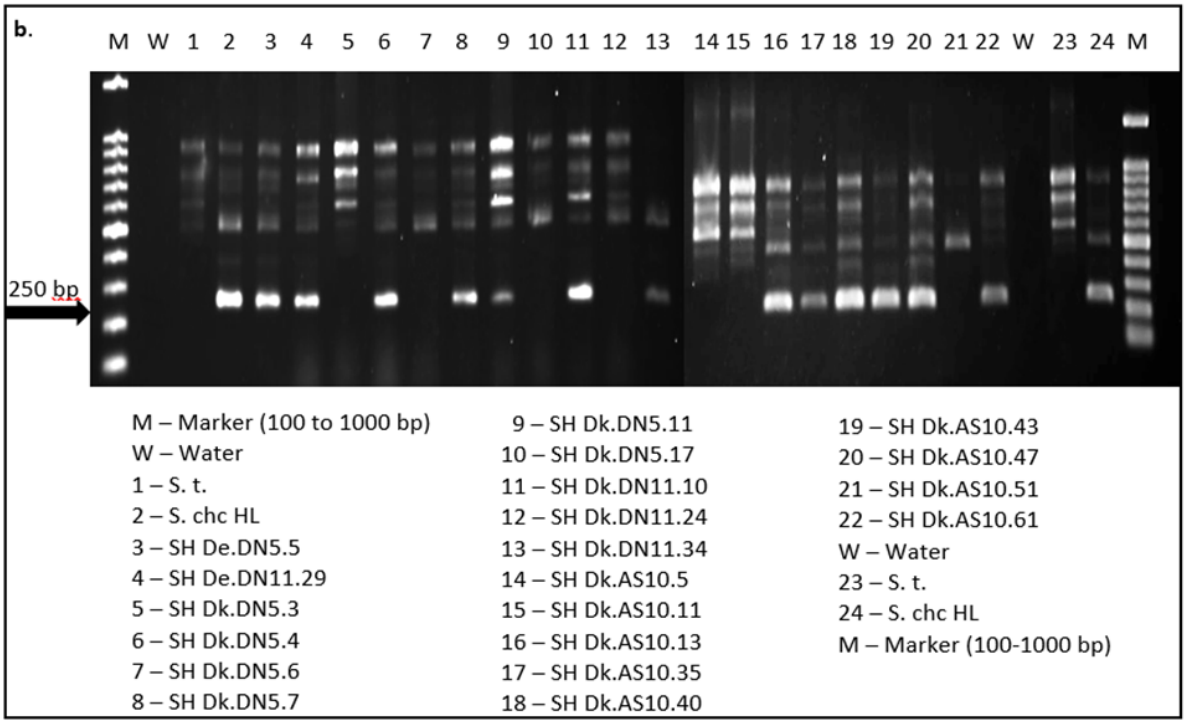

Fig. 3 Selection of leptine producing MMR-deficient SHs based on OPT-20 RAPD marker linked to leptine biosynthesis

IV.2. SCAR marker design

The main drawback of the RAPD technique is their low reproducibility mainly among different laboratories (Penner *et al.* 1993). The main advantage of SCAR markers is based on their stability, and they are also reliable with high reproducibility rate and not lastly they are locus-specific. These markers are often used in gene mapping and in marker assisted selection. In order to increase the stability of molecular identification of leptine producer *Solanum* genotypes, the effective RAPD primer conversion into SCAR marker was required.

The specific 250 bp length DNA sequence amplified by OPT-20 marker in *S. chacoense* was extracted and was sequenced, which was further analyzed with Blast searches to identify homology with other sequences. Only one of the extracted sequence showed 88% similarity with a sequence of *Solanum pennelli* located on the 9th chromosome. The obtained sequences were used in designing and synthetizing specific primers for the leptine trait (191I1, 220I3, 242I4). Unfortunately none of the designed SCAR markers were effective in identifying leptine producer genotypes. Neither of the primers recognized specific DNA sequences exclusively in *S. chacoense*. In the case of 191I1, 220I3 and 242I4 marker, no specific amplification products were observed, while in the case of 242I2 marker, a 250 bp length DNA sequence was only recognized in the cultivated potato.

In the future, further experiments are required in order to develop a specific leptine trait recognizer marker. First of all, we want to shorten the SCAR primers's length, which may increase the success rate of SCAR marker conversion. If that is not achieved successfully, designing new markers will be required.

V. BIOCHEMICAL ANALYSIS OF *SOLANUM* SOMATIC HYBRIDS AND DERIVATES

Potato glycoalkaloids are considered to have an indispensable role in plant chemical defense system against herbivores, pests and pathogens (Bennett and Wallsgrove 1994; Friedman and McDonald 1997). Glycoalkaloids have an antibiotic effect (Gubarev *et al.* 1998), inhibit spore germination and fungal growth (Fewell and Roddick 1993). Glycoalkaloids also have inhibitory effect on the behavior and development of insects: antifeedant and a deterring effect against herbivore insects (Sanford *et al.* 1997; Yencho *et al.* 2000). The main glycoalkaloids of potato,

which represents 95% of the total glycoalkaloid content are α-solanine and α-chaconine (Friedman 2006; Fewell and Roddick 1997).

Leptines are rare glycoalkaloids and they could only be found in a few accessions of *S. chacoense* Bitter (Sinden *et al.* 1986; Ronning *et al.* 1999). Four different leptine glycoalkaloid are known, which are formed through a combination of two aglycones: leptinidine and acetylleptinidine and two carbohydrate groups: chacotriose and solatriose. High leptine contents have been found to reduce Colorado potato beetle feeding (Coombs *et al.* 2002; Sinden *et al.* 1984), and to have a toxic effect on larval development. Due to the fact that these glycoalkaloids are synthetized only in the aerial tissues of plants, *S. chacoense* is considered to be a valuable host plant for a source of resistance by potato breeders. Introgression of leptines coding genes into the cultivated potato's germplasm should replace the chemical insect management with a more natural and long-term defense strategy.

In our experiments the steroidal glycoalkaloids pattern of SHs and derivates between cultivated potato and *S. chacoense* 138 were analyzed using HPLC. Additionally, FTIR spectroscopy was used to determine the chemical differences between CPB resistant and susceptible plants.

V.1. Steroidal glycoalkaloid determination by HPLC method

Nowadays, HPLC became the primary analytical separation tool for steroidal glycoalkaloids. The reasons for the widespread use of this method are its high sensitivity and its adaptability to punctual quantitative determinations of glycoalkaloids. The separation of glycoalkaloids is difficult because of their structural similarity and lack of chromophores. In the case of *S. chacoense*'s chromatograph a specific peak at 11.53 minutes was detected, from which we considered to be one type of leptine glycoalkaloid. In addition, other specific peak on *S. chacoense* chromatograph were not observed, which is presumably due to the lower concentration of the other leptine glycoalkaloids and probably the used HPLC method was not sensitive enough to detect them.

Based on our results we can conclude that the lack of leptine glycoalkaloid standards and the used HPLC method were not suitable to determine the presence of different leptine glycoalkaloid in *Solanum* genotypes. Only one specific glycoalkaloid was determined in the extracts, which could be one of the leptine glycoalkaloids. We can only surely conclude that those

genotypes that contained this specific glycoalkaloid at retention time around 11.5 minutes were resistant to CPB.

In the future, we would like to analyze the *Solanum* genotypes glycoalkaloid content with LC-MS spectroscopy, which allows determining with high confidence the leptine glycoalkaloid composition of the extracts without standards and quantitative analysis of glycoalkaloids is also possible with this method.

V.2. Total chemical content determination by FTIR

FTIR spectroscopy is a simple and a rapid technique, based on the measurement of a molecule excited by IR radiations at a specific wavelength range. To compare the spectra and to visualize the clustering of genotypes, the principal component analysis (PCA) method was used.

After PCA analysis, we observed that the first two created principal factors (PCs) preserved 89.91% information from the original data. These two factors (PC_1 and PC_2) were used to visualize the analyzed genotypes clustering tendency based on their chemical composition.

After PCA analysis we observed that SHs, BC clones and parent lines formed well separated clusters according to their resistance to CPB. Thereby we can conclude that the two groups have different chemical composition and the difference between plants affect their resistance against CPB.

After our results we can conclude that FTIR spectroscopy is an effective method in determining biochemical factors that have a high impact on resistance of plants to CPB. PCA analysis demonstrated that resistant SHs and BC clones had similar chemical compound like *S. chacoense*, while total chemical compound of susceptible plants were more similar to cultivated potato. Based on our results we concluded that the identified specific bands are presumably related to leptine glycoalkaloids contents of the plants, because intense absorbance values in the specific wavelengths were only observed in the case of resistant plants.

VI. RESISTANCE AND DEFENSE RESPONSE OF *SOLANUM* SOMATIC HYBRIDS AND BACKCROSS PROGENIES TO COLORADO POTATO BEETLE

(Molnár *et al.* 2016 [a,b])

VI.1. Colorado potato beetle

Colorado potato beetle (CPB) has the biggest enemy of the cultivated potato worldwide. Due to their destructive feeding habits, CPB can reduce potato yields and can even cause total tuber loss (Hare 1990). Despite of relatively young evolutionary age of CPB, they possess high intraspecific polymorphism, which provides them a broad range of ecological plasticity and adaptability to different biotic and abiotic condition changes (Udalov and Benkovskaya 2011; Hare 1990). The high capability of CPB to adapt rapidly against different insecticides encourages breeders to find a different type of control to CPB attacks. The only long-term solution for CPB management would be to integrate different control techniques. One of the most important ways to control this voracious and adaptable pest is to use host plant resistance. *S. chacoense* is of interest to potato breeders because it is highly resistant to CPB (Sinden *et al*. 1986).

The insect resistance of *S. chacoense* is due to their specific steroidal glycoalkaloids: the leptines. CPB larvae fed on *S. chacoense* HL leaves, which are known to produce the largest amount of leptine glycoalkaloids, developed slowly and none reached the adult stage. For CPB larvae *S. chacoense* HL leaves are toxic. SH 1552/1 used as a female parent in backcrossing with *S. tuberosum* cv. Sonate (♂) is highly resistant to CPB. Only one larva survived 23 days but it did not reach the adult stage. Backcrossing cultivated potato with highly resistant SH 1552/1 led to a one-to-three segregation of resistance in the descendants.

SHs with MMR deficiency performed better in the resistance test. (**Fig. 4**). Of the twenty SHs analyzed just seven genotypes were similar to potato, in that the larvae survived well, which indicate these genotypes are susceptible to CPB.

The mortality of CPB larvae caused by the resistant plant group was significantly greater than that of the susceptible plants. Larvae fed on leaves of resistant plants developed more slowly: larvae fed on leaves of *S. tuberosum* reached the fourth larval stage (L4), while most of the larvae on resistant plants reached only L3 or L2 (on SHs: De.DN11.29, Dk.DN5.11, Dk.AS10.40), or even remained in the first larval stage (on SHs: Dk.DN5.4, Dk.DN5.7).

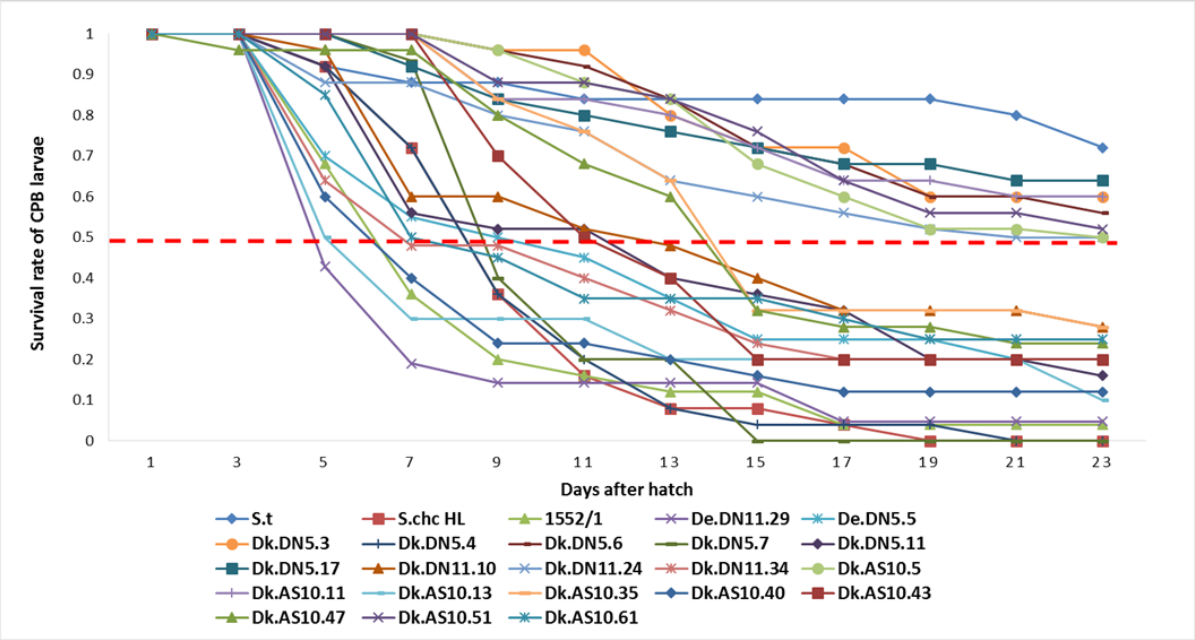

Fig. 4 Percentage* of CPB larvae (n=25) that survived for 23 days after hatching when fed on *Solanum chacoense* (S. chc 138), derived SH 1552/1 with *Solanum tuberosum* (S. t) and BC_1 progenies. The red dashed line is the dividing line between susceptible and resistant plants. In the case of susceptible plants, over half of the larvae survived 23 days, while in the case of resistant plants this value is far below 50%; * value of 1 means that all larvae survived (100%)

Due to decreased viability, significantly fewer adults developed from larvae fed on resistant plants than on susceptible hybrids. On SHs: Dk.DN5.4, Dk.DN5.7 and De.DN11.29 with MMR deficiency none of the larvae reached the adult stage. In the case of female beetles, the mean difference in weight was significant when compared with that of adults reared on susceptible plants and were not very fertile, with several of the beetles malformed and not capable of reproducing (those reared on SHs: De.DN5.5 or Dk.AS10.47).

Among these resistant hybrids both types of MMR-deficient plants: DN (7 genotypes) and AS (6 genotypes) were recorded. Three of them (SHs: Dk.DN5.4, Dk.DN5.7 and De.DN11.29) had strong toxic effects on CPB larvae similar to *S. chacoense* HL. In the case of SH De.DN11.29 larvae reached the second stage (L2) but not the third instar (L3), with the last dying 29 days after hatching.

SHs Dk.DN5.4 and Dk.DN5.7 proved to be the most resistant genotypes: no larvae survived for 23 days, which is similar to that recorded for the larvae fed on *S. chacoense* HL leaves. On day 13 when 100% of the larvae fed on *S. tuberosum* leaves reached the L4 stage and had an LDI=84, larvae fed on these two SHs and *S. chacoense* HL had an LDI = 1, which indicates strong

inhibition of larval development. These larvae did not reach the second larval stage, L2. In addition, mortality was very high: only two larvae survived when fed leaves of *S. chacoense* HL and SH Dk.DN5.4, and only one survived until day 13 when reared on SH Dk.DN5.7.

In the second experiment beetles had to choose between one parent lines (cultivated potato or *S. chacoense*) and one of the SHs (with or without MMR deficiency) or BC_1 clones. SHs and BC_1 clones that bioassays indicate are resistant to CPB larvae also deterred adult beetles from feeding. In these cases, beetles preferred to consume the leaves of cultivated potato to those of SHs or BC_1 clones. Significant differences were recorded between the biomass of leaf consumed of *S. tuberosum* and that of the SHs and BC_1 clones marked with ***b*** in **Fig. 5**.This implies a very strong deterrent effect.

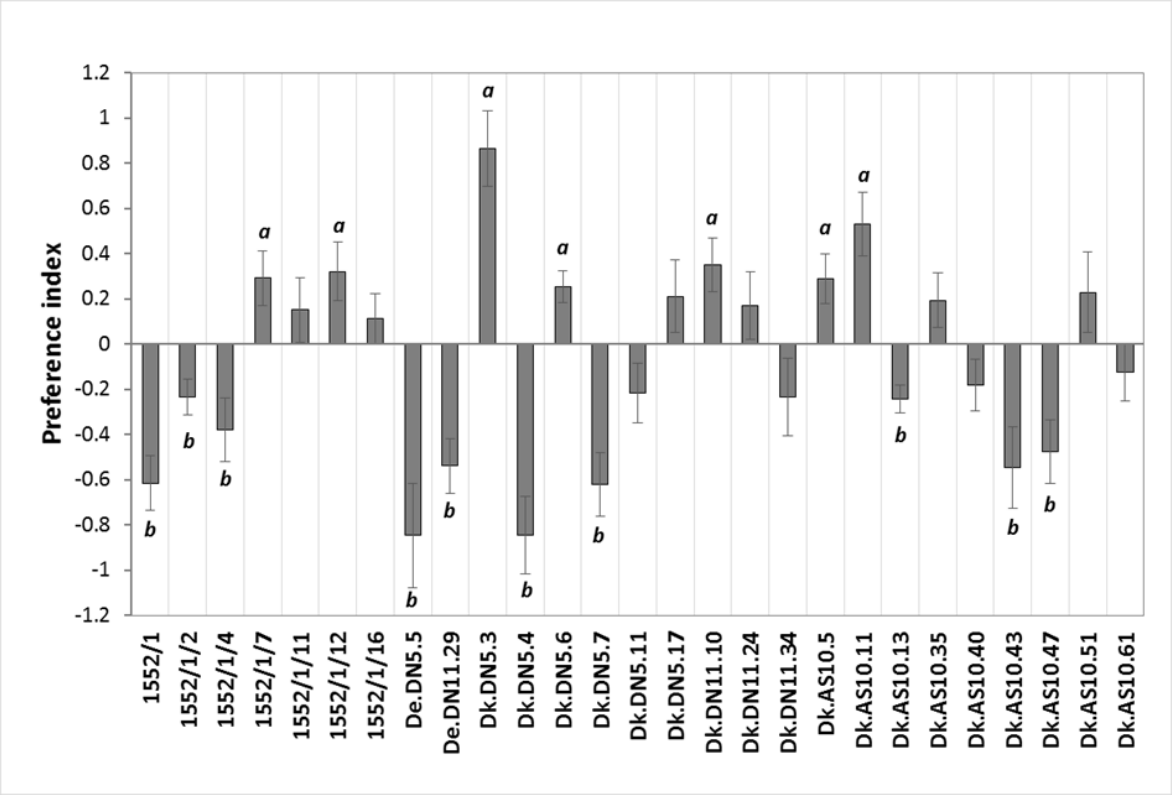

Fig. 5 Preference indices (mean ± SE, n=9) for adult CPBs feeding on SH with or without MMR deficiency and some BC_1 descendant compared with *S. tuberosum* (a). Letter ***a*** indicates significantly higher phago-stimulant effect of SHs and BC_1 clones than *S. tuberosum*, while letter ***b*** marks plant genotypes with strong deterrent effect (the consumed leaf area were significantly lower than in the case of *S. tuberosum*) (t. test, $p<0.05$)

The genotypes 1552/1, Dk.DN5.4, Dk.DN5.7, Dk.DN5.11 and Dk.AS10.43 had the strongest deterrent effect. Beetles did not consume when provided with leaves of these genotypes, which supports the observations on the performance of the CPB larvae in the bioassays.

Among the deterrent genotypes, both types of SHs with or without MMR deficiency were recorded. The majority of the most resistant SHs are MMR-deficient SHs containing a dominant negative sequence of *AtMSH2* gene. These results confirm the hypothesis that the mutant allele of the *MSH2* gene can compete in a dominant way with the four normal alleles of the potato parent, resulting in an increased probability of transferring the resistance genes.

VI.2. Plant defense response to herbivore attacks

Reactive oxygen species (ROS) are involved in plant defense against different pathogens. Superoxide anion, hydrogen peroxide and hydroxyl radical are the most frequently generated forms of ROS. In plants the herbivore beetle attack is generally associated with wounding. Both herbivore attack and mechanical injury induce modification of plant's wound response (Kessler and Baldwin 2002). Immediately after wounding, plants accumulate reactive oxygen species.

The role of reactive oxygen species in plant defense against herbivores is not clear, but the importance of ROS signaling in the generation of plant defense responses is supported with numerous experiments. The quantity of ROS accumulation has a positive correlation with plants's resistance against attackers (Moloi and van der Westhuisen 2006).

In plants ROS has a general physiological response. Under stress condition large amounts of ROS is generated, which has an important role in plant defense response but also could affect the health of plants. Therefore, a system, which stabilizes the concentration of ROS is essential. Stabilization of ROS levels after pathogen attack in *Arabidopsis thaliana* is controlled by ascorbic acid and also by anthocyanin generation (Nagata *et al.* 2003), which has ROS scavenging effect (Sanz *et al.* 1994).

Wounding as an experimental procedure is often used to investigate plant defense responses against herbivore attacks (Bruxelles and Roberts 2001). Quantitative analysis of H_2O_2 accumulation in wounded plants was performed using DAB staining, which produces brown coloration at accumulation zones of H_2O_2 in plant tissue.

In our experiments the concentration of H_2O_2 varied between 34.5 and 45.5 µM/g FW in wounded leaves. Intense accumulation of H_2O_2 was observed after wounding in the case of *S.*

chacoense, 11 SHs and 2 BC_1 clones. (**Fig. 6**). Both types of MMR deficient somatic hybrids (DN and AS) was represented in this group.

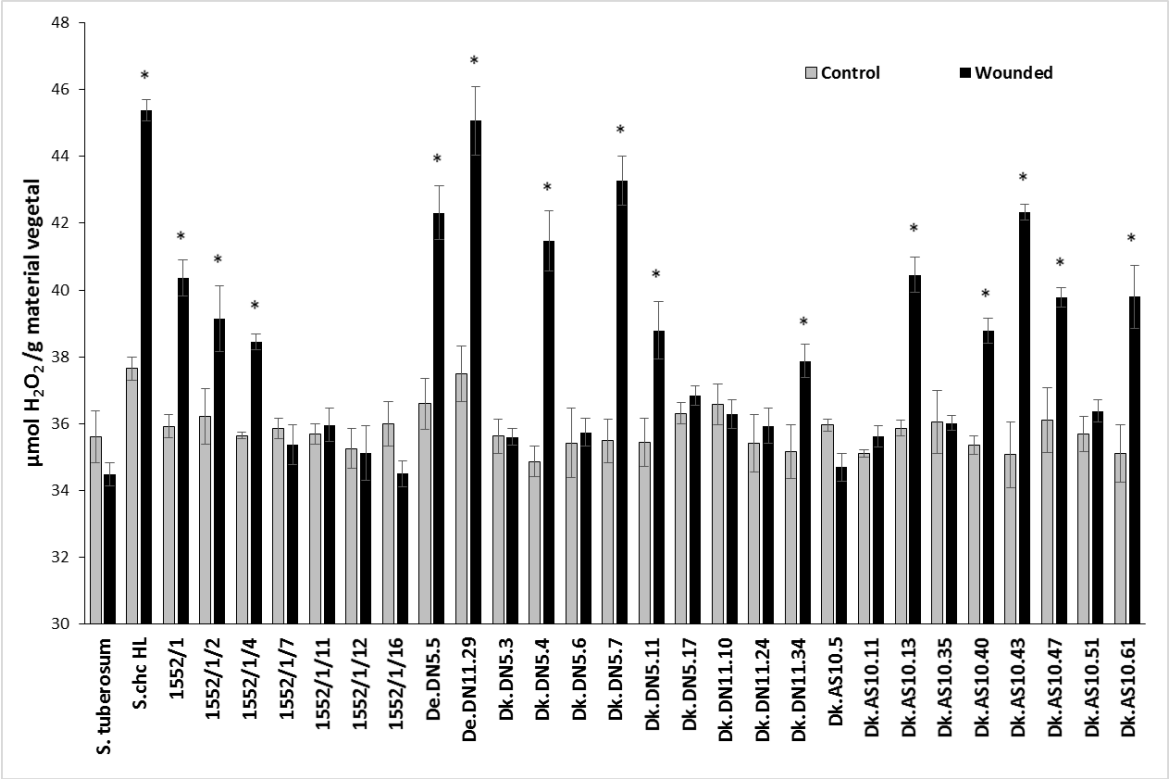

Fig. 6 Quantitative evaluation of H_2O_2 in control and wounded somatic hybrids with and without MMR deficiency, their progenies and parental lines (*S. tuberosum*, *S. chacoense*). Note: * - somatic hybrids and BC clones with significant differences (n=5, t-test, $p<0.05$) between wounded and control plants

In the case of marked genotypes (*) on **Fig. 6**, the produced H_2O_2 concentration was significantly higher than in control leaves. In the case of *S. tuberosum*, the wounded leaves produced a significantly less amount of H_2O_2 than the marked genotypes.

The intense accumulation of H_2O_2 as a result of wounding showed high correlation with plant resistance against CPB. Most of the somatic hybrids which proved to be resistant to CPB attacks produced high concentration of H_2O_2 during mechanical stress.

The generated ROS during different biotic and abiotic stresses need to be stabilized in order to avoid oxidative damages in cells, which may also affect the survival of plants. Plants can protect their cells by scavenging ROS with activating antioxidative systems like: superoxide dismutases, glutathione peroxidases, catalases or by production different antioxidants like ascorbate, flavonoids, anthocyanin, etc. (Nagata *et al.* 2003). Because the accumulation of anthocyanin takes

1-2 days after detection of stress, this antioxidant is effective against long-lived radicals like H_2O_2 (Nagata *et al.* 2003). The basic levels of anthocyanin varied between 4.8 and 10 µg/mg FW. The radical scavenging activity in wounded plants increases the anthocyanin level by a minimum of 20%, but even by 185% in affected leaves.

In addition, a positive correlation between the generated anthocyanin content and the accumulated ROS amount was observed. In those cases when highest amount of ROS was synthesized after mechanical injury of leaves a high quantity of anthocyanin was also produced. Presumably the accumulation of ROS therefore was followed by anthocyanin synthesis in order to stabilize ROS concentration in plants.

Based on the results presented above, one can conclude that wound-induced hydrogen peroxide accumulation plays an indispensable role in plant security system and can be associated with plants defense against herbivore attacks. Bi and Felton (1995) proposed that ROS accumulation affects plant-herbivore interaction.

The H_2O_2 accumulation ability of plants highly influences their response to insect herbivore attacks. In those cases when plants responded to mechanical injury with intense H_2O_2 accumulation they also possessed both of antibiosis and antixenosis properties against CPB.

In case of potato somatic hybrids anthocyanin has an important role as radical scavenger, which provides protection against oxidative stress generated after mechanical injury of leaves.

VII. DROUGHT STRESS EFFECTS ON *SOLANUM* SOMATIC HYBRIDS AND DERIVATES

Fresh water shortage became an increasing worldwide problem, which is the result of climate changes, increased pollution and increased human pretension and overuse of water. Cultivated potato uses water relatively efficiently, but it is considered to be sensitive to moderate levels of water deficit, which causes yield losses. The increase in drought periods, which affect the most agriculturally important areas, motivate breeders to select drought tolerant cultivars to avoid yield losses.

Generally, stress-selection of plants begins with *in vitro* prescreening of drought tolerant plants which is followed by an *ex vitro* selection. *Ex vitro* experiments imitate the naturally

occurring conditions. As selection agent in the case of *in vitro* selection experiments of drought-tolerant plants, Polyethylene glycol (PEG) is frequently used (Hassanpanah 2010; Pino *et al*. 2013). Mustata *et al.* (under publication) observed that high proline content in stressed tissues have beneficial effects on water deficit induced stress toleration. Proline is involved in reducing the photodamage in thylakoid membranes by reducing the production of $^1O_2$ in drought conditions (Chaves *et al*. 2009).

Phenotypic characterization of drought stressed plants helps to determine the morphological and physiological effects of the induced stress. Phenotyping platforms made it possible to monitor the development of plants during water deficit by determining biomass accumulation of plants without physiological damaging (Feher-Juhasz *et al.* 2014).

As an effect of drought stress, the photosynthesis rate, as well as the CO_2 accumulation decreases in plants (Kaiser 1987; Chaves *et al*. 2009). This response can be attributed to stomatal closure (Cornic 2000).

The goal of our research was to assess drought tolerance ability of somatic hybrids and backcross progenies between potato and *Solanum chacoense*, respectively *S. chacoense* with MMR deficiency using *in vitro* stress-selection with PEG. Plant response to drought stress was also evaluated using phenotypic characterization and photosynthesis of drought stressed plants.

VIII.1. *In vitro* stress-selection of drought tolerant plants

In the case of *in vitro* stress-selection, drought stress was induced with different concentration of PEG (5% and 15%), which simulated mild and severe drought conditions. In the case of cultivated potato and *S. chacoense* HL, only the root system were developed normally, their shoot did not grow as efficiently as the shoots of the control plants. Based on the morphological differences of the stressed plants, we can conclude that the some genotypes managed the water deficit efficiently during moderate drought stress. In their case, only the number of leaves was significantly lower than in the control group, which means that these plants highly tolerate the moderate drought condition. Sensitive genotypes survived the induced moderate drought stress, but they were not capable of overcoming the negative effects of water deficit. In this case, if the water deficit had persisted for a longer time, the plants would have most probably died. The shoot and root systems of the plants did not developed, which is essential for surviving, and in many instances the bottom leaves and the edges of the upper leaves were withered.

In the second experiment where the plants were exposed to severe drought conditions, induced with 15% PEG supplementation in culture media, all of the genotypes developed weaker, their shoot lengths were significantly lower than in case of control plants. The stressed plants root system was not so rich in ramification than the control ones, and a large part of the analyzed genotypes did not developed more than 0.5 cm long root.

During severe drought condition the newly grown leaves were visibly smaller than those leaves, which the plants possessed at inoculation and smaller than control plants' leaves. Shao *et al*. (2008) observed that leaf area was negatively affected during water stress, which lead to decreased crop yield due to photosynthesis reduction. Also greater degree of leaf senescence was observed at severe stressed plants than in the case of moderate stress condition.

The degree of proline accumulation during drought stress influences the plant's stress tolerance ability. The majority of the analyzed genotypes accumulated significantly more (t. test, $p< 0.05$) proline as a response of moderate water stress, but in the accumulation of proline levels visible differences were observed. Some hybrids accumulated 7-8 fold more proline during water deficit, while others accumulated only 2-3 fold more proline than control plants.

During severe water deficit a large part (two-thirds) of the stressed plants were not able to efficiently manage their resources, which would have helped in overcoming the negative effects of drought, therefore these genotypes became susceptible to severe water stress.

The drought tolerant group accumulated significantly more proline than susceptible genotypes (t. test, $p< 0.05$). Among both type of somatic hybrids with or without MMR deficiency can be found in the drought-resistant group, which supports our hypothesis that somatic hybridization has a greater influence on the ability to develop drought-tolerance than the deficiency in the DNA repair system.

Based on the obtained results we can conclude that proline accumulation during water stress greatly contributed to plants's tolerance ability. During moderate stress, the resistant plants developed normally, while in severe drought condition the tolerant plants were able to grow roots, which are essential to achieve water storages in natural conditions. Proline accumulation allowed the plants to survive and develop relatively well in the water deficit period.

VIII.2. Plants biomass accumulation under drought condition

In our experiment, the impact of drought on morphological traits of stressed plants were determined using biomass accumulation differences between control and drought stressed plants. Plants with higher amounts of green pixels represent an extended surface area of their shoot, which is directly proportional to the drought tolerance ability of plants.

As an effect of drought stress the majority of the analyzed plants, accumulated significantly less biomass (ANOVA, $p< 0.05$) than control ones. Obidiegwu *et al*. (2015) obtained similar results: drought negatively affected plant development, which resulted in reduced foliage extension and decreased tubers yield quantity and quality.

Drought stress negatively affects foliar extension of plants, which led to tuber yield reduction. Comparing tuber yield and biomass accumulation changes during water stress, we observed the plants that suffered less from drought and accumulated approximately as much green biomass than control plants, were able to develop good quality tubers, while plants with reduced foliar extension developed small-sized tubers.

Photosynthesis is the most important biological process of plants, which is essential in biomass accumulation (Arabzadeh 2013). Drought stress proved to reduce the growth and yield of the potato by affecting the kinetics of chlorophyll fluorescence (Jefferies 1992). After both measurements, we observed that the maximal quantum efficiency of stressed plants were not decreased under drought conditions. In the next step the effectiveness of the photosynthesis was evaluated by calculating the performance index (PI), which proved to react more sensitively to drought stress than the maximal quantum efficiency (Cavender-Bares and Bazzaz 2004). As was with the other calculated parameters, in this case the PI value of drought stressed plants was also similar to that of the control group in the first investigation, and was significantly higher (t. test, $p<0.05$) in the second measurements. The increase of PI index in the second measurements suggests that the analyzed plants were able to adapt, with time, to the applied moderate drought stress. Therefore if this parameter was not reduced during water stress, it is possible that the induced water scarcity was not strong enough to have a negative impact on this trait.

Non-photochemical quenching (NPQ) of chlorophyll fluorescence indicates the level of light-energy dissipation through fluorescence re-emission, or heat releasing in the PSII reaction center (Fracheboud and Leipner 2003). NPQ levels of drought stressed plants were significantly higher in the early stage of water scarcity (2^{nd} week) than during second measurements. This

observation explains the lack of similar amount of biomass accumulation in stressed plants compared to control ones. A large amount of the harvested light energy was lost by non-photochemical quenching, therefore the effectiveness of carbon fixation was reduced. Non-photochemical quenching was the plants' necessarily evil solution to protect themselves from photo damaging, thereby the stressed plants were able to survive and to maintain their vital functions without noticeable losses.

Based on the second measurement's results we can conclude that stressed plants adapted to the induced stress conditions. Non-photochemical quenching level was reduced, which yielded visible results in biomass accumulation. Carbon fixation of stressed plants began to work more efficiently, in some cases stressed plants biomass level caught up or approached to those of the control plant.

VIII. GENERAL CONCLUSIONS

The main objective of my research was to characterize the *Solanum* somatic hybrids between cultivated potato and *S. chacoense*. These SHs and derivates are genetically unique plant material, therefore they need to be characterized separately in order to find those genotypes which possess valuable traits. My experiments were focused on determining the resistance of somatic hybrids and derivates to biotic and abiotic stresses.

The specific conclusions by chapters are as follows:

1. **Genetic constitution determination of S*olanum* somatic hybrids using classical and modern cytogenetic methods**

First of all, the genetic stability of somatic hybrids and derivates were investigated. The ploidy level of the analyzed genotypes varied between tetraploid and hexaploid level. SHs eliminated chromosomes during regeneration, which can be explained by somatic incompatibility of the parental species. The majority of the first generation backcross progenies and all of the BC_2 clones were tetraploids, therefore we can state that the hybrids became genetically stable at tetraploid level. Flow cytometry proved to be a rapid and easy, but also a precise technique.

Chromosome number of *Solanum* plants could be predicted with high accuracy using the above described method.

Genomic *in situ* hybridization method was used to establish the genetic composition of somatic hybrids. GISH analyses revealed that SHs eliminated more chromosomes from wild species than from cultivated potato, which can be explained by ploidy level differences or asynchrony of mitosis process of parental lines.

2. Determination of somatic hybrids which possess leptine glycoalkaloid encoding genes by using molecular markers

RAPD analysis with leptine-specific markers, described by Bouarte-Medina *et al.* (2002) and Ronning *et al.* (1999) were used to select leptine producer SHs and BC clones. Among these markers, only OPT-20 showed specific polymorphism. Based on RAPD-PCR analysis the leptine synthesis ability showed a 1:2 segregation in descendants. Among the analyzed MMR deficient SHs (20 SH), 13 amplified the 250 bp length DNA sequence. SHs with *AtMSH2* gene in antisense orientation (AS) possessed the specific DNA sequence in a higher proportion (54.54%), than SHs with dominant negative mutant *AtMSH2* gene (40%).

In our experiments the effective OPT-20 RAPD marker was converted into SCAR marker. Unfortunately, none of the newly designed SCAR markers were effective in recognizing specific DNA sequence exclusively in leptine-producer *Solanum* genotypes.

3. Biochemical analysis of *Solanum* somatic hybrids and derivates

The glycoalkaloid composition of parent lines, SHs with or without MMR deficiency and BC clones were determined using HPLC method. All of the *Solanum* genotypes synthetized the common α-chaconine with retention time (RT) at 11.7 minutes and α-solanine with RT at 11.2 minutes. Because commercial standards for leptine glycoalkaloids were not available, the determination of leptine glycoalkaloids was not possible. In the future, we would like to analyze the *Solanum* genotypes glycoalkaloid content with LC-MS spectroscopy.

FTIR spectroscopy was performed to compare the chemical composition of SHs and derivates with parental lines. To visualize the clustering of genotypes, principal component analysis was used. The analyzed genotypes formed well-separated clusters, according to the

second principal component. FTIR spectroscopy was a reliable method in determining biochemical factors that have a high impact on the resistance of plants.

4. Resistance and defense response of *Solanum* somatic hybrids and backcross progenies to Colorado potato beetle

A laboratory bioassay was performed to evaluate the antibiosis effect of somatic hybrids against CPB. This long-term monitoring was effective in selecting resistant genotypes, which had toxic effects and affected the normal development of the CPB larvae. “Choice test” experiments were used to determine *Solanum* genotypes with antixenosis properties. Based on food preference of adult CPBs, SHs and derivates with repellent effect were successfully selected.

More of the SHs with MMR deficiency were resistant to CPB than in the case of wild type SHs. MMR deficient SHs had stronger toxic effect, some of them were as toxic as *S chacoense*, in these cases no larvae survived to adulthood. Percentage survival, mean weight of larvae were more reduced in SHs with deficiency in DNA repair system. Only in the case of MMR deficient SHs were emerged adults with malformation observed which were not able to reproduce. Based on our results we concluded that DNA repair system deficiency increases the transfer of resistance genes from *S. chacoense* into cultivated potato.

SHs and BC progenies with resistant properties amplified the specific 250bp DNA sequence, which was linked to leptine production, in RAPD analysis, therefore the resistant of these genotypes were attributed to leptine glycoalkaloid synthesis.

Wounding were used to evaluate the defense response of *Solanum* plants against herbivore attacks. After mechanical injury, high amounts of H_2O_2 were accumulated in leaves. The H_2O_2 accumulation ability of plants highly influenced their response to insect herbivore attacks. In those cases when plants responded to mechanical injury with intense H_2O_2 accumulation, they also possessed both of antibiosis and antixenosis properties against CPB.

In case of potato somatic hybrids, anthocyanin has an important role as radical scavenger, which provides protection against oxidative stress generated after mechanical injury of leaves.

5. Drought stress effects on *Solanum* somatic hybrids and derivates

In vitro stress-selection proved to be an effective method in selecting moderate and/or severe drought tolerant *Solanum* genotypes. Parental lines were sensitive to water shortage, but

despite this, drought tolerant SHs with or without MMR deficiency and BC clones was observed. A higher proportion of MMR deficient SHs effectively tolerated the induced severe drought stress than *Solanum* genotypes without MMR deficiency. Based on the obtained results we concluded that somatic hybridization process and deficiency in DNA repair system influenced the new properites development in somatic hybrids.

Moderate drought condition negatively affected the development of *Solanum* genotypes. After *ex vitro* stresselection, all of the analysed genotypes tolerated the induced moderate drought stress, but based on the tuber yield and biomass accumulation changes, plants that suffered less from drought and accumulated approximaltey as much green biomass than control plants can be selected. These genotypes were also able to develop better quality tubers than the other genotypes.

The induced moderate drought conditions did not affected the maximum quantum yield of photosynthesis, which shows that the PSII photosystem was not damaged. The increased performance index values during the second measurements suggest that the stressed plants were able to adapt with time to the moderate drought condition. Non-photochemical quenching (NPQ) was higher in the early stage of water scarcity, than during second measurements. NPQ level reduction with time contributed to the increase of carbon fixation in stressed plants, which resulted in visible increase in biomass accumulation. Based on the photosynthesis analyses we concluded that stressed plants were able to adapt to the induced water stress condition.

Based on the obtained results, we concluded, that somatic hybrids between *S. tuberosum* and *S. chacoense* with or without MMR deficiency, and their BC progenies represent a valuable plant material for potato breeders and also for experimental biologists. The unique character of these genotypes lies in the fact that these plants were obtained by the fusion of two genetically different plant cells, which causes mixing of the parent lines's genetic material in the SHs.

Combining different biotechnological tools (protoplast electrofusion, genetic transformation for MMR deficiency) - which can be termed as combinatorial biotechnology (Rakosy-Tican 2012; Rakosy-Tican *et al.* 2013) - to increase the possibility of integrating stress resistance genes into potato gene pool were used to produce Colorado potato beetle resistant somatic hybrids between cultivated potato and *S. chacoense*. The application of DNA mismatch repair system deficiency in the somatic hybridization processes was the first time used by our research group to produce potato somatic hybrid plants (Rakosy *et al.* 2004; Rakosy *et al.* 2015).

After thorough characterization of SHs and BC clones with cytological, molecular, biochemical methods and also with CPB laboratory bioassay as well as with choice test, the CPB resistant genotypes were selected. More SHs with MMR deficiency possessed the CPB resistance than the wild-type SHs. Our results confirmed the potential of combinatorial biotechnology: the genetic transformation which induced DNA mismatch repair deficiency increased the homeologous recombination between the two related species and thereby it increased the transfer of resistance genes into cultivated potato.

Moreover, SHs with or without MMR were exposed to different drought conditions in order to select drought-tolerant plants by using *in vitro* and *ex vitro* stress-selection methods. Despite of the sensibility of parental species to drought some of the somatic hybrids - mostly from MMR deficient genotypes - were tolerant to the induced drought stress. This result reveals a new role of the concept of combinatorial biotechnology because this strategy may be important in new resistance properties formation during somatic hybridization process.

A total of 5 SHs with and one without MMR deficiency and also one BC_1 line were selected, which combined all of the tested resistance traits. These genotypes might also be resistant to other biotic or abiotic stresses because plants use the same signaling pathways for activating defense responses to different stress. Therefore, exposure to a mild stress trigger the plant's immune responses and enhances their resistance against a range of stress factors (Foyer *et al*. 2016).

These results open new possibilities for the integration in breeding programs of these somatic hybrid clones.

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