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Reactive oxygen species and anthocyanin are involved in plant response to wounding as part of insect feeding – the case of the somatic hybrids *Solanum tuberosum* + *Solanum chacoense*

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SUMMARY. The cultivated potato ranks third in agricultural crop production on global scale. Being the target of attacks by various insects it requires increased attention and protection as well as breeding for resistance. Meanwhile the Colorado potato beetle (*Leptinotarsa decemlineata*, CPB) has become the biggest enemy of the cultivated potato worldwide. An alternative method of controlling the growth of CPB population would be the use of resistant potato varieties. One of the most effective sources of host-resistance mechanisms is the wild *Solanum chacoense*. Somatic hybridization *via* protoplast fusion made it possible the introgression of valuable traits from *S. chacoense* into cultivated potato.

Adequate responses to biotic stresses are crucial for plant survival. The formation of reactive oxygen species during microbial infection is a well-known process but their role against herbivore attacks is still not outlined. The reactive oxygen species (ROS) concentration in plants is controlled by different antioxidative mechanisms in order to maintain the normal function of cells.

The aim of our study was to determine the role of ROS signaling as defense response induced by mechanical wounding in potato somatic hybrids. The correlation between CPB resistance and H_2O_2 accumulation rate were determined, as well. Also the role of anthocyanin as a ROS scavenger was established.

Based on our results we conclude that H_2O_2 accumulation highly influences somatic hybrid response to insect herbivore attacks therefore this type of ROS plays an important role in plant defense mechanism against CPB.

Hydrogen peroxide accumulation led to anthocyanin generation therefore anthocyanin plays a role as radical scavenger in potato somatic hybrids. This study is the first to point out the role of reactive oxygen species and anthocyanin biosynthesis in response to mechanical wounding in the somatic hybrids resistant or sensitive to Colorado potato beetle.

Keywords: anthocyanin, hydrogen peroxide, somatic hybrids

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Introduction

Potato (*Solanum tuberosum* L.) is considered as an extremely valuable crop especially now, when Earth's nutritional problems are increasing. Potatoes are grown in 160 countries and more than 4000 cultivars are known (Camire *et al.*, 2009). Romania is ranked on the sixth place as largest harvested area potato grower in Europe: in 2014 more than 3.5 million tons of potato were produced (FAOSTAT, 2014).

Most of the wild relatives of 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. *Solanum chacoense* is a diploid (2n=2x=24), tuber-bearing *Solanum* species. This wild *Solanum* species attracted the attention of potato breeders because possesses broad resistance against different pathogens (Sato *et al.*, 2006; Simko *et al.*, 2007). Some accessions of *S. chacoense* are highly resistant to Colorado potato beetle (CPB) (Sinden *et al.*, 1986). In order to introgress insect resistance from *S. chacoense* Thieme *et al.* (unpublished data) and Rakosy-Tican *et al.* (2004) produced somatic hybrids and backcross progenies between cultivated potato cv Delikat and Désirée and *S. chacoense* with/without DNA mismatch repair system (MMR) deficiency. The MMR system is involved in maintaining genome stability. Harfe and Jinks-Robertson (2000) observed that *MSH2* mutations, a key gene of MMR, significantly increases the rate of homeologous recombination. The high degree of MMR system effectiveness makes difficult the incorporation of useful genes into agronomic important crops. Therefore Rakosy-Tican *et al.* (2004, 2015) used MMR deficient *S. chacoense* to increase the chance of introgression of wild species genetic material into cultivated potato.

In plants the herbivore beetle attack is generally associated with wounding. Both herbivore attack and mechanical injury induce modifications of plant's wound response (Kessler and Baldwin, 2002). Immediately after wounding, plants accumulate reactive oxygen species, like superoxide anion, hydrogen peroxide and hydroxyl radical.

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. Van Breusegem *et al.* (2001) observed that plants after wounding begin to produce superoxide anion in damaged tissue and H_2O_2 throughout the plant. The quantity of ROS accumulation has a positive correlation with plants's resistance against attacker (Moloi and van der Westhuisen, 2006). Hypersensitive response (HR) like symptoms were observed in the case of resistant willow to the *Dasineura marginemtorquens* (Hoglund *et al.*, 2005), resistant rice to the *Orseolia oryzae* (Bentur and Kalode, 1996) and bean resistant to *Apion godmani* (Garza *et al.*, 2001).

In plants ROS represent a general physiological response. Under stress condition large amounts of ROS are 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 effects (Sanz *et al.*, 1994).

The aim of our study was to evaluate the importance of ROS signaling as defense response induced by wounding potato somatic hybrids with/without MMR deficiency and BC clones. The correlation between CPB resistance and H_2O_2 accumulation rate as a response to mechanical injury of SHs and BC clones was investigated, along with the ROS scavenging role of anthocyanin in potato somatic hybrids.

Materials and methods

Plant Material

Somatic hybrids were produced by using protoplast electrofusion technique. Thieme *et al.* (unpublished) used mesophyll protoplasts 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) to produce somatic hybrid plants (ex. SH 1552/1). BC1 plants were obtained after sexual backcrossing of somatic hybrids with S. *tuberosum* cv. Sonate (ex. 1552/1/2). Rakosy-Tican *et al.* (2004) used potato Delikat and Désirée cultivars in somatic hybridization as tetraploid parents and the highest leptine producer diploid *S. chacoense* accession PI 458310 (*S. chc* HL) from NPGS Sturgeon Bay, USA. MMR deficient *S. chacoense* was obtained by using *Agrobacterium*-mediated transformation. 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 Gly codon at position 697 to an Asp codon (Ispas, 2004). One transgenic line for AS and two for DN (DN5 and DN11) and *S. tuberosum* cv. Delikat and Désirée were used in protoplast electrofusion to produce MMR deficient somatic hybrids. The hybridity nature of regenerated plants with/without MMR deficiency was validated using SSR (Besenyei *et al.*, personal communication) and RAPD molecular markers.

The obtained SHs and BC clones resistant ability against Colorado potato beetle were evaluated using laboratory bioassay and food preference test, also known as adult choice test (Table 1) (Molnar *et al.*, 2016). Several genotypes possessed antibiosis and antixenosis ability to CPB. Somatic hybrids marked with red in Table 1 proved to be as effective as *S. chacoense* in CPB resistance analysis.

Four weeks old *ex vitro* cultured *Solanum* genotypes were used in evaluating plant response to mechanical wounding (Table 1). Injury-free plants were selected and were divided into two categories: stressed and control groups. The third and fourth mature leaves of different genotypes belonging to stressed group were wounded with a sterile perforator (hole diameter = 0.55 cm), which has to simulate the physical injury during herbivore attacks. The experiments were done in triplicate.

Table 1.

Resistance of somatic hybrids between cultivated potato and *S. chacoense* with/without MMR deficiency and BC progenies to Colorado potato beetle.

	Resistant	**Susceptible**
Wild type SHs + BC$_1$	**1552/1**	1552/1/7
	1552/1/2	1552/1/11
	1552/1/4	1552/1/12
		1552/1/16
MMR deficient SHs	**De.DN5.5**	Dk.DN5.3
	De.DN11.29	Dk.DN5.6
	Dk.DN5.4	Dk.DN5.17
	Dk.DN5.7	Dk.DN11.24
	Dk.DN5.11	Dk.DN11.34
	Dk.DN11.10	Dk.AS10.5
	Dk.AS10.13	Dk.AS10.11
	Dk.AS10.35	Dk.AS10.51
	Dk.AS10.40	
	Dk.AS10.43	
	Dk.AS10.47	
	Dk.AS10.61	

Note: Red marked SHs proven to be the most toxic to CPB larvae and also had strong repellent effect against adult CPB, being as resistant as the wild species *S. chacoense*.

ROS determination

Six hours after wounding, the damaged third leaves and the same third undamaged leaves from control plants were collected and weighted for hydrogen peroxide determination. These detached leaves were placed into a 3,3 -diaminobenzidine solution (DAB 1 mg/ml, pH 7.5). The samples were incubated for 3 hours in light, then the leaves chlorophyll content was removed by heating them at 80°C in 80% ethanol. The quantity of hydrogen peroxide was determined using the protocol described by Kotchoni *et al.* (2006). After the ethanol treatment the leaves were almost transparent, only the produced polymerization products were brown. The leaves were homogenized in 5x volume compared to leaf weight of 0.2 M perchloric acid and the mixture was incubated for 5 minutes on ice and then was centrifuged (10000 RPM) for 10 minutes at 4°C. The supernatant absorbance of samples was measured at 450 nm using UV/VIS JASCO V-530 spectrophotometer. The concentration of hydrogen peroxide was determined for 1 g of plant tissue using standard calibration with 0.2 M $HClO_4$ containing different concentration of H_2O_2 (100 µM, 1 mM, 5 mM, 10 mM, 25 mM, 50 mM).

Anthocyanin content determination

Changes in anthocyanin production during wounding was determined using wounded fourth leaves from stressed group and untouched fourth leaves of control plants. Total anthocyanin content of leaves was measured using pH differential method described by Lapornik *et al*. (2005). 72 hours after wounding the leaves were collected and weighted. Detached leaves were homogenized in 5x volume compared to leaf weight of 70% methanol and were incubated for 48 hours in dark at room temperature. After two days 1 ml of the filtered extract was transferred in two sterile Falcon tubes (noted as A and B). 1 ml of 0.01% HCl in 95% ethanol was added into each tube. In A tubes 10 ml of 2% HCl (pH 0.8), in B tubes 10 ml of citrate buffer (pH 3.5) were then added. The samples were mixed and the absorbance of both types of probes (A and B) was measured at 520 nm against 70% methanol as blank. The total anthocyanin content was calculated using the formula: TAC = (A-B) x f, TAC = total anthocyanin content expressed as μg/g cyanidine, A = sample absorbance in 2% HCl (pH 0.8), B = sample absorbance in citrate buffer (pH 3.5), f =(MW x DF x CF1 x CF2)/(ε x l), MW = molecular weight of cyanidine-3-glucoside (449 g/mol), DF = dilution factor (5): 1:5 volume leaf weight: extraction solution (70% methanol), CF1 = conversion factor 1 (106 μg/g), CF2 = conversion factor 2 (1 l/ 1000 ml), ε = molar extinction coefficient of cyanidine-3-glucoside (26900 l/mol*cm), l = path length (1 cm).

Statistical analysis

Statistical analysis was performed using R statistical software. Pairwise comparison of ROS accumulation and anthocyanin content of control and wounded plants was compared using Student t-test. During this study if the P value fell below 0.05 it was interpreted as significant difference between the compared data.

Results and discussion

Because plants are sessile organisms and are exposed to different pathogen or pest attacks, they have developed various defense strategies. Inducible defenses of plants have three major levels: surveillance, signal transduction and defensive chemicals production (Dangl and McDowell, 2006).

When insect herbivores attack a plant, they first produce wounds on the plant's tissue, then inject elicitors through the generated hole. Wound- and herbivore specific elicitors activate different signaling pathways in plants (Kessler, 2002). The earliest signaling responses include ion fluxes through the plasma membrane, calcium concentration changes in cytoplasm, generation of active oxygen species (ROS) and changes in protein phosphorylation and immobilization (de Bruxelles and Roberts, 2001).

The accumulation of ROS during defense is biphasic. In the first minutes after wounding a rapid but weak transient burst of $^1O_2$ and H_2O_2 production occurs and after a few hours a second, more sustained, massive accumulation of ROS can be observed (Liu *et al.*, 2010). The highest concentration of ROS accumulation can be observed after four-six hours from wounding (Orozco-Cardenas and Ryan, 1999). After wounding plants produce ROS locally in damaged tissue and systemically (mostly H_2O_2) throughout the plant (Kessler and Baldwin, 2002). H_2O_2 level in plants are maintained at high concentration as long as the herbivore attack persist (Orozco-Cardenas and Ryan, 1999).

The role of ROS in plant defense against herbivores is still unclear, but the quantity of ROS accumulation positively correlates with the plant's resistance to herbivore attacks. HR-like symptoms, which were induced by ROS signaling, have been observed in different resistant plants attacked by insects (Chen, 2008; Liu, 2010). Hypersensitive response to Colorado potato beetle egg masses were observed in resistant somatic hybrids between *S. chacoense*, *S. berthaultii* and *S. tuberosum* (Balbyshev and Lorenzen, 1997). In the places were CPB eggs where attached onto leaves, plants generated necrotic zones.

Bi and Felton (1995) proposed that ROS accumulation affects plant-herbivore interaction. ROS accumulation in plants during herbivore attacks also have direct effects on the insects's health. ROS is responsible of direct oxidative damages to the insect's midguts. Besides ROS damages the nutritive components of the plants, therefore the nutritive value as food decreases during herbivore attacks (Orozco-Cardenas and Ryan, 1999).

ROS level changes after wounding

ROS accumulation of wounded somatic hybrids with or without MMR deficiency and backcross progenies were evaluated during our experiments. Wounding as an experimental procedure is often used to investigate plant defense responses against herbivore attacks (de 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.

During optimal conditions, plant cells produce various ROS, which play important role in normal metabolic processes. In case of non-stressed plants (control group) the average concentration of H_2O_2 ranged between 35 and 37.5 μM/g FW in our investigation.

After DAB treatment, brown coloration appeared on the wounded leaves around the induced wound (hole) and across major (and rarely on minor) veins of the leaves. Orozco-Cardenas and Ryan (1999) observed similar distribution of H_2O_2 accumulation after wounding in tomato leaves. 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. 1). Both type of MMR deficient somatic hybrids (DN and AS) were represented in this group.

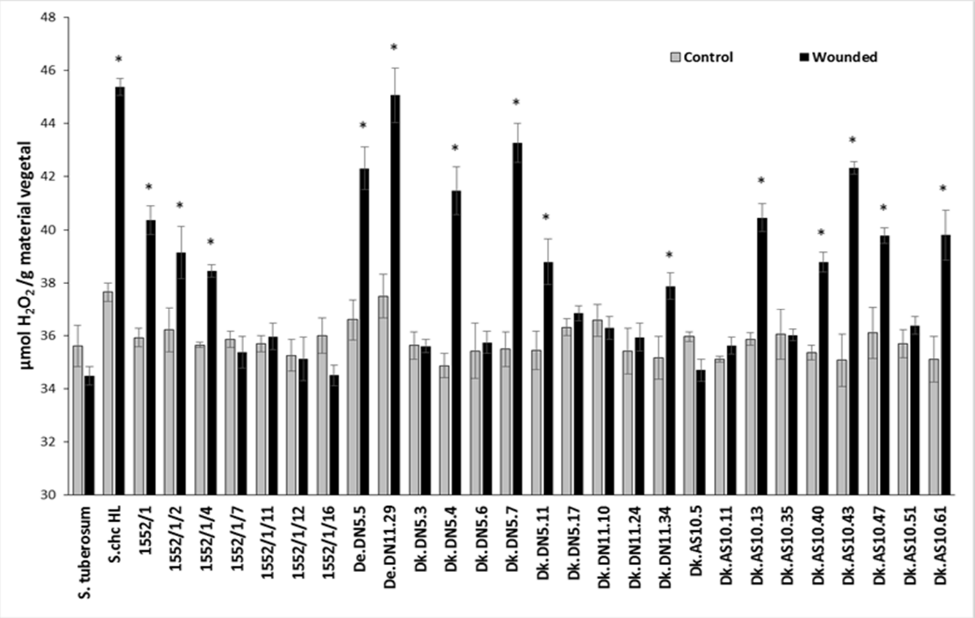

Figure 1. Quantitative evaluation of H_2O_2 in control and wounded somatic hybrids with and without MMR deficiency, their progenies and parental lines (*S. tuberosum* and *S. chacoense*); Note: * - somatic hybrids and BC clones with significant differences (n=5, t-test, $p<0.05$) between wounded and control plants

In case of marked genotypes (*) in Fig. 1, the produced H_2O_2 concentration was significantly higher than in control leaves. In case of *S. tuberosum* wounded leaves produced significantly less (t. test, $p<0.05$) amount of H_2O_2 than the wounded leaves of 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. Only CPB resistant Dk.DN11.10 and Dk.AS10.35 hybrids have similar H_2O_2 pattern like susceptible genotypes, but these hybrids also show contradictory results after two types of CPB resistance analyses. Both SHs had antibiosis properties being toxic to CPB larvae but did not have repellent effect against adult CPB.

Resistant MMR deficient SHs produced larger amounts of H_2O_2 than resistant wild-type SHs but resistant SHs without MMR deficiency accumulated a significantly higher amounts of H_2O_2 than susceptible plants. *S. chacoense* and MMR deficient SHs with the strongest resistance against CPB like De.DN5.5, De.DN11.29, Dk.DN5.4 and Dk.DN5.7 produced the highest concentrations of H_2O_2 after wounding. Somatic hybrids which contained dominant negative sequence of *MSH2* gene accumulated higher amounts of H_2O_2 compared to SHs with antisense orientation of *MSH2* gene but these differences between the two types of SHs were not significant.

Anthocyanin accumulation

The generated ROS during different biotic and abiotic stresses need to be stabilized in order to avoid oxidative damage of the cells, which may also affect the survival of plants. Plants can protect their cells by scavenging ROS through the activation of antioxidative systems like: superoxide dismutases, glutathione peroxidases, catalases or by production of different antioxidants like ascorbate, flavonoids, anthocyanin, etc. (Nagata *et al.*, 2003). These enzymes and antioxidants optimize cellular redox state, which are essential in maintaining the homeostasis of plants (Shao *et al.*, 2008). 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).

In order to evaluate the antioxidative effect of anthocyanin against the accumulated active oxygen species the concentration of anthocyanin was determined and compared between wounded and control plant leaves. The basic levels of anthocyanin varied between 4.8 and 10 µg/mg FW. In all cases of tested *Solanum* genotypes, mechanical injury of leaves induced accumulation of anthocyanin in different concentration levels (Fig. 2).

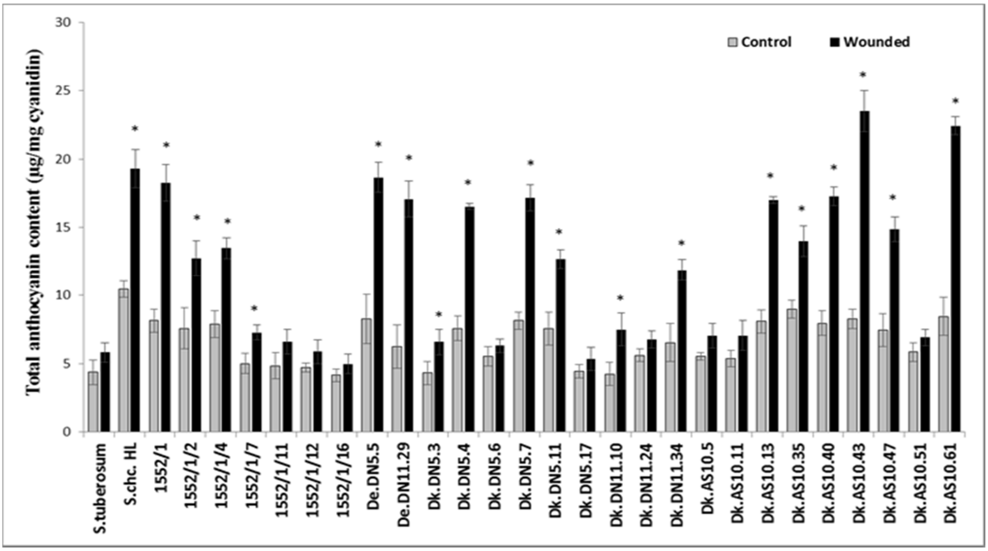

Figure 2. Anthocyanin accumulation level in control and wounded wild-type and MMR deficient somatic hybrids, backcrosses and parental lines (*S. tuberosum*, *S. chacoense*); Note: *-somatic hybrids and BC clones with significant differences (n=5, t-test, $p<0.05$) between wounded and control plants

The radical scavenging activity in wounded plants increases the anthocyanin level by a minimum of 20%, but even by 185% in affected leaves. Significant differences between stressed and control plant anthocyanin content was observed in case of genotypes marked with * in Fig. 2. Nagata *et al.* (2003) obtained similar result: they observed a 10 fold increase of anthocyanin content, which had intensified radical scavenging activity in *A. thaliana* plants irradiated with γ-rays.

In addition, a positive correlation between the generated anthocyanin content and the accumulated ROS amount was observed (Pearson correlation coefficient: 0.731). In those cases when highest amount of ROS was synthesized after mechanical injury of leaves, a high quantity of anthocyanin was also produced. Therefore, in the case of somatic hybrids the accumulation of ROS was followed by anthocyanin synthesis in order to stabilize ROS concentration in plants.

Conclusion

Based on the results presented above, one can conclude that wound-induced hydrogen peroxide accumulation plays an indispensable role in plant response to mechanical injury and can be associated with plants defense against herbivore attacks.

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 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.

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