Response of organically and conventionally produced potatoes to a controlled attack of a pathogen

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Summary

Organic agricultural products have increasing share of the food market in Europe. The questions related to benefits of organic versus conventionally produced agricultural crops are of increasing meaning. In our paper we have focused on assessment of the reaction of the immunity system of potato tubers from both agricultural systems on an external controlled attack of *Phytophthora infestans*, a pathogenic microorganism. The reaction of organic and conventional potato tubers was expressed via determination of chlorogenic acid. The content of chlorogenic acid in organic potato tubers increased 1.3 times after an attack, compared to potato tubers without a microbial attack. In conventional potato tubers, the increase was 4 times higher in the same way of comparison. The observed increase in conventional tubers is an evident exaggerated protection reaction to the attack of a natural potato pathogen. It is evident that there is a difference between the reaction of the immunity system of organic versus conventional potato tubers – phytoallergy.

Keywords

organic and conventional potatoes; controlled pathogenic attack; *Phytophthora infestans;* chlorogenic acid; plant immunity system; phytoallergy

Plants are exposed to various potential pathogens during their life. Plant diseases significantly influence world economy. Of all pathogens, fungi probably cause the most devastating damage [1]. The estimated loss caused by pathogens is typically 10–20% [2]. Activation of the defence mechanism is related to suppression of the primary plants mechanism. It is supposed that plants have innate immune system, which is different to the mammals' adaptive one [3–7]. After an attack, plants defend themselves by consecutive steps of complex defence mechanisms. An attack by a pathogenic microorganisms recognised and immune response activated by microbe-associated molecular pattern recognition, based on molecular structures unique to microbes. Receptor-mediated recognition at the place of infection initiates cellular and systemic signalling processes activating multicomponent defence responses on local and systemic levels [2]. The active or induced defence responses include a hypersensitive response (cell control dying), production of phytoalexins and pathogenesis-related proteins, ion flux across the plasma membrane, reactive oxygen and nitrogen species production, lignifications (suberization – parts of the plants under the soil) and re-inforcement of the cell wall through crosslinking of cell wall structural proteins, callose deposition [5] and production of other secondary plant metabolites (polyphenolic acids, flavonoids). Other components of the signal network are the induction of phospholipases, which act on lipid-bound unsaturated fatty acids within the membrane, resulting in releasing jasmonate, methyl jasmonate and related molecules [2]. Recent evidence confirmed the role of jasmonate, ethylene and salicylic acid in the signal pathway leading to up-regulation of pathogen defence-related genes in plants [2, 8]. Defence response can be primed for amplified expression upon a pathogen attack. Priming has the benefit of keeping defence potential dormant until the pathogen infection [6]. Primed plants

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have a higher level of disease protection with only marginal reduction in growth, while the induction of direct defence causes much stronger reduction in plant growth and reduced seed production [6]. Benzothiadizole and beta–aminobutyric acid are well known priming agents [6] and chitosan was reported as good priming agent for carrot [9]. However, the function of many proteins and other molecules that interact with resistance genes during defence mechanisms are yet unknown. Additionally, proteins are likely to participate in avirulent perception and subsequent signal transduction [2]. There is still a long way to detailed understanding of the immunity response of plants to external attack of natural pathogens.

Since the beginning of industrial revolution, massive application of artificial fertilizers and pesticides was aimed to increase the yield of agricultural production. Just a few years later movement called organic agriculture started its activities in India and its ideas were spread also to Europe and around the world. It was based on an idea that, in agricultural production, all off-farm materials should be avoided. Simply said, no pesticides and artificial fertilizers should be used. Now the organic agriculture is growing around the world and legal rules are valid [10].

The aim of this work was to evaluate the reaction of the immunity system of a model agricultural plant, namely potatoes, coming from organic and conventional agriculture. The immunity system was activated by controlled inoculation with a selected microorganism and the reaction of the immunity system of potatoes was evaluated via determination of the content of phenolic acids.

Phenolic acids could be present in plants either free or in various bound forms [11–16], mostly esters or glycosides [11, 12]. The stability of phenolic acids is rather limited, due to transformation activities of enzymes [17].

Phenolic acids determination is an important task suitable for evaluation of potential health benefit of various fruits and vegetables [18, 19]. Estimation of their content was also used for discrimination between organically and conventional produced agricultural products [20–22]. The analysis of phenolic acids in plants and plant products attracts considerable interest [13, 18, 23].

Phenolic acids are studied using different extraction and subsequently separation and determination procedures [24, 25]. Various solvents and solvent mixtures were used for free phenolic acids determination [26]. The most widely used mixture to extract free phenolic acids is methanol : water (80 : 20, v/v) [18, 27, 28]. Bound phenolic acids are afterwards hydrolysed with acidic or alkaline solutions, and extracted for subsequent chromatographic separation [18, 29]. MATTILA and KUMPULAINEN [30] determined free phenolic acids in an aliquot of the extraction mixture and used the residual mixture together with the solid food residue for alkaline hydrolysis. After alkaline hydrolysis, the mixture was acidified and phenolic acids were extracted with diethylether : ethylacetate (50 : 50, v/v). The residual water : methanol portion was subsequently hydrolysed with acid and extracted with diethylether : ethylacetate (50 : 50, v/v) again. Organic solvents in both cases were evaporated, residue diluted and analysed by liquid chromatography.

In order to hydrolyse phenolic acids, strongly acidic or alkaline media are used. The stability of phenolic acids at such extremes has to be known. Reversible changes in UV absorption spectra were observed for ferulic acid when pH values of model solutions changed from seven to eleven and back to seven; for gallic and caffeic acid changes were irreversible [31].

In general, the mostly applied technique for final content determination of phenolic acids is high performance liquid chromatography (HPLC) linked to a UV or diode array detector (DAD) [21, 22, 32]. Depending on the nature of the phenolic acids that have to be analysed, gradient elution is the method of choice for ascertaining successful separation [27, 28, 33]. Gas chromatography with mass-selective detection (MSD) or thin layer chromatography were also used [34, 35].

In the present paper, the content of phenolic acids was determined by simple acidic extraction with HPLC and UV detection. The method was used for determination of 12 selected phenolic acids. The change in contents of phenolic acids after a controlled microbiological attack in potato tubers from organic and conventional agricultural regimes is considered as a tool for immunity system evaluation.

MATERIALS AND METHODS

Samples of potatoes

History of potatoes samples has roots in the stationary field experiment established in the year 1990 at Borovce (in the west of the Slovak Republic) on a loamy luvi-haplic Chernozem. The territory has a continental climate with a mean annual precipitation of 593 mm per year and with an annual temperature average of 9.2 °C. The area is classified as a maize-barley-growing region. The experimental design involved a split plot arrangement with two replicates. Crop rotation: alfalfa-

alfalfa-winter wheat–potatoes-spring barley-maize for grain.

Farmyard manure at a rate of 40 t per hectare was applied to the organic and conventional variants. The agro-technical operations were conducted in accordance with Commission Regulation (EC) No. 889/2008 of 5 September 2008 [10] on organic production and labelling of organic products with regard to organic production, labelling and control. The selected potatoes were the variety Mozart in both agricultural regimes. Full record of used chemicals in conventionally grown potatoes is available. In autumn 2009, artificial fertilizer with 22 kg of phosphorus per hectare and 20 kg of potassium per hectare were used for the conventional field. In 2010 before planting, 60 kg per hectare of ammonium nitrate was used and, after the growth of plants appeared, other 30 kg of ammonium nitrate per hectare were applied. Fields of conventional and organic production were in a distance of 0.5 km. Potato tubers used for experiments were from the 2010 growing season. During vegetation, there was no observed attack by *Phytophthora infestans.* Both organic and conventional fields were regularly controlled. The harvest was done at the mature stage of the plants.

After another careful visual inspection, 8 potato tubers were selected from both agricultural systems, which were not naturally contaminated. These were freeze-dried and the content of phenolic acids was determined separately for each tuber individually in both groups, organic and conventional. These data were considered as reference. The other part from the selected inspected tubers (organic and conventional – 8 again) was used for the controlled inoculation.

Selected microorganisms

As a fungal pathogen of potatoes, *Phytophthora infestans* was selected. The selected strain was purchased from Výskumný a šľachtiteľský ústav zemiakársky (Veľká Lomnica, Slovakia), registered number NŠ-18-1.

Controlled inoculation

PGA (Potato Glucose Agar, Imuna, Šarišské Michaľany, Slovakia) agar in Petri dish was used for inoculation and cultivation of *Phytophthora infestans* at 18 °C with daylight access. Within 10 days, the whole surface of PGA agar was covered with the inoculated fungus.

In the germ on the potato tuber, a hole (diameter, 10 mm; depth, 6 mm) was done with a cutter. The material taken from the potato was stored. Afterwards, with the same cutter, circles of agar with the fungus (diameter, 10 mm) were placed into the hole in the potato tuber. The removed piece of the potato tuber was placed on the top of the agar plate with fungi.

Incubation of inoculated potatoes

Inoculated potato tubers were incubated at 15 °C without light access. The weight loss was controlled in 24 h intervals and recorded. After two month of incubation, the potato tubers were removed from the incubator and inspected. In each tuber in spot of inoculation, a dark patch of suberin, larger than the inoculation spot, was observed. It was evident that there was no progress of the fungus into the body of the potato tuber. The immunity reaction of the potato tuber prevented the tuber from degradation caused by the inoculated fungus.

Sample preparation

After completing the incubation, the inoculated part of the potato tuber was removed (approx. 10% of the tuber) and the residual part was freeze-dried and homogenized. Phenolic acids were extracted with a mixture methanol:water (80:20, v/v) – 1 g of the freeze-dried sample was mixed with 9 ml of the extraction mixture in a dark vial and sonicated for 30 min. The mixture was filtered (Whatman 40; Whatman, London, United Kingdom) and the filtrate was ready for injection to HPLC.

Extraction of phenolic acids

Twelve phenolic acids were selected to be analysed and their pure forms as standards were purchased – gallic, 3.4-dihydroxy benzoic, 4-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, salicylic all from Fluka (Buchs, Switzerland), *trans*-cinnamic from Aldrich (Steinheim, Germany) and chlorogenic from Sigma (St. Louis, Missouri, USA). Purity of the acids was better than 95%.

Phenolic acids were extracted from 1 g of the freeze-dried sample with 8 ml of mixture of methanol:water (80:20, v/v) with addition of 2 ml of 30% solution of HCl overnight. After filtration (Whatman 40, Whatman, London, United Kingdom) extracts were ready for HPLC analysis.

Water (resistance, 18.2 MW) was prepared by a Milli Q 185 system from Millipore (Billerica, Massachusetts, USA). Methanol of HPLC purity grade was provided by Merck (Darmstadt, Germany).

HPLC measurement

Phenolic acids were quantified with an Agilent Technologies HPLC system (Agilent Technolo-

gies, Palo Alto, California, USA). Samples were injected by an autosampler cooled to 5 °C. A variable wavelength detector was used for quantification at 280 nm wavelength for all phenolic acids. A quaternary pump was used for gradient formation. A Zorbax SB-C18 (Agilent Technologies) 4.6×250 mm column with 5 mm particles was used for separation. A binary gradient was used for separation: A: 0.01 mol·l^{-1} solution of phosphoric acid; B: methanol – start with 100% A, linear gradient to 95% A in 1.5 min, stable up to 2nd minute, linear decrease to 83% A in 3rd minute, stable up to 23rd minute, linear decrease A to 74% up to 30th minute, stable up to 35th minute, linear decrease A to 30% in 45th minute. Column was flushed for 7 min with methanol and afterwards equilibrated for 10 min with 100% A. External standard procedure was used for phenolic acids quantification. Example chromatograms of a mixed phenolic acids standard and extract from potatoes are given in Fig. 1 and Fig. 2, respectively.

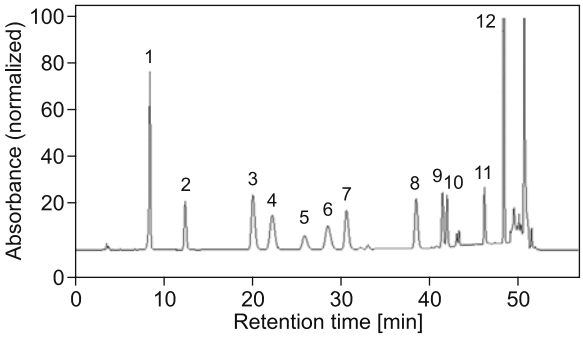

Fig. 1. Chromatogram of selected phenolic acids in standard solutions.

1 – gallic acid, 2 – 3,4-dihydroxibenzoic acid, 3 – 4-hydroxybenzoic acid, 4 – chlorogenic acid, 5 – vanillic acid, 6 – caffeic acid, 7 – syringic acid, 8 – *p*-coumaric acid, 9 – ferulic acid, 10 – sinapic acid, 11 – salicylic acid, 12 – *t*-cinnamic acid.

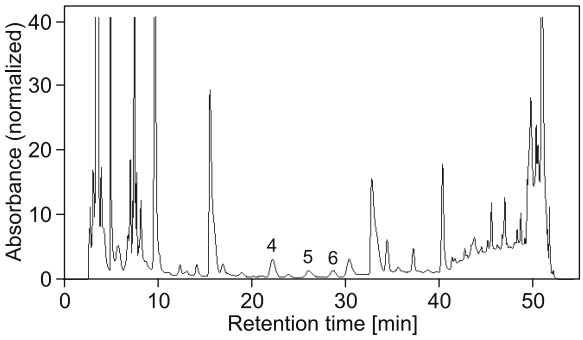

Fig. 2. Chromatogram of phenolic acids in the extract from potatoes.

4 – chlorogenic acid, 5 – vanillic acid, 6 – caffeic acid.

RESULTS AND DISCUSSION

In reference samples as well as in samples after controlled pathogenic attack, chlorogenic, vanillic and caffeic acids were determined. Recovery was assessed by repeated analysis of ten identical samples. Standard deviation of recovery for chlorogenic acid at level 87.3 mg·kg^{-1} was ± 2.9 mg·kg^{-1}, which is ± 3.4%. For vanillic acid at level 12.2 mg·kg^{-1}, it was ± 1.5 mg·kg^{-1} (12%) and for caffeic acid at level 21.6 mg·kg^{-1}, it was ± 2.3 mg·kg^{-1}(11%).

The contents of phenolic acids in both groups, organic and conventional potato tubers in reference and inoculated samples, were evaluated with single factor ANOVA test. No significant difference ($P > 0.05$) was observed for vanillic and caffeic acids between organic and conventional groups of reference and inoculated samples (data not shown).

No significant difference was further observed in contents of chlorogenic acid in group of organic potatoes tubers between reference and inoculated potato tubers, $P = 0.3$. Clear difference was identified in conventional potato tubers between reference and inoculated samples, $P = 2\times10^{-5}$. Reaction of the immunity system of conventional potato tubers was different in comparison to organic ones.

The quantitative evaluation of different reaction of the immunity system of conventional and organic potato tubers was expressed via relative values. They were calculated as the ratio between average content of chlorogenic acid in group of organic potato tubers after inoculation and the average content of chlorogenic acid in the respective reference group of potato tubers. The value of relative number for chlorogenic acid in organically grown potatoes was 1.3, while the respective value of relative number in conventional potatoes was 4. The data for chlorogenic acid are listed in Tab. 1.

Chlorogenic acid is one of the defence agents in the immune response of plants to external attack of microbial pathogens. Its content was remarkably increased in conventionally grown potatoes in comparison to organically grown ones after a controlled microbial attack. Since the same variety of potatoes was used as well as climatic and soil impact could be considered as equal, the only reason for the observed phenomenon might be the different growing conditions. In the conventional technological system, chemical fertilizers

were applied in addition to farmyard manure, and pesticides were applied as well as. This difference should have impact on the plant immune system.

Growing conditions in organic agriculture could be considered as standard from the point of view of the plant immune system. Neither artificial fertilizers nor synthetic chemicals for protection were applied. The plants were grown on their own without any artificial external support except for the farmyard manure. The increased content of chlorogenic acid by the factor of 1.3 reflected the activity of the immune system, which could be considered as a standard defence response.

In case of conventional agricultural technology, artificial chemical fertilizers were added and plants were protected against external biological attack with pesticides. These two factors were reflected by a change in the immunity response to the controlled microbial attack expressed by factor 4 for chlorogenic acid content. Protection of the conventional potato tubers was much more intensive, expressed in relative numbers, in comparison to the organic potato tubers.

The value of relative numbers depends highly on how the controlled microbial attack is carried out. Selection of suitable microorganisms is vital. It is highly recommended to test pure pathogenic strains from the point of view of their aggressiveness. A strain has to be selected, which does not destroy the selected plant material during the incubation period, but the plant immune system has to limit the growth of the microorganism during the incubation period. This aim can be achieved only when the controlled microbial attack is similar to a natural microbial attack of the selected agricultural plant in the field. It is well known that germs of pathogenic microorganisms are distributed by the air or are persistent in the soil and infect the agricultural plants. The microorganisms can be identified and the level of contamination of the plant recorded after careful inspection. For our experiments, only potato tubers free of microbial contamination were used, for measurement of reference values as well as for the controlled microbial attack.

It is supposed that plants have innate immunity system. If the applied agricultural system has no impact on the immunity system of plants, the defence response of plants against a controlled microbial attack should be the same. It means, no remarkable difference in the contents of protective chemicals (chlorogenic acid) should be observable. Our results evidence that the applied agricultural system has impact on the immune system of the agricultural plants. Potatoes from conventional agriculture have a perturbed immunity system in comparison to potatoes from organic agriculture. The marked increase in the content of chlorogenic acid could be considered as “phytoallergic” reaction. The observed difference in the content of chlorogenic acid expressed as a relative change was due to the applied agricultural technology in the plant growing period. It is not known, which part of the multicomponent defence response system was perturbed. The answer would require a detailed study and appearing of new question is obvious. What is the impact of agricultural plants with a perturbed immunity system on the immunity system of mammals? The increase in the reported cases of human allergy indicates that there could be some influence.

Tab. 1. Content of chlorogenic acid in potato tubers.

Sample number	Organic	Organic after controlled microbiological attack	Conventional	Conventional after controlled microbiological attack
	[mg·kg^{-1}]			
1	47	81	18	212
2	69	42	32	199
3	112	65	19	86
4	59	67	38	239
5	55	97	89	325
6	90	131	79	298
7	28	156	71	283
8	18	41	74	195
Average	60	77	53	232
Variability between samples [%]	52	55	55	32

CONCLUSIONS

It is generally supposed that the immunity system of plants is innate. Based on the presented results it is evident that plant immunity system is influenced by the growing conditions. Plants grown in conventional agriculture react in a different way to the controlled external pathogenic attack in comparison to organically grown plants. A four-fold increase in the content of chlorogenic acid in conventionally grown potato tubers compared to 1.3 increase in organic potato tubers after a controlled microbial attack were observed. The exaggerated increase in the content of chlorogenic acid in conventional potato tubers could be considered as phytoallergy. The immunity system of other conventional agricultural plants has to be also assessed. It is required to give the answer on question: Have all conventional agricultural plants phytoallergy?

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Received 1 December 2011; 1st revised 5 March 2012; 2nd revised 22 March 2012; accepted 23 March 2012.