

Establishing biotic stress tolerance of maize (*Zea mays* L.) by measuring hydroxamic acid contents

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SUMMARY

Cyclic hydroxamic acids are the most considerable secondary metabolites in grasses and their main task is to protect these species from pathogens and pests. The cyclic hydroxamic acid content and common smut susceptibility were examined in our experiments. 27 maize hybrids were used for experimental plants in a climate room, where the plants were grown on a nutrient solution. An infiltration method was used for the inoculation of the plants. The total quantity of cyclic hydroxamic acids was determined and the ratio of infected plants and the ratio of inhibition was determined, too. Based on our results, on the basis of all hybrids' data, the total hydroxamic acid content of the infected plants was higher than in the control. On the level of individual hybrids, only 9 of them had higher cyclic hydroxamic acid content in the case of infection. Increase in cyclic hydroxamic content induced by the fungus in this case is a tool for the fungus to suppress other pathogens and pests. Amongst the hybrids' cyclic hydroxamic acid contents, significant differences were detected in the control and in the infected treatment, too. The so-called "sweetcorn" hybrids showed high level of cyclic hydroxamic acid content. According to the differences amongst hybrids, homogenous groups were created which groups differed in the case of control and infected treatment, because of the difference in increase of cyclic hydroxamic acid content. The examined hybrids showed different levels of infection and different rate of growth inhibition for the effect of inoculation. According to the infection caused damage hybrids were ranked. Infection caused notable damage for hybrids Prelude, Desszert 73, DKC5276 and DK440.

Keywords: common smut, sensitivity, maize hybrids, cyclic hydroxamic acid, biotic stress

INTRODUCTION

In addition to chemical plant protection, which means the usage of pesticides, there are several possibilities for reducing the damage caused by pathogens and pests. Integrated pest management (IPM) apply various procedures for plant protection in concert (Bozsik 2014). Elements of IPM are: proper choice of agricultural land for a given crop; good agricultural practices, fit to the demand of this crop; breeding work for preventing against abiotic and biotic stress factors; application of the natural enemies; sustaining the enemies natural habitats or applying artificial devices for the enemies' survive; mechanical plant protection (e.g. land cultivation); on small areas producing companion crops beneficial for each other. There are great reserves in the choice of variety/hybrid holding resistance/tolerance and suitability for producing on the given land.

For selecting and improving new varieties/hybrids it is necessary to reveal the factors of resistance, the chemical, morphological and ontogenetical properties, which influence the colonization and harmfulness of pathogens and pests. Chemicals produced by the crop plants themselves can protect these plants properly. These chemicals or their analogues are nowadays used in the chemical plant protection too.

There are several cultivated species, frequent and harmful weeds in the plant family of grasses. Cyclic hydroxamic acids are „self-protecting” secondary metabolite chemicals produced by most of the grasses' species. These chemicals have important role in protection against several pathogens and pests. Their concentration in the plant connected with the resistance

and tolerance at most of the harmful pathogen and pest species (Niemeyer 1988, 2009; Bravo and Lazo 1996).

Till now there are only few data in connection with the cyclic hydroxamic acid content and common smut infection (Basse 2005, Tanaka et al. 2014). It would be important to make clear the factors determining the severity of damage caused by this pathogen and the role of cyclic hydroxamic acids in this process. For this purpose the quantity of hydroxamic acids was measured, the effect of infection was evaluated and the connection of hydroxamic acid content and severity of damage were examined.

Common smut can be a considerable disease of maize depending on the hybrid and weather conditions. The severity and appearance of symptoms caused by this biotrophic parasite effected by several factors. The exploration of these factors can help to understand the background of susceptibility/tolerance.

MATERIALS AND METHODS

For experimental plants 27 hybrids of maize (*Zea mays* L.) were used. The hybrids, with considerable Hungarian cultivated area are presented in *Table 1*. Plants were grown in climate room on nutrient solution. The inoculation with the pathogen happened when the plants coleoptile was 1.0–1.5 cm long. Until the coleoptile elongation to 3.5–4.0 cm, the plants were kept in darkness. 3–3 pots with 10 plants in each from inoculated and healthy (control) plants made the experimental plant material. The nutrient solution was prepared according to Treeby et al. (1989). Iron was added in the form of Fe(III)-EDTA, in quantity of 0.1 mM L⁻¹. Nutrient solution was constantly aerated and

changed in every 3rd days. Light intensity was 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, temperature 25/20 °C (day/night), relative humidity 65–75%, ratio of light and darkness 16 h/8 h. Plants were grown in climate room for a week, so taking the time for coleoptile elongation into consideration, at the time of sampling they were 12 days old.

Common smut can be a considerable disease of maize depending on the hybrid and weather conditions. The severity and appearance of symptoms caused by this biotrophic parasite effected by several factors. The exploration of these factors can help to understand the background of susceptibility/tolerance. The common smut spores used for inoculation originated from infected plant parts from the field. Growing of pathogen from its spores was carried out on solid and liquid culture medium. Compatibility of pathogen strains, originated from a single spore, was evaluated according to establishment of aerial mycelia. Before the inoculation in the experiment the compatible pathogen cultures were tested. Inoculation was carried out by the method of infiltration, after cutting off the coleoptile tips. The control plants were infiltrated too, with sterile distilled water. For inoculation, 24 hours old liquid culture media of compatible pathogen strains, in 1:1 ratio was used, with 500 pc. ml^{-1} spores.

For examining the cyclic hydroxamic acid content 1 plant was used from every experimental pot (3 plants from every hybrid and treatment). Sampling was carried out from the second leaf of 12 days old plants. Samples fresh weights were measured, after samples were labelled and packed. Till the further preparation the samples were stored on -80 °C temperature. Sample preparation was carried out by the method of Lyons et al. (1988). The total cyclic hydroxamic acid content was measured by the method of Long et al. (1974). The measurement was facilitated by preliminary isolation of pure hydroxamic acids according to Hartenstein et al. (1992) and construction of standard curves (Makleit 2004).

The ratio of infection means the ratio of infected plants. That was counted from the next equation: (infected plants number/total plants number) \times 100. The ratio of inhibition was calculated by the length of infected plant compared to the healthy ones. It can be describe with the next equation: $100 - [100 \times (\text{length of infected plants} / \text{length of control plants})]$. For the evaluation of the damage caused by the infection, an index was created. This index, the damage, caused by infection, was calculated from the indexes mentioned before. It can be describe with the next equation: (ratio of infection \times ratio of inhibition)/100. All the indexes' unit is percent.

Statistical analysis was performed by using SPSS 23.0 version. For the evaluation, whether the data of a same subject were normal distribution or not, the Kolmogorov-Smirnov test was used. Differences were measured by t-test or Mann-Whitney and Kruskal-Wallis tests. The differences between groups were performed by Duncan test. The connection between the factors was measured by using correlation or regression analyses.

RESULTS

Cyclic hydroxamic acid content of hybrids

The second leaf's hydroxamic acid content varied between 175 and 614 mg kg^{-1} fresh weight in the control plants, and between 214 and 662 mg kg^{-1} fresh weight in the infected plants (*Table 1*).

According to all hybrids' data, the control and infected plant's hydroxamic acid content in the second leaf differed. The infected plants had higher hydroxamic acid content (443 mg kg^{-1} fresh weight), than the control ones (379 mg kg^{-1} fresh weight). Examining the differences between control and infected plants' hydroxamic acid content hybrid by hybrid lead to controversial results. The infection increased hydroxamic acid content only in 7 hybrids. These hybrids were: PR37N01, PR37Y12, PR38A24, P9494, DKC4490, DKC5007, NK Octet. On the level of $P=10\%$, in case of hybrids DK440 and Prelude the infection increased hydroxamic acid content too. In case of other examined hybrids, there was no difference between the control and infected plants' hydroxamic acid content (*Table 1*).

Comparison of hybrids' hydroxamic acid content

Examining the homogeneity of hybrids' hydroxamic acid content in control plants according to Duncan's test resulted that the 27 hybrids were divided into 10 groups. Groups are labelled with same small letters in the *Table 1* 'Control' column. Hybrids have very diverse hydroxamic acid content. PR37Y12 had the lowest (175 mg kg^{-1} fresh weight) (Group: a), NK Lucius, DKC5276 and Noa had high hydroxamic acid content (601–611 mg kg^{-1} fresh weight) (Group: f). Hybrids with lower (211–235 mg kg^{-1} fresh weight) hydroxamic acid content were DKC4490 and PR38A24 (Groups: ab and abc). Hybrids with higher (401–512 mg kg^{-1} fresh weight) hydroxamic acid content were Jumbo, Desszert 73, Boks-R, Prelude, Occitan, DKC4964, and NK Columbia (Groups: cde, def and ef). The other hybrids had intermediate level (270–398 mg kg^{-1} fresh weight) of hydroxamic acid content: P0017, P9494, Amandha, P9578, PR37N01, DKC4014, DKC4795, DKC4590, PR36V52, DKC4717, NK Thermo, DK440, DKC5007, NK Octet (Groups: abcd, abcde, bcde). All the examined sweet maize hybrids had high hydroxamic acid content in control samples (*Figure 1*).

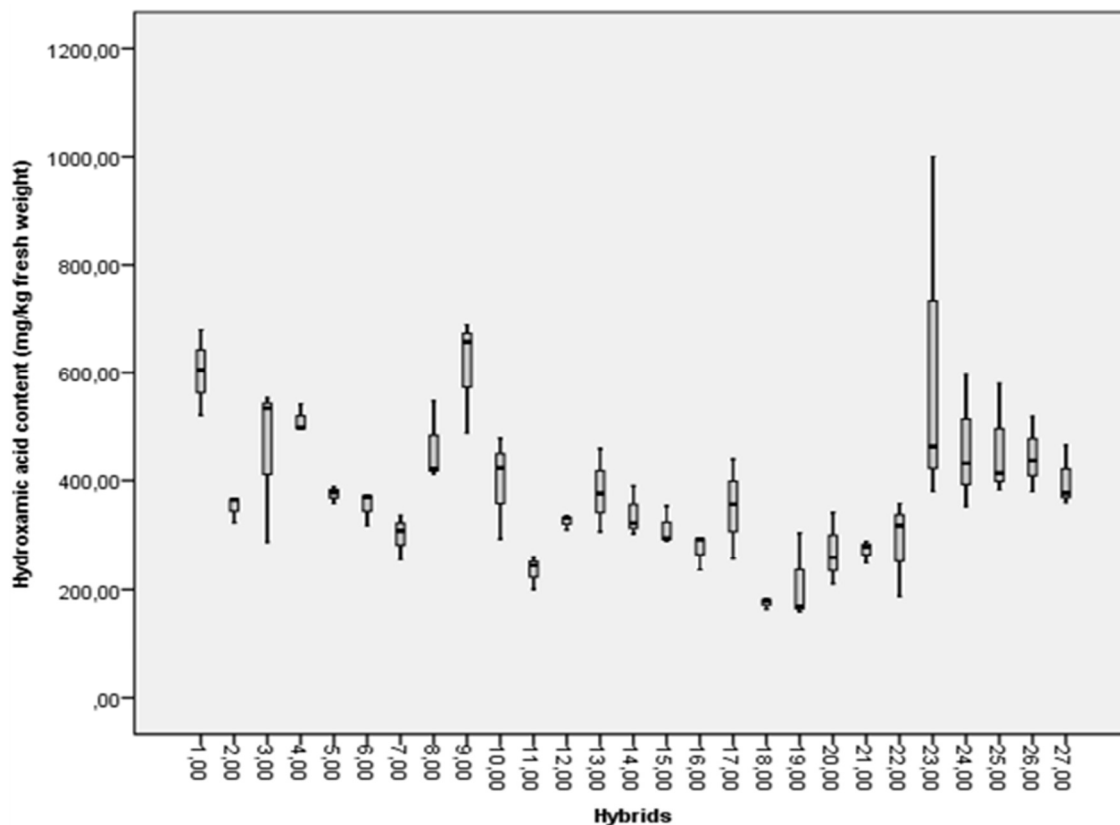
Examining the homogeneity of hydroxamic acid content of infected hybrids according to Duncan's test resulted that most of the hybrids were at the same group. Groups are labelled with same small letters in the *Table 1* 'Infected' column. The 16 hybrids with intermediate level (356–527 mg kg^{-1} fresh weight) of hydroxamic acid content of the most populous group were the following: NK Lucius, Occitan, NK Columbia, NK Octet, DKC4964, DKC5276, DKC4490, DKC4795, DKC4590, P9578, PR37N01, PR36V52, PR38A24, P0017, P9494, Desszert 73, Boks-R, Jumbo (Group: abc).

Table 1

Total cyclic hydroxamic acid content of various hybrids' second leaf (mg kg ⁻¹ fresh weight)			
Hybrid	Control	Infected	Significance
PR37N01	274.63±18.84 ^{abcd}	354.97±52.67 ^{abc}	S
PR36V52	351.78±52.86 ^{abcde}	397.04 ±30.77 ^{abc}	NS
PR37Y12	175.69±5.72 ^a	300.04±10.33 ^{ab}	S
PR38A24	211.27±46.68 ^{ab}	365.84±51.95 ^{abc}	S
P0017	270.93±38.09 ^{abcd}	356.43±18.29 ^{abc}	NS
P9494	272.69±11.90 ^{abcd}	480.26±2.60 ^{abc}	S
P9578	313.23±35.09 ^{abcd}	481.90±260.10 ^{abc}	NS
DKC4490	235.25±17.65 ^{abc}	371.70±57.15 ^{abc}	S
DKC4795	326.08±7.74 ^{abcde}	377.02±57.41 ^{abc}	NS
DKC5007	381.15±43.92 ^{bcde}	662.35±4.41 ^c	S
DKC4590	339.04±26.17 ^{abcde}	352.85±5854 ^{abc}	NS
DKC4717	354.26±31.13 ^{abcde}	568.20±214.34 ^{bc}	NS
DKC4014	300.44±41.40 ^{abcd}	214.99±75.32 ^a	NS
DKC4964	460.63±75.09 ^{def}	416.11±186.91 ^{abc}	NS
DKC5276	611.56±107.47 ^f	527.76±358.07 ^{abc}	NS
DK440	398.48±94.84 ^{bcde}	656.39±127.83 ^c	S P=10 %
NK Lucius	601.73±79.21 ^f	416.03±323.25 ^{abc}	NS
NK Thermo	351.74±24.30 ^{abcde}	286.36±76.82 ^{ab}	NS
NK Columbia	512.16±25.78 ^{ef}	517.86±153.62 ^{abc}	NS
NK Octet	375.25±15.51 ^{bcde}	514.17±52.16 ^{abc}	S
Occitan	458.53±147.43 ^{def}	464.34±118.03 ^{abc}	NS
Amandha	288.07±89.13 ^{abcd}	326.69±49.84 ^{ab}	NS
Noa	614.88±336.44 ^f	658.42±256.34 ^c	NS
Desszert 73	460.52±123.67 ^{def}	474.82±241.28 ^{abc}	NS
Boksz-R	458.91±105.93 ^{def}	409.99±90.10 ^{abc}	NS
Prelude	445.48±69.56 ^{def}	566.51±49.72 ^{bc}	S P=10 %
Jumbo	401.25±56.38 ^{ede}	466.33±179.33 ^{abc}	NS

Note: n=3, ±SE. Homogenous subjects labelled with superscript letters; S: significant; NS: not significant P=10%: significant on P=10%.

Figure 1: Total cyclhydroxamic acid content of hybrids in control samples



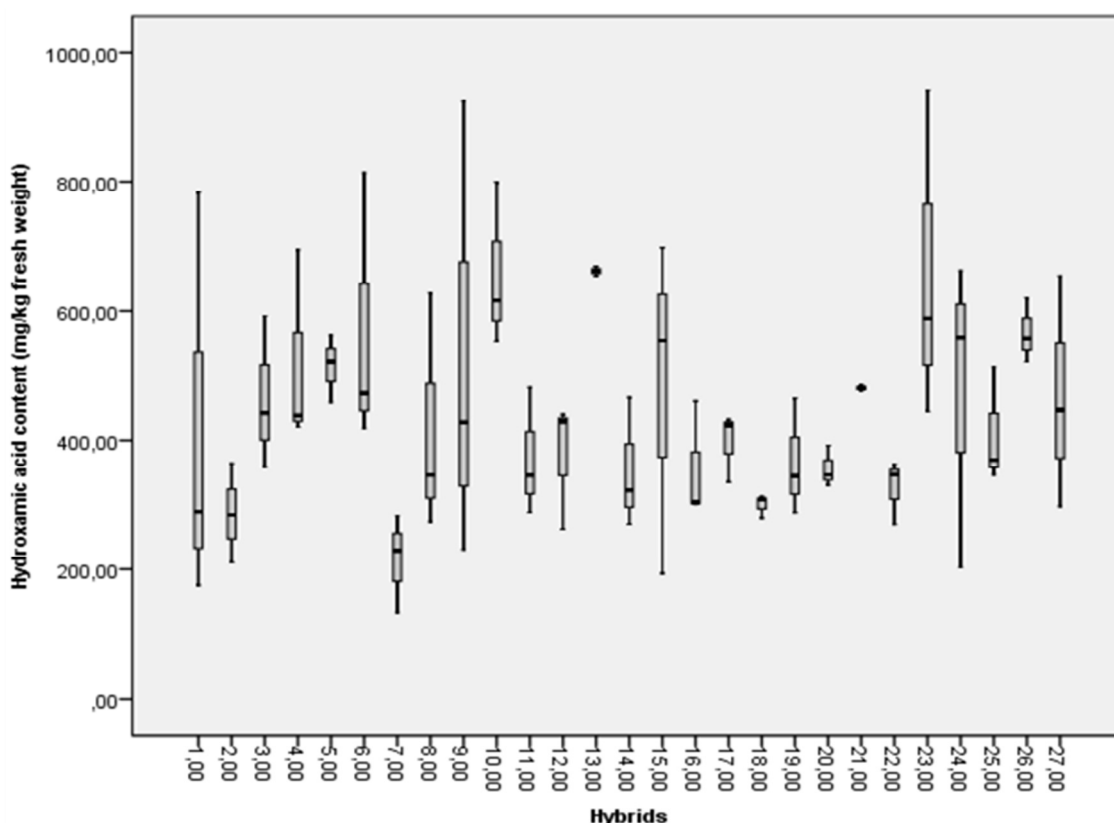
Note: n=3, ±SD, SE. Hybrid numbers: 1: Nk Lucius; 2: NK Thermo; 3: Occitan; 4: NK Columbia; 5: NK Octet; 6: DKC4717; 7: DKC4014; 8: DKC4964; 9: DKC5276; 10: DK440; 11: DKC4490; 12: DKC4795; 13: DKC5007; 14: DKC4590; 15: P9578; 16: PR37N01; 17: PR36V52; 18: PR37Y12; 19: PR38A24; 20: P0017; 21: P9494; 22: Amandha; 23: Noa; 24: Desszert 73; 25: Boksz-R; 26: Prelude; 27: Jumbo.

The lowest hydroxamic acid content had hybrid DKC4014 (214 mg kg⁻¹ fresh weight), (Group: a), the highest (656–662 mg kg⁻¹ fresh weight) had hybrids DK440, DKC5007 and Noa (Group: c). Hybrids with lower hydroxamic acid content (286–326 mg kg⁻¹ fresh weight) were NK Thermo, Amandha and PR37Y12 (Group: ab), and hybrids with higher

hydroxamic acid content (566–568 mg kg⁻¹ fresh weight) were DKC4717 and Prelude (Group: bc).

According to these results the DKC hybrids have the most versatile hydroxamic acid content in infected plants, because there were DKC hybrids in almost all above mentioned groups. Notable that infected “sweet corn” hybrids had at least intermediate, or higher hydroxamic acid content (Figure 2).

Figure 2: Total cyclichydroxamic acid content of infected hybrids



Note: n=3, ±SD, SE. Hybrid numbers: 1: Nk Lucius; 2: NK Thermo; 3: Occitan; 4: NK Columbia; 5: NK Octet; 6: DKC4717; 7: DKC4014; 8: DKC4964; 9: DKC5276; 10: DK440; 11: DKC4490; 12: DKC4795; 13: DKC5007; 14: DKC4590; 15: P9578; 16: PR37N01; 17: PR36V52; 18: PR37Y12; 19: PR38A24; 20: P0017; 21: P9494; 22: Amandha; 23: Noa; 24: Desszert 73; 25: Boksz-R; 26: Prelude; 27: Jumbo.

Differences amongst hybrids in degree and effect of infection

Table 2 summarizes the ratio of infected plants, the ratio of growth inhibition and the severity of damage at the examined hybrids. Ratio of infected plants in the second column of Table 2 shows the percent of the plants infected for the effect of inoculation. The degree of infection kept from 40 to 100%, depending on the hybrid. The higher was the degree of infection, the higher was the sensitivity of the hybrid, so the most sensitive hybrids to common smut from the examined hybrids was Prelude, and the less sensitive hybrid was DKC5007. According to our results Syngenta hybrids were sensitive to the artificial inoculation according to the degree of infection. At least 87% of the plants were infected in case of these hybrids. In contrast, Pioneer hybrids were less susceptible. The degree of infection of Pioneer hybrids

kept from 60 to 80%. Surprisingly amongst so called “sweet corn” hybrids more and less sensitive ones were, too. DeKalb hybrids had various degree of sensitivity, which kept from 40 to 97%.

The third column of Table 2 shows the effect of infection. The effect of infection can be demonstrated with the ratio of inhibition, which was calculated from the means of length of shoots of control and infected plants. The ratio of significant inhibition kept from 9 to 56%. The more severe was the effect of infection, the more severe was the degree of significant inhibition. Surprisingly hybrid Occitan had longer shoots in case of infection. In contrast of the degree of infection, the effect of infection at Syngenta hybrids’ was lower than in case of other types of hybrids. Pioneer, DeKalb, and “sweet corn” hybrids had various degree of infection’s effect. The ranking of hybrids according to the combination of ratio of

infected plants and ratio of growth inhibition (fourth column of *Table 2*) results the damage caused by infection. The infection caused notable damage at the next hybrids: Prelude, Desszert 73, DKC5276 and

DK440. As at some hybrids the growth inhibition was not measurable, the damage of infection wasn't relevant. These hybrid were Occitan, Amandha, NK Lucius and P9578.

Table 2

Ratio of infected plants, ratio of growth inhibition, damage, caused by infection and the ranking of hybrids

Hybrid	Ratio of infected plants (%) (A)	Ratio of inhibition (%) (B)	Damage (AxB)/100 (%)	Rank based on the damage caused by the infection
PR37N01	70	12.51*	8.76	22.
PR36V52	76.67	32.88*	25.21	5.
PR37Y12	66.67	18.21*	12.14	19.
PR38A24	70	13.27*	9.28	20.
P0017	60	9.01*	5.41	23.
P9494	63.34	28.47*	18.03	11.
P9578	80	3.38	it is not relevant	24-27.
DKC4490	63.34	25*	15.84	15.
DKC4795	60	25.69*	15.41	16.
DKC5007	40	30.96*	12.38	18.
DKC4590	56.67	34.11*	19.33	10.
DKC4717	90	21.72*	19.55	9.
DKC4014	86.67	27.31*	23.67	6.
DKC4964	80	20.23*	16.18	14.
DKC5276	96.67	33.04*	31.94	3.
DK440	86.67	32.48*	28.15	4.
NK Lucius	100	1.29+	it is not relevant	24-27.
NK Thermo	86.67	15.23*	13.2	17.
NK Columbia	86.67	22.91*	19.86	8.
NK Octet	93.34	21.64*	20.2	7.
Occitan	93.34	13.42+*	it is not relevant	24-27.
Amandha	93.34	9.38	it is not relevant	24-27.
Noa	56.67	30.71*	17.4	13.
Desszert 73	96.67	43.58*	42.13	2.
Boksz-R	90	19.97*	17.97	12.
Prelude	100	55.72*	55.72	1.
Jumbo	56.67	15.7*	8.9	21.

Note: *significant difference.

Surveying the hybrids' data on degree and effect of infection it can be stated that only a part of the hybrids had similar level in these indexes. For example hybrids Prelude, DKC4717, DK440 and Boksz-R had higher and similar level, while for example PR38A24 and PR37N01 had lower level and similar level in the degree and effect of infection. In case of the most hybrids in our experiments, there was no connection between these indexes. For example hybrid Jumbo's was ranked almost the worst in the effect of infection, but was ranked almost the best in the degree of infection.

Connection between cyclic hydroxamic acid content and sensitivity to common smut, and connection between the effect and degree of infection

No correlations were found between the total cyclic hydroxamic acid content of control or infected plants and the various parameters indicating the infection (ratio of infected plants, ratio of growth inhibition, damage, caused by infection; ranking of hybrids according to damage, caused by infection). No correlation was found between the ratio of infected plants and ratio of inhibition as well. Cluster analysis could have been proper method for performing groups

with similar properties, but its' requirement, the existence of correlation had not come true in our cases.

DISCUSSION

According to our results, on the basis of all hybrids data, the total hydroxamic acid content of the infected plants was higher than in the control. On the level of individual hybrids 9 hybrids had higher cyclic hydroxamic acid content in case of infection. Basse (2005) also experienced, that *Ustilago maydis* infection increased the cyclic hydroxamic acid content of infected plants compared to the healthy ones. The surprising result of Basse is that in *in vitro* conditions *Ustilago maydis* decomposed the most frequent cyclic hydroxamic acid, so this pathogen insensitive for this self-protecting chemical. Interestingly the decomposition product of hydroxamic acid was toxic for common smut. That is no clear whether the decomposition product develops or not in the plant in case of infection.

Examining the homogeneity of hybrids' hydroxamic acid content it can be stated that there were significant differences between the hybrids in case of the control and infected treatments, too. The infection itself rearranged the groups of the hybrids because of its different effect on changing the

hydroxamic acid content. In contrast, hybrid DKC5007 had high hydroxamic acid content in case of the control and infected plants too and P37Y12 hybrid showed a lower level of hydroxamic acids in case of these treatments.

Difficult to explain the detrimental effects caused by common smut on young plants. The fresh or dry weight of infected plants sometimes even higher than the control, because the fungus formed galls have higher weight, than the normal leaf tissue has. That is why the index, "damage, caused by the infection" was introduced. The infection doesn't always produce lower plants, what makes more difficult to demonstrate the effect of infection. Frommer et al. (2016) lead to different result with the susceptibility of hybrids compared to the present findings, but in their examination the examined hybrids were compared to each other, and the number and types of the hybrids were less, than in the present investigation.

There were no measurable correlations between the cyclic hydroxamic acid content of the examined hybrids and the indexes of infection. The reason of this result is that the susceptibility of a plant to a given pathogen depends on many factors. The actual general condition of the plant, the strength and types of stress factors, morphological, physiological and ontogenetical features together regulate the connection of a plant and its pathogen (Engelberth 2010). The level of cyclic hydroxamic acids as a factor itself can't be the only

reason of the susceptibility or tolerance. The rising hydroxamic acid content for the effect of infection in hybrids was an unusual result. According to opinion of Tanaka et al. (2014), common smut induce not "only" the gall formation, but also the cyclic hydroxamic acid synthesis. As this pathogen insensitive to the most common hydroxamic acid, the elevated level of this chemical can be useful, because it decreases the effect of other, competing pathogens and pests. Their other explanation that the elevated cyclic hydroxamic acid content would regulate the development of galls.

Based on the fact that the edible form of common smut – cuitlacoche – is produced especially on "sweet corn" hybrids (Pataky 1991), it can be stated that "sweet corn" hybrids usually more susceptible to common smut than the other types of hybrids, produced especially for animal feeding purposes. The relative high cyclic hydroxamic acid content of "sweet corn" hybrids in the control and the infected plants can't be the obstacle of infection, because the above mentioned feature of con smut.

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