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RESPONSE OF WINTER WHEAT ON LONG-TERM MONOCULTURE IN DIVERSIFIED CONDITIONS OF CHEMICAL PROTECTION

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Key words: crop rotation, fungicide, herbicide, monoculture, winter wheat, yields.

Abstract

The appearance of herbicides and fungicides significance in reducing damage in yields of winter wheat cultivated for a 38–46-year monoculture was the purpose of that paper. In 9-year researches (2005–2013) at the Production and Experimental Station in Bałcyny near Ostróda town, the response of winter wheat on a 38−46-year monoculture in different conditions of chemical protection of a wheat canopy: 0 – without protection; *H* – protection from weeds, and HF – protection from weeds and diseases was assessed. The comparative object was winter wheat crop in 6-field crop rotation: sugar beet – maize – spring barley – pea – winter rape – winter wheat. In the case of crop rotation the average yield of winter wheat grains amounted to 7.72 t ha^{-1}. The crop in a 38–46-year monoculture has decreased winter wheat yielding by 37% on average. The biggest productivity decline took place on the object without protection – 61%. The application of herbicides has limited the aforementioned decline to 25% while the combined application of herbicides and fungicides to 27%. Worse yielding of winter wheat in a monoculture are conditioned by thinning out density of ears per 1 m^2, and the decrease of grains weight of an ear and the weight of 1.000 grains.

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REAKCJA PSZENICY OZIMEJ NA WIELOLETNIĄ MONOKULTURĘ W WARUNKACH ZRÓŻNICOWANEJ CHEMICZNEJ OCHRONY ŁANU

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Słowa kluczowe: płodozmian, fungicyd, herbicyd, monokultura, pszenica ozima, plonowanie.

Abstrakt

Celem pracy była ocena zastosowanych herbicydów oraz fungicydów jako czynników redukujących spadek plonów pszenicy ozimej uprawianej w 38–46-letniej monokulturze. W 9-letnich badaniach (2005–2013), prowadzonych w ZPD Bałcyny na glebie płowej, wytworzonej z gliny piaszczystej, oceniono reakcję pszenicy ozimej na 38−46-letnią monokulturę w warunkach różnej chemicznej ochrony łanu: 0 – bez ochrony; *H* – ochrony przed chwastami i HF – ochrony przed chwastami i chorobami. Obiektem porównawczym była pszenica ozima w 6-polowym płodozmianie: burak cukrowy – kukurydza − jęczmień jary – groch siewny – rzepak ozimy – pszenica ozima. W płodozmianie średnia wydajność ziarna pszenicy ozimej wyniosła 7,72 t ha^{-1}. Uprawa w 38–46-letniej monokulturze obniżyła plonowanie pszenicy ozimej średnio o 37%. Największy spadek produkcyjności uzyskano w obiekcie bez ochrony – 61%. Zastosowanie herbicydów ograniczyło te straty do 25%, a łączna aplikacja herbicydów i fungicydów do 27%. Gorsze plonowanie pszenicy ozimej w monokulturze ma swoje uwarunkowania w przerzedzeniu obsady kłosów na 1 m^2 oraz w zmniejszeniu masy ziaren z kłosa i masy 1000 ziaren.

Introduction

The crop rotation in a field production of plants is a basic yield-protecting factor against a mass appearance of pests and at the same time it stabilizes plant yielding. The species and biological diversity of cultivated plants are accompanied by variable in years, and appropriate agrotechnology. It ensures high production potential of soil, good health, low weed infestation degree, and good and stable yielding level for plants. When we give up natural interest of a correct crop rotation in favour of cultivation of plants carried out in monocultures, it causes numerous biocoenosis disorders and as the final result the significant decrease of yields (BERZSENYI et al. 2000, JOŃCZYK and KAWALEC 2001, LEMAŃCZYK 2002, BLECHARCZYK et al. 2004, WOŹNIAK 2006, BURACZYŃSKA and CEGLAREK 2008). Among basic cereals taking into account their response on continuous sowing in a monoculture, which was measured by grain yield decline, the winter wheat was put first (NORWOOD 2000, ADAMIAK 2007, WESOŁOWSKI et al.

2007). Its great sensitivity to that crop sequence system results from little competitiveness towards weeds (MARSHALL et al. 2003, ADAMIAK 2007) and great susceptibility to stem base disease (LEMAŃCZYK 2002, KUROWSKI and BRUDEREK 2009).

The appearance of herbicides and fungicides significance in reducing damage in yields of winter wheat cultivated for a 38–46-year monoculture was the purpose of that paper.

Materials and Methods

The field experiment on plots concerning the crop of 10 species of plants in a monoculture was set up in autumn 1967 at the Production and Experimental Station in Bałcyny village near Ostróda town. The soil composition was as follows: 2.8–3.2% of fractions with their diameters below 0.002 mm; 30.4–31.9% of fractions with their diameters range – 0.002–0.050 mm, and 64.9–66.8% of fractions with their diameters range 0.050–2.00 mm, respectively. Till 1992 the cultivation of 10 species of plants in two 5-field crop rotations was as a comparative object. During the growing season 1992/1993 the winter triticale and pea were introduced and from that time two 6-field crop rotations were as a comparative object.

This paper presents results of a 9-year experiment (2005–2013) concerning winter wheat response on a 38–46-year monoculture.

Before starting the present researches, the soil was characterized by the following chemical properties: pH value in 1M KCl solution was equal 5.7–6.0; the organic carbon content amounted to 0.90–0.96%, the content of available microelements as follows: phosphorus – 8.9–9.6 mg; potassium – 15.0–20.7 mg; calcium – 48–56 mg, and magnesium – 6.4–7.0 mg in the sample of 100 g of the soil.

The following research factors in 3 replications in this experiment have been considered:

I. Crop sequence system:

a) the cultivation of winter wheat in 6-field crop rotation: sugar beet – maize – spring barley – pea – winter rape – winter wheat;

b) The cultivation of winter wheat in a 38–46-year monoculture.

II. Chemical protection.

0 – without protection;

H – protection from weeds;

HF – Protection against weeds and diseases.

Weeds were controlled with the following herbicides: Cougar 600 SC (a.s. – diflufenican + isoproturon) – 2005, 2006 and 2007; Chisel 75 WG

(a.s. – thifensulfuron-methyl + chlorosulfuron) + Trend 90 EC (a.s. – isodecyl alcohol) – 2007; Dicuran Forte 80 WP (a.s. – chlorotoluron + triasulfuron) – 2008; Granstar 75 WG (a.s. – tribenuron-methyl) + Puma Universal 069 EW (a.s. – fenoxaprop-P-ethyl + mefenpyr-diethyl) – 2005, 2006 and 2008; Lancet Plus 125 WG (a.s. – piroxysulam + aminopyralid + florasulam) + Atpolan 80 EC (a.s. – paraffin oil) -2009; Maraton 375 SC (a.s. – pendimethalin + isoproturon) – 2009 and 2010; Komplet 560 SC (a.s. – diflufenican + flufenacet) – 2011, 2012 and Legato Pro 425 SC (a.s. – diflufenican + chlorotoluron) – 2013.

Fungal diseases were controlled through 2-3-fold spraying in the growing season of winter wheat with the following fungicides: Alert 375 SC (a.s. – fusilazole + carbendazim); Amistar 250 SC (a.s. – azoxystrobin); Amistar 250 SC + Artea 330 EC (a.s. – ropioconazole + cyproconazole); Capalo 337,5 (a.s. – fenpropimorph + metrafenone + epoxiconazole) and Fandango 200 EC (a.s. – prothioconazole + fluoxastrobin). Winter wheat was sown in the quantity of 500 sprouting grains per 1 m^2 between 14^{th} and 24^{th} September of every year.

Mineral fertilization was identical in both crop sequence systems and amounted to 238 kg ha^{-1} of NPK (N-120, P-35 and K-83). Every 3 years the winter wheat monoculture was additionally fertilized with manure, i.e. 15 t ha^{-1} to balance an organic fertilization applied in a crop rotation prior to sugar beet (30 t ha^{-1}).

In the analysed 9-year experiment, the influence of its factors on the yield of grains from one hectare, and the number of ears on the surface of 1 m^2, length of an ear, the number and a mass of grains from an ear, the mass of 1.000 grains and the contribution of small than 2,0 mm of diameter have been carried out.

Rainfalls in the summer and autumn period and their distribution were favourable for winter wheat (Table 1, Table 2). Such conditions allowed preparing carefully the soil and carrying out sowing on time as well as on the even emergence and good initial growth of winter wheat. As regards wintering of crops in the case of all growing seasons were good, except for the growing season 2009/2010. In the last growing season the winter wheat after wintering was thinned out and weakened. In stages of the biggest demand for water, the rainfalls were optimal in general. The discussed growing seasons differed in thermal conditions. In comparison with the long-term average values (1961–2000), warmer growing seasons were the years 2006/2007 (the warmest); 2007/2008 and 2011/2012; colder ones the years– 2005/2006 and 2010/2011.

Table 1

Mean air temperature [°C] in the years 2004–2013
(data according to the Research Station at Bałcyny)

Year	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Av. IX–VIII
2004/2005	13.0	9.2	2.4	2.3	0.6	-3.2	-1.4	7.7	12.5	14.9	18.9	16.8	7.8
2005/2006	15.3	8.3	2.8	-1.1	-8.7	-3.3	-2.5	7.8	12.5	16.0	21.0	17.3	7.1
2006/2007	15.7	10.1	5.6	4.2	2.4	-2.0	5.4	7.3	13.7	17.5	17.5	18.2	9.6
2007/2008	12.6	7.4	1.0	0.4	0.7	2.3	2.9	7.8	12.3	16.6	18.3	17.8	8.3
2008/2009	11.8	8.7	4.0	-0.1	-3.7	-1.5	1.9	9.7	12.2	14.7	18.9	18.5	7.9
2009/2010	14.7	5.9	5.2	-1.7	-8.9	-2.9	2.1	7.9	12.0	15.7	20.8	19.3	7.5
2010/2011	12.2	5.3	4.4	-6.9	-1.6	-6.1	2.0	9.7	13.6	17.5	18.0	18.1	7.2
2011/2012	14.6	8.6	3.1	2.4	-2.0	-7.5	3.5	8.4	13.9	15.2	19.0	17.9	8.1
2012/2013	14.0	7.9	4.9	-3.3	-4.5	-0.8	-4.0	6.3	15.0	17.4	17.9	17.4	7.4
1981–2012	13.0	8.1	2.8	-1.0	-2.4	-1.6	1.8	7.7	13.2	15.8	18.3	17.7	7.8

Table 2

Sum of rainfall [mm] in the years 2004–2013
(data according to the Research Station at Bałcyny)

Year	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sum IX–VIII
2004/2005	41.9	77.6	27.8	39.5	50.3	21.4	29.3	22.0	68.2	35.4	83.9	39.6	537
2005/2006	17.9	19.3	31.1	82.9	15.3	26.7	3.1	24.2	93.2	83.5	27.1	141.7	566
2006/2007	105.6	34.3	107.3	60.0	110.2	14.6	27.9	26.8	79.7	60.8	176.5	81.0	885
2007/2008	65.4	48.9	50.0	9.0	30.8	33.9	47.1	33.8	48.4	27.8	47.0	103.1	545
2008/2009	17.0	104.6	40.5	29.4	16.2	14.7	68.0	3.7	89.6	133.1	82.2	25.7	625
2009/2010	15.6	58.5	40.8	29.6	13.3	14.2	23.8	9.4	105.5	73.7	87.8	99.3	572
2010/2011	45.0	11.2	110.4	39.2	29.6	20.5	8.6	33.7	41.5	56.2	171.9	83.6	651
2011/2012	38.9	29.9	9.6	46.0	87.7	24.9	21.3	44.7	42.5	107.2	11.2	25.7	490
2012/2013	41.0	57.6	48.5	15.1	34.6	21.3	14.0	22.5	46.2	45.4	163.8	37.6	548
1981–2012	56.2	51.2	46.1	42.6	30.1	23.1	30.7	29.8	62.3	72.9	81.2	70.6	597

The results were statistically processed with STATISTICA 13.0 software (StatSoft, Tulsa, Oklahoma, USA). The statistical calculations were performed using a two-way ANOVA. Coefficients of linear correlation (Pearson's r) were calculated.

Results

In the crop rotation without chemical protection in the period 2005–2013, the average yield of winter wheat grains amounted to 6.88 t ha^{-1} (Table 3). Nevertheless in particular years it was strongly diversified, the highest yield took place in 2008 while the least one in 2012. In the case of unprotected monoculture in the tested 9-year experiment, the average yield amounted to 2.65 t ha^{-1} of grains i.e. by 61% less than in the analogical object of crop rotation. The biggest yield decline i.e. by 84% was stated in 2007, in the 40th-year of winter wheat cultivation in a monoculture. After this deep decrease, in the next 2008 the yield in the monoculture has increased up to 57% of a crop rotation yield but in the next 2009 it has decreased by 65%.

In the years 2010 and 2011 the productivity of a monoculture has stabilized on the level 48% and 45% of the crop rotation yields, while in two last years of researches the aforementioned yield level amounted to 25% and 28% only of that what took place in the crop rotation. In essence according to a trend of yielding in the years 2005–2013 (Figure 1) with a prolonged time of winter wheat crop in a monoculture, differences in yielding of winter wheat in comparable crop sequence systems expanded for the worse of the monoculture.

The application of protection from weeds (*H*) significantly reduced the negative effect of a monoculture on winter wheat yielding. According to data in Table 3 in the case of this monoculture object, the yields of winter wheat depending on a year of researches were by 12–58% lower than in the case of crop rotation treated with herbicides and were on average by 25% less. Reducing losses in yields of a monoculture on the aforementioned object was the result of a higher increase of wheat productivity in the monoculture than in the case of crop rotation through the influence of herbicides activity. First of all herbicides significantly have reduced the competition of such weeds as: *Apera spica-venti*, *Centaurea cyanus*, *Galium aparine*, *Matricaria perforata* and *Veronica hederifolia* that numerously occurred on a plants canopy of the unprotected monoculture (data not shown). The final result of herbicides application has increased winter wheat yielding in a monoculture in the range from 61% up to do 202%, on average by 123% (i.e. by 3.25 of t ha^{-1}). In the crop rotation, the yield-creative effect of herbicides varied from its negative value i.e. (-8%) up to the maximum level i.e. 43%. The average value of the yield increase within the 9-year assessed time amounted to 14% (1.0 t ha^{-1}). Despite better yield--creative activity of herbicides in the monoculture they were not able to stop that decreasing trend (Figure 1). Therefore in the case of this object, the difference in winter wheat yielding has also expanded for the worse of the monoculture with its continuation.

Table 3

Grain yields of winter wheat [t ha^{-1}]

Year	Crop rotation				Monoculture				Monoculture in relative to crop rotation [in]				$LSD_{0.05}$**
	0*	*H**	HF*	means	0	*H*	HF	means	0	*H*	HF	means	
2005	6.55	8.19	8.26	7.67	2.92	6.88	6.91	5.57	44.6	84.0	83.7	72.6	I – 0.23; II – 0.26; I × II – 0.37
2006	7.45	8.00	8.92	8.12	3.30	6.47	6.71	5.49	44.3	80.9	75.2	67.6	I – 0.24; II – 0.13; I x II – 0.18
2007	7.22	6.64	7.75	7.20	1.16	2.77	2.74	2.22	16.1	41.7	35.4	30.8	I – 0.26; II – 0.22; I x II – 0.53
2008	8.46	8.76	8.35	8.52	4.80	7.75	8.10	6.88	56.7	88.5	97.0	80.8	I – 0.10; II – 0.46; I x II – 0.35
2009	6.21	6.40	6.62	6.41	2.14	5.41	5.63	4.39	34.5	84.5	85.1	68.5	I – 0.53; II – 0.13; I x II – 0.18
2010	5.31	6.86	7.58	6.58	2.57	5.64	6.36	4.86	48.4	82.2	83.9	73.9	I – 0.61; II – 0.23; I x II – 0.33
2011	7.63	9.81	10.55	9.33	3.44	8.13	8.05	6.54	45.1	82.9	76.3	70.1	I – 0.67; II – 0.51; I x II – 0.72
2012	5.94	8.51	8.86	7.77	1.46	3.93	3.94	3.11	24.6	46.2	44.5	40.0	I – 0.39; II – 0.17; I x II – 0.32
2013	7.18	7.63	8.83	7.88	2.02	6.11	6.49	4.87	28.1	80.1	73.5	61.8	I – 0.26; II – 0.26; I x II – 0.37
means	6.88	7.87	8.41	7.72	2.65	5.90	6.10	4.88	38.5	75.0	72.5	63.2	I – 0.56; II – 0.48; I x II – 0.54

* 0 – without plant protection; *H* – protection from weeds; HF – protection from weeds and disease

** $LSD_{0.05}$: I – crop sequence system; II – plant protection; I × II – interaction

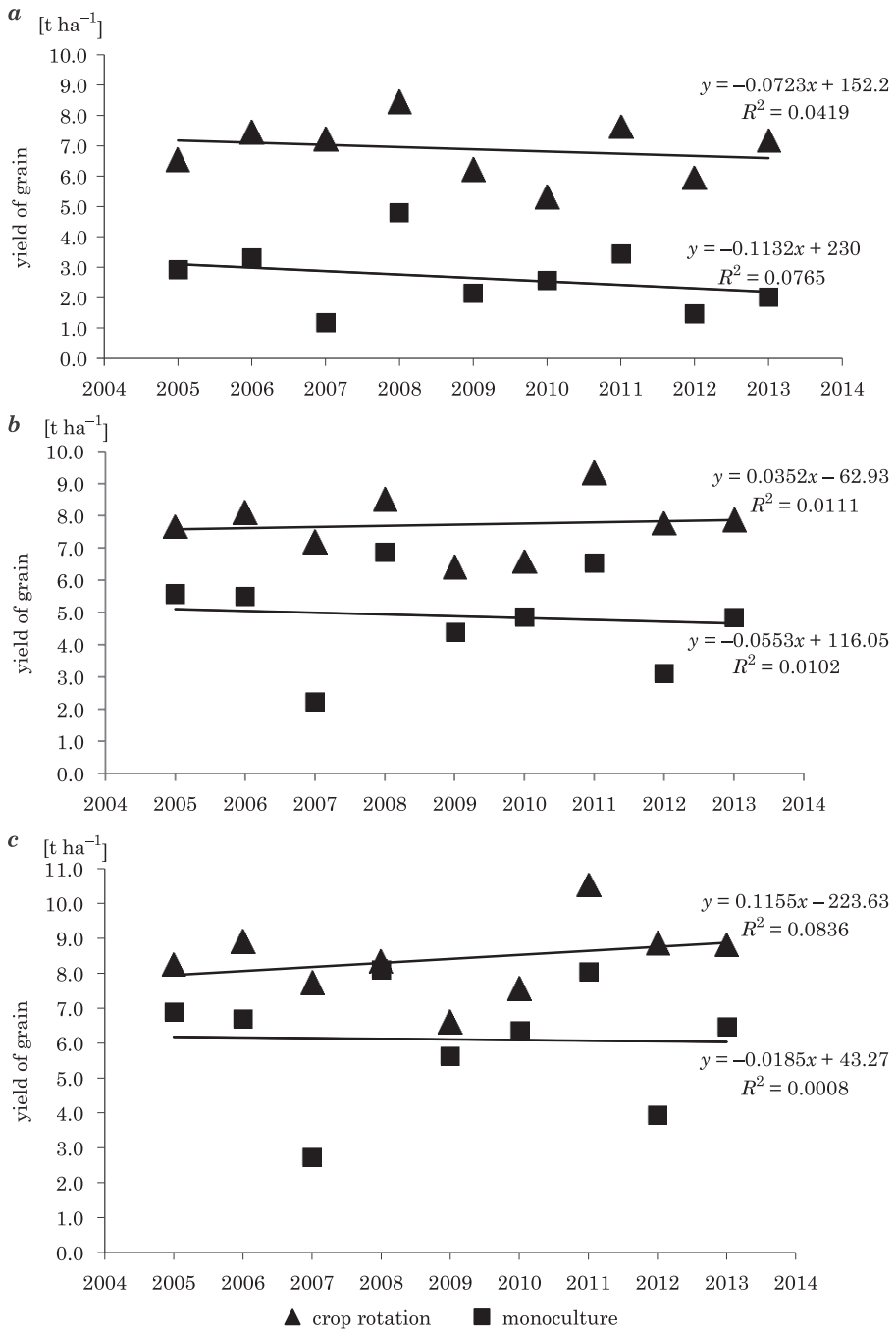

Fig. 1. Yield grain trends of winter wheat in 2005–2013: *a* – without protection (0); *b* – protection from weeds (*H*); *c* – protection from weeds and disease (*H* + *F*)

The combined application of the protection against weeds and diseases (HF) has reduced the decrease of winter yield in the monoculture in particular years of its crop in the range 3–65%, on average by 27% (Table 3). So the function of the protection variant in order to compensate the negative position because of a monoculture has appeared less effective than in the case of the application only herbicides. It results from the fact that fungicides applied in the monoculture in relation to the object treated solely with herbicides have increased the productivity of winter wheat in average by 3.4% (by 0.20 of t ha^{-1}). The significant and positive effect of herbicides has been stated in the years 2006, 2008, 2009, 2010 and 2013. In the crop rotation the yield-creative effect of fungicides has caused the capacity increase of winter wheat i.e. on average by 6.9% (by 0.54 of t ha^{-1}). Except for 2005 and 2008 in all remaining growing seasons the fungicides significantly increased winter wheat productivity. Better efficiency of fungicides in the crop rotation was not the surprise because in that position both thick and close density of stalks created more favourable condition for diseases of leaves and ears occurrence than a thinned out canopy of a monoculture. The applied fungicides were very effective while they weakly reduced the occurrence of stem base diseases that more strongly infected winter wheat in the monoculture. The analysis of the winter wheat yielding trends in the discussed years on the aforementioned protected object has shown increased tendency in crop rotation and practically invariable in the case of monoculture (Figure 1). It meant that also on that object of protection, the difference in yields of winter wheat among positions minimally increased in favour of the crop rotation with the time of the experiment continuation. In total, regardless of the protection levels, the 38–46-year winter wheat monoculture has shown on average 37% of yielding decrease. In individual years the decrease of yields varied from 19% in 2008 to 69% in 2007.

The yielding of winter wheat in crop rotation considering all protection levels was significantly correlated with the weight of 1.000 grains, and additionally on the object without protection was correlated with the number of weeds per 1 m^2 and the weight of grains of an ear. As regards the objects chemically protected it was also correlated with the number of ears per 1 m^2 and the contribution of small grains (Table 4). In the case of a monoculture a significant effect on winter wheat yielding on all objects had the weight of weeds per 1 m^2, the mass of grains from an ear, and the weight of 1.000 grains while in the case of the object without protection such effect had the number of weeds and the number of ears per 1 m^2. The crop sequence system the most strongly diversified the number of ears per 1 m^2 and next the mass of grains of an ear and the mass of 1.000 grains (Table 5).

Table 4

Simple correlation factors of winter wheat yields vs. weed infestation and with elements of structure and biometry, average values for 2005–2013

Specification	Crop rotation				Monoculture			
	0*	*H**	HF*	means	0	*H*	HF	means
Number of weeds per 1 m^2	-0.46	ns	ns	ns	-0.73	ns	ns	-0.58
Weight of weeds per 1 m^2	ns	ns	ns	ns	-0.41	-0.43	-0.43	-0.42
Number of ears per 1 m^2	ns	0.57	0.52	0.50	0.44	ns	ns	ns
Number of grains in ear	ns	ns	ns	ns	ns	ns	ns	ns
Weight of grains per ear [g]	0.48	ns	ns	ns	0.46	0.55	0.49	0.53
Weight of 1000 grains [g]	0.52	0.66	0.58	0.67	0.56	0.51	0.51	0.55
Unripe grains [%]	ns	-0.72	-0.41	-0.52	ns	ns	ns	ns

*0 – without plant protection; *H* – protection from weeds; HF – protection from weeds and disease

Table 5

Crop yield structure and biometry of winter wheat, average values for 2005–2013

Specification	Plant protection	Crop rotation (CR)	Monoculture (M)	Relative values M : CR [%]
1. Number of ears per 1 m^2	0*	555	397	72
	*H**	610	572	94
	HF*	639	557	87
2. Ear length [mm]	0	77	76	99
	H	81	80	99
	HF	80	80	100
3. Number of grains in ear	0	32	28	88
	H	34	35	103
	HF	34	36	106
4. Weight of grains per ear [g]	0	1.36	1.05	77
	H	1.48	1.34	91
	HF	1.50	1.45	97
5. Weight of 1000 grains [g]	0	42.3	36.7	88
	H	41.3	37.0	90
	HF	42.8	40.1	94
6. Unripe grains [%]	0	1.1	2.0	182
	H	1.3	2.3	177
	HF	1.1	1.7	155

$LSD_{0.05}$ for:	1	2	3	4	5	6
I. Crop sequences system:	51	ns	ns	0.12	1.1	0.5
II. Plant protection:	52	2.2	1.4	0.13	1.5	ns
III. Interaction I × II:	73	2.9	1.9	0.19	1.8	ns

*0 – without plant protection; *H* – protection from weeds; HF – protection from weeds and disease

The aforementioned parameters have achieved the least values on unprotected object of a monoculture. They were by 12–28% smaller than on the analogical object of a crop rotation. Intensification of chemical protection has decreased differences among positions because herbicides

and fungicides more favourably increased their values in the case of a monoculture than in the case of a crop rotation. It was the most visible in the formation of the number of grains in an ear and the mass of grains of an ear as well as in density of ears per 1 m^2. In essence, the chemical protection has decreased differences between crop rotation systems in the case of discussed parameters up to 0–13%.

Discussion

In a light of scientific literature, the decline in this study of winter wheat yielding was by 37%, on average in its 38−46-year monoculture and it should be recognised as a big one. Most of researches admittedly carried out in a shorter time (10–17-year) of monocultures have informed about less decline of winter wheat yield i.e. in the range 13–27% (PANSE et al. 1994, PARYLAK and PYTLARZ 2013). In turn, in researches carried out by BERZSENYI et al. (2000) in a 38-year monoculture, the decline of winter wheat grain yield depending on the fertilization level varied in the range 24–40%. In the case of the oldest in the world monoculture of wheat in Rothamsted carried out in the years 1979–1984, its average decline of yields amounted to 10% (POULTON 1996) while in the years 1985–1990 that decline amounted to 22% (JOHNSTON 1994). The aforementioned papers have shown the declines of yields in a monoculture apart from its continuation time depended on yield conditions, agrotechnology factors as well as weather course in subsequent years. Especially variability of weather course caused these declines of yields in a monoculture duration taking on the character of fluctuation (SIELING and HANUS 1990). It has been confirmed by previous results of carried out researches presented in the current paper. The average winter wheat yield decline in 6–15-year monoculture amounted to 36% (ADAMIAK 2007).

The negative response of winter wheat on its crop in a monoculture forces to the application of different soil sickness preventing factors inhibiting the yield declines. From among factors compensating the faulty position, the intensification of mineral and organic fertilization was applied the most often (JOHNSTON 1994, POULTON 1996, BERZSENYI et al. 2000, ADAMIAK et al. 2002, KIRKEGAARD et al. 2008). In the carried out own researches, the chemical protection from weeds and fungal diseases served as a compensating factor. The application of herbicides due to the competition elimination of weeds has increased the yields of wheat in a monoculture by 123% while in the case of a crop rotation by 14% only. As a result of that application the regress of wheat yielding has limited from

61% to 25% on the object without protection. So the anti-tired activity of herbicides amounted to 36%. In previous researches carried out by ADAMIAK (2007) the compensating activity of herbicides has achieved 8–19%.

The intensification of that protection through additional application of fungicides gave worse different soil sickness preventing factors effect than the application of herbicides only. That protection variant (herbicides + fungicides) has reduced losses of wheat yields by 34% compared to the object without protection. However, taking into account the activity of fungicides solely, it should be stated they did not play any anti-tired role in a monoculture, minimum even (by 2%) they reduced compensating effect of herbicides. It results from the fact that thanks to the effective control of diseases of leaves and ears by fungicides, they more raised yields of wheat in the case of crop rotation than in the case of monoculture. Other authors have shown that the application of fungicides did not have any effect on the decrease of yields in a monoculture (PANSE et al. 1994) or limited the regress of wheat yielding only by 1–3% (SIELING and HANUS 1990).

Lower yielding of cereals in the monoculture than in the case of crop rotation has been caused by deterioration in phytometric factors, especially the density of ears on 1 m^2 and the number of grains from an ear, and a smaller weight of 1.000 grains (PARYLAK and PYTLARZ 2013). In presented researches, especially the monoculture without protection has shown the biggest reduction of ears density on 1 m^2 and then the mass of grains from one ear and the weight of 1.000 grains.

Conclusion

1. The cultivation in a 38–46-year monoculture has reduced the productivity of winter wheat by 37% on average. The biggest yield decline took place in the case of unprotected object of monoculture i.e. 61%. The chemical protection from weeds has decreased the damage to 25% while the combined protection against weeds and diseases has decreased the damage to 27%.

2. The chemical protection had significant effect on yielding of winter wheat in the case of monoculture. In this position the application of herbicides has increased yields of winter grains by 123% (i.e. by 3.25 t) while in the case of combined application of herbicides and fungicides by 130% (i.e. by 3.45 t). In the case of a crop rotation the increase of winter wheat yield amounted to 14% and 22% respectively.

3. Crop sequence system diversified the parameters of winter wheat yield structure. The long-term crop in a monoculture has caused the biggest decrease of ears density per 1 m^2 (by 15%), a little bit smaller in the case of the weight of grains from an ear (by 12%) and the mass of 1.000 grains (by 10%). The significant and positive effect of chemical protection on the formation of the aforementioned parameters in winter wheat monoculture has been proved.

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VARIABILITY OF SOIL PROPERTIES IN CONDITIONS OF THE SUSTAINABLE SOIL UTILIZATION IN CARPATHIAN REGION*

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Key words: Carpathian region, ecological agriculture, soil properties, sustainable utilization.

Abstract

The work deals with the assessment of the basic physical and chemical soil properties in the conditions of ecological farming system in chosen area of Carpathian region. The aim of this work is based on time variability of soil properties to consider sustainability of ecological farming management and its role in sustainable agriculture and development. The observation was carried out in 1996–2000, 2013–2015 in the farm with the ecological farming system in north--east of Slovakia. The paper confirmed that ecological agriculture has positive influence on chemical and physical soil properties and enables its sustainable utilization.

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Słowa kluczowe: region karpacki, rolnictwo ekologiczne, właściwości gleby, zrównoważone użytkowanie.

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Abstrakt

Praca dotyczy oceny podstawowych fizycznych i chemicznych właściwości gleby w systemie rolnictwa ekologicznego na wybranym obszarze Karpat. Celem pracy jest analiza zmienności czasowej właściwości gleb z uwzględnieniem zrównoważonego zarządzania ekologicznym gospodarstwem rolnym i jego roli w zrównoważonym rolnictwie i rozwoju. Obserwacje przeprowadzono w latach 1996–2000 i 2013–2015 w gospodarstwie z systemem rolnictwa ekologicznego w północno-wschodniej Słowacji.

W badaniach potwierdzono, że rolnictwo ekologiczne ma pozytywny wpływ na chemiczne i fizyczne właściwości gleby oraz umożliwia jej zrównoważone wykorzystanie.

Introduction

There is a need to assess the impact of different farming methods on soil quality and fertility in specific ecoregions. Many authors have been investigated the impact of different managements on soil properties, mainly biological, such as enzyme and microbial indices (BOBUĽSKÁ et al. 2015, CASTILLO and JOERGENSEN 2001, HOSSAIN et al. 2002). Sustainable farming systems in Slovakia is mainly represented by the incorporation of organic fertilizers and crop residues that not only increase soil quality and fertility, but also affect soil organisms (ZAHARIA et al. 2010). Application of organic fertilizers can also significantly increase the level of plant-available nutrients and soil chemical and physical properties that affect soil health and quality. The sustainable soil utilization responds to concrete soil-ecological conditions and is realized in a specific way and intensity that does not affect any negative changes in the soil and on soil properties. The basic principle of the sustainable soil utilization philosophy is in its protection against any nature degradation and human impact (FAUCON et al. 2017, LI et al. 2017, DARWISH et al. 2015). The sustainable development of soil utilization involves protection of the land area at the scale that follows the needs to sustain all soil functions. From the ecological farming point of view on the soil, information about quality changes or soil degradation are very important (JIANG et al. 2017).

To the soil development evaluation, the basics characteristics of the natural environment (physical, chemical and biological) are employed. As the physical soil properties are involved bulk density, porosity, water holding capacity, soil temperature, etc. The chemical properties are characterized by total carbon and nitrogen content, soil pH and nutrients availability.

The paper contributes to knowledge within the problems of changes of soil properties in ecological farming system in time horizon. Therefore, the main objective of this work is a) to determine selected soil properties

(physical and chemical) in the long-term monitoring in less favourable soil-climatic conditions and b) to assess the ability of ecological farming system influence the quality and health of soil ecosystems.

Material and Methods

The research was carried out in 1996–2000 and 2013–2015 at the production conditions on the model farming enterprise Liptovská Teplička (48º57'N; 20º05'E), which has been in the system of ecology agriculture since 1996. The study period was performed at the very beginning of ecological management application on this site with due to show the impact of this system on soil properties after almost 20 years. The studied territory is a part of the National Park Low Tatras. The altitude of the area is 846 till 1492 m a.s.l. From the geomorphologic point of view, the area comes under sub-unit Kráľovohoľské Tatry. Climatic conditions are relatively homogenous. The whole site belongs to mildly cold area, the average daily temperature is higher than 10˚C ranging from 1600–2000 mm and average precipitations of 800–1100 mm. The soil conditions are relatively homogenous. The biggest land area is formed by Cambisol, medium heavy, heavily skeleton mostly under topsoil. The second widespread soil type is Rendzina soil, medium heavy, shallow and skeleton. There is some Histosol in the territory. Mostly of the soil in the area are located on the slopes. The soil samples for determination of physical properties were collected two times per year from six sites, in spring (connected vegetation) and in summer (before the harvest), from the layer 0,5–0,15 m in three replicates.

Bulk density [t m^{-3}] and total porosity [%] were determined in Kopecký cylinder (FIALA et al. 1999) during years 1996–2000 and 2013–2015. The soil samples for chemical properties determination were collected once a year. Soil pH, humus content [%], available Mg, K, P [mg kg^{-1}] (analysed during years 1997–2000 and 2013–2015), total nitrogen content [mg kg^{-1}] (analysed during years 1998, 1999 and 2013–2015) were observed using the common available laboratory methods (FIALA et al. 1999). For time and spatial demonstration of soil properties, the results in 1996–2000 are compared to the results in 2013–2015.

Obtained soil physical and chemical data were tested by mathematical-statistical methods from which analysis of variance and regression analysis were used (the Statgraphics software package).

Results and Discussion

Table 1 and Table 2 show the basic physical and chemical properties of soil in farming system in Liptovská Teplička. Bulk density and total porosity are closely related to each other. Values of porosity correspond with the values of bulk density and there was a strong negative correlation (Table 3) between those two parameters that correspond with several authors (PAUL OBADE and LAL 2014, LIU et al. 2013, KOTOROVÁ 2007). Higher bulk density changes ratio between water and air capacity in behalf of water capacity. Also porosity is lower and in parallel capillary pores ratio is higher. That induce favourable water mode and plant water supply during vegetation (KOTOROVÁ 2007). Therefore, bulk density and porosity values increase. Average values of bulk density in 1996 were higher (1.48 t m^{-3} in spring and 1.46 t m^{-3} in summer) than over than 10 years later (1.07 t m^{-3} in spring and 1.09 t m^{-3} in summer). This parameter stabilized its values over the time horizon and increased total porosity (ranged from 44.18–63.33% in spring and 44.86–59.01% in summer).

Table 1

Basic soil physical properties in the farming system in Liptovská Teplička

Year	Season	Ho* [t m^{-3}]	P** [%]
1996	spring	1.48 ± 0.11	44.18 ± 5.40
	summer	1.46 ± 0.13	44.86 ± 5.35
1997	spring	1.15 ± 0.09	56.68 ± 6.05
	summer	1.22 ± 0.10	53.92 ± 5.96
1998	spring	1.15 ± 0.08	56.65 ± 5.25
	summer	1.12 ± 0.08	57.71 ± 6.10
1999	spring	1.18 ± 0.09	55.68 ± 5.55
	summer	1.28 ± 0.11	51.94 ± 5.20
2000	spring	1.10 ± 0.06	63.33 ± 6.32
	summer	1.15 ± 0.05	56.78 ± 5.84
2013	spring	1.12 ± 0.09	57.75 ± 5.39
	summer	1.14 ± 0.10	56.90 ± 5.98
2014	spring	1.06 ± 0.05	59.76 ± 5.95
	summer	1.09 ± 0.06	59.01 ± 5.87
2015	spring	1.20 ± 0.09	54.78 ± 5.34
	summer	1.22 ± 0.10	54.06 ± 5.67
Mean 1996–2000	spring	1.21 ± 0.15	55.30 ± 6.90
	summer	1.25 ± 0.13	53.04 ± 5.12
Mean 2013–2015	spring	1.12 ± 0.07	57.43 ± 2.50
	summer	1.15 ± 0.07	56.66 ± 2.48

* bulk density; ** porosity

Table 2

Basic soil chemical properties in the farming system in Liptovská Teplička

Year	pH	Humus [%]	Mg [mg kg^{-1}]	N [mg kg^{-1}]	P [mg kg^{-1}]	K [mg kg^{-1}]
1997	6.3 ± 0.06	5.34 ± 0.50	246 ± 25	–	57 ± 4	306 ± 11
1998	6.3 ± 0.06	5.26 ± 0.46	240 ± 22	2932 ± 211	57 ± 6	327 ± 10
1999	6.4 ± 0.00	5.07 ± 0.53	227 ± 34	3023 ± 200	54 ± 6	261 ± 10
2000	6.3 ± 0.06	5.06 ± 0.54	274 ± 40	–	64 ± 9	260 ± 13
2013	6.3 ± 0.04	6.24 ± 0.33	283 ± 35	3680 ± 263	64 ± 8	254 ± 15
2014	6.6 ± 0.00	5.87 ± 0.41	284 ± 45	2223 ± 199	59 ± 5	233 ± 20
2015	6.4 ± 0.03	5.66 ± 0.26	295 ± 44	2085 ± 155	66 ± 8	271 ± 18
Mean 1996–2000	6,3 ± 0.05	5,48 ± 0.21	247 ± 20	2978 ± 64	58 ± 4	289 ± 33
Mean 2013–2015	6,4 ± 0,15	5,9 ± 0.29	287 ± 7	2662 ± 884	63 ± 4	253 ± 19

Table 3

Correlation coefficients (*r*) for relationship of soil physical and chemical parameters

Parameter	Bulk density	Porosity	pH	humus	Mg	N	P	K
Bulk density	–	-0.99**	-0.13	-0.54**	-0.06	-0.13	-0.14	-0.14
Porosity	-0.99**	–	0.13	0.53**	0.06	-0.13	0.14	0.14
pH	-0.13	0.13	–	0.16	0.42*	-0.15	0.54**	0.29
Humus	-0.54**	0.53**	0.16	–	0.26	0.05	0.61**	0.38*
Mg	-0.06	0.06	0.42*	0.26	–	-0.12	0.37*	0.08
N	-0.13	-0.13	-0.15	0.05	-0.12	–	-0.13	0.12
P	-0.14	-0.14	0.54**	0.61**	0.37*	-0.13	–	0.56**
K	-0.14	-0.14	0.29	0.38*	0.08	0.12	0.56**	–

** $P < 0.01$, * $P < 0.05$

Long-term research has shown that ecological farming system regulates bulk density in the soil ecosystem during the years and within the seasons. Soil reaction, which ranged between 6.3 and 6.6, is one of the most important factors of soil fertility, in spite of the fact, that its value is dynamic and changes in dependence on external and internal factors (SHEAHAN et al. 2012). During the research, soil reaction changed on model area only minimally due to ecological agriculture without application of acid mineral fertilizers. On the other hand, application of organic material significantly affects soil reaction and thus maintain soil ecosystem to become sustainable (LI et al. 2014). In acid soil, calcium, magnesium, potassium, phosphorus, or molybdenum may be deficient, and the decomposition of the soil organic matter is slowed down, causing a decreased mineralization of nitrogen (WYSZKOWSKI and SIVITSKAYA 2016, MAGDOFF and VAN ES 2009). Soil reaction also affects the availability of some nutrients (Table 3) and content of heavy metals in soil systems which was also shown in our study.

Organic matter positively influences soil buffer capacity and thus, soil reaction changed only minimal. It is important continuously pay attention on soil reaction, because soil naturally change due to acid atmospheric fallout and calcium taking off by crops. It is less probable that the increase of total nitrogen (2223–3680 mg kg^{-1}) would positively influence the soil fertility. For soils with low productivity, to which also our researched area belongs, non-directly comparable relationship between total nitrogen content and fertility is typical. In soil-ecological conditions of researched area mineralization of nitrogen runs little intensively (optimal temperature for intensive process is 28–30°C). Therefore, within high content of total nitrogen, content of mineral – nitrogen immediately available for plants does not have to be high. Phosphorus is relatively firmly fixed (54–66 mg kg^{-1}) and its content is relatively stable and depends on soil reaction. Therefore content of available phosphorus changed minimally and within the common interval. Content of potassium (233–327 mg kg^{-1}) and magnesium (227–295 mg kg^{-1}) was relatively high during focused period. With the regard to soil granularity, these nutrients can be firmly fixed on soil particles and therefore are not flowed out from plough-land in spite of high rainfalls during the year. Content of humus is changing markedly during the long time period in comparison of two time periods (Table 4).

Table 4

Analysis of variance of soil physical and chemical parameters

Parameter	Source of variability	Degree of freedom	F-value calculated	*P* significance
Bulk density	year	7	8.33	**
Porosity	year	7	8.24	**
pH	year	6	0.82	-
Humus	year	6	1.99	*
Mg	year	6	17.52	**
N	year	6	12.77	**
P	year	6	2.06	*
K	year	6	5.87	**

** $P < 0.01$, * $P < 0.05$

Percentage of humus has increased from 5.06 to 6.24%. The concentration of soil organic matter is corresponding with the average values for Cambisols (Barančíková et al. 2016) and their study also shows the positive effect of ecological farming system on soil ecosystem. Our research confirmed suitability of the area for ecological farming, at the same time the positive influence of the applied system on humus balance in soil during the long term ecological farming management.

Conclusion

Research confirmed that physical soil conditions have positively changed in ecological system of agriculture. Measured values of bulk density and porosity are continuously modified and stabilized during the focused time period. Chemical soil properties (soil reaction, humus, available nutrients, nitrogen) were also markedly changed. Based on the introduced results we can conclude, that stability and sustainable utilization of agroecosystem can be achieved thought biodiversity of maintained areas and by returning of organic matter into soil.

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ALLELOPATHIC MANAGEMENT OF SOME NOXIOUS WEEDS BY THE AQUEOUS EXTRACTS OF *PARTHENIUM HYSTEROPHORUS* AND *CARTHAMUS OXYACANTHA*

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Key words: allelopathy, early seedling growth, germination, phytotoxicity, *Triticum aestivum*, weed management.

Abstract

Weed occurrence in cultivated fields may cause significant losses of agricultural crops as a result of competition and allelopathic stress. In this study, 100 g l^{-1} aqueous extracts of leaves and roots of two wild plants *Parthenium hysterophorus* and *Carthamus oxyacantha* were evaluated for their effect on germination, seedling growth and biomass of commonly occurring weeds in wheat fields (*Chenopodium album*, *Lepidium didymium*, *Phalaris canariensis* and *Rumex dentatus*). Negative allelopathic effect of *P. hysterophorus* and *C. oxyacantha* were observed on test weeds, however, 100 g l^{-1} leaf and root extracts of *P. hysterophorus* had a more drastic effect on the weeds than *C. oxyacantha*. The sensitivity of subject weeds to allelopathic stress were recorded in the order *Lepidium didymium* > *Rumex dentatus* > *Chenopodium album* > *Phalaris canariensis*. The study suggests that *P. hysterophorus* possesses phytotoxic activities and may serve as a potential candidate in natural weed management strategies.

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ALLELOPATYCZNE ZWALCZANIE NIEKTÓRYCH SZKODLIWYCH CHWASTÓW ZA POMOCĄ WODNYCH EKSTRAKTÓW *Z PARTHENIUM HYSTEROPHORUS* I *CARTHAMUS OXYACANTHA*

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Słowa kluczowe: allelopatia, wczesny wzrost siewek, kiełkowanie, fitotoksyczność, *Triticum aestivum*, zwalczanie chwastów.

Abstract

Występowanie chwastów na polach uprawnych może powodować znaczne straty plonów roślin uprawnych w wyniku konkurencji i stresu allelopatycznego. W badaniu oceniano wpływ wodnych ekstraktów z liści i korzeni w stężeniu 100 g l^{-1} dwóch dziko rosnących roślin *Parthenium hysterophorus* i *Carthamus oxyacantha* na kiełkowanie, wzrost siewek i biomasę powszechnie występujących chwastów na polach pszenicy (*Chenopodium album*, *Lepidium didymium*, *Phalaris canariensis* i *Rumex dentatus*). *P. hysterophorus* i *C. oxyacantha* oddziaływały allelopatycznie negatywnie na testowane chwaty. Przy czym ekstrakty w stężeniu 100 g l^{-1} z liści i z korzeni *P. hysterophorus* wykazywały silniejszy negatywny wpływ na chwasty niż z *C. oxyacantha*. Wrażliwość chwastów na stres allelopatyczny wyglądała następująco: *Lepidium didymum*> *Rumex dentatus*> *Chenopodium album*> *Phalaris canariensis*. Z badań wynika, że *P. hysterophorus* ma działanie fitotoksyczne i może być używany w strategiach zwalczania chwastów metodami naturalnymi.

Introduction

Weeds are undesirable plants that appear in cultivated fields and which impart drastic effects on the growth and production of agricultural crops corresponding to more than 30% annual yield losses (WALSH et al. 2012, GABA et al. 2014). In the large agro-farming system, mechanical control of weeds seems impractical and farmers have no choice but to use herbicides for suppression of weeds. However, in recent years increased resistance shown by weeds to herbicides and their effect on ecosystem has drawn grave concerns about the sustainability of agricultural practices (TRANEL and WRIGHT 2002, SINGH et al. 2003, SJOLLEMA et al. 2014). Thus there is an increasing shift in research for working out the natural solution of weed control than the exhaustive use of herbicides.

Allelopathy, which primarily manipulates the release of secondary compounds from certain plant parts with interactive potentials with other plants, seems an ideal natural solution to herbicide application for weed control (JABRAN et al. 2015). Plants which possess allelopathic properties may serve as ideal sources for weedicides compounds of natural origin. WESTON (2005) outlined that all plants possess diverse allelochemicals with structural and functional diversity and they may be potentially exploited as herbicides. In earlier studies, allelopathic suppression of different weeds by sorghum extracts (CHEEMA and KHALIQ 2000), turnip-rape mulch (PETERSEN et al. 2001), barley and rye (DHIMA et al. 2006), rice, mustard, sorghum and sunflower (JABRAN et al. 2010), rye mulch (SMITH et al. 2011), spring vetch, mustard and radish (KUNZ et al. 2016), maize, barley and sorghum (JABRAN 2017), velvet bean (TRAVLOS et al. 2018) and buckwheat (WIRTH et al. 2018) have been well established, canary grass (*Phalariscanariensis*), swine cress (*Lepidium didymium*), goosefoot (*Chenopodium album*) and toothed dock (*Rumex dentatus*) are noxious weeds commonly prevalent in fields of cultivated crops particularly in wheat fields. These weeds may serve as potential competitors for resources with cultivated crops and may correspond to significant yield losses. Owing to hazardous effects of herbicide application, ecological friendly approaches are needed to suppress these weeds. The aim of this experiment was therefore to evaluate the allelopathic potentials of two allelopathic plants namely *Parthenium hysterophorus* and *Carthamus oxyacantha* on germination and growth of four weed species.

Materials and Methods

Whole plant specimen of *Parthenium hysterophorus* and *Carthamus oxyacantha* were collected at the flowering stage during 2017 from different fields in Peshawar. Plants were separated into leaves and roots and were dried in shade. Dried materials were finely ground with an electric grinder and sieved through a fine muslin cloth. 100g l^{-1} aqueous extracts of leaf and root parts of *P. hysterophorus* and *C. oxyacantha* were prepared by soaking the appropriate amount of dried powder in distilled water for 24 hours at room temperature (25±2°C).

Viable seeds of four weed species (*Phalaris canariensis, Lepidium didymium, Chenopodium album and Rumex dentatus* were obtained from Weed Science Research Department, Agriculture University Peshawar. 15 seeds of each weed species were put in Petri dishes containing filter paper. The aqueous extracts of dry leaf and roots were separately provided

to Petri dishes @ 5ml. Distilled water (5 ml) was used as control treatment. Treatments were: T1LE (leaf extract of *P. hysterophorus*), T1RE (root extracts of *P. hysterophorus*), T2LE (leaf extracts of *C. oxyacantha*) and T2RE (root extracts of *C. oxyacantha*).Petridishes were kept at room temperature (25±2°C). at Department of Botany, Qurtuba University during September 2017. The experimental arrangement was completely randomized, each Petri dish being replicated five times. Germination percentage, seminal root length, and hypocotyl lengths were recorded in each treated-Petri dish. Seeds were considered as germinated when radicle length attained a length of 2 mm.The experiment lasted for ten days since the initial recording of germination data. 10-day old seedlings were dried in an oven and dry biomass of each weed species was calculated against the applied aqueous extracts. Data analysis was carried out through Excel sheet using Analysis of variance. Least significant difference test was used at $p \leq 0.05$ to differentiate between significant and non-significant results.

Results

Germination of four weeds species in control treated Petri dishes was 100% but significant retardation was observed for each weed species in respect to leaf and root aqueous extract application of the two allelopathic plants. Germination was reduced to 35, 60, 73 and 90% in *L. didymium*, *R. dentatus*, *C. album* and *P. canariensis* respectively by leaf extract of *P. hysterophorus* (T1LE) followed by almost same pattern of inhibition by root extracts (T1RE) which revealed 41, 68, 75, 98% germination of stated weeds (Figure 1).

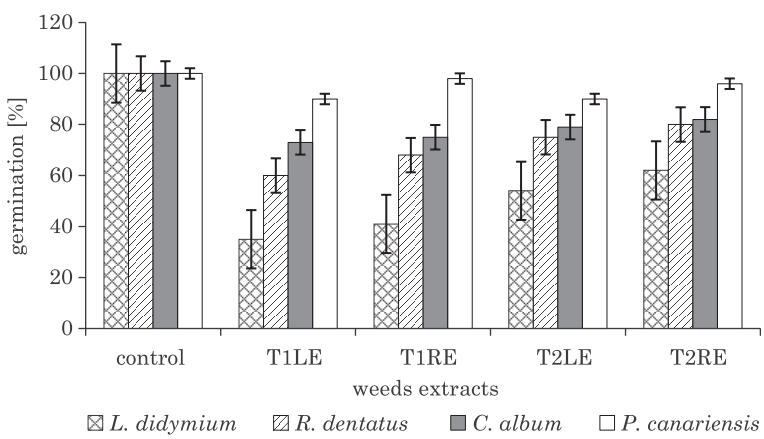

Fig. 1. Effect of aqueous extracts of allelopathic plants on germination four weeds: T1LE (dry leaf extracts of *P. hysterophorus*), T1RE (dry root extracts of *P. hysterophorus*), T2LE (dry leaf extracts of *C. oxyacantha*), T2RE (dry root extracts of *C. oxyacantha*)

Leaf extracts of *C. oxyacantha* (T2LE) resulted in 54, 75, 79 and 90% while root extracts (T2RE) caused 62, 80, 82 and 96% germination in *L. didymium*, *R. dentatus*, *C. album* and *P. canariensis* respectively when compared to control where germination was maximum (100%). It was clearfrom the results that leaf extracts of *P. hysterophorus* were more phytotoxic than other parts and *L. didymium* was the most susceptible species than other weeds.

Seminal root length (SRL) of four weeds showed variation in control conditions as well as in extracts treated conditions. Control condition revealed that maximum SRL (22 mm) was recorded in *P. canariensis* followed by *R. dentatus* (17 mm) while *C. album* and *L. didymium* showed 15 and 12 mm respectively. In each weed species, SRL was significantly lowered by the applied aqueous extracts except for leaf and root extracts of *C. oxyacantha* which did alter the studied parameter to a significant extent. Lowest SRL 4 and 7 mm were observed in L. didymium by leaf and root extracts of *L. didymium* respectively when compared to control 12 mm (Figure 2).

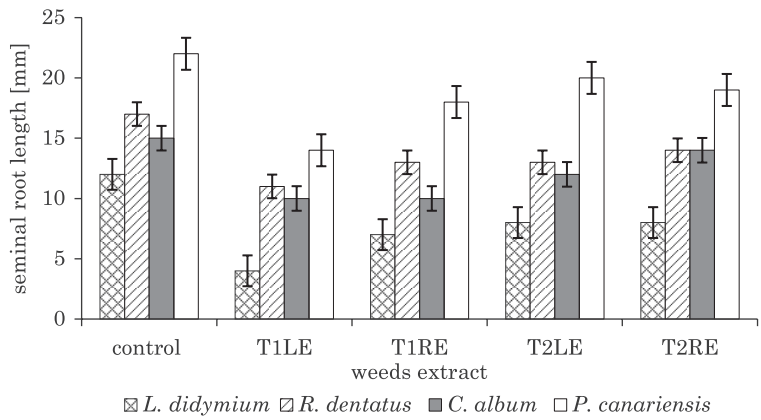

Fig. 2. Effect of aqueous extracts of allelopathic plants on seminal root length (SRL) of four weeds: T1LE (leaf extracts of *P. hysterophorus*), T1RE (root extracts of *P. hysterophorus*), T2LE (leaf extracts of *C. oxyacantha*), T2RE (root extracts of *C. oxyacantha*)

Different results were obtained for hypocotyl growth of four weeds under different treatments. Under control conditions, hypocotyl length for *L. didymium*, *R. dentatus*, *C. album* and *P. canariensis* was recorded as 9, 13, 10 and 18 mm respectively which was drastically reduced to 3,7, 6 and 12 mm by T1LE while to 6,9,7 and 15 mm by T1RE respectively (Figure 3). Leaf and root extracts of *C. oxyacantha* had also a negative effect on this parameter of weeds such as *L. didymium*, *R. dentatus,* and *C. album*, however, T2LE and T2RE had no effects on hypocotyl growth of *P. canariensis* where results were nearly consistent with control treatment. Again, highest phytotoxicity was exhibited by T1LE and maximum resistance was shown by *P. canariensis*.

Results for dry biomass of four weed seedlings are depicted in Figure 4. It was evident that the studied parameter was significantly declined by T1LE and T1RE in all weed species. In contrast, T2LE and T2RE had only drastic effects on dry biomass of *L. didymium* and *R. dentatus* but caused no changes in the other two weeds. Maximum dry biomass 98, 131, 122 and 143 mg for *L. didymium*, *R. dentatus*, *C. album* and *P. canariensis* respectively were found in control which gradually decreased under T1LE and T1RE. On the other hand, T2LE and T2RE were found comparatively less toxic to weeds for their dry biomass when compared to T1LE and T1RE. Profound effects were evident in weed *L. didymium* where dry biomass was reduced to 70 and 78 mg respectively against 98 mg in control (Figure 4).

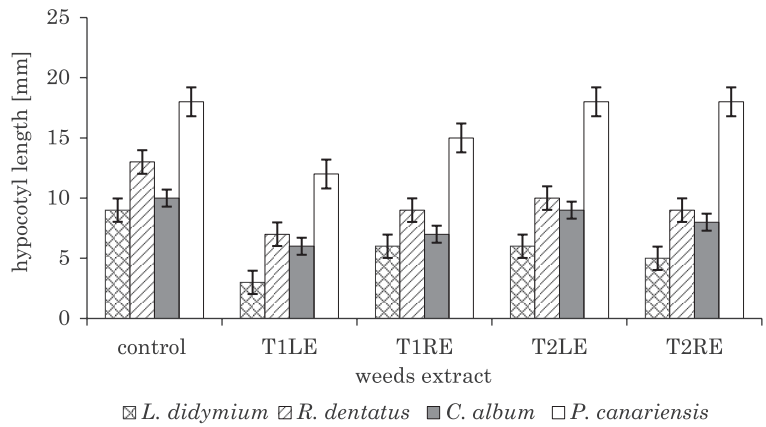

Fig. 3. Effect of aqueous extracts of allelopathic plants on hypocotyl length of four weeds: T1LE (leaf extracts of *P. hysterophorus*), T1RE (root extracts of *P. hysterophorus*), T2LE (leaf extracts of *C. oxyacantha*), T2RE (root extracts of *C. oxyacantha*)

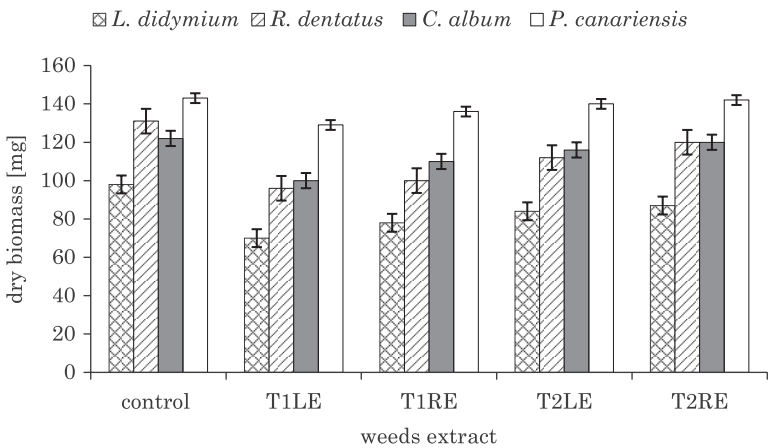

Fig. 4. Effect of aqueous extracts of allelopathic plants on dry biomass of four weeds: T1LE (leaf extracts of *P. hysterophorus*), T1RE (root extracts of *P. hysterophorus*), T2LE (leaf extracts of *C. oxyacantha*), T2RE (root extracts of *C. oxyacantha*)

Discussion

Decreased germination, seminal root and hypocotyl length and retarded biomass of four weeds in response to aqueous leaf and root extracts of *P. hysterophorus* and *C. oxyacantha* suggest that these plants are actively allelopathic. The greater phytotoxic activity of *P. hysterophorus* than *C. oxyacantha* also indicate that former plant possesses toxic and inhibitory compounds presumably in higher concentration in leaves than roots. It may be asserted that potential allelopathic compounds present in *P. hysterophorus* and *C. oxyacantha* were water soluble and capable of causing retardation in germination and other growth characters of test weeds. Germination of plants is affected by many factors. External conditions such as water availability, pH, temperature, and light while internal factors such as the state of embryo, hormones, and enzymes play a key role in seed germination (KOGER et al. 2004, NANDULA et al. 2006). Thus it could be assumed that the imposed allelopathic stress in this study could have influenced one or more factors controlling germination of seeds. Our results are generally supported by earlier works which demonstrated that extracts of *P. hysterophorus* had detrimental effects on seed germination weeds and cultivated crops (BATISH et al. 2002, MAHARJAN et al. 2007, SHIKHA and JHA 2016).

Successful germination of seeds results in the emergence of seminal root and hypocotyl. Seminal root is the first part of the seedling to respond to chemical changes in the prevailing conditions which will influence the growth of hypocotyl as a result of water and mineral absorption. If seminal root finds its surrounding environment appropriate, it will proceed to normal water and mineral uptake resulting in boosted growth which could be observed in hypocotyl as well. However, if the surrounding environment imposes stress conditions, water and mineral absorption capacity of seminal root will be challenged with consequent abnormal growth patterns. In our result, allelopathic extracts of both plants suppressed seminal root length, hypocotyl growth and dry biomass of four weeds which may be assigned to the presence of allelochemicals in extracted parts and their toxic effect on the studied parameters. It may be elucidated that allelochemicals of donor plants cause changes in the permeability of cell membrane of the receiving plants, pH changes in the surrounding of radicle, enzymatic dysfunction and abnormalities in mineral and water absorption which could result in reduced root and shoot length (GATTI et al. 2010, MAJEED et al. 2012, MUHAMMAD and MAJEED 2014, MAJEED et al. 2017, SIYAR et al. 2017a,b). Reduced seminal root and hypocotyl length may also be correlated to reduced dry biomass. In previous studies, suppressed radicle, hypo-

cotyl growth and dry biomass of chickpea and radish (SINGH et al. 2003), sugarcane (FERNANDEZ et al. 2015), different weeds of wheat and rice fields (AFRIDI and KHAN 2015), *Rumex dendatus* and *Avena fatua (*ANWAR et al. 2016) have been observed in response to allelopathic effects of *P. hysterophorus*, although reports on the allelopathic activities of *C. oxyacantha*are lacking in review.

Conclusion

The results of this study conclude that germination, seminal root length, hypocotyl length and dry biomass of *P. canariensis, L. didymium*, *C. album* and *R. dentatus* were significantly retarded by the application of aqueous leaf and root extracts of *P. hysterophorus* and *C. oxyacantha*. The most sensitive weed to the allelopathic stress was found to be *L. didymium* while *P. canariensis* exhibited some tolerance. Leaf and root extracts of *P. hysterophorus* exhibited maximum phytotoxic effects on the studied attributes than and *C. oxyacantha* which suggests its potential role in natural weed management strategies.

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ERYSIPHE FLEXUOSA (FUNGI, ERYSIPHALES) – LIFE STRATEGIES AND THREATS TO CHESTNUT TREES INCLUDING *CAMERARIA OHRIDELLA* (LEPIDOPTERA, GRACILLARIIDAE) PEST IN THE URBAN ENVIRONMENT

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Key words: *Erysiphe flexuosa*, powdery mildew, *Aesculus spp.*, *Cameraria ohridella,* host infestation.

Abstract

This study was aimed at analyzing the occurrence and at monitoring the developmental cycle of *Erysiphe flexuosa* (Peck) U. Braun & S. Takamatsu (syn. *Uncinula flexuosa*) on leaves of chestnut tree in the urban environment in 2013–2014, as affected by the presence of horse-chestnut leaf miner *Cameraria ohridella* Deschka & Dimic. A high occurrence of both these species and high infestation of plants were noted in each study year. Powdery mildew was infesting both *Aesculus hippocastanum* L. and *A. x carnea* Hayne, with a clear preference for the second host, whereas *C. ohridella* occurred mainly on *A. hippocastanum*. The highest number of recorded cases of species presence was found in the samples wherein these organisms cooccurred. It was demonstrated that *E. flexuosa* underwent a complete developmental cycle and accomplished its life strategies in the presence of *C. ohridella*, however pest presence was found to affect the disease index and the number of chasmothecia developed by *E. flexuosa*.

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ERYSIPHE FLEXUOSA – STRATEGIE ŻYCIOWE I ZAGROŻENIE DLA KASZTANOWCÓW Z UWZGLĘDNIENIEM SZKODNIKA *CAMERARIA OHRIDELLA*

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Słowa kluczowe: *Erysiphe flexuosa*, mączniaki prawdziwe, *Aesculus spp.*, *Cameraria ohridella,* stopień porażenia.

Abstract

Celem pracy była analiza występowania oraz prześledzenie cyklu rozwojowego *Erysiphe flexuosa* (Peck) U. Braun & S. Takamatsu na liściach kasztanowca w środowisku miejskim w latach 2013–2014, z uwzględnieniem obecności szrotówka *Cameraria ohridella* Deschka & Dimic. W każdym roku badań notowano wysoki udział tych gatunków i wysokie porażenie roślin. Mączniak prawdziwy atakował zarówno *Aesculus hippocastanum* L., jak i *A. x carnea* Hayne, z wyraźną preferencją drugiego żywiciela, natomiast *C. ohridella* wystąpił głównie na *A. hippocastanum*. Najwięcej notowań dotyczyło prób, w których organizmy te występowały razem. Stwierdzono, że *E. flexuosa* przechodzi pełny cykl rozwojowy i realizuje w pełni swoje strategie życiowe w obecności *C. ohridella*. Jednak obecność owada *C. ohridella* wpływa na indeks chorobowy oraz liczbę owocników tworzonych przez *E. flexuosa*.

Introduction

Powdery mildew of a chestnut tree – *Erysiphe flexuosa* (Fungi, Erysiphales) is an obligatory parasite developing on plants from the genus *Aesculus*. Symptoms of infestation with this pathogen were described on various species of chestnut trees: *A. arguta* Buckl., *A. georgiana* Sarg., *A. glabra* Willd, *A. hippocastanum* L., *A. pavia* L., and *A. x carnea* Hayne. *E. flexuosa* occurs in the North America (GLAWE and DUGAN 2006), it was also described in Eastern Asia (BRAUN 1987) and contemporarily also in Europe, where it had been brought into at the end of the XXth century. This pathogen turned out to be a highly invasive species, fast spreading in many countries (ALE-AGHA et al. 2000, BOLAY 2000, DENCHEV 2008, ING, SPOONER 2002, NALI 2006 ZIMMERMANNOVA-PASTIRČÁKOVÁ and PASTIRČÁK 2002, KISS et al. 2004, MILEVOJ 2004, STANKEVICIENE et al. 2010, TOZLU and DEMIRCI 2010, TALGO et al. 2012). In Poland, first communications concerning the occurrence of *E. flexuosa* have appeared in the year 2002 (ADAMSKA 2002, PIĄTEK 2002). So far, this species has been

recorded in Poland on *A. hippocastanum, A pavia*, *A.* × *carnea* in such cities as: Szczecin (ADAMSKA 2002), Tarnów (PIĄTEK 2002), Lublin (WOŁCZAŃSKA and MUŁENKO 2002), Poznań, Inowrocław, Ciechocinek (WERNER 2007, WERNER et al 2009, KAROLEWSKI et al. 2010), and Olsztyn (SUCHARZEWSKA et al. 2011, 2012). The aforementioned works provide important information not only about the occurrence of *E. flexuosa* but also about its morphological traits, about the presence of a powdery mildew hyperparasite *Ampelomyces quisqualis* Ces., and about the susceptibility of different species of chestnut tree to this parasite.

Apart from powdery mildew, chestnut trees may also be infested by *Guignardia aesculi* (Peck) V.B. Stewart, a fungus inducing the chestnut leaf blotch as well as by a butterfly *Cameraria ohridella* Deschka & Dimic. Considering that the chestnut tree constitutes a permanent and important element of the Polish dendroflora (SENETA 1994), especially much attention has been paid to *C. ohridella* pest being very harmful to chestnut trees, the presence of which in Poland was first documented in the year 1998 (ŁABANOWSKI and SOIKA 1998). This insect feeds on leaves, which leads to damage of the assimilation tissue and, consequently, to tissue death (BARANOWSKI et al. 2002). Fungi and pests may simultaneously colonize the same surface of a leaf and differently act on one another. For instance, it has been demonstrated that the presence of chemical compounds, produced during mycotic infections, affected a reduction in the number of eggs laid by horse-chestnut leaf miner, and that the possibility of mutualistic interactions between these organisms is small (JOHNE et al. 2006, 2008).

In Poland, investigations addressing the issues linked with *Erysiphe flexuosa* parasite and *Cameraria ohridella* pest are mainly focused on their occurrence, evaluation of their harmfulness to a host plant expressed by the diseases index, and on determination of the susceptibility of a chestnut species or variety to infestation (WERNER et al. 2002, DZIĘGIELEWSKA et al. 2005). In turn, little information is available regarding the biology of *E. flexuosa* development in the presence of *C. ohridella*.

The objective of this study was to analyze the life cycle and vital strategies of *Erysiphe flexuosa* on chestnut trees occurring in the urban environment, considering *Cameraria ohridella* as a biotic factor significant for the condition of a host plant.

Material and Methods

Determination of the extent of leaves infestation with *Erysiphe flexuosa* and leaves damage by *Cameraria ohridella*

The study was carried out on the area of the city of Olsztyn on *Aesculus hippocastanum* and *A.* x *carnea* in two vegetative seasons: 2013 (55 stations), 2014 (64 stations). The analyzed trees, at different age, were growing in various parts of the city (communication tracts, parks, neighborhoods). In 2013, samples were collected once – in September, whereas in 2014 the material was collected four times at the beginning of the following months: June, July, August, and September, in order to analyze the developmental cycle of powdery mildew within the entire vegetative season. One sample included 10 leaves collected randomly from the bottom part of a head of each tree.

The following research tasks were planned:

1. Determination of the extent of leaves infestation with *Erysiphe flexuosa* and leaves damage by *Cameraria ohridella.*

2. Analysis of the developmental cycle of *Erysiphe flexuosa* considering the presence of *Cameraria ohridella.*

3. Determination of the odds ratio OR.

The extent of host plant infestation with *Erysiphe flexuosa* was calculated for each sample according to the Mc Kinney's formula (Dynowska 1994):

$$R = \frac{\Sigma(a \cdot b) \cdot 100\%}{N \cdot 4}$$

R – disease index expressed in per cents (index)

$\sum (a \cdot b) \cdot 100\%$ – sum of products obtained by multiplying the number of analyzed organs of plants (a) by the infestation degree (b)

N – total number of analyzed leaves (leaves)

4 – the highest degree of infestation in a five-point scale (from 0 to 4):

– 0 – no infestation;
– 1 – infestation of up to 10% of leaf surface;
– 2 – infestation of 11–25% of leaf surface;
– 3 – infestation of 26–50% of leaf surface;
– 4 – infestation of 51–100% of leaf surface.

Ultimately, the R value considered in the analysis of results was computed based on the arithmetic mean and described as a mean degree of infestation.

In addition, the extent of leaf damage by *Cameraria ohridella* was evaluated for each sample according to a five-point scale adopted by Baranowski et al. (2002):

– 0 – no damage;
– 1 – weak damage (up to 10% of damaged leaf surface);
– 2 – medium damage (10–25%);
– 3 – severe damage (25% to 70%);
– 4 – very severe damage (70%).

In the Results section, arithmetic mean was provided for each sample and described as the mean degree of infestation/ mean degree damage.

Infestation degree and damage degree vs. age of host plant. Analysis of the developmental cycle of *Erysiphe flexuosa* considering the presence of *Cameraria ohridella*

The average degree of host plant infestation with *E. flexuosa* and the average degree of host plant damage by *C. ohridella*. The age of the analyzed tress was determined based on a table developed by MAJDECKI (1980–1986).

An SZX9 stereoscopic microscope (Olympus) was used to determine:

a) developmental stage of *E. flexuosa* (anamorph, teleomorph);

b) number of chasmothecium-type fruiting bodies, mature and immature, per 1 cm^2 of the surface area of each infested leaf in the sample; the number of chasmothecia presented in the Results section was computed based on an arithmetic mean;

c) BX41 optical microscope (Olympus) was used to analyze 10 randomly chosen, morphologically mature chasmothecia; a 3^o scale, adopted in a previous own study (Sucharzewska 2009), was applied to evaluate:

– degree of development of appendages: 0 – chasmothecia without appendages, I – chasmothecia with not fully developed appendages, and II – chasmothecia with fully developed appendages,

– degree of maturity of chasmothecia: 0 – chasmothecia without sacks and ascospores, I – chasmothecia with sacks but without formed ascospores, and II – chasmothecia with sacks and ascospores.

Determination of the odds ratio OR in order to:

– compare the degree of plant infestation with *Erysiphe flexuosa* in the samples with and without horse-chestnut leaf miner. The odds ratio indicates the ratio of the likelihood of severe infestation (> 60%) in group *A* (without horse-chestnut leaf miner) to the likelihood of low infestation in group *B* (with horse-chestnut leaf miner),

– the number of chasmothecia of *E. flexuosa* in the samples with and without horse-chestnut leaf miner. The odds ratio indicates that ratio of the likelihood producing chasmothecia in group *A* (without horse-chestnut leaf miner) to the likelihood of producing chasmothecia in group *B* (with horse-chestnut leaf miner).

Results

In total, there were analyzed 311 samples (3110 leaves) collected from *A. hippocastanum* (246 samples) and *A. x carnea* (45 samples). The presence of both *Erysiphe flexuosa* and *Cameraria ohridella* was noted on chestnut tree leaves in each study year. The percentage of the samples infested with the fungus only reached 13%, whereas the percentage of the samples invaded by the pest only reached 26%. In turn, samples colonized by these two organisms together constituted as much as 59% of all samples (Figure 1). The parasite and the pest showed preferences for different species of chestnut tree: *E. flexuosa* attacked both *A. hippocastanum* and *A. x carnea* with preference for *Aesculus x carnea*, whereas *C. ohridella* was more frequently foraging leaves of *A. hippoccastanum* (Table 1).

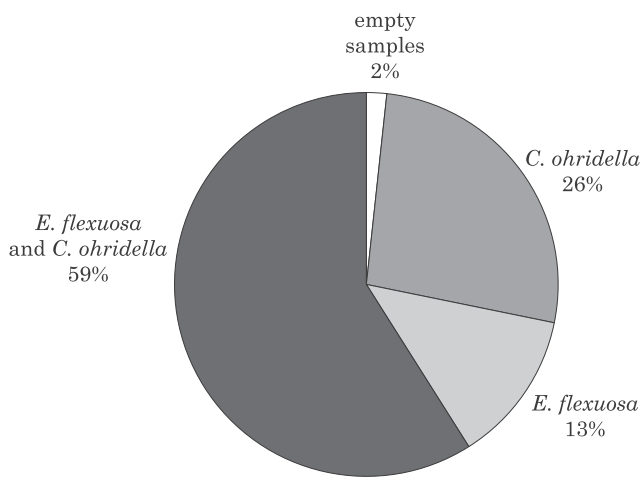

Fig. 1. The percentage contribution of samples with the analyzed species *Erysiphe flexuosa* and *Cameraria ohridella* in the study period (2013, 2014)

Table 1

The percentage contribution of *A. hippocastanum* and *A.* × *carnea* infested by *E. flexuosa* and *Cameraria ohridella* in the study years (2013, 2014)

Plant	2013		2014	
	E. flexuosa	*C. ohridella*	*E. flexuosa*	*C. ohridella*
A. hippocastanum	47%	82%	60%	76%
A. x carnea	100%	11%	100%	22%

When analyzing the mean infestation/damage degree from September of 2013 and 2014, it was found that the plants were to a greater extent damaged by *C. ohridella* – 62% and 73% respectively, whereas in the case

of *E. flexuosa* the disease index reached 28% and 31%, respectively (Figure 2). The study demonstrated the effect of insect presence on the degree of plants infestation by the powdery mildew. If only *E. flexuosa* was noted in the samples, the mean degree of infestation was two times higher than in the samples invaded by both *E. flexuosa* and *C. ohridella* (Figure 3). The odds ratio reached 23.59 for the compared groups. It means that in group A the likelihood of severe plant infestation by *E. flexuosa* was almost twenty four times higher than in the samples with co-occurrence of the fungus and the insect (group *B*).

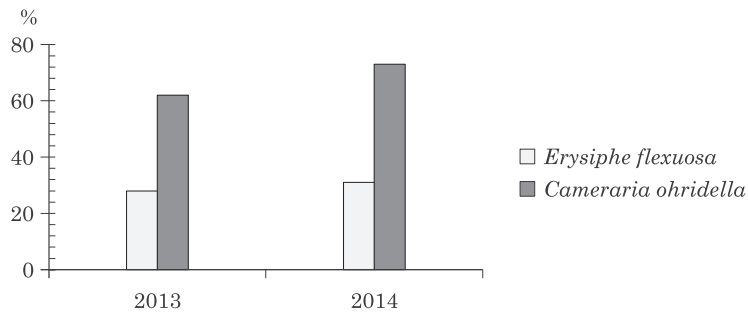

Fig. 2. Mean infestation degree [%] of host plants by *Erysiphe flexuosa* and *Cameraria ohridella* in the study period (2013, 2014)

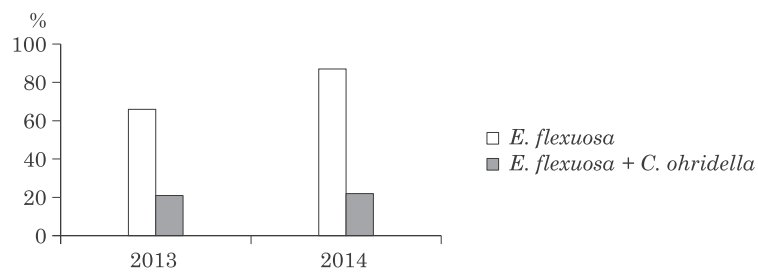

Fig. 3. The effect of the presence of horse-chestnut leaf miner *Cameraria ohridella* on the mean degree of plants infestation by *Erysiphe flexuosa* in study years (2013, 2014)

The age of chestnut trees was demonstrated to affect differences in the degree of their infestation/damage. The powdery mildew caused the strongest infestation of young trees up to 10 years, whereas the insect – the strongest damaged of the oldest trees (Figure 4).

The presence of *E. flexuosa* was noted already at the beginning of June. The percentage of positive samples in this month reached 21% and was successively increasing in the subsequent months – to 78% in September, with the simultaneous, stable and high contribution of *C. ohridella* reaching over 80% (Figure 5).

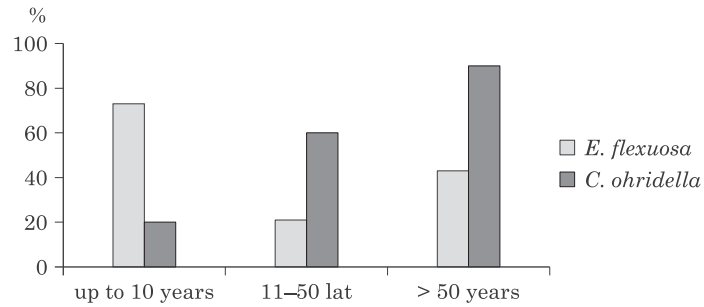

Fig. 4. The effect of tree age on the mean degree of samples infestation by *Erysiphe flexuosa* and *Cameraria ohridella*

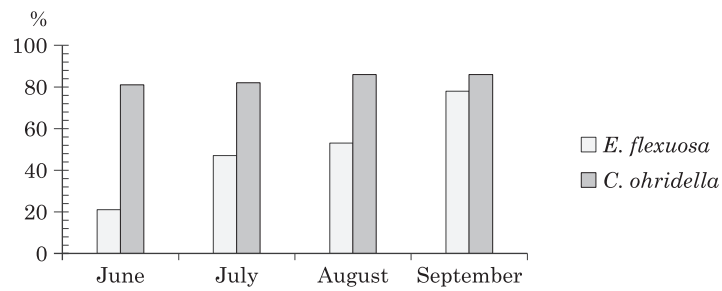

Fig. 5. Percentage of contribution of samples with *Erysiphe flexuosa* and *Cameraria ohridella* in particulars months in 2014

Fungus *Erysiphe flexuosa* was observed to undergo a complete developmental cycle. Its anamorphous and teleomorphous stages were noted in each studied month, also in the samples infested by the pest. Chasmothecia of the parasite were appearing already in June and were produced until the end of the vegetative season, mainly at the bottom part of leaf. The chasmothecia were always at a different developmental stage – from young ones (white, yellow and orange) to mature ones (brown, dark-brown or black). The mature chasmothecia were prevailing through the entire study period (Figure 6). The highest number of chasmothecia was produced in August and September. Differences were observed in the mean number of produced chasmothecia in the samples without horse-chestnut leaf miner (group *A*) compared to the samples attacked by the insect (group *B*). In the samples with co-occurrence of both organisms, the mean number of *E. flexuosa* chasmothecia was lower (Figure 7). The odds ratio calculated for the compared groups reached 1.82, which means that in group A the chance for producing chasmothecia was less than twofold higher than in the samples attacked simultaneously by the fungus and by the insect (group *B*). The highest number of chasmothecia (497/cm^2) was determined on *A. x carnea* in the sample with 100% infestation, without the presence of *C. ohridella*.

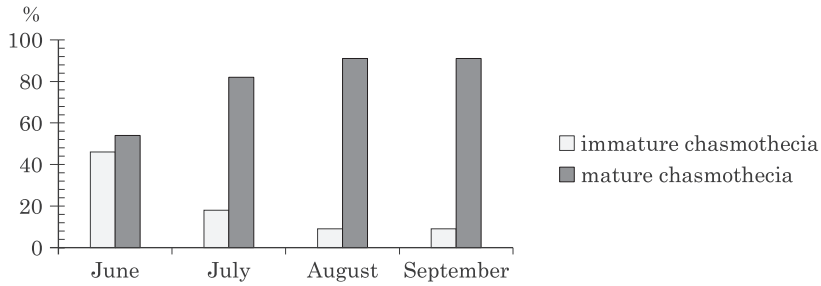

Fig. 6. Mean percentage of *Erysiphe flexuosa* chasmothecia per 1 cm^2 of the surface of infested leaves in particular months in 2014

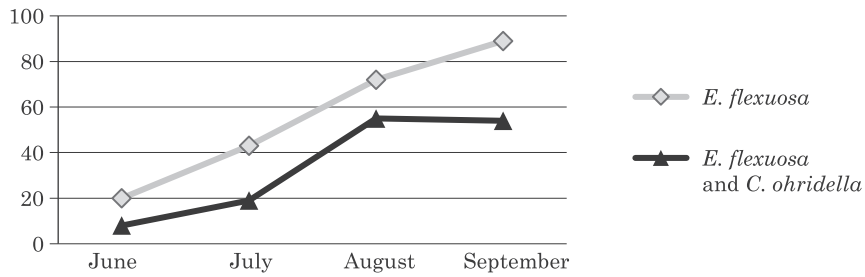

Fig. 7. The mean number of chasmothecia of *Erysiphe flexuosa* with and without *Cameraria ohridella*

Throughout the vegetative season, chasmothecia of *E. flexuosa* had appendages being at various developmental stages, however already in July most of the chasmothecia had fully developed appendages at stage II of development (Figure 8). The process of formation and development

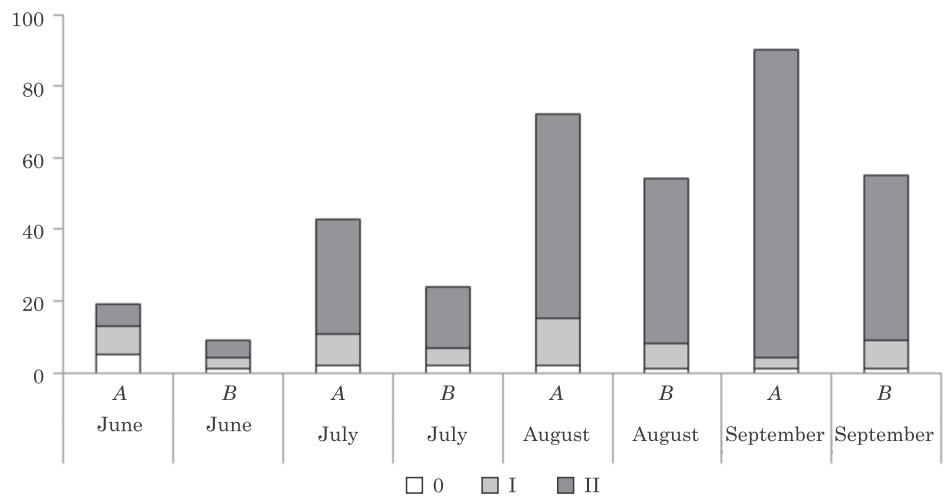

Fig. 8. Mean number of mature chasmothecia of *Erysiphe flexuosa* with appendages in various developmental stages in particular months of the study in the samples infested by the fungus alone (*A*) and in the samples co-infested by the fungus and by the insect (*B*) in 2014 year

of chasmothecia was not impaired by the presence of *C. ohridella*. Asci with developed ascospores were noted already in June. In each studied month, the highest number was determined for the chasmothecia at the II stage of development. Empty chasmothecia, without developed asci nor and spores, constituted 7%.

Discussion

The most important element of the life strategy of parasites, whose diaspores are present in the entire biosphere, is to meet an appropriate host that would ensure their survivability in various environmental conditions (MUŁENKO 1998). An urbicenosis is a type of habitat wherein many species of parasitic fungi fully accomplish their vital roles (BERNADOVIČOVÁ and IVANOVÁ 2008, DYNOWSKA and SUCHARZEWSKA 2005, HOŁOWNIA and KOSTRZEWSKA 1991, JARVIS et al. 2002, SUCHARZEWSKA and DYNOWSKA 2005, SUDNIK-WÓJCIKOWSKA 1998, SUCHARZEWSKA 2009, 2010). It was also confirmed in the presented study, wherein *Erysiphe flexuosa* occurred on chestnut trees in the urban environment in each year of the study. The high prevalence of powdery mildew and a high infestation degree of host plants are indicative of the invasive character of this species. It confirms earlier findings reported by: DZIĘGIELEWSKA et al. (2005), STANKEVICIENE et al. (2010), WERNER et al. (2009, 2012), WOŁCZAŃSKA and MUŁENKO (2002) or KAROLEWSKI et al. (2010). The spreading of powdery mildews results from, i.a., their capability to infect plants in a wide range of temperature and humidity. In most species, the germination of spores may occur even at the minimal relative air humidity, owing to the sufficient water content in the cell. For these reasons, they easily extend their geographical range (BRAUN 1995). An important strategy of parasites is their high reproductive capability, expressed by the formation of structures enabling their spreading, and their ability to survive in unfavorable environmental conditions. In the case of powdery mildews, these roles are played by conidiospores and chasmothecia (BRAUN 1987). Their production throughout the vegetative season allows the parasites accomplishing these strategies. In the reported study, we observed a complete developmental cycle of *E. flexuosa* – the fungus was producing both conidiospores and chasmothecia. According to literature data, powdery mildews undergo the anamorphous stage at the early phase of development, whereas they enter into the teleomorphous stage usually in the autumn period, i.e. at the end of the developmental cycle (SAŁATA 1985). In turn, in our study, the chasmothecia of *E. flexuosa* were beginning to

appear already in June and were produced throughout the vegetative cycle. A similar observation was made for *Erysiphe palczewskii* infesting *Caragana arborescens* (SUCHARZEWSKA and DYNOWSKA 2005), also an extrinsic and invasive species of powdery mildew (MUŁENKO et al. 2010). The tasks of chasmothecia include both producing ascospores as a source of infection as well as surviving winter. The strategy of potentiating and improving reproduction is an important trait of parasites. The production of chasmothecia by *E. flexuosa* over the entire vegetative season is indicative of the vast reproductive potential of this species and perhaps, it provides secondary infections and a high likelihood of surviving unfavorable conditions. It emphasizes the invasive character of this parasite, which from its appearance in Europe has been displaying the capability of undergoing the reproductive stage (ALE-AGHA et al. 2000, PIĄTEK 2002). In most cases, the extrinsic species spreading over new areas are noted at the asexual stage, for example *Erysiphe deutziae* (Bunkina) U. Braun & S. Takam or *Erysiphe russellii* (Clinton) Braun & Takam (BOLAY et al. 2005). In the case of the first, the sexual stage has never been noted, whereas in the case of the latter – already ca. 80 years after its first record (GELJUTA and MARCHENKO 1987). Chestnut trees are host plants also to *Cameraria ohridella*. The reported study confirmed the presence of this dangerous insect. Its percentage contribution in the samples and the degree of plant damage were significantly higher compared to powdery mildew. The overlapping of ecological niches of parasites makes some interactions between organisms likely. These may include competing for habitat and feeding areas or entering into synergistic reactions that result in the establishment of a new system, the so-called "disease complex". Such a phenomenon was observed in the case of the Dutch elm disease, induced by *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* fungi as well as by *Scolytus* spp. and *Hylurgopinus rufipes* bark beetles (after JOHNE et al. 2008). In the case of *E. flexuosa* and *C. ohridella*, works of German scientists demonstrated chemical-and-ecological predispositions for antagonistic interactions during co-colonization of a host plant (JOHNE et al. 2006, 2008). These investigations showed the capability of *C. ohridella* to detect volatile chemical compounds synthesized by fungi (1-Octen-3-ol and 3-octanone) and produced by plants (5-ethyl-2 (5H) –furanone) in response to the microbiological attack, which results in a reduced number of eggs laid by *C. ohridella*. Perhaps, this factor which minimizes the occurrence of horse-chestnut leaf miner on leaves also ensures *E. flexuosa* the possibility of colonizing a healthy, undamaged plant tissue and realizing its vital functions. It could be confirmed by, demonstrated in this study, significantly higher number of the samples with co-occurrence of these organisms (59%) and

production of well-developed chasmothecia with asci and spores by *E. flexuosa*. It is feasible due to the fact that both organisms attacking chestnut trees were noted since the beginning of June, which suggests concomitant development of these parasites at the beginning of the vegetative season. The presence of the analyzed pathogenic factors was also reported in other works (DZIĘGIELEWSKA et al. 2005, JOHNE et al. 2008, WERNER 2007, WERNER et al. 2012). Results of this study and findings of other authors (DZIĘGIELEWSKA et al. 2005, KUKUŁA-MŁYNARCZYK and HUREJ 2007, WERNER et al. 2012) show some preferences of both parasites for infesting different species of chestnut trees. The damage degree of red chestnut trees by *C. ohridella* is very low, whereas *E. flexuosa* attacks both white and red chestnut trees, with a very high occurrence on *Aesculus* x *carnea* (100% infestation in all samples). Such a high degree of plant infestation by this fungus, without the presence of the insect, may be due to both host susceptibility and high availability of a feeding base which is limited when the insect feeds on the same plant. It is indicated by the calculated odds ratio (OR), according to which the degree of leaves infestation by *E. flexuosa* would be significantly higher without the presence of *C. ohridella*.

Conclusions

Results achieved in this study enable concluding that the *Erysiphe flexuosa* fungus fully accomplishes its life strategies in the urban environment. Despite insect's presence *Cameraria ohridella*, the fungus has the possibility of developing and producing a high number of chasmothecia with spores, which – as a result of the sexual process – ensure the parasite the possibility of adjusting to variable environmental conditions that are especially dynamically changing in the urban environment. Therefore, investigations focused mainly on eradication of horse-chestnut leaf miner (BARANOWSKI and DANKOWSKA 2012, KROPCZYŃSKI et al. 2006, ŁABANOWSKI et al. 2008), should also take into account the presence of *E. flexuosa* which poses great threat to chestnut trees considering its invasive character.

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CONTROVERSY OVER DIETARY SOURCES OF CALCIUM

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Key words: bioavailability, calcium, milk, vegetables, bones.

Abstract

This study addresses recent controversies regarding calcium, in view of the dietary intake and sources of protein (animal, vegetable). The results of a meta-analysis, which suggested that milk increases the risk of prostate cancer in men and atherosclerosis in elderly women, were discussed and compared with other research findings. It was demonstrated that prostate cancer is caused by long-term deficiency of vitamin D_3 and vitamin K_2. The bioavailability of calcium appears to be more important than its dietary intake. Various dietary sources of calcium and the health benefits of a balanced diet that adequately meets daily calcium needs were also described.

It was found that plant foods are not good sources of calcium because they contain compounds that limit calcium absorption. Dairy products, in particular full-fat ripened cheese, are the best sources of calcium due to their high calcium content and the presence of vitamins D_3 and K_2.

KONTROWERSJE WOKÓŁ ŹRÓDEŁ WAPNIA W DIECIE

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Abstract

Praca dotyczy kontrowersji wokół wapnia w kontekście ilości białka w diecie oraz źródła jego pochodzenia (zwierzęce, roślinne). Odniesiono się do wyników metaanalizy, w której wykazano, że mleko zwiększa ryzyko raka prostaty u mężczyzn oraz miażdżycy u starszych kobiet. Korzystając z licznej przedmiotowej literatury wykazano, że rzeczywistą przyczyną raka prostaty są długotrwałe niedobory witaminy D_3 oraz K_2. Wynika z tego, że bardziej istotna jest biodostępność wapnia niż jego ilość w diecie. Opisano spektrum prozdrowotnych właściwości diety pokrywającej zapotrzebowanie na wapń. Scharakteryzowano ponadto różne źródła wapnia w diecie człowieka.

Wykazano, że produkty roślinne nie są najlepszym źródłem wapnia z powodu różnych składników ograniczających jego biodostępność. Najlepszym źródłem wapnia, ze względu na jego wysoką zawartość oraz obecność witaminy D_3 i K_2, są pełnotłuste produkty mleczarskie, zwłaszcza sery dojrzewające.

Introduction

The effect of different dietary sources of protein on calcium bioavailability

Until recently, even the most ardent opponents of milk regarded dairy products as a good source of bioavailable calcium. However, this belief has been recently called into question. Attempts are being made to demonstrate that proteins of animal origin decrease calcium absorption by increasing urinary calcium excretion (BRANDOLINI et al. 2005). Various authors have suggested that milk and dairy products increase the risk of vascular calcification and prostate cancer (AUNE et al. 2015, DUARTE-SALLES et al. 2014, GAO et al. 2005). The aim of this review is a comparison of calcium content in selected plant and dairy products including ingredients which reduce or stimulate its bioavailability. In this study an attempt was made to can get recommended amounts of calcium while eliminating dairy products from a diet.

Several authors have postulated that proteins of animal origin lead to the loss of the bone mineral phase, whereas plant proteins enhance calcium absorption (BRANDOLINI et al. 2005, GAFFNEY-STOMBERG et al. 2014). This hypothesis suggests that diets rich in animal proteins and, consequently, sulfur-containing amino acids cause acidification, which allegedly increases urinary calcium excretion and leads to the loss of the bone mineral phase. Sulfur-containing amino acids are catabolized through oxidation to produce acid-forming SO_3^{2-}. The hydrogen ions released during this process are bound by blood buffer systems and are excreted by the kidneys. Buffer systems maintain the pH of blood at a constant level (7.35–7.45) and decrease the pH of urine (below 5.5). Increased urine acidity promotes calcium loss, however, there is no published evidence to indicate that excre-

ted calcium is liberated by increased bone resorption (BRANDOLINI et al. 2005). Previous studies (MUNGER et al. 1999) demonstrated that higher intake of animal proteins decreases the risk of femoral fracture, whereas plant proteins exacerbate that risk. A study of post-menopausal women revealed that urinary calcium loss was not reduced when dietary meat proteins were replaced with soy proteins (MASSEY et al. 2001). In a cohort study, the source of dietary protein was not significantly correlated with the incidence of bone fractures in men or women (MEYER et al. 1997). Recent research (SHAMS-WHITEET al. 2016, LANGSETMO et al. 2015, FENTON et al. 2011) did not present any evidence to support this hypothesis, either. Both vegetable and animal proteins are characterized by considerable buffer capacity. Unlike meat and cereal products, milk contains mainly alkalizing mineral compounds. For these reasons, the presence of sulfur-containing amino acids in animal proteins does not lead to acidification or the loss of skeletal calcium (MASSEY et al. 2003, 2001).

There is no reliable evidence to indicate that plant proteins supersede animal proteins with regard to their influence on calcium metabolism, the risk of bone fracture or osteoporosis. The claim that sulfur-containing amino acids have a detrimental impact on calcium and bone metabolism is completely unfounded (ROUGHEAD et al. 2005). Contrary findings of studies investigating the influence of proteins on bone density and bone susceptibility to fracture could have resulted from variations in the protein content of the analyzed diets.

In a study of young women, a low-protein diet decreased calcium adsorption (18.4%) as compared with a high-protein diet (26.3%). The absorption of dietary calcium from the gastrointestinal tract increased only after 5–9 weeks into the high-protein diet (KERSTETTER et al. 2003). In post-menopausal women (50–75 years), and in men and women younger than 50 years, a high-protein diet increased calcium absorption and transfer to the bones (ROUGHEAD et al. 2005). In elderly subjects whose diets were supplemented with calcium and vitamin D, higher protein intake improved bone mineral density (BMD) (DAWSON-HUGHES and HARRIS 2002). An adequate supply of protein stimulates the bioavailability of calcium because amino acids are involved in calcium transport across the intestinal wall (KERSTETTER et al. 2005). A positive correlation was observed between the dietary intake of plant and animal proteins and the levels of insulin-like growth hormone (IGF-1) which promotes bone growth (RIZZOLI and BONJOUR 2004, BONJOUR 2005).

The risk of bone fracture associated with osteoporosis increases with age. Observations of elderly patients with orthopedic injuries revealed that a high-protein diet contributed to muscle growth and physical reco-

very. High muscle mass and adequate physical fitness levels contribute to healthy bone structure (WOLFE 2012, BEASLEY et al. 2010), minimize the risk of fractures and alleviate the consequences of injuries (SJÖBLOM et al. 2013). In a cohort study, the susceptibility to bone fractures increased with a decrease in protein intake (<15% in the daily ration) in subjects older than 50 (LANGSETMO et al. 2015). Diets deficient in protein lead to loss of muscle mass and reduce IGF-1 levels. A diet rich in milk proteins delivered beneficial effects for patients with hip fractures in comparison with patients whose protein intake was not monitored. A high-protein diet had a more beneficial influence on BMD when it was supplemented with the appropriate amounts of vitamin D_3 (GUNN et al. 2014, ZHOU et al. 2013, BERGER et al. 2012).

Studies analyzing intestinal absorption of calcium as well as parathormone (PTH) and IGF-1 levels which determine calcium metabolism did not confirm the hypothesis that a high-protein diet contributes to calcium deficiency. However, high protein intake accompanied by low calcium intake could compromise the bioavailability of the analyzed nutrient. KERSTETTER (1994) demonstrated that protein intake of 50 g decreased the supply of bioavailable calcium by 60 mg, but only in calcium-deficient diets. The influence of protein on the calcium balance should be analyzed based on the calcium-to-protein ratio (mg calcium : g protein). When the said ratio is below 20:1, high protein intake could have a negative effect on the calcium balance. The calcium-to-protein ratio is estimated at 36:1 in milk and fermented milks, 33-36:1 in ripened cheese, 48:1 in white cabbage and 16:1 in broccoli. However, most food products are characterized by an adverse calcium-to-protein ratio which is determined at 7–12:1 in cottage cheese, 2–5:1 in bread and only 0.7:1 in pork (ZMARLICKI 2009, ZITTERMAN 2002). Therefore, protein, calcium and vitamin D_3 are independent determinants of bone metabolism when consumed in the appropriate amounts. A balanced diet that meets protein, calcium and vitamin D_3 requirements is a very important consideration, in particular in children, adolescents and the elderly (GRILLENBERGER et al. 2006).

Calcium and the risk of prostate cancer and vascular calcification

A meta-analysis revealed that high calcium intake increases the risk of prostate cancer by 39% on average (GAO et al. 2005). A cohort study demonstrated a correlation between the intake of milk and dairy products and the incidence of prostate cancer. Daily calcium intake in excess

of 1500 mm (diet and supplements) increased the risk of prostate cancer relative to daily intake levels below 500 mg (AUNE et al. 2015, MICHAËLSSON et al. 2014). According to some studies, excess calcium inhibits the conversion of vitamin D_3 to 1,25 $(OH)_2D_3$, a derivative with anticarcinogenic properties (GILBERT et al. 2011).

The above findings suggest that prostate cancer is most probably caused by long-term deficiency of vitamin D_3 which inhibits proliferation, stimulates cell differentiation and induces apoptosis, thus preventing carcinogenesis. Vitamin D_3 also controls hormonal metabolism, decreases progesterone and estradiol levels, and prevents the estrogen-induced proliferation of cancer cells (JEONG et al. 2015, FELDMAN et al. 2014). A clinical study demonstrated that the risk of prostate, lung, breast and colon cancer decreased proportionally with plasma levels of $1,25(OH)_2D_3$ (FRIEDMAN and BACHOW 2013). Similar conclusions can be derived from an epidemiological study which revealed that the risk of prostate, breast and colon cancer is significantly lower in populations with higher vitamin D_3 levels (CHEN et al. 2010, HUNCHAREK et al. 2009).

Vitamin K_2 also delivers numerous health benefits. Menaquinone controls the activity of proteins responsible for calcium deposition in bodily organs. Calcium is transported from the cardiovascular system to the skeleton by osteocalcin, also known as bone gamma-carboxyglutamic acid--containing protein (BGLAP). In the human body, vitamin D_3 is essential for the synthesis of osteocalcin. Osteocalcin has to undergo carboxylation in the presence of vitamin K_2 before it can be bound to skeletal minerals (GUNDBERG et al. 2012). Osteocalcin has a number of other biological roles: it stimulates insulin secretion from the pancreas, influences insulin sensitivity at the cellular level, and determines the number and activity of spermatozoa (DI NICOLANTONIO et al. 2015).

Vitamin K_2 is also active in arteries where it regulates the activity of matrix GLA protein (MGP). MGP removes calcium from arteries and other soft tissues, and it is activated through carboxylation in the presence of vitamin K_2. In vitamin K_2 deficiency, osteocalcin and MGP remain inactive (noncarboxylated), which increases the risk of osteoporosis and vascular calcification. Calcium accumulated in soft tissues cannot be removed or transported to bones and teeth by any other factor. Unlike osteocalcin which is present in bone tissue, MGP is active throughout the entire body (WALLIN et al. 2008).

Prostate cancer is not caused by dairy products or dairy calcium (PARODI 2003, PARODI 2005). Calcium requirements can be adequately met by consuming a calcium-rich diet, but only when the supplied calcium is bioavailable. Calcium metabolism is determined by milk fat compo-

nents, in particular vitamins D_3 and K_2. Fat-free dairy products and calcium supplements can pose certain risks. Research indicates that the risk of prostate cancer associated with high calcium intake is, in fact, exacerbated by a deficiency of vitamins D_3 and K_2 (Di Nicolantonio et al. 2105, Chen et al. 2010, Li et al. 2007). Increased intake of vitamin K_2 minimizes that risk (Lamson and Plaza 2003, Nimptsch et al. 2008).

Dietary components limiting the bioavailability of calcium

Foods of plant origin contain components that significantly reduce the bioavailability of mineral compounds, including calcium (Buchowski 2015, Guéguen and Pointillart 2000). A diet rich in fiber improves peristalsis, decreases the risk of obesity and colon cancer (Schlemmer et al. 2009). However, fiber creates insoluble chelate compounds that limit the bioavailability of mineral nutrients and trace elements. Very strong chelate bonds are formed between calcium and phosphate groups found in oatmeal, which can decrease the calcium bioavailability by up to 65% (Kłobukowski et al. 2014, Skibniewska et al. 2010, Weaver et al. 1991). Chitosan supplements also lower the bioavailability calcium by more than 20% (Rodríguez et al. 2008). The bioavailability of calcium from *Brassica oleracea* vegetables is relatively high due to high concentrations of uronic acids, despite the presence of insoluble fiber fractions (Müller-Maatsch et al. 2016, Weaver et al. 1991). Uronic acids, whose content ranges from 10% in non-cellulosic plant fiber to 40% in fruits and vegetables, inhibit calcium absorption (360 mg of calcium can be absorbed daily from a vegetarian diet). However, up to 80% of uronic acids are fermented in the intestines, therefore, significant amounts of calcium are released and absorbed in the colon (Louis et al. 2016, Pieper et al. 2015). Colonic calcium absorption is also enhanced by organic acids in *Brassica oleracea* plants which form highly available low-molecular-weight complexes with calcium ions. For these reasons, white cabbage and broccoli are abundant sources of calcium whose bioavailability is similar to that of milk calcium (Park at al. 2013, Lucarini et al. 1999).

Calcium absorption is inhibited by methylated pectins (Bosscher et al. 2003, Powell et al. 1982) which are widely used in the production of juice, jam, jelly and baby food. However, according to Cummings et al. (1979), even high levels of dietary pectins do not compromise calcium absorption. Methylene groups bind around 80% of uronic acids, thus preventing the formation of insoluble complexes with calcium ions. Therefore,

the inhibitory effect of dietary fiber on calcium bioavailability is determined by the content of uronic acids (PIEPER et al. 2015, GUÉGUEN and POINTILLART 2000).

Table 1
The mean content of calcium and compounds limiting calcium bioavailability from plant foods

Specification	Total calcium content [mg/100 g]	Calcium available [%]	Fiber [g/100 g]	Phytic acid [g/100 g]	Oxalic acid [mg/100 g]
Kale	300	38,9	3	7,9	1,3
Nut (almond)	250	nd	8.8	4.88	0.3
Parsley	245	nd	6.1	nd	0
Nut (brazil)	150	nd	8.5	3.3	0.1
Celery	138	36.9	1.8	5.2	8.4
Cabbage	40	24.8	3.3	traces	traces
Seed of sunflower	100	nd	10.1	nd	0
Nut (walnut)	89	nd	6.4	3.44	0
Spinach	92	5.1	6.3	0.22	870
Green bean	49	nd	3.1	nd	0
Carrot	42	nd	3.9	nd	0
Red cabbage	35	nd	3.4	nd	0
Broccoli	33	22.9	2.4	0.16	traces
Garlic	30	nd	16.9	nd	0
Leek	30	nd	3.2	nd	0
Cucumber	28	nd	1.4	nd	0
Kohlrabi	25	nd	3.3	nd	0
Onion	23	nd	1.9	nd	0
Pumpkin	22	nd	2.6	nd	0
Lettuce	19	nd	1.6	nd	0
Cauliflower	22	23.4	2.1	traces	1
Pumpkin	18	nd	2.6	nd	0
Red bean	12	26.5	9.1	0.75	0
Nut (pine)	11	nd	5.1	0.2	0.1
Tomato	9	nd	1.7	nd	0

nd – not detected, USDA 2016; BUCHOWSKI et al. (2015)

Cereal products contain phytates which are composed of numerous phosphate groups (Bong et al 2016) that form insoluble complexes with Ca^{2+} ions (Israr et al. 2017). Oxalates, which are found in large quantities in tea, in particular red tea, and coffee, form insoluble salts with calcium. Solvents present in coffee also inhibit mineral absorption (Higdon and Frei 2006).

Many foods of plant origin (nuts, cereals, fruits and vegetables) are characterized by high calcium content (Table 1), but they are not ample sources of dietary calcium due to the presence of compounds that inhibit calcium absorption (fiber, uronic acids, phytates and oxalates). The calcium content of 1 glass of milk is equivalent to that of 8 cups of spinach, 5 cups of red beans or 2 cups of broccoli (Weaver and Boushey 2003, Miller et al. 2001). The bioavailability of calcium from most plant-based products, including cereals, generally does not exceed 10%.

Phosphates are widely used in the production of convenience foods (soft drinks, processed meats, sweets, bread), and their consumption usually exceeds the recommended levels. Excess dietary phosphates compromise the healthy Ca:P ratio, which increases PTH levels, inhibits the synthesis and release of 1,25(OH)2D, and limits the bioavailability of calcium (Lenton et al. 2015, Guéguen and Pointillart 2000).

Raw foods with high calcium content are not always abundant sources of this nutrient due to an unfavorable Ca:P ratio. Soybeans contain 240 mg of calcium per 100 g, but they are characterized by an undesirable Ca:P ratio of 1:3. Herrings are abundant in calcium at 86 mg Ca/100 mg, but their Ca:P ratio is 1:5. Dairy products are characterized by the most favorable Ca:P ratio of 1.3:1 (Aljewicz et al. 2018). Milk and dairy products do not contain compounds that interfere with calcium absorption such as phytates, oxalates, uronic acids or insoluble fiber, which are plentiful in cereals, fruits and vegetables (Baye et al. 2015, Wolf et al. 2000).

Milk and dairy products: the best source of bioavailable calcium

Milk and dairy products are the best sources of dietary calcium. They are abundant in bioavailable calcium and are characterized by optimal Ca:P and calcium:protein intake rations. The bioavailability of calcium is additionally enhanced by fat-soluble vitamins D_3, K_2 and selected peptides that are produced during digestion. In lactose-intolerant subjects, undigested lactose consumed as a component dairy products arrives in the large intestine without being absorbed in the small intestine. This undi-

gested lactose stimulating growth of saccharolytic bacteria in the intestine, decrease in ammonia content and increase acidity in intestinal digesta (ALJEWICZ et al. 2018, KŁOBUKOWSKI et al. 2004, 2014). The bioavailability of calcium from milk, fermented milks and ripened cheeses generally reaches 30–45%, but it can be as high as 75% in pregnant women and athletes due to metabolic adaptation. Two glasses of milk or yogurt meet daily calcium requirements in 60%. Ripened cheese is an even more abundant source of dietary calcium, and 50 g of cheese meets daily calcium needs in around 40%. Ripened cheese has a high content of bioavailable calcium (Table 2).

Table 2

Selected dairy products ranked by calcium content; the mean content of vitamin K, calcium and protein in different chesses

Cheese	Calcium [mg/100 g]	Protein [mg/100 g]	Vitamin D [ug/100 g]	Menaquinone-4 [µg/100 g]	Menaquinone-7 [µg/100 g]
Parmesan	1253	36	0.475	7.1	0.215
Swiss	961	27	0.500	7.4	nd
Gruyere	950	30	0.600	45.5	0.022
Edam	770	25	0.500	0.033	0.012
Gouda	740	25	0.500	nd	0.006
Mozzarella	716	22	0.375	4.1	nd
Cheddar	711	23	0.600	nd	0.025
Brie	540	21	0.500	nd	0.014
Blue	500	22	0.525	nd	0.223
Cammbert	350	21	0.450	nd	0.017
Buttermilk	115	3.21	1.300	0.2	0.1
Milk	113	3.5	0.100	0.8	nd
Yoghurt	107	3.47	0.100	0.6	nd
Cream	98	7	0.625	19	nd
Cottage	53	11	0.075	0.9	nd
Butter	24	0.84	0.000	15	nd

Source: own compilation based on USDA 2016, MANOURY et al. 2013, HOJO et al. 2007, SCHURGERS et al. 2000

The bioavailability of cheese calcium is additionally enhanced by proteins, bioactive peptides, fat-soluble vitamins D_3 and K_2, as well as short-chain saturated fatty acids (butyric, acetic and propionic acids). Fatty acids present in milk fat and synthesized by gut microbiota increase: acidity of cecal content, ionization of minerals and permeability of colonocytes

by changing osmotic pressure, and they stimulate the absorption of mineral nutrients, including calcium (DEN BESTEN et al. 2013, CANANI et al. 2011). Ripened cheese also contains long-chain saturated fatty acids which limit calcium bioavailability by forming soaps that are not digested in the human gastrointestinal tract. The amount of precipitated calcium ions increases proportionally to the length of the fatty acid chain and its saturation (ALJEWICZ et al. 2014, GUÉGUEN and POINTILLART 2000). Calcium from cottage (acid-set) cheese is more easily absorbed due to high ionization and lower fat content. Despite the above, cottage cheese contains approximately 10-times less calcium than ripened cheese (SIEMIANOWSKI et al. 2014).

The protein content of cheese ranges from 7% to 36% (Table 2). Protein influences parathyroid glands which stimulate the production of PTH responsible for calcium adsorption. Cheese proteins are digested to produce biologically active peptides, including phosphopeptides, which enhance calcium availability by preventing the formation of insoluble phosphate. Calcium is transported across the intestinal wall with the involvement of amino acids (TANG and SKIBSTED 2016). High protein intake always improves bone mineral density (ALJEWICZ et al. 2018, O'CALLAGHAN et al. 2017, BUCHOWSKI 2015, GUÉGUEN and POINTILLART 2000).

Ripened cheese is the richest source of bioavailable calcium whose content ranges from 350 to 1253 mg/100 g (Table 2). Vitamins D_3 and K_2 are responsible for the absorption of calcium in the small intestine and its deposition in bones. Vitamin D_3 is essential for the healthy function of parathyroid glands and kidneys. It is also required for the synthesis of osteocalcin, a protein that transports calcium ions to bones. Osteocalcin has to undergo carboxylation in the presence of vitamin K_2 before it can be bound to the bone mineral phase. Vitamin D_3 and K_2 deficiency inhibits the synthesis of proteins which are responsible for depositing calcium in bones and teeth (SAHNI et al. 2015, HUANG et al. 2015).

Ripened cheese and other dairy products contain calcium that is highly bioavailable on account of the optimal Ca:P ratio and the presence of compounds that enhance calcium absorption (proteins, peptides, amino acids, vitamins D_3 and K_2, short-chain saturated fatty acids) (KŁOBUKOWSKI et al. 2004, GUÉGUEN and POINTILLART 2000).

Health benefits of calcium

A healthy diet that meets daily calcium requirements is one of the pillars of health protection programs. The recommended daily calcium

intake is determined by age, gender and health status. It is set at 800–1000 mg Ca for healthy adults, 1300 mg Ca for pregnant and breastfeeding women, and up to 1200 mg Ca for menopausal women. In women with chronic calcium deficits, pregnancy and breastfeeding increase the risk of osteoporosis because lactation decreases bone mineral density. In children, calcium requirements are very high (1200 mg daily) during periods of intensive growth which involve the growth of new bone tissue. An increase in young people's peak bone mass by only 10% could decrease the risk of osteoporosis-related bone fractures by 50% in adulthood (HOSKING et al. 2016, ARONOW 2011, KERSTETTER et al. 2003).

Diets rich in calcium decrease the risk of dental caries and periodontitis. The hard tissue of teeth develops between the fourth week of fetal life and 20 years of age. The building blocks of hard tissue (calcium as well as fluoride and magnesium) contribute to healthy tooth development and prevent tooth decay (MOYNIHAN and PETERSEN 2004). During prolonged calcium deficiency, the missing amounts of calcium are "borrowed" from the jaws, which can lead to gum disease. The calcium intake of patients (aged 29–40 years) suffering from periodontitis was low at 400 mg Ca daily or less (the recommended daily intake is around 800 mg Ca). The problem was resolved by supplementing the studied subjects' diets with calcium in the daily amount of 1000 mg for 180 days (NISHIDA et al. 2000, ADEGBOYE et al. 2016).

Epidemiological research indicates that diets deficient in calcium increase the body mass index (BMI) in both children (BERKEY et al. 2005) and adults (KEAST et al. 2015, ARRUDA and HOTAMISLIGIL 2015, WEAVER AND BOUSHEY 2003). Dairy products are much more likely to promote weight loss than calcium supplements. The above was demonstrated by a study of 32 obese men and women who were placed on low-calcium (400–500 mg Ca/day), high-calcium (1200–1300 mg Ca/day, including 800 mg from calcium supplements) and high-dairy (3–4 servings of dairy products containing 1200–1300 mg Ca/day) diets for 24 weeks. Their daily energy intake was reduced by 500 kcal. The high-dairy diet led to the greatest reduction in body mass among respondents with identical daily calcium intake levels (HUTH et al. 2006).

Milk proteins are responsible for the higher bioavailability of calcium from dairy products than calcium supplements. Whey proteins are abundant in branched-chain amino acids, such as leucine, which regulate the flow of energy from adipose tissue to muscles. They contribute to muscle growth and exert anabolic effects during weight loss (ZHU et al. 2013, ABARGOUEI et al. 2012). Calcium reduces the energy value of high-fat dairy products by forming soaps with long-chain saturated fatty acids. Synergis-

tic interactions between calcium and bioactive fat components, including vitamins D_3 and K_2 and conjugated linoleic acid (CLA), also contribute to weight loss (KAMYCHEVA et al. 2003, ZEMEL and MILLER 2004).

Diets that meet daily calcium needs also reduce the risk of insulin resistance. The Cardia program demonstrated that the above risk was 72% lower in obese subjects consuming dairy products than in obese respondents with a low intake of milk and milk products. Dairy products contain bioavailable calcium which influences insulin secretion and controls tissue resistance to insulin (PEREIRA et al. 2002). Magnesium, biologically active peptides, n-3 α-linolenic acid and, indirectly, vitamins D_3 and K_2 deliver similar effects (RALSTON et al. 2012, ZEMEL et al 2004). Dairy foods are highly effective in the prevention and treatment of obesity and type 2 diabetes because they contain highly bioavailable calcium which enhances lipolysis and the release of triglycerides from adipocytes, regulates insulin secretion and insulin resistance at the cellular level (MENSINK 2006).

Calcium deficiency increases vascular resistance, whereas diets rich in calcium decrease vascular resistance and lower blood pressure. High intake of calcium increases urinary sodium excretion and inhibits neurotransmitters, such as noradrenaline, which induce vascular contraction (TRIALISTS'COLLABORATION, BLOOD PRESSURE LOWERING TREATMENT et al. 2015, HOFMEYR et al. 2014, MASSEY 2001). Biologically active peptides also play an important role in blood pressure control by inhibiting the enzyme that converts angiotensin I to angiotensin II and by inactivating bradykinin. Whey protein tetrapeptides as well as phosphopeptides have hypotensive properties, and they enhance the absorption of calcium, magnesium and potassium (ENGBERINK and HENDRIKSEN 2009). The effectiveness of milk and dairy products in treating high blood pressure was demonstrated by the DASH diet (Dietary Approaches to Stop Hypertension). Two weeks into the program, the DASH diet contributed to a reduction in blood pressure, which was more pronounced in hypertensive patients than in subject with healthy blood pressure. The reported reduction in blood pressure and the time required to achieve a therapeutic effect were comparable to the outcomes of pharmacological treatment. The DASH diet also delivered additional health benefits that cannot be achieved with pharmacological treatment, including a reduction in homocysteine levels, decrease in body mass (by 4.9 kg on average) and the absence of side effects (LIN et al. 2003).

A clinical study demonstrated that higher calcium intake reduces the risk of colon cancer and slows down tumor growth in humans. Calcium exerts anticarcinogenic effects by inhibiting hyperproliferation of intesti-

nal epithelial cells, i.e. an abnormally *high rate* of their *proliferation* by rapid *division*. Calcium also binds bile acids, fatty acids and phosphates into insoluble salts, thus minimizing their irritating effects and their ability to stimulate the proliferation of intestinal epithelial cells (FLEET 2006, LARSSON et al. 2006). The Nurses' Health Study performed on around 88,000 women and 44,000 men demonstrated that the risk of colon cancer in subjects consuming 700–800 mg of calcium daily was 40–50% lower than in subjects whose daily calcium intake was limited to 500 mg (WU et al. 2002, KESSE et al. 2005). A meta-analysis performed by KEUM et al. (2014) also revealed that the risk of colon cancer was reduced by 9% per every 300 mg of ingested dietary calcium.

According to epidemiological research, the incidence of cancer is significantly lower in countries with a high consumption of ripened cheese (France, Italy, Greece) than in countries with lower cheese intake (Belgium, the Netherlands, Great Britain). The anticarcinogenic properties of ripened cheese can be attributed to its high calcium content as well as the presence of potent antioxidants (CLA, α-tocopherol, β-carotene, vitamins A and D_3, phospholipids, ether lipids), medium-chain and short-chain saturated fatty acids that exert protective effects on the intestinal mucosa (GALLUS et al. 2006, GROSS 2005).

Conclusions

A healthy calcium balance is more likely to be determined by the bioavailability of calcium than its dietary intake. Plant foods, including products with high calcium content (vegetables, nuts, cereals, fruits), are not always good sources of calcium. They lack vitamins that control calcium metabolism, and they contain compounds (fiber, phytates, oxalates, uronic acids) that limit calcium absorption. Due to the presence of the bioactive peptides as well as vitamins D_3 and $K_{2,}$ dairy products are much more effective in preventing diet-dependent diseases than calcium supplements. The DASH diet provides robust evidence that milk and dairy products are effective in the treatment of obesity, atherosclerosis and hypertension.

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THE CONTENT OF BIOLOGICALLY ACTIVE SUBSTANCES AND ANTIOXIDANT ACTIVITY IN COFFEE DEPENDING ON BREWING METHOD

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Key words: coffee, antioxidants, brewing method, caffeine, reactive oxygen species.

Abstract

Coffee is one of the world's most popular beverages. It is rich in biologically active compounds possess antioxidant activities. The aim of the study was to determine the influence of coffee brewing method to the total antioxidant activity, the phenolic acid, flavonoid and caffeine content of coffee infusion from beans available in local markets.

Three methods of brewing coffee were evaluated: pouring hot water over coffee beans, using coffee percolator where the water is boiling through ground coffee and collecting as coffee above and preparing in an automatic coffee maker. Three species of coffee: arabica, robusta and green coffee beans infusions were analyzed.

Total antioxidant activity of the infusion was measured using 2,2-*diphenyl*-1-picrylhydrazyl radical (DPPH). Polyphenol, flavonoid and caffeine contents were determined by spectrophotometry.

The results showed that antioxidant activity of analyzed infusion was no significant changes depending on coffee species and beverage preparing method in roasted coffee beans. It has been also shown that the method of brewing unroasted coffee beans significantly affects the antioxidant potential of infusion, as well as the brewing time (first, second, third). Methods of brewing did not make a difference to the total polyphenol content. The caffeine concentration and total flavonoid content in the coffee infusion changed depending on the conditions of brewing.

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ZAWARTOŚĆ ZWIĄZKÓW BIOLOGICZNIE CZYNNYCH W KAWIE ORAZ ICH AKTYWNOŚĆ PRZECIWUTLENIAJĄCA W ZALEŻNOŚCI OD SPOSOBU PARZENIA

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Słowa kluczowe: kawa, antyoksydanty, sposoby przygotowywania naparu, kofeina, wolne rodniki tlenowe.

Abstract

Kawa jest jednym z najpopularniejszych napojów na świecie. Charakteryzuje się dużą zawartością związków biologicznie czynnych o właściwościach antyoksydacyjnych. Celem pracy było określenie wpływu metody parzenia kawy na całkowitą aktywność antyoksydacyjną, zawartość kwasów fenolowych, flawonoidów i kofeiny w naparach kawy przygotowanych z ziaren dostępnych w lokalnych sklepach.

Oceniano trzy sposoby parzenia kawy: zalanie zmielonych ziaren kawy gorącą wodą, przygotowanie naparu z użyciem kawiarki,w której gorąca woda obmywa zmielone ziarna i do górnego zbiornika skrapla się napar, oraz przygotowanie naparu za pomocą ekspresu do kawy. Analizowano napary przygotowane z trzech gatunków kawy: arabiki, robusty i zielonych ziaren kawy.

Całkowitą aktywność przeciwutleniającą naparu oceniano, stosując rodnik 2,2-difenylo-1-pikrylhydrazylowy (DPPH). Całkowitą zawartość polifenoli, flawonoidów i kofeiny ustalono spektrofotometrycznie.

Wykazano, że aktywność antyoksydacyjna naparów z palonych ziaren kawy nie uległa istotnym statystycznie zmianom w zależności od gatunku kawy i sposobu przygotowywania napoju. Dowiedziono również, że przygotowywanie naparów z niepoddanych procesowi palenia ziaren kawy ma znaczący wpływ na jego potencjał utleniający, który zależy także od liczby zaparzeń. Metody przygotowywania naparów nie miały wpływu na całkowitą zawartość polifenoli. Stężenie kofeiny i całkowita zawartość flawonoidów w naparze kawy zmieniały się w zależności od metody przygotowywania.

Introduction

Coffee remains one of the most commonly consumed beverages across the world (MUSSATTO et al. 2011). The homeland of coffee is Ethiopia (MIRAN 2012), from where it spread though Arabic countries, reaching Europe a few hundred years later. The most popular species are *Coffea arabica* and *Coffea canephora* var. Robusta (KLEIN 2003).

Coffee contains more than 1,000 biologically active substances. Besides caffeine (1–2%), green coffee beans comprise carbohydrates (59–61%), lipids (10–16%), proteins (10%), chlorogenic acids (7–10%), minerals (4%), aliphatic acids (2%), trigonelline (1%) and free amino acids (<1%). Roasted

coffee beans have a slightly different composition: carbohydrates (38–42%), proteins (8%), chlorogenic acids (3–4%), and free amino acids, lipids (11–17%), minerals (5%), aliphatic acids (3%), and trigonelline (1%). Some of these compounds, such as polyphenols, possess antioxidant activity (LUDWIG et al. 2014).

Although polyphenols are known for their vital antioxidant properties, the same is true for caffeine. The antioxidant activity of phenolic compounds proceeds by a variety of mechanisms of action. They are capable of reducing a substrate by donating electrons or a hydrogen atom to bind free radicals, stabilize or delocalize unpaired electrons, activating chelating enzymes that can bind metal ions catalyzing oxidation reactions, inhibiting the action of oxidase, interrupting radical chain reactions, and stabilizing free radicals through their hydrogenation or complexion (FERNANDEZ-PANCHON et al. 2008). Polyphenols can be classified into four categories: phenolic acids and flavonoids, stilbenes and lignans. The most abundant polyphenols in human diet, flavonoids are composed of two aromatic rings connected by a three-carbon bridge, making a total of 15 carbon atoms. An important group of phenolic compounds formed as secondary plant metabolites are the hydroxycinnamic acids, represented by caffeic acids (CA) and their esters, chlorogenic acids (CGA). Apart from their antioxidant activity (SATO et al. 2011), both compounds demonstrate a range of properties. For example CA and CGA inhibit the activity of α-amylase and α-glucosidase, key enzymes linked to type 2 diabetes (OBOH et al. 2015), and acetylcholinesterase and butyrylcholinesterase, which are linked to Alzheimer's disease (OBOHet al. 2013). It has also been reported that CA and CGA possessanticancer properties (ROCHA et al. 2012), anti-metastatic activity (TANAGORNMEATR et al. 2014) and anti-inflammatory effects (SERGENt et al. 2010). They also possess the ability to inhibit the activation of transcription factor NF-κB (MAet al. 2013) and can inhibit DNA methylation (VUCIC et al. 2008). Studies have shown that CA has greater antioxidant activitythan CGA (CHEN and HO 1997), which is positively correlated with the number and position of hydroxyl groups bound to the aromatic ring, as well as the nature of its substituents (RICE-EVANSet al. 1996).

Caffeine (1,3,7-trimethylxanthine), a natural alkaloid, is the most extensively studied compound in coffee. It is composed of two fused rings whose chemical properties are closely related to those of purines (NUHU 2014). The caffeine content in coffee beans depends on the species. Robusta has approximately twice the caffeine content as Arabica (FOX et al. 2013). In general, the daily caffeine intake for adults has been found to be approximately 3–4 mg kg^{-1} body weight day^{-1}(BARONE and ROBERTS 1996). It

reaches maximum concentration in the blood after about 15–120 minutes and remains steady for about four hours. The physiological effect of caffeine is stimulation of central nervous system. High doses of caffeine, of up to 6 mg kg^{-1} body weight day^{-1} have adverse effects such as overstimulation of central nervous system and other harmful properties such as general toxicity, cardiovascular effects, effects on bone status and calcium balance, changes in adult behavior, increased incidence of cancer and impaired male fertility (NAWROt et al. 2003). In addition, it has been also reported that caffeine demonstrates antioxidant activity against lipid peroxidation caused by reactive oxygen species (LEE 2000).

Around over the world many brewing methods may be used to prepare coffee brews. Brewing techniques are classified inthree main groups:

1. Original Italian method under high pressure.
2. Infusion, by pouring hot water on ground coffee followed by a filtration.
3. Decoction or boiling method (PETRACCO 2001).

Brewing coffee by boiling was the earliest method. It is prepared by adding the water to the grinding coffee beans and bringing it to the boil for no more than an instant in a pot.

Another common brewing technique of coffee using a percolator applies both aspects of the pressure and gravity methods. The percolator has two chambers: water is placed inside the percolator's lower chamber, while coffee grounds is placed inside the filter basket within the percolator's upper chamber. The water in the chamber boils and is forced, through a tube, to the top of the percolator and drips down over the coffee grounds.

The most popular pressure brewing method is espresso. Espresso is obtained by pushing hot water, slightly under boiling temperature, with pressure, through ground coffee (CAPRIOLI 2015).

The aim of this work was to analyze the main biologically active substances in coffee with regard to brewing method. For this purpose, four commercially available types of roasted coffee and one type of green coffee were examined, together with the following methods of coffee brewing: pouring hot water over the coffee beans, making coffee using a percolator and using an automatic coffee maker. The total antioxidant activitywas measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Total polyphenol content was measured using the by Folin-Ciocalteu assay and flavonoid content by the Dowd method. The quantity of caffeine was determined by spectrophotometry following extraction from the coffee infusion using polar-nonpolar solvent extraction techniques.

Materials and Methods

Chemicals.2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), catechin, quercetin were provided from Sigma-Aldrich (Poznan, Poland). Ethanol, Folin-Ciocalteu reagent, chloroform, nitrate (III) sulfate, aluminum chloride, sodium carbonate, potassium acetate and ferric chloride were obtained from Avantor Performance Materials Poland S.A. (Gliwice, Poland).

Coffee Material. Four roasted coffee samples, blends of Arabica and Robusta, numbered 1–4, and one sample of green coffee (Table 1) obtained from retail stores in Lodz (Poland) were analyzed. The most popular brands of pre-grounded roasted coffee and whole beans of unroasted coffee were selected for the analysis.

Table 1

Characterization of selected coffee

Number of coffee	Species of coffee	Roasted/unroasted	Country of the beans origins
1	arabica	roasted (medium)	South and Central America, Brazil
2	arabica	roasted	–
3	arabica	roasted	Brazil, Colombia and Central America
4	robusta	roasted	–
Green coffee beans	–	unroasted (green coffee)	–

Sign "–" means that the information has not been provi

Preparation of beverages. The five types of coffee including roasted and green beans were prepared in three different ways. In the first method, 100 ml of hot water (90°C) was poured onto 2 g of ground coffee beans and dripped through paper filter after 10 minutes. In the second one, the extract was prepared using a percolator (Domotti, model 32704 Vella, Poland): 100 ml of cold water was added to the reservoir and then 2 g of ground coffee to the basket. The percolator was placed over a heat source. After complete brewing, coffee solutions were dripped through a paper filter. The final method was to brew a 100 ml measure of coffee from 2 g ground coffee beans using a coffee machine (De'Longhi, model EC145, Italy) and drip the infusion through paper filter.

For the green coffee, the beans were ground to a powder in a coffee grinder (Siemens, model MC23200, Germany) for 30 s immediately before sample preparation. Green coffee was brewed three times, according to the

instructions on the package. The extract was filtered and the seeds were poured once more with water. Freshly prepared coffee brews were taken to analyses. All analyses were performed using a UV-Vis spectrometer (Cary 100 UV-Vis, Agilent Technologies, Santa Clara, USA).

Antioxidant activities by DPPH assay. Theantioxidant activity of coffee beverages was determined according to (BRAND-WILLIAMSet al. 1995) using the synthetic radical DPPH. The absorbance of the solution was measured at λ = 517 nm. Following this, 0.5 mM DPPH alcoholic solution stored in the dark was prepared.

The spectrophotometer was first calibrated using ethanol, before 100 ul of ethanol and 100 ul diluted coffee samples (10, 15, 25, 35, 50, 75,100, 150, 200 x dilutions) were added to 1 ml DPPH solution. After 30 minutes from the initiation of the reaction, the absorbance of the DPPH radical solution (A_0) and samples (A) were monitored. Each measurement was done in triplicate and meanvalues (A_{mean}) were calculated. The ability of the antioxidant to counteract the oxidation reaction were calculated by the formula (MOLYNEUX 2004):

$$\text{Inhibition [\%]} = 100\ (A_0 - A_{mean}) / A_0$$

when:

A_{mean} – the meanabsorbance of test the solution containing antioxidant

A_0 – absorbance of DPPH radical solution.

The total phenolic content of the extract was determined by the Folin--Ciocalteu method [11]. Briefly, 200 µL of crude extract (1 mg ml^{-1}) were made up to 3 ml with distilled water, mixed thoroughly with 0.5 mL of Folin-Ciocalteu reagent for 3 min, followed by the addition of 2 ml of 20% (w/v) sodium carbonate. The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Total polyphenol content. The content of total phenolic compounds was determined by spectrophotometry using the Folin-Ciocalteu reagent and caffeic acid as a standard (range 0–200 µg ml^{-1}). 100 µl of coffee extract solutions (2 g/100 ml) were placed in a 10 ml volumetric flask, then 1 ml of 0.2 M Folin-Ciocalteu reagent and 6 ml of distilled water were added and mixed. After a three minute pause, 1.5 ml 20% sodium carbonate was added and made up to 10 ml with distilled water, followed by incubation in darkness at room temperature for 120 min. Following this, the solution was mixed again and the absorbance of the samples were measured at a wavelength of 765 nm against an ethanol blank.

The total polyphenol content was calculated using a linear equation based on a calibration curve ($y = 0.11x + 0.0558$, $r = 0.9935936$) and expressed in μg of caffeic acid/ml (SINGLETON and ROSSI 1965).

Total flavonoid content. The flavonoid content was determined spectrophotometrically according to the Dowd method (KAŠKONIENIE et al. 2009), using quercetin as a standard in the range 0–200 μg ml^{-1}. The coffee extracts were placed in a 10 ml volumetric flask. The mixtures were made up to 5 ml with distilled water, and 0.3 ml of 5% aqueous solution of $NaNO_2$ was added. 1 ml of the samples (2 g/100 ml) were then mixed and left for five minutes before 0.6 mL of 10% hexahydrate solution of $AlCl_3$ was added and mixed again. After five minutes, 2 ml of 1 M aqueous solution of NaOH was added and made up to 10 ml with distilled water. The solution was mixed again and the absorbance of the samples wasmeasured at a wavelength of 510 nm against a ethanol blank. The total flavonoid content was calculated using linear equations based on the calibration curve ($y = 0.1125x - 0.1027$, $r = 0.998203$) and expressed in μg of quercetin/ml. (KAŠKONIENIE et al. 2009).

Caffeine content. The contentof caffeine was determined by spectrophotometry at 276 nm, after prior extraction of the caffeine by chloroform in alkaline solutions of coffee (pH 12.5–12.7). The alkaline extract solutions were transferred to a separatory funnel and the caffeine was extracted with three further portions of chloroform (10ml/5ml/5ml). The samples were mixed for one minute and the solutions were allowed to separate for five minutes at room temperature. The organic layer of the extracted caffeine was collected in a 25 ml flask. The solution was made up to 25 ml with chloroform, and the samples were dried by adding anhydrous $MgSO_4$ to the organic phase and swirl with the dual purpose of removing water and breaking any emulsion and then filtered. The absorbance was measured at a wavelength of 277 nm (maximum absorption for caffeine) against a chloroform blank. Commercially obtained caffeine at concentrations ranging from 0 to 0.2 mg ml^{-1} was used as a standard solution. The caffeine content was calculated using a linear equation based on calibration curve ($y = 0.1335x - 0.1094$, $r = 0.998233$) and expressed in mg of caffeine per 1 ml (PARADKAR and IRUDAYARAJ 2002).

Statistical Analysis.The results were expressed as mean ± standard deviation (SD). Each parameter was examined in triplicate. One-way analysis of variance (ANOVA) was used to access the statistical significance of any difference between the coffee brews. A p-value less than 0.05 was considered as significant. All statistical calculations were performed using Statistica software (StatSoft, Inc., Tulsa, OK, USA).

Results and Discussion

Coffee is one of the most popular beverages throughout the world. The present study focuses on the antioxidant activity, total polyphenol content, flavonoid content and caffeine content of coffee extracts prepared in three different ways (Table 2 and Table 3).

Table 2

Total polyphenols, flavonoids and caffeine contents of roasted coffee depending on the species and the method of preparing infusion

Coffee species	Method of preparing infusion	Total polyphenols	Total flavonoids	Caffeine
1	hot water	498±12,4	269±4.84	0.41±0.05*
	percolator	540.4±35.8	355.8±6.67	0.34±0.02*
	coffee machine	593.9±12.6	308.2±2.65	0.39±0.01*
2	hot water	481±15.8	276±5.12	0.47±0.05*
	percolator	533±56.7	355.5±5.64	0.42±0.04*
	coffee machine	519.9±30.02	29.71±5.27	0.33±0.02*
3	hot water	578.4±46.9	297.4±0.62	0.70±0.05*
	percolator	623±58.5	331.6±0.62	0.65±0.05*
	coffee machine	529.4±1.7	298±19.2	0.41±0.02*
4	hot water	587.7±13.6	340.7±1.32	0.76±0.06*
	percolator	600.3±27.2	403.8±13.88	0.69±0.03*
	coffee machine	573.4±19.7	374.3±1.82	0.15±0.01*

* Statistically significant between groups

Table 3

Total polyphenols, flavonoids and caffeine contents of green coffee depending on the species and the method of preparing infusion

Brewing	Method of preparing infusion	Total polyphenols	Total flavonoids	Caffeine
I	hot water (*H*)	380.9±6.77*	153.8±1.37*	0.28±0.02
	percolator (*P*)	384.9±4.46*	212.8±6.39*	0.13±0.02
	coffee machine (*M*)	271.7±7.15*	86±7.05*	0.16±0.02
	statistically significant between:	*P* and *M*, *H* and *M*	*H* and *P* and *M*	*H* and *P* *H* and *M*
II	hot water (*H*)	138.4±7.95	55.5±2.54*	0.09±0.03
	percolator (*P*)	174.2±14.4	102.6±0.85*	0.05±0.02
	coffee machine (*M*)	125.2±5.9	32.7±4.32*	0.09±0.00
	statistically significant between:	*H* and *M* *P* and *M*	*H* and *P* and *M*	–
III	hot water (*H*)	55.8±8	26±2.65*	0.06±0.00
	percolator (*P*)	68.1±0.1	36.6±4.51*	0.02±0.01
	coffee machine (*M*)	47.6±12.6	5.6±2.83*	0.05±0.00
	statistically significant between:	–	*H* and *P* and *M*	*H* and *P* *M* and *P*

I – first brew, II – second brew, III – third brew

* Statistically significant between groups

The analyses of the different brewing processes are given for roasted coffee in Figure 1, and for green coffee in Figure 2.

Generally, no significant changes were found between the method of preparing the beverages and the percentage of inhibition of DPPH radicals. For the beverages from roasted coffee beans, the mean inhibition values were 64.35±3.98% for pouring hot water over the ground beans; 67.33±4.87% for the percolator and 64.4±2.35% for the coffee machine, for 120 μg ml^{-1} concentration of the coffee beans. The concentration 120 μg ml^{-1} was chosen as an example to illustrate no differences in the antioxidant properties of the analyzed coffee extracts depending on the method of brewing.

For the green coffee beans (120 μg ml^{-1} concentration), the mean inhibition values of DPPH radical scavenged by the antioxidants included in the coffee beveragesare 36.20±0.40% by pouring hot water, 46.46±4.87% for percolator coffee and 21.93±0.66% for the coffee machine for first brewing, 10.01±0.06, 20.51±3.07, 13.63±0.72 for second brewing, 5.86±0.84, 11.91±2.01, 5.96±0.47 for third brewing, respectively.

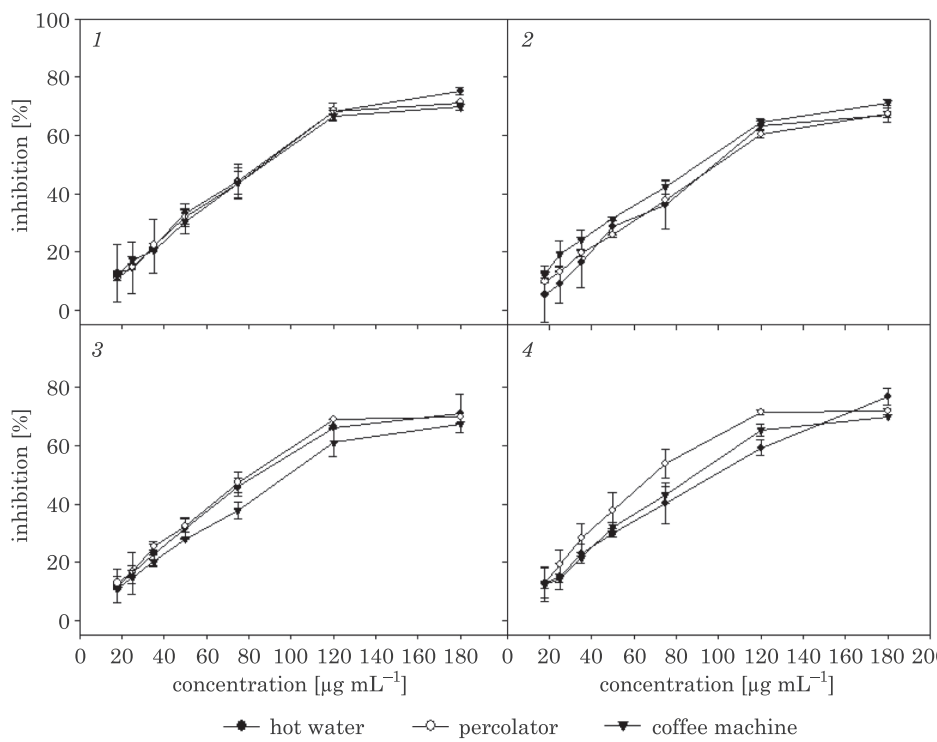

Fig. 1. Scavenging effect (percentage of remaining DPPH radical) of four roasted coffee extracts depending on three different brewing methods during the DPPH test, as measured by changes in absorbance at 517 nm. The numbers *1*, *2*, *3* and *4* represent types of coffee

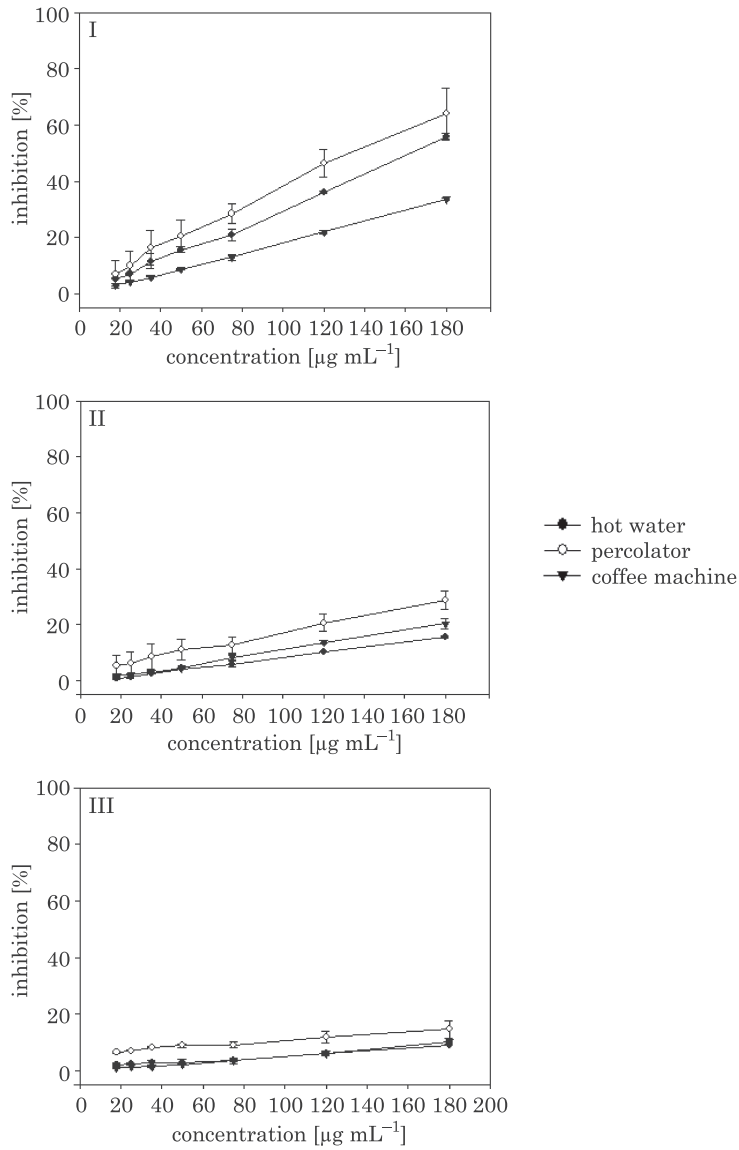

Fig. 2. Scavenging effect (percentage of remaining DPPH radicals) of green coffee extracts depending on three different brewing methods during the DPPH test, as measured by changes in absorbance at 517 nm: I – first brew, II – second brew, III – third brew

These results indicate that the best way to brew coffee to obtain the largest number of antioxidants is by using a coffee percolator, with pouring hot water over ground coffee beans being less effective, followed by using a coffee machine for coffee number one and two. For coffee number 3

and 4, the percentage values are very similar for all preparation methods. However, no statistically significant difference was found between these methods.

In addition, it is noteworthy that the studied extract of green coffee beans demonstrated significantly lower antioxidant activity than roasted coffee beans. Similar results were reported by GUNALAN et al. (2012), who note a 52% mean percentage of inhibition for two tested roasted coffee bean extracts at a concentration of 100 µg coffee ml^{-1}. RAMADAN-HASSANIEN (2008) reports the mean DPPH inhibition to be 33.2% for coffee prepared by pouring hot water over coffee beans, but it should be noted that this study uses half the content of coffee (1 g) and DPPH solution (0.5 ml), and a larger quantity of water (200 ml) to prepare the brew, compared to the present study. Lower antioxidant potential, ranging from 15.2% to 24.3%, were observed by DE OLIVEIRA et al. (2014) for 100 µg ml^{-1} extract for selected types of coffee. Regarding the antioxidant activity of a sample of green coffee, PRIFTIS et al. (2015) report that the roasted beans exhibited greater antioxidant activity than their green counterparts in eight of 13 tested varieties, with the opposite being the case in the remaining five varieties. GORNAS (2016) demonstrated that the green coffee samples exhibited in turn the highest antioxidant capacity, decreasing with the rise in the degree of coffee bean. WOLSKA et al. (2017) show that the antioxidant activity of infusions was high and dependent on the species of coffee used and the condition of brewing. However, significant differences were found only between green coffee and arabica, and green coffee and robusta. Our research also has indicated, that, depends on brewing method, the greatest difference in antioxidant potential was found in green coffee infusions with reference to roasted coffee beans extracts.

Total polyphenol content

The results according to brewing process, expressed as µg caffeic acid/100 µl sample of coffeeextract (2 g/100 ml), are shown in Figure 3 for the roasted coffee, and Figure 4 for the green coffee.

Generally, no significant changes were found regarding brewing method or total polyphenol content for roasted coffee. For the beverages from roasted coffee beans, the mean polyphenol content were as fallow: 536.28±16.54 µg caffeic acid ml^{-1} for pouring hot water over coffee beans, 574.18±15.49 µg caffeic acid ml^{-1} for percolator coffee and 554.15±11.92 µg caffeic acid ml^{-1} for the coffee machine. The green coffee beans were examined after three consecutive brews. For the first brew, mean polyphenol content

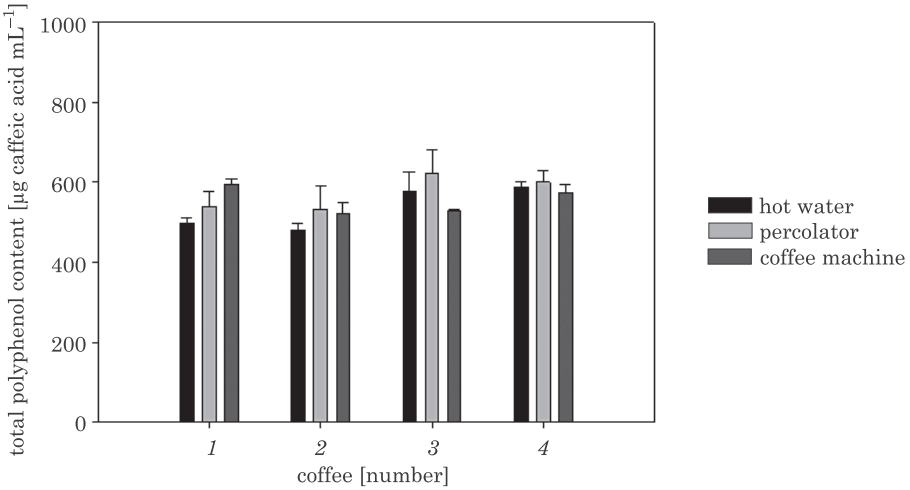

Fig. 3. Total polyphenol content in four roasted coffee beverages depending on three different methods of brewing

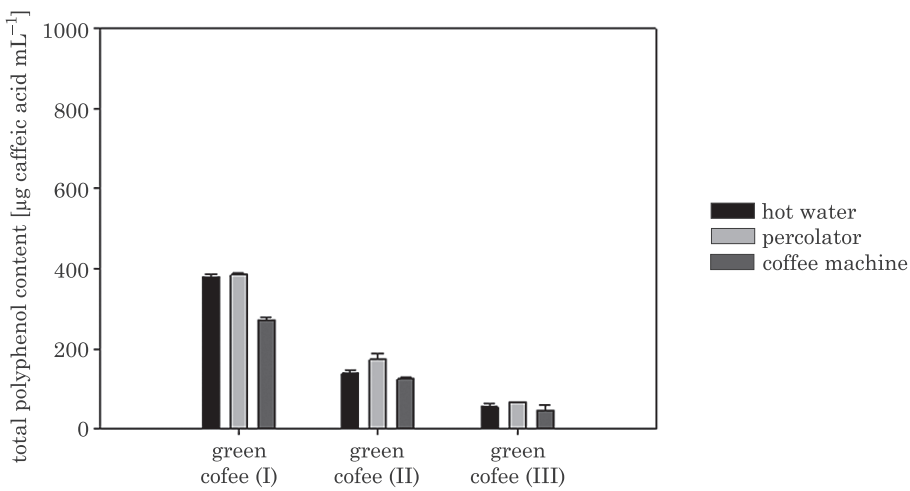

Fig. 4. Total polyphenol content in green coffee beverages depending on three different brewing methods: I – first brew; II – second brew; III – third brew

were 380.9±6.77 µg caffeic acid ml^{-1} for pouring hot water over coffee beans, 384.9±4.46 µg caffeic acid ml^{-1} for the percolator and 271.7±7.15 µg caffeic acid ml^{-1} for the coffee machine. For the second brew, the respective values were 138.4±7.95, 174.2±14.4 and 125.2±5.9 µg caffeic acid ml^{-1}, according to brewing method. For the third brew, the respective values were 55.8±8, 68.1±0.1 and 47.6±12.6. Significant differences were found in first and second brew and the highest polyphenol content was in percolator

beverages. The extract of green coffee beans was found to have lower concentrations of biologically active compounds. Caffeic acid is one of the most important polyphenols in coffee beans responsible for their antioxidant activity. No differences in polyphenol content were reported in a similar study (KREICBERGS et al. 2011). However, in a study of 13 coffee varieties, green coffee beans were found to have higher amounts of polyphenols in seven varieties and the roasted beans in six varieties (PRIFTIS et al. 2015).

As reported by DEROSSI 2017 the American coffee presented higher values of total polyphenol content than espresso and Turkish coffees. In this case, different amounts of coffee powder was used for the experiments.

Total flavonoid content

The results, based on the brewing process, expressed as percentage of quercetin ml^{-1} of coffee beverage, are shown in Figure 5 for the roasted coffee beans, and in Figure 6 for the green coffee beans.

No significant differences in total flavonoid content between different species of roasted coffee were measured while using simple infusion, coffee machine and percolator (Figure 5). For the beverages from roasted coffee beans, the mean flavonoid content was found to be 295.78±2.34 µg quercetin ml^{-1} for pouring hot water over coffee beans, 361.68±5.47 µg quercetin ml^{-1} for the percolator coffee and 252.55±8.11µg quercetin ml^{-1} for the coffee machine. For the beverages from green coffee beans, the mean flavonoid content was found to be 153.80±1.37 µg quercetin ml^{-1} for hot water poured

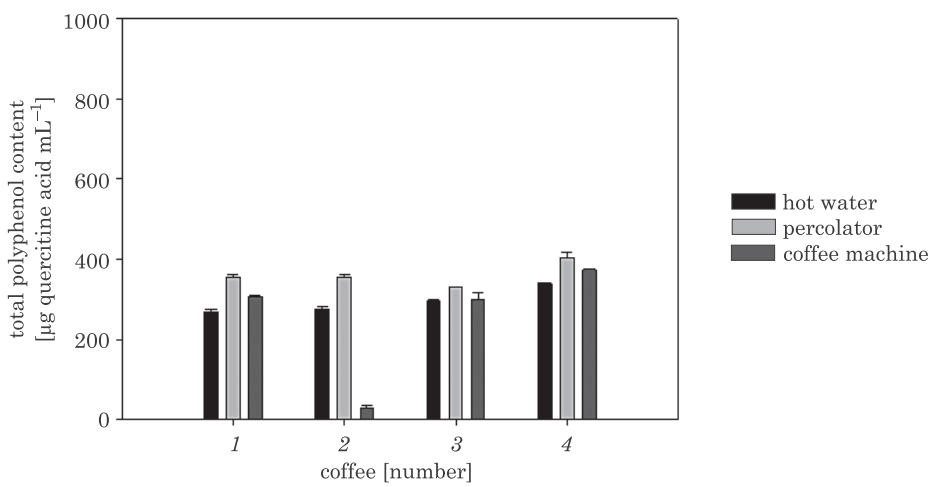

Fig. 5. Total flavonoid content in four roasted coffee beverages according to the three different brewing methods

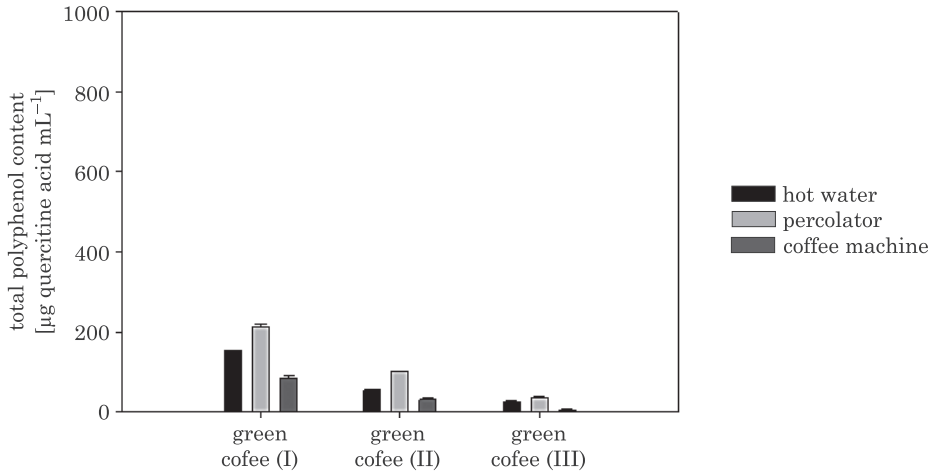

Fig. 6. Total flavonoid content in green coffee beverages depending on three tested brewing methods: I – first brew; II – second brew; III – third brew

over the coffee beans, 212.80±6.39 µg quercetin ml^{-1} for the percolator and 86.00±7.05 µg quercetin ml^{-1} for the coffee machine (first brew); 55.50±2.54 µg quercetin ml^{-1}, 102.60±0.85 µg quercetin ml^{-1} and 32.70±4.32 µg quercetin ml^{-1} (second brew); 26.00±2.65 µg quercetin ml^{-1}, 36.6±4.51 µg quercetin ml^{-1} and 5.6±2.83 µg quercetin ml^{-1} (third brew). Significant differences were found in first, second and third brew between different beverages. Quercetin is a flavonol, the most wide spread sub-class of flavonoids. As with total antioxidant activity and polyphenol content, flavonoid content was highest in green coffee brewed using a percolator; lower levels were observed in the coffee prepared by pouring hot water over coffee beans, and finally the coffee machine. On average, flavonoids comprised 44% of total polyphenol content, for both roasted and green beans (HEČIMOVIĆ et al. 2011).

Caffeine content

Caffeine is the most important active ingredient responsible for the stimulatory effect of coffee, and its levels may vary depending on the type of bean and length of roasting process. The results according to brewing process are shown in Figure 7 for the roasted coffee and Figure 8 for the green coffee.

For the beverages from roasted coffee beans, the average content of caffeine [mg ml^{-1}] were 0.59±0.05 mg caffeine ml^{-1} from pouring hot water over the beans, 0.53±0.03 mg caffeine ml^{-1} for percolator coffee and 0.32±0.02 mg caffeine ml^{-1} for coffee machine coffee. For the beverages

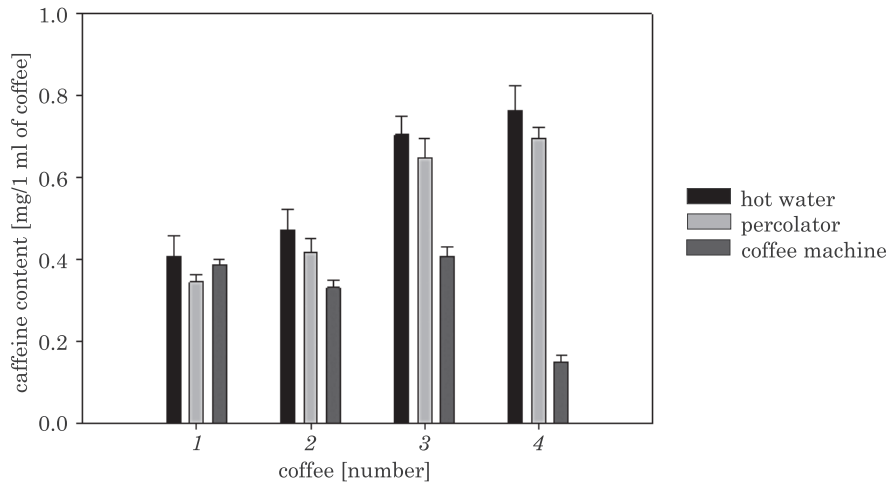

Fig. 7. Caffeine content of four roasted coffee beverages depending on three different brewing methods

from green coffee

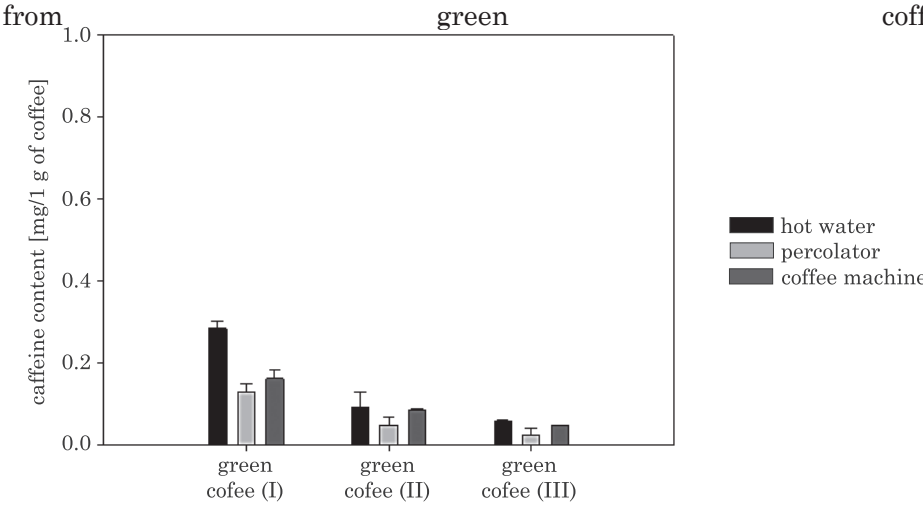

Fig. 8. Caffeine content of green coffee beverages depending on three different brewing methods: I – first brew; II – second brew; III – third brew

beans, the respective values were 0.28±0.02, 0.13±0.02 mg caffeine ml^{-1} and 0.16±0.02 mg caffeine ml^{-1} after the first brew, 0.09±0.03, 0.05±0.02 mg caffeine ml^{-1} and 0.09±0.0004 mg caffeine ml^{-1} after the second brew, and 0.06±0.005, 0.02±0.1 mg caffeine ml^{-1} and 0.05±0.0008 mg caffeine ml^{-1} after the third brew. A slightly higher level of caffeine was found in roasted and unroasted coffee beverages prepared by pouring hot water over the beans, than by using a coffee percolator or coffee machine. The caffeine content of green coffee beans varies between 0.9% and 1.3% dry

matter for Arabica and 1.5% and 2.5% for Robusta coffees. Typical caffeine levels in a cup of coffee vary between 50 and 100 mg (LUDWIG et al. 2014). Caffeine content differs based on brewing method and were ceive similar results (BELL et al. 1996).

The beneficial influence of coffee consumption has been highlighted in previous studies. Coffee intake appears to be associated with a lower risk of type 2 diabetes mellitus (van DAM and HU 2005,van DAM 2006), some cancers (BØHN et al. 2014, NKONDJOCK 2009) and Parkinson's disease (HERNÁN et al. 2002).

Conclusions

Antioxidant activity of infusions was high for roasted and green coffee (first brew) but independent on the condition of brewing. No significant differences in total polyphenol and total flavonoids content between roasted coffee were measured while using simple infusion, coffee machine and percolator. However, significant differences in total polyphenol and total flavonoids content were found for green coffee infusions prepared in percolator. Statistically significant differences in caffeine content have been noticed for roasted and unroasted coffee beans in the following infusions: hot water, percolator and coffee machine for roasted coffee and hot water, coffee machine and percolator for unroasted coffee. Our findings indicate also that infusions from green coffee beans have lower antioxidant activity, as well as lower phenolic acid, flavonoid and caffeine content. Consecutive brews, performed according to the manufacturer's instructions, result in significantly lower levels of bioactive components.

To summarize, conducted experiments did not unequivocally answer the question, if the type of brewing method affects significantly the antioxidant activity in coffee beverages.

Simple infusion allows to obtain the highest content of biologically active substance in coffee. The results depend on type of selected coffee and, among other things, the method of its storage, the degree of grinding.

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PHYSICOCHEMICAL AND ANTIOXIDANT PROPERTIES OF ALGERIAN HONEYS AND THEIR ANTIBACTERIAL POTENCY AGAINST THREE STRAINS OF *E. COLI**

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Key words: Algerian honey, physicochemical, antioxidant, antibacterial properties, entero-pathogenic, bacteria.

Abstract

The aim of the present study was to investigate the physicochemical, the antioxidant and the antibacterial properties of three Algerian honeys (*Eucalyptus*, Wild carrot and Multifloral). Several physicochemical parameters including moisture content, pH, electrical conductivity (EC), Hydroxymethylfurfural (HMF), invertase number and diastase number were measured. Total phenolic contents, reducing power and ABTS scavenging activity were determined. The agar incorporation method was used to determine the antibacterial activity of honeys against three strains of *E. coli* isolated from diarrhea in young calves. The results showed that moisture contents vary from 15.4% to 18.0%, pH values ranged between 4.19 and 4.34, HMF contents ranged between 11.2 and > 100 mg kg^{-1}, invertase number showed values of 3.2 and 20.7, electrical conductivity ranged between 0.38 and 1.1 mS cm^{-1} and diastase number was detected only in Wild carrot honey (11.3). This honey showed the highest level of polyphenols (850.48 ±167.29 mg gallic acid/kg) and the highest reducing power (0.771 ± 0.141), while *Eucalyptus* honey showed the best ABTS scavenging activity (1.7637 ± 0.8596 mmol Eq Trolox/L^{-1}). A strong

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correlation was found between total phenolic content and reducing power (r value was 0.875 and $P < 0.001$). All honeys were effective against all the tested strains with Minimum Inhibitory Concentrations (MIC) of 7% and 8%. *Eucalyptus* honey was bactericide against all the tested strains. This study demonstrated remarkable variation in antioxidant properties of honey depending on its botanic or geographic origin. It also revealed that Algerian honeys exhibit a strong antibacterial activity.

FIZYKOCHEMICZNE I ANTYOKSYDACYJNE WŁAŚCIWOŚCI ALGIERSKICH MIODÓW ORAZ ICH POTECJAŁ ANTYBAKTERYJNY WOBEC TRZECH SZCZEPÓW *E. COLI*

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Słowa kluczowe: miód algierski, właściwości fizykochemiczne, antyoksydacyjne, antybakteryjne, enteropatogenne szczepy bakterii.

Abstract

Celem badań było określenie fizykochemicznych, antyoksydacyjnych oraz antybakteryjnych właściwości trzech gatunków algierskich miodów (eukaliptusowego, z dzikiej marchwi oraz wielokwiatowego). W miodach oznaczono: zawartość wody, pH, przewodność elektryczną, zawartość hydroksymetylofurfuralu (HMF), aktywność inwertazy, liczbę diastazową, całkowitą zawartość związków polifenolowych oraz aktywność przeciwutleniającą. Określono również właściwości antybakteryjne analizowanych miodów wobec trzech szczepów *E. coli*. Stwierdzono, że zawartość wody w badanych miodach wynosiła od 15.4% do 18.0%, a wartości pH – od 4.19 do 4.34, natomiast zawartość HMF – od 11.2 do > 100 mg kg^{-1}. Liczba inwertazowa wynosiła od 3.2 do 20.7, a przewodność elektryczna od 0.38 do 1.1 mS cm^{-1}, natomiast liczbę diastazową określono jedynie dla miodu z dzikiej marchwi (11.3). Ten gatunek miodu charakteryzował się najwyższą zawartością polifenoli ogółem (średnio 850.48 mg kg^{-1} w przeliczeniu na kwas galusowy) oraz najwyższą siłą redukującą (średnio 0.771). Z kolei miód eukaliptusowy wykazał najwyższą aktywność przeciwrodnikową w układzie ABTS (średnio 1.7637 mmol Eq Trolox L^{-1}). Silne związki korelacyjne stwierdzono między całkowitą zawartością związków fenolowych i siłą redukującą (r = 0.875; $P < 0.001$). Wszystkie badane miody wykazywały aktywność antybakteryjną wobec testowanych szczepów bakterii (MIC 7% i 8%), przy czym miód eukaliptusowy charakteryzował się aktywnością antybakteryjną wobec wszystkich trzech szczepów *E. coli*. Wykazano zróżnicowanie właściwości przeciwutleniające badanych miodów zależne od ich pochodzenia botanicznego i geograficznego. Stwierdzono również, że algierskie miody wykazują silne właciwości antybakteryjne.

Introduction

Antibiotic-resistant bacteria pose a very serious threat to public health. Nowadays, the most critical problem facing modern medicine is the rapid emergence of many strains of antibiotic-resistant bacteria (WANG et al. 2012). In addition, the problem of drug resistance is not restricted to pathogenic bacteria but it also involves the commensal bacterial flora that may become a major reservoir of resistant strains (ERB et al. 2007). A number of epidemiological studies demonstrated that resistance does not only concern hospitals but resistant bacteria continue to occur among various groups in the community including pig-breeding, chicken, and cattle, ... etc.

Diarrhea of neonatal calves is a major problem in breeding farms because they often recorded heavy losses and higher rate of morbidity and mortality during calving period (AKAM et al. 2011). The most commonly pathogens incriminated in neonatal calf scours include viral (*rotavirus* and *coronavirus*), protozoal (*Cryptosporidium parvum*) and bacterial pathogens (enterotoxigenic *Escherichia coli* K99 and *Salmonella* spp.) (IZZO et al. 2011). Among bacteria, enterotoxigenic *Escherichia coli* (ETEC) can cause severe diarrhea in newborn calves via the production of a heat-stable enterotoxin (STa). The most common observed fimbriae on ETEC in calves with diarrhea are K99 (F5) and F41, although, strains with F17 fimbriae have been also isolated (NGUYEN et al. 2011).

The use of honey as a traditional remedy for microbial infections dates back to ancient times (BOUKRÂA and BELLIK 2011). The Holy Hadith records the Muslim prophet Mohammed instructing a man afflicted with diarrhea to take honey. The Roman physician Celsus, (c. 25 AD) used honey as a cure for diarrhea (MOLAN 1999). The healing property of honey is due to its chemical composition (ARVANITOYANNIS et al. 2005). Honey contains abundant amounts of polyphenols and flavonoids which confer it good antimicrobial properties. The antimicrobial action is due to its high osmolarity, low pH, hydrogen peroxide content, and some minor uncharacterized compounds (ALZAHRANI et al. 2012a, BERETTA et al. 2005).

The antibacterial nature of honey is dependent on various factors working either singularly or synergistically, the most salient of which are: hydrogen peroxide (produced by the glucose oxidase added to honey by bees), phenolics and aliphatic hydroxy acids of royal jelly and unsaturated dicarboxylic acids, acidity of honey, and the osmotic pressure exerted by honey (ISODOROV et al. 2015). KWAKMAN et al. (2010) reported that the bactericidal activity of honey is due to its high sugar concentration, H_2O_2, the 1,2-dicarbonyl compound methylglyoxal (MGO), the cationic antimicrobial peptide bee defensin-1 and the low pH.

The objective of the present study is to investigate the physicochemical, the antioxidant properties and the antibacterial activity of three varieties of Algerian honeys from different botanical and geographical origin.

Material and Methods

Honey samples

Three local Algerian honey samples (AH1, AH2 and AH3) were purchased from beekeepers in three different geographic area of Algeria (Tlemcen district, Mostaganem district and Chelef district, respectively), and classified according to their botanical origin using acetolysis according to the Erdtman acetolysis method (ERDTMAN 1969). The studied honeys were *Eucaluptus* honey (AH1), Multifloral honey (AH2) and *Daucus carota* or Wild carrot honey (AH3). The three honey samples used in this study were stored at room temperature (22–24°C) in airtight plastic containers until analysis.

Physicochemical analysis

Physicochemical analysis was realized in CARI ASBL (Beekeeping Center for Research and Information, Louvain-la-Neuve, Belgium). Briefly, moisture in honeys was determined using a refractometer at 20°C. The pH value was measured by a pH meter. Hydroxymethylfurfural (HMF) content was measured according to the method of Winkler and the results were expressed in milligrams per kilogram [mg kg^{-1}]. Invertase number was determined by a spectrophotometer at 400 nm. Diastase number was measured according to the method of Phadebas and electrical conductivity was determined with a conductivity meter, the result was expressed in mS cm^{-1}.

Bacterial analysis

Bacterial strains and inoculum standardization

The antibacterial properties of the honeys samples were tested against *Escherichia coli* F5, *Escherichia coli* F17 and *Escherichia coli* CS31A isolated from neonatal calves with diarrhea. Prior to the experiment the strains were inoculated onto the surface of Mac Conkey agar media; the inoculum suspensions were obtained by taking five colonies from 24 h cul-

tures. The colonies were suspended in 5 mL of sterile saline (0.85% NaCl) and shaken for 15 seconds. The density was adjusted to the turbidity of a 0.5 McFarland Standard (equivalent to $1–5 \cdot 10^8$ cfu mL^{-1}).

Minimum Inhibitory Concentration (MIC) measurement

By using the incorporation method, concentrations of honey between 5% and 10% (v/v) were added into Mueller Hinton agar media to test their efficiency against bacteria. The final volume of honey and media in each plate (60 mm) was 5 ml. The plates were inoculated and incubated at 37°C for 24 h. The minimum inhibitory concentration (MIC) was determined by recording the plates with the lowest concentration of honey on which the strain would not grow. Tests were achieved in triplicate. All MIC values are expressed in percentage (v/v).

Antibiotic susceptibility test

Susceptibility to a panel of antimicrobial agents was determined by the standardized disc diffusion assay on Mueller-Hinton agar using commercial antimicrobial susceptibility discs according to the recommendations of the Standardization of Antimicrobial Susceptibility Testing in the Veterinary Medicine at the national level, according to WHO recommendations (MOARD 2008 and 2011). The plates were inoculated and the antibiotic discs were placed on their surface. The tested antibiotics and their corresponding disc concentrations were as follows: Amoxicillin+ acid clavulanic (20/10 µg), Ampicillin (10 µg), Gentamicin (10 µg), Tetracycline (10 µg), Colistin (10 µg), Trimethoprime/sulfamethoxazole (1.25/23.75 µg), Ofloxacine (5 µg) and Cifotaxime (30 µg). The plates were then incubated at 37°C for 24h to 48h. The zone of inhibition was recorded and the data was interpreted using the Standardization of Antimicrobial Susceptibility Testing in the Veterinary Medicine at the national level, according to WHO recommendations (MOARD 2008 and 2011).

To establish whether the antibacterial activity of the tested honey samples was bacteriostatic or bactericidal, the plates where bacterial growth, with the corresponding concentration of honey, was inhibited were scraped by sterile swabs and plated on to nutriment agar. Plates with visible colony growth were considered to correspond to bacteriostatic honey activity while those with no growth were recorded as representing bactericidal honey activity.

Antioxidant activity

Total Phenol Content (TPC)

Total phenolic content was determined using Folin-Ciocalteu method as described by BERETTA et al. (2005). One g of honey was treated with distilled water (10 mL), mixed and filtered using a qualitative filter (filter paper Whatman No. 40, Cambridge, England). An aliquot of this solution (200 μL) was mixed with Folin-Ciocalteu reagent (500 μL, 10%) for 5 min, and then a solution of Na_2CO_3 (1500 μL, 7.5%) was added. All samples were incubated at room temperature in the dark conditions for 30 min, and the absorbance of blue mixtures was recorded at 765 nm using a double beam UV-Visible spectrophotometer (Shimadzu UV-Vis 1202, Japan). Total phenolic content was expressed as mg gallic acid equivalents (GAE)/kg of honey from a calibration curve using the equation: $y = 0.0094\,x + 0.029$ ($R^2 = 0.998$) where y is absorbance and x the concentration.

Reducing power

Ferric reducing power of honey varieties was determined by the method of YEN and DUH (1993). Each sample of honey was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated for 20 min at 50 °C. After incubation, 2.5 ml of trichloroacetic acid (10%) was added to the mixture followed by a centrifugation at 3000 rpm for 10 min. The upper layer (1 ml) was mixed with 1 ml of distilled water and 0.5 ml of ferric chloride (0.1%). The absorbance of the obtained solution was measured by spectrophotometer (Shimadzu UV-Vis 1202, Japan) at 700 nm. A higher absorbance indicates a higher reducing power.

Total antioxidant status (ABTS scavenging activity)

Total antioxidant status was measured by using radical cation decolorization assay (RE al. 1999). This assay based on the inhibition by antioxidants of the absorbance of the free radical cation from ABTS (2,2'-Azinobis-(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt. ABTS was incubated with potassium persulfate in order to produce the free radical cation ($ABTS^{\circ+}$). In brief, ABTS was dissolved in deionized water to make a 7 mmol L^{-1} concentration solution. $ABTS^{\circ+}$ was produced by mixing ABTS stock solution with 2.45 mmol L^{-1} potassium persulfate (final concentration) and the mixture was allowed to stand in the dark at room temperature for 12–16 h before use. In our study, the $ABTS^{\circ+}$ solution was

diluted with PBS (Phosphate buffer solution), pH 7.4, to an absorbance of 0.70 (± 0.02) at 734 nm. After addition of 2 mL of diluted $ABTS^{\circ+}$ to 20 µL of honey sample in PBS, the absorbance reading was taken exactly 6 min after initial mixing. PBS blank were run in each assay. Trolox was used as standard. Radical scavenging activity was expressed as mmol Eq Trolox L^{-1}.

Statistical analysis

All experiments were carried out in triplicate and the results were expressed as the mean values with standard deviations (SD). The significant differences were obtained by a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test ($P < 0.05$). Correlations were established using Pearson's correlation coefficient (r) in bivariate linear correlations ($P < 0.01$). These correlations were calculated using Systat 12 (version 12.00.08).

Results

The results of the pollen analysis obtained by acetolysis according to the Erdtman acetolysis method (ERDTMAN 1969) for three honey samples are shown in Table 1. The frequency classes of pollen grains are given as predominant pollen (> 45%), secondary pollen (16–45%) and minor pollen (< 15%). The botanical families Myrtaceae, Apiaceae, Cistaceae and Asteraceae were the most frequent in the honey samples.

Table 1

Pollen types present in the honey samples [%]

Samples	Samples predominant pollen > 45%	Secondary pollen 10–45%	Minor pollen < 10%
AH1 : Tlemcen district	Eucalyptus (75%)	–	Acacia, Brassicaceae, Ericaceae, Cistaceae, Lamiaceae, Asteraceae Type Dandelion, Rosaceae, Apiaceae, Asteraceae
AH2 : Mostaganem district	–	Apiaceae (42%), Asteraceae (22%) and Cistaceae (16%)	Chenopodiaceae, Plantaginaceae, Poaceae, Fabaceae, Myrtaceae, Rosaceae, Brassicaceae
AH3 : Chelef district	Wild carrot (74%)	Myrthacées (12%)	Acacia, Chenopodiaceae, Cistaceae, Plantain, Poaceae, Renunculaceae, Asteraceae, Brassicaceae, Rosaceae, Apiaceae

Table 2 summarizes the physicochemical values of the studied honey varieties. AH1 (*Eucalyptus* honey) presented the lowest moisture and HMF contents and the highest values of pH and electrical conductivity. On the contrary, AH2 (Multifloral honey) showed the lowest pH and the highest value of moisture content, HMF and invertase number. While AH3 (Wild carrot honey) presented the lowest invertase number and moisture content and the highest amount of HMF and diastase number.

Table 2

Physicochemical properties of the studied honey

Honey Variety	pH		Moisture content [%]		HMF mg kg^{-1}	
	Value	Difference	Value	Difference	Value	Difference
AH1	4.34	AH1-AH2	15.4	AH1-AH2	11.2	AH1-AH2***
AH2	4.19	AH1-AH3	18.0	AH1-AH3***	18.2	AH1-AH3***
AH3	4.31	AH3-AH2	15.4	AH3-AH2	> 100	AH3-AH2***
Honey variety	Invertase number		Diastase number		Electrical conductivity mS cm^{-1}	
	Value	Difference	Value	Difference	Value	Difference
AH1	16.7	AH1-AH2	ND	AH1-AH2	1.1	AH1-AH2***
AH2	20.7	AH1-AH3	ND	AH1-AH3	0.81	AH1-AH3***
AH3	3.2	AH3-AH2***	11.3	AH3-AH2	0.38	AH3-AH2***

ND – no determined
In each column, difference with "***" indicate significant differences at $P < 0.001$.

Table 3 summarizes the results concerning total phenolic contents, reducing power and total antioxidant status of the tested honeys. AH3 presented the highest phenolic content and ferric reducing capacity, but the lowest scavenging activity against $ABTS^{\circ+}$ free radical. We noticed that AH1 showed the best scavenging activity against $ABTS^{\circ+}$ free radical even its total phenolic content and reducing power were the lowest when compared to the other honey samples (AH2 and AH3)

Table 3

Total phenolic contents (TPC), reducing power (PR) and total antioxidant status (TAS) of the tested honeys

Honey Variety	TPC [mg gallic acid kg^{-1}]		Reducing power (PR) [$ABS_{700\ nm}$]		TAS [mmol Eq Trolox L^{-1}]	
	Mean ± SD	Difference	Mean ± SD	Difference	Mean ± SD	Difference
AH1	679.49 ± 90.17	AH1-AH2	0.6819 ± 0.052	AH1-AH2	1.7637 ± 0.8596	AH1-AH2
AH2	736.95 ± 115.09	AH1-AH3*	0.7372 ± 0.081	AH1-AH3	1.3678 ± 0.3369	AH1-AH3*
AH3	850.48 ± 167.29	AH3-AH2	0.771 ± 0.141	AH3-AH2	0.9623 ± 0.2691	AH3-AH2

SD – standard deviation
In each column, difference with "*"indicate significant differences at $P < 0.05$.

A positive and statistically significant correlation ($P < 0.001$) was observed between total phenolic content and reducing power (Table 4).

Table 4

Correlation matrix showing the interrelation between total phenolic content, reducing power and total antioxidant status

Specification	TPC	PR	TAS
TPC	1.000	–	–
PR	0.875***	1.000	–
TAS	-0.112	-0.239	1.000

*** Correlation is significant at the $P < 0.001$.

In terms of the antibiotic susceptibility, all tested strains were susceptible to Cifotaxime but they exhibited resistance to the majority of the tested antibiotics (Table 5).

Table 5

Antibiotic susceptibility of the tested strains

Antibiotic	*E. coli* F5	*E. coli* F17	*E. coli* CS31A
Ampicillin (10 µg)	R	R	R
Amoxicillin + acid clavulanic (20/10 µg)	R	R	R
Gentamicin (10 µg)	R	R	S
Tetracycline (10 µg)	R	R	R
Colistin (10 µg)	R	R	R
Trimethoprime/sulfamethoxazole (1.25/23.75 µg)	R	R	R
Ofloxacine (5 µg)	R	R	R
Cifotaxime (30 µg)	S	S	S

Table 6 summarizes the MIC [%] of the studied honey varieties against three strains of *E. coli* responsible of diarrhea in young calves. The MIC of all honey varieties against the tested strains was 7%, except that AH2 on *E. coli* CS31A which was 8%. The bactericidal or the bacteriostatic effect of honeys varied considerably according to the variety of honey (Table 7). AH1 showed a bactericidal effect against the three strains of bacteria. Whereas AH2 exhibited a bactericidal effect against *E. coli* F5 and *E. coli* F17 and a bacteriostatic effect against *E. coli* CS31A. AH3 exerted a bactericidal effect only on *E. coli* F17 and a bacteriostatic effect against *E. coli* F5 and *E. coli* CS31A.

Table 6

The antibacterial potency of honeys against the tested strains

Honey variety	MIC% of the three varieties against the tested microbes		
	E. coli F5	*E. coli* F17	*E. coli* CS31A
AH1	7	7	7
AH2	7	7	8
AH3	7	7	7

Table 7

Bacteriostatic/bactericidal activity of honeys against the tested strains

Honey variety	Bacteriostatic/bactericidal activity of honeys against the tested microbes		
	E. coli F5	*E. coli* F17	*E. coli* CS31A
AH1	bactericidal	bactericidal	bactericidal
AH2	bactericidal	bactericidal	bacteriostatic
AH3	bacteriostatic	bactericidal	bacteriostatic

Discussion

Honey is rich in properties that result from its chemical composition. The variation of the physicochemical properties of honey depends on the nectar and pollen of the plant source, color, moisture, and protein and minerals contents (WHITE 1978). The pH value has great importance during honey extraction and storage, due to influence on texture, stability and endurance (TERRAB et al. 2002). In our study, all of the investigated honey samples were acid (pH 4.19–4.34). Among all the honey types, Multifloral honey was the most acidic (pH 4.19). Our results are similar to those reported by other researches for Algerian (OUCHEMOUK et al. 2007), Moroccan (NAMAN et al. 2005), Slovak (KASPEROVÁ et al. 2012), Indian (SAXENA et al. 2010), and Portuguese (GOMES et al. 2010) honeys with values ranging between 3.49–4,43, 3.8–4.5, 3.83–4,72, 3.7–4.4 and 3.7–4.3, respectively. The obtained results were slighthly higher to those reported by MONIRUZZAMAN et al. (2013a) and MONIRUZZAMAN et al. (2013b) with values ranging between 3.53–4.03 and 3.83–4.10 and KHALIL et al. (2012) who obtained pH values of 3.7–4.0.

Moisture is a parameter related to the maturity degree of honey and temperature. In the present study, moisture values ranged between 15.4% and 18%. All tested honeys showed moisture contents below 20%, which is the maximum prescribed limit (≤ 20%) by European Community regulations (The Council of the European Union 2002). The moisture contents

of the analyzed honey samples were consistent with those previously reported for Algerian honeys with values ranging between 14.64 to 19.04% (OUCHEMOUK et al. 2007) and 11.59 to 14.13% (KHALIL et al. 2012), Malaysian honey (14.16 to 17.53%) (MONIRUZZAMAN et al. 2013b), Portuguese honey (15.9–17.2%) (GOMES et al. 2010), Moroccan honey (14.3 to 20.2%) (CHAKIR et al. 2016), and Indian honey (17.2–21.6%) (SAXENA et al. 2010). It is worth noting that the moisture content of honey can be affected by climate, season and moisture content of plant source (OUCHEMOUK et al. 2007).

Hydroxymethylfurfural (HMF) content is widely recognized as a parameter of honey samples freshness because it is absent in fresh honeys and tends to increase during processing and/or aging of the product. Several factors influence the levels of HMF such as temperature and time of heating, storage conditions, pH and floral source; therefore HMF provides an indication of overheating and storage in poor conditions (GOMES et al. 2010, MONIRUZZAMAN et al. 2013a). AH3 contained a high level of HMF (>100 mg kg^{-1}), which exceeded the limit established by Codex Alimentarrius (ALINORM 01/25 2000); this honey was probably stored for more than one year. The concentrations of HMF for AH1 and AH2 were 11.2 mg kg^{-1} and 18.2 mg kg^{-1}, respectively; these concentrations were within the recommended range set by the Codex Alimentarrius at 80 mg kg^{-1}. Others studied Algerian honeys and reported higher concentrations of HMF (15.23–24.21 mg kg^{-1}) (KHALIL et al. 2012). Likewise, MAKHLOUFI et al. (2010) recorded a higher concentration of HMF for *Eucalyptus* honey (25.63 mg kg^{-1}) and a low concentration of HMF for Multifloral honey (17.18 mg kg^{-1}).

In the present study, diastase number (DN) was not detected in *Eucalyptus* (AH1) and Multifloral (AH2) honeys but in Wild carrot honey (AH3) it was 11.3. Similar result regarding diastase number of Wild carrot honey (8.3) was reported by ALZAHRANI et al. (2012a). However, MAKHLOUFI et al. (2010) obtained higher levels of DN (18.0 and 15.9 for Multifloral and *Eucalyptus* honeys, respectively). Besides, the results showed an invertase number of 16.7, 20.7 and 3.2 for *Eucalyptus*, Multifloral and Wild carrot honeys, respectively. These results were higher to those obtained by MAKHLOUFI et al. (2010) for *Eucaluptus* honey (9.64) and Multifloral honey (7.9). The possible explanation for variation in the diastase and invertase number could be attributed to variations in geographical origin of honey as well as the time of harvest.

Electrical conductivity (EC) is a key physicochemical parameter for the authentication of unifloral honeys. The EC value depends on the ash and acid content in honey. According to the EU directive (EUROPEAN COM-

MISSION 2002) nectar honey should have a conductivity of no more than 0.8 mS cm^{-1}. Higher values are considered as belonging to honeydew honey or mixtures of honeydew and nectar honey. There are however some exceptions to this limit, *Eucalyptus* honey being one of them.

EC values of all honey samples were 0.38–1.1 mS cm^{-1} (Table 2). Two of honey samples (AH1 and AH2) were upper to the allowed parameters set by Codex Alimentarius. The EC values of some Algerian honeys were reported to be 0.21–1.61 mS cm^{-1} (OUCHEMOUK et al. 2007). Our results were higher to the findings reported by KHALIL et al. (2012) and SAXENA et al. (2010).

Polyphenols are an important group of compounds that were reported to influence not only the appearance but also the functional properties of honey (MONIRUZZAMAN et al. 2013b, CIMPOIU et al. 2013). The concentration of phenolic compounds in honey is highly dependent on its plant source (KHALIL et al. 2012). The total phenolic contents in the studied honeys varied greatly according to the type of honey. Wild carrot honey contained the highest level of polyphenols (850.48 ± 167.92 mg gallic acid/kg) (Table 3). This concentration is higher than that reported by ALZAHRANI et al. (2012a) for the same type of honey (503.09 ± 8.29 mg gallic acid/kg). In general, the levels of total phenolic content of all tested honeys were higher than that reported by KHALIL et al. (2012) for some Algerian honeys with values ranging between 411.10 ± 1.55 to 498.16 ± 1.32 mg gallic acid/kg, but lower than that reported by OUCHEMOUK et al. (2007) with values ranging between 64 mg gallic acid/100 g to 1304 mg gallic acid/100g. Many researchers found that a honey with high level of total phenol content indicates their high antioxidant proprieties.

Reducing power is a widely used method for antioxidant determination of plants and natural products and has been successfully applied for the assessment of the antioxidant capacity of honey. The reducing power gives a direct estimation of the antioxidants or reductants present in a sample based on its ability to reduce the Fe^{3+}/Fe^{2+} couple (ISLAM et al. 2012). A relatively higher absorbance value corresponds to a more reduction of ferric ions to ferrous ions. The reducing power of honey samples varied from 0.6819 ± 0.052 to 0.771 ± 0.141. The obtained results were greatly higher than those reported by SAXENA et al. (2010) and ALZAHRANI et al. (2012a).

ABTS is a measure of antioxidant activity in contrast to antioxidant concentration, which might include a proportion of biologically inactive antioxidants. ABTS permits the measurement of antioxidant activity of mixtures of substances, hence helping to distinguish between additive and synergistic effects (MONIRUZZAMAN et al. 2012). Despite the fact that hon-

eys showed important phenolic contents, which involves the presence of many hydroxyl groups capable of chelating free radicals, the studied honeys showed low scavenging activity against ABTS free radical. Our results disagree those reported by ALZAHRANI et al. (2012b) and other previous studies dealt on TAS of honey.

Statistical tool is a useful complimentary approach to investigate the relationship between the antioxidant activities of honey and its biochemical composition. From the Table 3, it can be seen that total phenol content was strongly correlated with reducing power (r = 0.885), indicating that polyphenol compounds also contribute to the antioxidant capacity of honey. This statistically significant correlation was in agreement with the previous findings (MONIRUZZAMAN et al. 2013a, ALZAHRANI et al. 2012a, ISLAM et al. 2012).

The important finding from this study is that all tested bacterial strains were inhibited by the three honey samples. While most of the used strains were resistant to the tested antibiotics (Table 3). It could be pointed out that, except for Cifotaxime (30 μg), which was active on the majority of strains, all the other antibiotic did not show an inhibition activity on the most of the isolates. The antibacterial activities of different brands of honeys were proved to be efficient against all strains of *E. coli*. The average MIC value was 7% (v/v). This antibacterial effect is greatly higher than those reported in the literature for *E. coli* strains (JIMENEZ et al. 2016, SHERLOCK et al. 2010, TAN et al. 2009). Regarding the bactericidal and bacteriostatic effect of the different varieties of honey, it was found that *Eucalyptus* honey exhibited bactericidal effect against all the tested strains of *E. coli*. Multifloral honey was bactericide on *E. coli* F5 and *E. coli* F17, while Wild carrot honey was bactericide only on *E. coli* F5.

Conclusions

In conclusion, this study showed that Algerian honeys contained a significant amount of polyphenols that can produce the high antioxidant activity. In addition, these honeys (*Eucalyptus*, Multifloral and Wild carrot) exhibited a potent antibacterial activity against pathogenic *E. coli* causing diarrhea in young calves. These findings may afford useful basis for the alleged therapeutic effects of honey and support its application as an alternative treatment, however, further *in vivo* studies are needed to confirm the findings of the present study.

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EVALUATION AND COMPARISON OF IDW, RBF, GPI AND KRIGING METHODS FOR GENERATING SPATIAL DISTRUBUTIONS OF HEAVY METALS FOR SMALL SURFACE AREAS*

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Key words: geostatistical analysis, interpolation, heavy metals, degraded areas, industrial areas.

Abbreviations: IDW (*Inverse Distance Weighted),* RBF (*Radial Basis Function),* GPI (*Global Polynomial Interpolation).*

Abstract

In the work, the problem of presenting the spatial distribution of contamination with heavy metals in small-scale areas has been described. Two study areas have been chosen, located near industrial areas in south Poland – The "Miasteczko Śląskie" Zinc Smelter and the "Częstochowa" Steel Mill. A network of measurement points was planned, where from the top layer of soil, total of 108 samples have been taken. The content of three heavy metals was determined and included: Zn, Pb, Ba. Six methods of generating spatial models were selected: IDW, RBF, GPI, Ordinary Kriging, Simple Kriging and Universal Kriging. Two main criteria of evaluation were adopted: accuracy and visual quality of interpolation methods. In terms of accuracy analysis, no significant differences between the different methods have been observed. In the case of visual evaluation, it was found that the most suitable methods are IDW and RBF.

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PORÓWNANIE METOD KRIGINGU, IDW, RBF I GPI W GENEROWANIU ROZKŁADU PRZESTRZENNEGO METALI CIĘŻKICH DO OPRACOWAŃ MAŁOOBSZAROWYCH NA PRZYKŁADZIE PRZEMYSŁU HUTNICZEGO

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Skróty: IDW (ang. *Inverse Distance Weighted*), RBF (ang. *Radial Basis Function*), GPI (ang. *Global Polynomial Interpolation*).

Abstract

Przeprowadzone badania dotyczyły problemu generowania rozkładów przestrzennych zanieczyszczeń metalami ciężkimi do opracowań małoobszarowych. Wybrano dwa obszary badań usytuowane w okolicy terenów przemysłowych – Huty Cynku "Miasteczko Śląskie" i Huty Stali "Częstochowa". Zaplanowano sieć punktów pomiarowych, na podstawie której pobrano dwie próbki gleb z wierzchniej warstwy. Wyznaczono zawartość trzech metali ciężkich: Zn, Pb, Ba. Wybrano sześć metod interpolacji: IDW, RBF, GPI, kriging zwyczajny, kriging zwykły i kriging uniwersalny. Przyjęto dwa główne kryteria oceny – dokładności lokalizacji oraz jakości wizualnej. W przypadku dokładności nie zauważono znaczących różnić między metodami. Lepszą jakość wizualną uzyskano natomiast za pomocą metody IDW oraz RBF.

Introduction

The problem of soil pollution is an important issue mainly related to the growing industry, urbanization, agriculture and mining. The emission of pollutants, including heavy metals occurs over a wide range of processes, for example incineration, mining, processing, transport or storage. Due to high solubility of heavy metal compounds (e.g. Pb, Zn) they may pose a threat not only to the topsoil but also to the surface and groundwater (JÄRUP 2003, ROZPONDEK et al. 2016, SOLLITTO et al. 2010). Heavy metals are not biodegradable, when incorporated into the soil they remain there permanently. The effects of this phenomenon pose a significant threat to the environment and humans (OCIEPA-KUBICKA and OCIEPA 2012, SINGH et al. 2011).

The pollution of soils is characterized by a high degree of spatial variation due to a combination of physical, chemical, biological processes, which impacts the soil with different intensity and at different scales

(GOOVAERTS 1993, Reza et al. 2010). Preparing spatial distribution of pollution is the basis for defining, monitoring and controlling environmental pollution. In particular, an important issue is the accuracy of the created pollution maps. Unfortunately, in many studies related to the environment, it is completely ignored (XIE et al. 2011).

Geostatistics is a set of methods of estimation, which uses a sophisticated set of statistical tools enabling the spatial analysis and interpolation. In addition to providing spatial distribution, geostatistic allows the assessment of the accuracy of the created models (CRESSIE 2015, ZAWADZKI 2011). The most commonly used techniques of interpolation are: IDW – Inverse Distance Weighted and a set of kriging methods. However, no superiority of one method over the other had been stated. The research conducted in 2005 on the content of arsenic in groundwater in Texas showed that the method of the weighted inverse distance achieved greater accuracy than the kriging method (GONGA et al. 2014). The thesis of the superiority of IDW was confirmed by determining the content of methane (ZHOU and MICHALAK 2009). However, in the studies on radioactive contamination, kriging proved to be more accurate (MABIT and BERNARD 2007). Similar results were obtained in studies of spatial distribution of mercury (HU et al. 2005). The discrepancy in the test results can point to the fact that the result of the estimation depends on the diversity of the studied phenomenon, its layout and the specific area of occurrence (ACOSTA et al. 2011). This leads to difficulty in obtaining an adequately accurate model. In addition, it is worth mentioning that the geostatistical methods usually require the input data to meet certain conditions (e.g. a normal distribution, a dense measurement network, independence of observation). Unfortunately, such conditions hardly exist in issues relating to environmental pollution (KISHNÉ et al. 2003, ROZPONDEK and WANCISIEWICZ 2016, XIE et al. 2011).

The aim of the study was to assess and identify the most accurate interpolation methods of spatial distribution in contaminated small surface areas. For purpose of this studies, three heavy metals were chosen (zinc, lead and barium). The selection was based on the laboratory results – high values of Zn, Pb and Ba occurs in both studied areas. Six available methods were selected for accuracy analysis in ArcGIS: Inverse Distance Weighted, Radial Basis Function, Global Polynomial Interpolation (GPI), Ordinary Kriging, Simple Kriging and Universal Kriging. In a previously conducted study, due to the much simpler application, interpolation was used with a method of Inverse Distance Weighted (ROZPONDEK et al. 2016, ROZPONDEK and WANCISIEWICZ 2016).

Materials and Methods

The study selected two small areas located near an industrial infrastructure. One of them is located in the vicinity of the "Miasteczko Śląskie" Zinc Smelter and the other in the area of the "Częstochowa" Steel Mill. The main slection criterias for the areas of research were as follow: the available information about the area, the state of vegetation, air images and the surrounding forms of land use.

The "Miasteczko Śląskie" Zinc Smelter is a facility working since the early 70s of the XX century. The plant produces zinc, lead, crude and refined and sulfuric acid. As a result of many years of emissions into the atmosphere, the areas around the plant were heavily contaminated with heavy metals (Pb, Zn, Cd) and gases (SO2, CO). As a result of the effect of metal-bearing dust in the closest vicinity of the plant, big surface areas have been created which are totally or largely devoid of vegetation (KACPRZAK 2007). On the selected part located in the neighbourhood of the Zinc Smelter, a network of 29 measuring points was planned (Figure 1), with particular emphasis on the area with the least amount of vegetation.

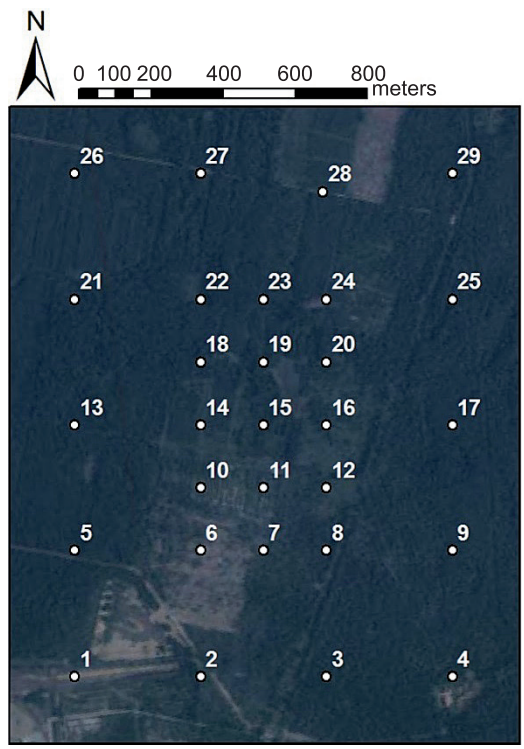

Fig. 1. Arrangement of measurement points for an area in the vicinity of the „Miasteczko Śląskie" Zinc Smelter" (ROZPONDEK et al. 2016)

The measurement network has the form of a grid of different edge lengths – for the central region it is 175 meters, and 350 meters for the external region. In March 2016, two samples of soil were taken from the each point of top layer of soil 0–20cm, respectively (ROZPONDEK et al. 2016, 2017).

The "Częstochowa" Steel Mill is the largest source of dust in the area of Czestochowa. It produces more than 65% of heavy plates produced in Poland. As a result of pollution emission into the atmosphere, on the surrounding area there are high concentrations of certain heavy metals. On the selected area, located in the neighbourhood of the Smelter, a network of 25 measuring points was planned (Figure 2). The measurement network has the form of a grid with the edge length of 140 meters. In June 2016, at each of the points two samples of soil were collected from the top layer of soil 0–20 cm.

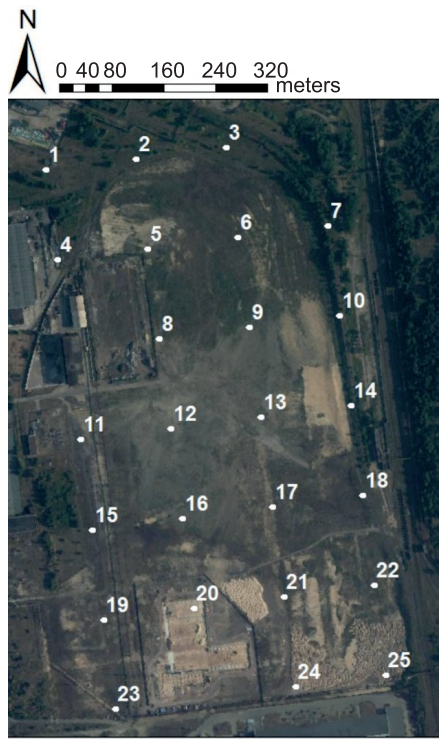

Fig. 2. Distribution of measuring points for an area in the vicinity of the "Częstochowa" Steel Mill

The collected soil samples were pre-dried at room temperature and sieved through a sieve with a mesh diameter of 2 mm. Then it was dried at a temperature of 105°C to solid mass and then triturated in a mortar. A six samples were prepared for analysis from each point. Subsequently, for the purpose of the designation of heavy metals, the extraction of metals with

aqua regia was made (a mixture of hydrochloric and nitric acid in a volume ratio of 3:1). Mineralization was carried out at 180°C for 30 minutes, using a high-pressure microwave mineralizer of the German company called Berghof (*Jakość gleby*… PN-ISO 11466:2002). The content of heavy metals was determined on an ICP-OES Thermo IRIS plasma spectrometer. The average results, as well as the standard deviation are presented in Table 1 (ROZPONDEK and WANCISIEWICZ 2016, ROZPONDEK et al. 2017).

Table 1

The content of Zn, Pb, and Ba in soils around the "Miasteczko Śląskie" Zinc Smelter and the "Częstochowa" Steel Mill

Zinc Smelter „Miasteczko Śląskie"					Steel Mill „Częstochowa"				
Point number	total content of heavy metals [mg kg^{-1}]				point number	total content of heavy metals [mg kg^{-1}]			
	parameter	Ba	Pb	Zn		parameter	Ba	Pb	Zn
1.1	mean	20.8	353.9	582.6	1.1	mean	92.5	81.9	223.9
	SD	2.33	11.67	11.25		SD	2.04	1.39	1.24
2.1	mean	254.9	1121.4	415.1	2.1	mean	134.0	171.7	382.6
	SD	13.82	15.34	15.91		SD	3.45	5.99	13.66
3.1	mean	19.0	26.3	125.0	3.1	mean	215.0	256.1	844.2
	SD	1.12	3.51	6.92		SD	12.12	6.99	28.65
4.1	mean	58.7	89.1	155.0	4.1	mean	101.9	169.7	336.6
	SD	4.87	4.24	8.32		SD	2.54	3.54	3.86
5.1	mean	65.5	360.2	721.3	5.1	mean	51.4	36.9	103.0
	SD	1.66	14.90	22.66		SD	1.76	2.69	3.99
6.1	mean	132.5	600.7	315.7	6.1	mean	50.8	22.2	32.8
	SD	8.56	13.05	12.00		SD	2.00	3.09	2.04
7.1	mean	173.0	509.8	218.2	7.1	mean	52.4	32.6	73.4
	SD	6.74	17.75	10.82		SD	0.86	1.00	1.63
8.1	mean	21.2	29.4	153.4	8.1	mean	48.1	35.5	84.7
	SD	1.58	2.14	7.71		SD	4.09	2.18	3.44
9.1	mean	18.3	31.8	64.1	9.1	mean	132.3	258.0	533.1
	SD	1.54	2.66	2.92		SD	8.68	4.60	24.90
10.1	mean	62.3	211.5	145.9	10.1	mean	46.3	33.5	70.0
	SD	8.09	22.96	18.64		SD	3.66	4.40	5.34
11.1	mean	13.9	21.9	45.1	11.1	mean	163.5	128.2	441.2
	SD	4.76	3.69	2.66		SD	10.60	8.55	18.18
12.1	mean	106.1	252.7	217.3	12.1	mean	460.5	277.7	776.9
	SD	15.88	31.80	30.72		SD	23.41	17.92	22.99
13.1	mean	752.0	1467.5	815.7	13.1	mean	97.8	89.8	290.9
	SD	26.52	44.67	19.73		SD	8.92	11.18	19.97

14.1	mean	224.4	435.7	273.6	14.1	mean	34.1	18.5	38.2
	SD	10.78	9.38	10.66		SD	2.08	1.66	2.55
15.1	mean	521.1	1710.8	586.1	15.1	mean	46.0	92.5	116.0
	SD	31.20	127.20	44.60		SD	4.46	10.03	8.25
16.1	mean	50.0	259.7	113.8	16.1	mean	66.3	128.1	251.1
	SD	4.29	6.75	5.64		SD	4.80	6.21	20.23
17.1	mean	81.3	175.0	90.8	17.1	mean	22.4	16.8	34.8
	SD	3.11	4.80	5.90		SD	1.85	1.55	4.01
18.1	mean	110.5	258.4	216.1	18.1	mean	74.9	92.9	431.1
	SD	8.98	14.13	10.24		SD	5.37	7.11	20.46
19.1	mean	803.7	1893,0	1376,7	19,1	mean	33,4	33,3	87,4
	SD	65.60	128.20	72.20		SD	2.06	2.76	4.40
20.1	mean	84.4	120.1	461.2	20.1	mean	16.9	16.4	23.3
	SD	4.19	7.56	12.96		SD	1.83	2.58	2.72
21.1	mean	86.8	158.6	223.2	21.1	mean	24.6	15.9	38.6
	SD	4.50	7.80	11.37		SD	3.19	2.18	4.77
22.1	mean	61.7	88.1	252.0	22.1	mean	40.1	27.1	68.2
	SD	2.47	6.05	10.51		SD	3.66	2.89	3.62
23.1	mean	680.3	1232.8	449.3	23.1	mean	45.9	38.4	83.7
	SD	16.21	29.76	21.74		SD	2.35	4.81	5.15
24.1	mean	115.8	229.3	238.9	24.1	mean	25.8	19.0	66.9
	SD	7.18	14.39	8.63		SD	4.95	2.10	2.70
25.1	mean	36.0	88.4	35.9	25.1	mean	17.5	11.9	25.1
	SmD	2.60	2.07	3.76		SD	2.18	2.18	1.75
26.1	mean	38.0	49.9	39.7	–	–	–	–	–
	SD	4.22	6.52	3.50	–	–	–	–	–
27.1	mean	298.1	439.6	733.8	–	–	–	–	–
	SD	10.68	17.95	18.36	–	–	–	–	–
28.1	mean	68.6	91.8	193.2	–	–	–	–	–
	SD	7.37	10.54	14.92	–	–	–	–	–
29.1	mean	404.8	833.5	361.3	–	–	–	–	–
	SD	17.98	34.24	20.49	–	–	–	–	–

After preparation of the descriptive statistics, on the basis of the obtained results, spatial distributions were carried out along with the assessment of the accuracy of the selected interpolation methods. For this purpose, an ArcGIS software was used.

Spatial distributions have been validated – using the parameters of the semivariogram, one of the values was excluded from the interpolation and its value was determined based on the remaining (Reza et al. 2010, http://desktop.arcgis.com). Due to the assessment of the models, the main

indicators were the Mean and the Root Mean Square. Due to the higher complexity of kriging methods, their validation were performed in more detail. The Root Mean Square Standardized and the Average Standard Error was additionally used. Using the relationship between these values, the following criteria was adopted:

– the average error should be zero,
– the mean square error should be as close to zero as possible,
– the mean square error should be equal to the average standard error,
– the standardized mean square error should be equal to one.

If the RMSS is greater than one or the ASE is greater than the RMS, then the model is underestimated (assumes lower values than in reality) and vice versa (JOHNSTON et al. 2001, KRAVCHENKO and BULLOCK 1999, MEHDI et al. 2013, http://desktop.arcgis.com). Another criterion of assessment was the visual quality.

Six selected interpolation methods were analyzed:

1. In the inverse distance weighted method (IDW), which can be assigned to the groups of kriging methods, the estimated points are determined on the basis of source points, found in its surroundings. The result is affected by several parameters such as: the search range, power factor and the number of points involved in the estimation (GONGA et al. 2014, ROBINSON and METTERNICHT 2006, ROZPONDEK et al. 2016).

2. The radial basis function (RBF) accommodates surfaces on the basis of measured points minimizing the curvature. The generated surface must contain all the source points. Then, the estimated points are projected on it. By this, it is possible to obtain higher values than the maximum measured and lesser values than the minimum (BHUNIAA et al. 2018, JOHNSTON et al. 2001).

3. The Global Polynomial Interpolation (GPI) is similar to RBF, however the fitted surface is more "smooth". It is determined by a polynomial mathematical function based on input points. Similarly, as in the RBF, values outside the range of input data may occur (http://pro.arcgis.com).

4. Ordinary, simple and universal kriging belong to the group of linear kriging. As in the method of the inverse distance weighted method, the interpolation is performed based on the relationship between the distance points. However, in contrast, the weights are allocated based on the semivariogram. The semivariogram is a characteristic of spatial continuity occurring in the tested data set. The semivariogram model is characterized by a mathematical function. Its main parameters are the scope of impact, the nuggets effect and threshold. The difference between the ordinary and universal kriging is not significant. It appears at the level of the disposal of the trend – again, universal kriging adapts the trend to

the data from which the trend has already been removed. It should be noted that due to the nature of the pollution of the environment, we quite often have to deal with a certain data trend. While the difference between ordinary and simple kriging is based on the assumption of stationarity, which expects that the average value and method of data distribution is constant for the tested region. Simple kriging accepts this assumption, while ordinary kriging calculates the average for the studied area. The kriging methods do not specify a minimum amount of source points, but other authors research indicates that there should be at least 30. In this study, due to the nature and area of study, the number of points was 29 and 25. This may result in obtaining less accurate results (GONGA et al. 2014, JOHNSTON et al. 2001, ZAWADZKI 2011).

Results and Discussion

The basis for performing the correct analysis is suitable geostatistical analysis of the input data. It enables a better understanding of the data and setting some initial relationships that could significantly affect the outcome of interpolation (CRESSIE 2015, ZAWADZKI 2011, http://pro.arcgis.com). The results of the determination of the total content of Pb, Zn and Ba in the soil analyzed is included in Table 1 (ROZPONDEK et al. 2017). Descriptive statistics were performed (Table 2, Table 3) and histograms were made (Figure 3, Figure 4).

Table 2

Statistics of the analyzed elements – the "Miasteczko Śląskie" Zinc Smelter

Zinc Smelter „Miasteczko Śląskie"									
Heavy metal	min.	max	mean	standard deviation	skewness	curtosis	Q1	median	Q3
Zn	36	1377	332	296.8	1.75	6.40	141	223	452
Ba	14	804	185	227.9	1.68	4.55	47	84	232
Pb	22	1893	453	529.3	1.51	4.11	89	253	533

Table 3

Statistics of the analyzed elements – the "Częstochowa" Steel Mill

Steel Mill „Częstochowa"									
Heavy metal	min.	max	mean	standard deviation	skewness	curtosis	Q1	median	Q3
Zn	23	844	218	234.4	1.37	3.98	60	87	348
Ba	17	461	84	92.7	2.91	12.08	34	51	99
Pb	12	278	84	83.0	1.19	3.19	21	37	128

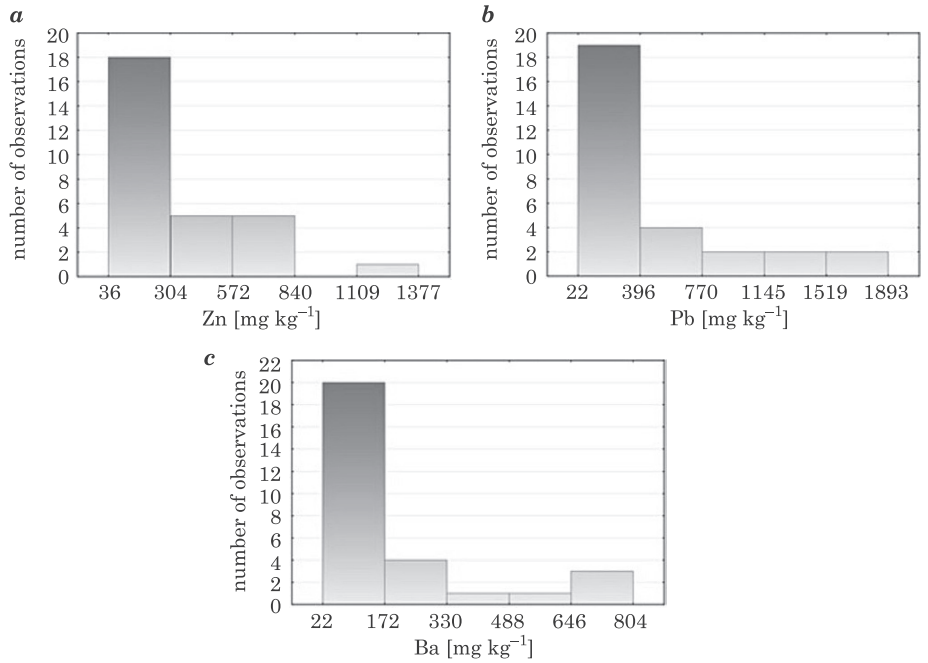

Fig. 3. Histograms of the analyzed elements – "Miasteczko Śląskie" Zinc Smelter: *a* – Zn [mg kg^{-1}]; *b* – Pb [mg kg^{-1}]; *c* – Ba [mg kg^{-1}]

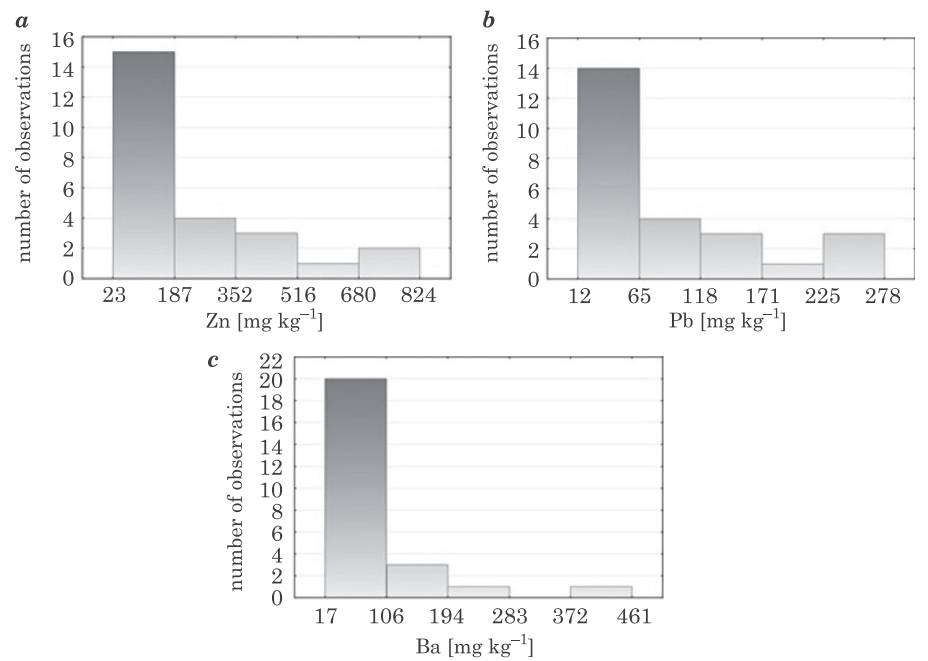

Fig. 4. Histograms of the analyzed elements – "Częstochowa" Steel Mill: *a* – Zn [mg kg^{-1}]; *b* – Pb [mg kg^{-1}]; *c* – Ba [mg kg^{-1}]

All the analyzed elements are characterized by similar descriptive statistics:

– strong right-sided asymmetry (high asymmetry value),
– high standard deviation relative to the mean value,
– the average value bigger than the median,
– a big difference between the maximum and minimum value,
– leptokurtic distribution – values are more concentrated than in the normal distribution,
– the occurrence of outliers.

IDW, RBF and the GPI methods are considered to be less complex because they do not require such a large amount of initial analysis. Here, descriptive statistics can only identify the outliers that should be reviewed in terms of credibility. The outliers were examined and no factors that might justify their removal were identified. The decomposition of soil is often complex and is characterized by sudden changes in the values (ACOSTA et al. 2011, ROZPONDEK et al. 2016, ZAWADZKI 2011).

The kriging method, however requires data with a normal distribution. The above analysis indicates the absence of such a distribution for the analyzed elements. Therefore, the data were subjected to logarithmic transformation, and after interpolation, the estimated values underwent reverse transformation (JOHNSTON et al. 2001, REZA et al. 2010). In the ArcGIS software, a geostatic tool was used to detect trends. In all the data, the trend of the first degree was detected, which is included in further studies (JOHNSTON et al. 2001, http://pro.arcgis.com).

After the descriptive analyzes, a total of 36 spatial distribution models were performed of the studied elements with the chosen interpolation methods. The results of the accuracy analysis were given in Table 4.

The differences in the results between the "Miasteczko Śląskie" Zinc Smelter and the „Częstochowa" Steel Mill may arise, not only because of the different physio-chemical values and the source of contamination, but also because of the different arrangement of the network of measuring points (ROZPONDEK et al. 2016, ZAWADZKI 2011). A comparison of the accuracy of interpolation is based on the established criteria for mainly RMS and the average value, but also RMSS and ASE (JOHNSTON et al. 2001, KRAVCHENKO and BULLOCK 1999, Mehdi et al. 2013, http://desktop.arcgis.com). After a thorough interpretation of the results it was observed that RMS obtained high values. This may result due to sudden value changes of a relatively small area.

Most of the kriging methods are characterized by underestimating the values – ASE greater than RMS and RMSS greater than one. Universal kriging obtained an average value most deviating from zero. In addition

for the area of the Zinc Smelter, RMSS value was quite high (close to 2) so that the resulting spatial resolution can be considered unreliable (JOHNSTON et al. 2001, ZAWADZKI 2011).

Table 4

The results of the accuracy analysis for the selected interpolation methods

Heavy metal	Interpolation method	Zinc smelter „Miasteczko Śląskie"				Steel mill „Częstochowa"			
		mean	RMS	RMSS	ASE	mean	RMS	RMSS	ASE
Zn	IDW	3.3	285.4	–	–	6.4	235.5	–	–
	GPI	1.8	297.5	–	–	2.3	232.2	–	–
	RBF	-3.6	261.9	–	–	1.1	248.8	–	–
	ordinary kriging	-5.0	316.2	1.17	305.3	10.7	260.0	1.16	362.5
	simple kriging	1.7	290.7	0.99	309.2	4.6	252.6	1.19	306.7
	universal kriging	-34.9	305.7	1.51	346.8	-25.5	229.9	1.08	301.0
Ba	IDW	10.6	229.8	–	–	0.1	91.8	–	–
	GPI	3.1	237.0	–	–	1.4	90.8	–	–
	RBF	-11.7	211.7	–	–	-1.8	94.1	–	–
	ordinary kriging	3.1	253.1	1.00	345.5	0.4	89.3	1.11	84.4
	simple kriging	-2.7	250.9	1.03	287.2	-3.3	89.5	1.26	72.1
	universal Kriging	-47.4	233.4	1.73	249.2	-9.8	89.8	1.40	85.0
Pb	IDW	22.4	557.5	–	–	3.5	79.6	–	–
	GPI	7.8	557.1	–	–	1.0	78.5	–	–
	RBF	-11.3	490.5	–	–	1.1	84.1	–	–
	ordinary kriging	10.5	601.6	1.00	828.2	-0.1	86.3	1.03	102.1
	simple kriging	-22.7	571.8	1.01	734.8	-3.5	85.1	1.23	84.0
	universal kriging	10.3	548.0	2.14	704.4	-5.4	86.8	1.21	100.4

In terms of RMS, for the area of the "Miasteczko Śląskie" Zinc Smelter, the most accurate method was the RBF method, and right after that IDW and ordinary kriging. In the case of the "Częstochowa" Steel Mill, the most accurate method proved to be GPI. However, the differences in RMS are relatively small. Only in the case of lead content in soils of the "Miasteczko Śląskie" Zinc Smelter, the advantage of the RBF method over the others was substantial. The results conducted by other researchers are often divergent, a part indicates the IDW method (GONGA et al. 2014, ZHOU and MICHALAK 2009), and some – the group of kriging methods (Hu et al. 2005, Mabit and Bernard 2007).

In the group of the kriging methods, the most stable results were obtained by the simple kriging method and the least reliable were obtained by universal kriging. Please note that apart from the criterion of accuracy, an important element of the assessment is visual quality. Accurate models in terms of RMS do not always meet the expectations of visualization of the studied environment. In Figure 4, Figure 5, and Figure 6 generated models are presented. It was observed, that the kriging method and the GPI method generate smooth surfaces. Often, from the point of view of the environmental test it is adverse – it can make it difficult to identify areas with a high degree of contamination. The omission of extreme values

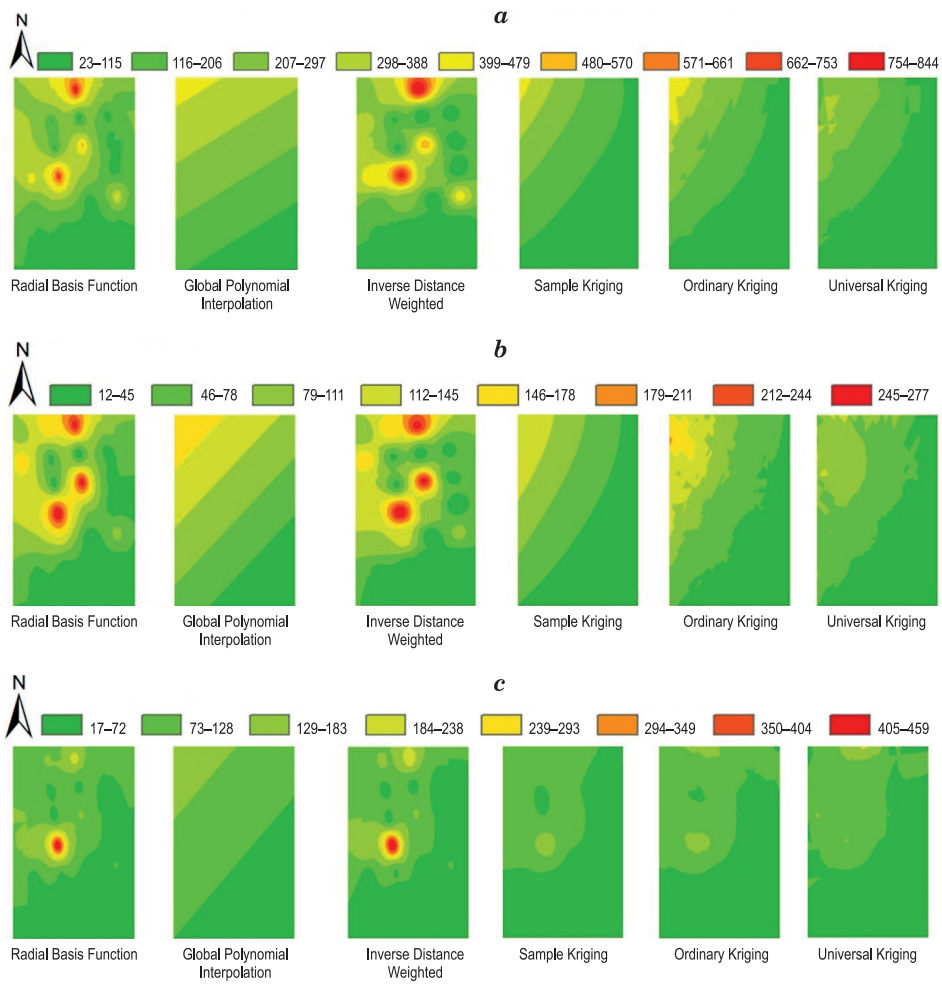

Fig. 5. Spatial distribution of heavy metals in vicinity of the "Częstochowa" Steel Mill generated by six interpolation methods: *a* – total content of Zn [mg kg^{-1}]; *b* – total content of Pb [mg kg^{-1}]; *c* – total content of Ba [mg kg^{-1}]

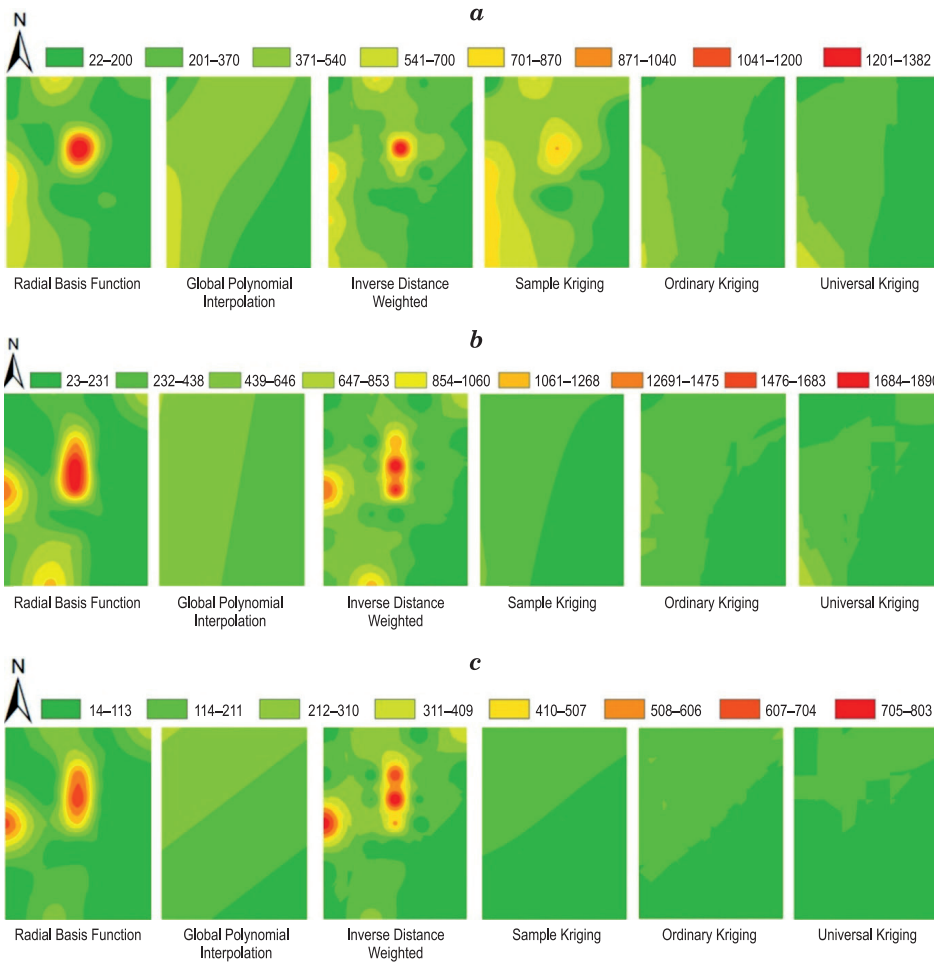

Fig. 6. Spatial distribution of heavy metals in vicinity of The "Miasteczko Śląskie" Zinc Smelter generated by six interpolation methods: *a* – total content of Zn [mg kg^{-1}]; *b* – total content of Pb [mg kg^{-1}]; *c* – total content of Ba [mg kg^{-1}]

in section 19 was found (for the area of "Miasteczko Śląskie") and in points 3, 9 and 12 (for the area of the "Częstochowa" Steel Mill). In contrast, the IDW and RBF methods are close to each other in terms of the spatial distribution. They definitely reflect the actual value of the surveyed points better. Although the models, in terms of accuracy of the algorithms used were similar, they differ considerably in terms of visualization. In terms of planning the processes of reclamation, remediation or monitoring, point area pollution may be important – in the future studies these areas should be taken into account and accurately analyzed. Therefore, the use of the IDW or RBF methods seems to be more appropriate.

Geostatistics is a relatively new, rapidly growing field. There are many programs that enable to perform it. Recently, the number and range of opportunities grow. It should be remembered, that due to the problems of geostatistics, even the best software will not be able to replace the knowledge of the environment. Understanding the studied issue is the basis for the proper conduct of the full research process (ROZPONDEK et al. 2016, ZAWADZKI 2011). Therefore, when choosing the appropriate method of interpolation accuracy can not be the only criteria, we should also pay attention to the possibilities of the use of the created spatial distribution. In addition, as it is apparent from the presented research, more advanced methods of kriging do not always achieve better results. A large number of parameters describing it make it much more difficult to handle. Additionally, in the case of environmental research, their characteristics are determined mainly experimentally. IDW and RBF methods that seem simpler in terms of use, do not necessarily achieve worse results in terms of visualization or accuracy. (ROBINSON and METTERNICHT 2006, REZA et al. 2010, ZAWADZKI 2011).

Conclusion

Because of the diverse spatial characteristics of soils, it is difficult to select the most appropriate interpolation method (GOOVAERTS 1993). This study did not provide a clear indication of the most accurate method. Analyzing only the methods of kriging, interpolation with the simple kriging method can be considered as the most stable. It generates a distribution, which pretty accurately represents the actual values in the entry points.

However, the kriging methods, even though they are more complex, in most cases obtained worse results than the others. This fact tends to support the thesis of the superiority of the IDW method. In addition, the kriging method requires much more knowledge about the analyzed area. During their use, it is necessary to perform accurate preliminary analysis. It should be mentioned that the kriging methods work better with large amounts of source points. In the studies, the number of measurement points was 25 and 29, due to which the kriging methods could have proven to be less reliable.

The presented research shows that by far the safer option for small-area environment research is the use of IDW and RBF methods. They generate possibly accurate spatial distributions, without losing a large amount of information about the area. In other methods, there is a greater risk of loss of some information about the area which can be very important in the processes of reclamation, remediation or monitoring.

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PAC COAGULANTS IN PULP AND PAPER WASTEWATER TREATMENT

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Key words: pulp and paper wastewater, coagulation, PACs.

Abstract

The coagulation/flocculation is a major process in the wastewater treatment. A study was conducted to compare the efficiency of two polymeric PAC coagulants (PAC, PAX 61) and two monomeric coagulants, $Al_2(SO_4)_3$ and $AlCl_3$, in pulp and paper wastewater treatment. PAC and PAX 61 showed the advantage over the traditionally used $Al_2(SO_4)_3$ and $AlCl_3$ in the removal of basic wastewater pollutants. The use of the optimum dose of 10.3 mg Al/dm^3 with PAC ensured over 90% removal of turbidity, color and around 50% COD reduction. A similar effect was obtained after the use of an around 50% higher PAX 61 dose.

KOAGULANTY TYPU PAC W OCZYSZCZANIU ŚCIEKÓW CELULOZOWO-PAPIERNICZYCH

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Słowa kluczowe: ścieki celulozowo-papiernicze, koagulacja, PAC.

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Abstract

Koagulacja/flokulacja jest głównym procesem w oczyszczaniu ścieków. Przeprowadzono badania porównania skuteczności działania dwóch polimerycznych koagulantów typu PAC (PAC, PAX 61) oraz dwóch koagulantów monomerycznych – $Al_2(SO_4)_3$ i $AlCl_3$ – w procesie oczyszczania ścieków celulozowo-papierniczych. PAC i PAX 61 wykazywały przewagę nad tradycyjnie stosowanymi $Al_2(SO_4)_3$ i $AlCl_3$ w usuwaniu podstawowych zanieczyszczeń ścieków. Zastosowanie optymalnej dawki 10,3 mg Al dm^{-3} z PAC zapewniało ponad 90-procentowe usunięcie mętności, barwy i ok. 50% obniżenie ChZT. Podobny efekt uzyskano po zastosowaniu ok. 50-procentowej wyższej dawki PAX 61.

Introduction

Industrial wastewater forms as a result of water consumption during raw material processing to industrial and consumer products. Progress and development lead to an increased amount of wastewater, often environmentally noxious due to their high pollutant load. The pulp and paper industry is distinguished by a high water consumption. Pulp and paper wastewater is characterized by a high content of fine and very fine wood fibers. The mean pollutant values corresponding to the production of 1 t paper are around 20 kg suspended solids, 18 kg settling suspended solids and 50 kg dissolved components. The waste liquor formed during the cellulose production process contains nearly 80% organic substances (these are mostly lignosulfonic acid compounds and lower amounts of sugars and resin) and 20% inorganic substances. Consequently, the formed wastewater is characterized by a high value of the COD and BOD indices. Pulp and paper wastewater reaching water bodies causes significant oxygen content reduction in them, along with water foaming, color and turbidity changes and sometimes even fish death or migration. Easily-decomposable compounds with high concentration contained in this wastewater favor the growth of wastewater fungi and secondary water body silting.

The increasingly used prepolymerised PAC coagulants show a much higher efficiency than conventional coagulants, such as $Al_2(SO_4)_3$ or $AlCl_3$ (Pernitzky, Edzwald 2006). The high effectiveness of PAC coagulants (Zoubolis, Tzoupanos 2010) is due to the presence in the coagulation system of aluminum poliyhydroxycations Al_{13}, which form in aqueous solutions as a result of rapid dilution and hydrolysis. High availability of flocs on an extensive surface with high load density causes increased pollutant adsorption.

The distinguishing feature of PAC coagulants is their alkalinity. It expresses the degree of hydrolysis, which is represented as the molar ratio

R = [OH]/[Al]. During pre-hydrolysis of Al-based coagulants, a number of soluble aluminum forms develop in the solution: Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_3$, $Al(OH)^{4-}$ monomers, $Al_2(OH)_2{}^{4+}$ oligomers, $Al_3(H)_4{}^{5+}$, $Al_{12}AlO_4(OH)_{24}{}^{7+}$ (Al_{13}) and even larger Al_{30} polymers (BOTTERO et al. 1980, PERNITZKY, EDZWALD 2006, WU et al. 2007). EXALL et al. (2003) proved that the $Al_2(SO_4)_3$ solution contains only monomeric Al forms (Al_1), while the occurrence of both Al_1 monomers, Al_2 dimers, oligomers and Al_{13} polymers was observed in solutions of prepolymerised PAC coagulants. Increasing PAC dilution (0.01 mol dm^{-3}) favors an increase in Al_{13} content in a solution with a reduced monomeric forms. In diluted solutions (0.01 mol dm^{-3}), a four times higher amount of polymeric forms of aluminum was observed than in solutions with higher concentration (0.05 mol dm^{-3}).

The aim of the study was to compare the efficiency of two polymeric coagulants PAC and PAX and of two monomeric coagulants $Al_2(SO_4)_3$ and $AlCl_3$ in removing the various pollutants of pulp and paper wastewater. This work sought to determine the effect of the dose and type of coagulant on the degree of wastewater treatment.

Materials and Methods

Pulp and paper wastewater from a pulp and paper plant in Ostrołęka were studied. Two-polymeric coagulants of the PAC type: PAC (51,6 gAl^{3+}/l), PAX 61 (107 gAl^{3+}/l)) and two monomeric coagulants $Al_2(SO_4)_3$ (9,1% as Al) i $AlCl_3$ (33,5 gAl^{3+}/l) were used. Chemical coagulation was conducted using the standard jar test method: rapid stirring (400 rpm) – 1 min., slow stirring (30 rpm) – 15 min. and 15 min. of sedimentation. The wastewater from a pulp and paper plant "Intercell" in Ostrołęka was tested. The raw wastewater was corrected to the pH = 5, using 0.1 m HCl. The determination of the particular parameters was performed using HACH 2000. The determinations of the particular parameters were performed using the following methods: turbidity was determined with the absorption method, color was determined with the platinum-cobalt method, COD was determined with the spectrophotometric method. The study was performed on a laboratory scale, using a 20-stand system for CAF (Computerised Automated Flocculation) testing.

Results and Discussion

Pulp and paper wastewater constitute a group of industrial wastes which are particularly cumbersome and difficult to treat. The purpose of the process of chemical coagulation of pulp and paper wastewater is to maximally remove the pollutants responsible for COD, turbidity, suspended solids and color. The wastewater from a pulp and paper plant "Intercell" in Ostrołęka was tested. The raw wastewater was corrected to the pH = 5, using 0.1 m HCl.

In Table 1, the values of the parameters illustrating the degree of treatment of the studied pulp and paper wastewater are summarized. The use of PAC in amounts from 10 mg Al dm^{-3} to about 40 mg Al dm^{-3} of wastewater enabled over 92–97% turbidity reduction, about 49–54% removal of the pollutants according to the COD scale, and 92–97% reduction in the content of substances responsible for the color of the coagulated wastewater.

Table 1

The results of pulp and paper wastewater coagulation using PAC

PAC dose [mg Al dm^{-3}]	Turbidity	COD	Suspended solids	Color
	[mg dm^{-3}]			
0.0	860	1754	680	4680
10.3	67	894	31	354
15.2	52	866	25	272
20.6	31	836	20	191
25.3	30	830	18	173
30.1	28	825	15	152
35.2	26	820	13	136
41.3	24	816	11	124

Analyzing the data summarized in the Table 1, it is possible to conclude that from the economical point of view, already the lowest of the applied doses of PAC, being 10.3 mg Al dm^{-3} of wastewater, seems to be optimal, as it ensures an over 90% removal of turbidity, suspended solids, color and an approximately 50% COD reduction. An increase in the dose of PAC above 10 mg Al dm^{-3} results in an undesirable pH reduction. Although the chemical coagulation of wastewater using PAC is effective at pH = 4.8 (BOTTERO et al. 1989) and even lower, under such conditions the solubility of the primary product of coagulation, i.e. $Al(OH)_3$ increases (STUMM, MORGAN 1970), which may result in an undesirable increase in the content of Al^{3+} ions and other ions containing Al (the so-called Al-rest) in the treated wastewater. According to BOTTERO and BERSILLON (1989)

depending on the pH, a wide range of PAC hydrolysis products are formed, with different charges and structures, which consequently has an effect on colloid sorption. In solutions with pH < 5.5–6, the coagulation-flocculation process happens through neutralization of the acid groups of organic pollutants, which results in their precipitation. At pH > 6.5 the treatment is performed by adsorption on a very developed surface of sorbent. According to RAKOTONARIVO et al. (1988), the optimal conditions of the aggregation process for polymer forms of Al_{13} are created by pH < 5. Under conditions of higher pH = 7–7.5, the concentration of Al_{13} polycations is too high and the sorption of surface pollutants is much more difficult. However, coagulation pH in the range of 6.0–7.0 are most common as it gives the best removal via a combination of mechanisms. Nevertheless, the experiments reported here are carried out much lower coagulation-pH resulted due to natural reduction of pH due to hydrolysis of aluminum ions. The intention was to avoid the use of pH adjusting chemicals which complicates the operations in practice.

Table 2

The results of pulp and paper wastewater coagulation using PAX 61

PAX 61 dose [mg Al dm^{-3}]	Turbidity	COD	Suspended solids	Color
	[mg dm^{-3}]			
0.0	860	1754	680	4680
5.0	208	1462	107	1155
10.0	78	1068	32	409
15.0	46	901	24	241
20.0	38	814	16	196
25.0	32	808	13	190
30.0	30	801	11	188
40.0	27	798	9	184

The Table 2 presents experimental data of the coagulation process of pulp and paper wastewater using PAX 61. A satisfactory level of 49% removal of the main pollutants responsible for COD was obtained using 15 mg Al dm^{-3}. This dose also ensured an over 94–96% reduction in the indices of turbidity, suspended solids and color at $pH_f = 4.8$ (pH_f– the final pH), which was attainable for PAC (Table 2) with a dose of 10 mg Al dm^{-3}. An increase in the dose of PAX 61 above 15–20 mg Al dm^{-3} did not result in significant changes in the values of particular parameters in the treated wastewater. Polymeric coagulants lower the pH in a similar way, though PAX 61 has a greater effect on the pH than PAC, which probably results from a lower alkalinity ([OH]/[Al]) of PAX 61, compared to PAC.

Table 3

The results of pulp and paper wastewater coagulation using $Al_2(SO_4)_3$

$Al_2(SO_4)_3$ dose [mg Al dm^{-3}]	Turbidity	COD	Suspended solids	Color
	[mg dm^{-3}]			
0.0	860	1754	680	4680
5.0	266	1492	162	1370
10.0	116	1102	56	626
15.0	68	899	31	346
20.0	64	860	26	336
25.0	62	858	27	320
30.0	58	856	24	306
40.0	55	852	22	290

Table 4

The results of pulp and paper wastewater coagulation using $AlCl_3$

$AlCl_3$ dose [mg Al dm^{-3}]	Turbidity	COD	Suspended solids	Color
	[mg dm^{-3}]			
0.0	860	1754	680	4680
5.4	292	1501	177	1605
10.7	121	1118	54	710
15.2	105	886	41	450
21.4	86	878	29	368
25.3	64	877	25	360
30.2	58	875	24	321
42.9	57	872	23	294

Table 3 and Table 4 present the results of two tests performed at the same time, of the wastewater treatment using $Al_2(SO_4)_3$ and $AlCl_3$. The values of pollution, measured by the COD index gradually increased with an increasing dose of Al. The obtained results indicate that following an addition of 15 mg Al dm^{-3} to wastewater with the pH = 5 there was a 49.5% reduction in the COD level. The use of the above dose produces a better result in removing the color (about 3%) and turbidity (about 4%) using $Al_2(SO_4)_3$, compared to $AlCl_3$. An addition of coagulant results in a reduction in the pH_f value. $AlCl_3$, had the most intensive effect on pH (from 4.7 to 3.8) in a dose from 5.4 to 42.9 mg Al/dm^3. The low value of the final pH = 4.34 could also have an effect on the lower effectiveness of treatment using $AlCl_3$. According to BOTTERO and BERSILLON (1989), sulfate ions, compared to chloride ions, have a greater affinity to the dispersed solid phase of $Al(OH)_3$.

As a result of sudden dilution and hydrolysis, PAC forms active forms of Al_{13} in a solution with very high valency (e.g. + 7) (BOTTERO et al. 1988, RAKOTONARIVO et al. 1985, 1988). The strongly-developed surface of octa-

hedral polycations of Al_{13} enables free access of organic substances to the adsorption interface, which creates the proper conditions for their complexing and adsorption. The characteristic property of aggregates formed in this way is their fractal nature (AXELOS et al. 1985, 1986).

The use of PAC- and PAX-type coagulants, compared to the traditional $Al_2(SO_4)_3$, ensures more effective removal of pollutants, lower "alkalinity consumption", broader range of pH for the optimum of coagulation-flocculation, lower susceptibility to low temperatures and a lower residual of Al in treated wastewater.

In this work, the efficiencies of monomeric and polymeric coagulants in removing particular pollutants were compared. The obtained results indicate much higher effectiveness of PAC, compared to other coagulants. Above 20 mg Al dm^{-3} the difference between the effects of PAC and PAX 61 gradually disappears, particularly in removing turbidity and suspended solids.

Conclusions

1. Polymeric coagulants have a great advantage over monomeric coagulants in removing the basic pollutants in pulp and paper wastewater.

2. The optimum PAC dose is 10.3 mg Al dm^{-3}, which ensures over 90% removal of turbidity, suspended solids and color and an about 50 % COD reduction. A similar effect was ensured by an approximately 50 % higher PAX 61 dose.

3. A similar coagulation efficiency in decreasing the levels of particular pollutants was displayed by the both studied monomeric coagulants, $Al_2(SO_4)_3$ and $AlCl_3$.

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THE INVESTIGATION OF DIFFERENT PATTERNS OF IN-FEED OR IN-WATER PROBIOTICS ADMINISTRATION METHODS ON PERFORMANCE, SMALL INTESTINAL MORPHOLOGY AND SRBC-REACTIVE IMMUNE RESPONSES OF JAPANESE QUAIL

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Key words: Japanese quail, probiotic, administration methods, performance.

Abstract

The aim of the current study was to investigate whether continuous consumption of probiotics is advantageous over intermittent consumption. A total of 336 1-d-old Japanese quail chicks were randomly divided into seven experimental groups and administered probiotics throughout the experiment in different manners via, *A* – no probiotics administered (control group), *B* – probiotics fed continuously, *C* – probiotics fed for 2 d on & 2 d off, *D* – probiotics fed for 1 d on & 4 d off, *E* – probiotics in drinking water throughout the experiment, *F* – probiotics in drinking water for 2 d on & 2 d off, *G* – probiotics in drinking water for 1 d on & 4 d off. Administration of probiotic as feed additive significantly increased body weight gain ($P < 0.01$). Feed intake was lower ($P < 0.01$) in group F compared with other groups. The birds in groups *C*, *D* and *G* had the lowest feed conversion ratio ($P < 0.01$). In comparison with control quails, ileum length and duodenum and ileum villus was higher in probiotic-received birds ($P < 0.01$). Crypt depth was increased ($P < 0.01$) by probiotics treatments. Number of goblet cells of duodenum and ileum increased in groups *B*, *C*, *E* and *F* ($P < 0.01$). There were no significant differences in heterophil: lymphocyte ratio among the groups. Consumption of probiotics increased the blood serum total immunoglobin ($P < 0.01$), IgM ($P < 0.05$) and IgY ($P < 0.01$) levels. It was concluded that administration of probiotic either in feed or in water improved the quail's performance and immunity. Regarding advantages of administration of probiotics in drinking water this method is recommended in quail production system.

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BADANIE WPŁYWU RÓŻNYCH METOD PODAWANIA PROBIOTYKÓW W PASZY LUB W WODZIE NA WYDAJNOŚĆ I MORFOLOGIĘ JELITA CIENKIEGO ORAZ SRBC REAKCYJNE ODPOWIEDZI ODPORNOŚCIOWEJ U PRZEPIÓREK JAPOŃSKICH

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Słowa kluczowe: przepiórka japońska, probiotyki, metody podawania probiotyków, wydajność.

Abstract

Celem pracy było sprawdzenie, czy ciągłe spożywanie probiotyków przez przepiórki japońskie jest korzystne w porównaniu z okresowym ich podawaniem. Grupę składającą się z 336 jednodniowych przepiórek japońskich podzielono losowo na siedem grup eksperymentalnych, którym w różny sposób podawano probiotyki w czasie trwania eksperymentu: *A* – brak podaży probiotyków (grupa kontrolna), *B* – probiotyki podawane w paszy stale, *C* – probiotyki podawane w paszy przez 2 dni oraz brak podaży przez 2 kolejne dni, *D* – probiotyki podawane w paszy przez 1 dzień oraz brak podaży przez 4 kolejne dni, *E* – probiotyki podawane w wodzie pitnej stale, *F* – probiotyki podawane w wodzie pitnej przez 2 dni oraz brak podaży przez kolejne 2 dni, *G* – probiotyki podawane w wodzie pitnej przez 1 dzień oraz brak podaży przez 4 kolejne dni. Stosowanie probiotyków jako dodatków paszowych znacząco wspomaga przyrosty masy ciała ($P < 0,01$). Spożycie paszy było niższe ($P < 0,01$) w grupie *F* w porównaniu z pozostałymi grupami. Ptaki z grup *C*, *D* i *G* wykazywały najniższe wskaźniki wykorzystania paszy ($P < 0,01$). W porównaniu z grupą kontrolną długości jelita biodrowego i dwunastnicy, a także kosmków w jelicie biodrowym były wyższe u ptaków, u których stosowano probiotyki ($P < 0,01$). Zastosowanie probiotyków skutkowało wzrostem głębokości krypt jelitowych ($P < 0,01$). Liczba komórek kubkowych w dwunastnicy i w jelicie biodrowym była wyższa w grupach *B*, *C*, *E* i *F* ($P < 0,01$). Nie zaobserwowano w badanych grupach znaczących różnic w stosunku heterofilii do limfocytów. Spożycie probiotyku przez przepiórki skutkowało wzrostem poziomu całościowej puli immunoglobulin ($P < 0,01$), IgM ($P < 0,05$) i IgY ($P < 0,01$) w surowicy krwi. Stosowanie u przepiórek probiotyków – zarówno w paszy, jak i wodzie pitnej – miało korzystny wpływ na wydajność i odporność ptaków. Biorąc pod uwagę zalety podawania probiotyków przepiórkom w wodzie pitnej, należy stwierdzić, że jest to zalecany system podawania u omawianego gatunku ptaków.

Introduction

Commercial poultry are reared under the stress of genetic selection for high performance, therefore, exogenous opportunistic bacteria or those that inhabit in bird's gastrointestinal tract such as *E. coli* could be pathogen in specific environmental situation. Sub-therapeutic doses of antibiotics in poultry diet are used as growth promoter for controlling bacterial population in gastrointestinal tract. Concerns about undesirable side

effects of growth promoter antibiotics, such as toxicity, allergy, cancer, drug resistance and retention in food (ARSLAN 2004, CAKIR et al. 2008) resulted in a global prohibition of feed additive antibiotics. Public pressures to reduce use of antimicrobial substances and consumer's tendency to organic products have influenced the development of alternative feed additives such as probiotics (HIGGINS et al. 2008).

Probiotics are beneficial bacteria that influence the host by improving intestinal health (FULLER 1989). Probiotics have been known to exert their beneficial effects by a variety of mechanisms including: competitive exclusion, immunomodulation, decrease of pH, production of anti-microbial substances, production of some enzymes and increase villus surface area, which makes them a *multi-purpose* feed additive. Supplementation of poultry feed with probiotics (or competitive exclusions) has been developed in order to encourage a protective barrier of bacteria in their digestive tract and prevent the colonization of growth-depressing or pathogenic microorganisms (GRIMES et al. 2008). Many researchers have obtained positive significant effects of using probiotics in broiler chickens (KALBANE et al. 1992, ECKERT et al. 2010, KARIMI-TORSHIZI et al. 2010), turkey (GRIMES et al. 2008, RAHIMI et al. 2011), gees (YAMAN et al. 2006) and quail (HOMMA and SHINOHARA 2004, VRANIC et al. 2006).

Probiotics are living organisms; therefore, their proliferation in digestive tract may guarantee their presence in adequate numbers over the lifetime. Thus the continuous supplementation of probiotics might not have more beneficial effects rather than intermittent supplementation of them. To assess this hypothesis, the present experiment was designed to investigate the effects of continuous or two intermittent administration patterns of probiotics in feed or drinking water upon the performance, small intestinal morphology and SRBC-reactive immune responses of Japanese quail.

Materials and Methods

Animal, management and experimental groups

Three hundred and thirty six 1-d-old (unsexed) Japanese quail (*Coturnix japonica*) chicks were randomly assigned into seven experimental groups with four replicates of 12 birds each. All the groups were maintained under similar management, nutritional and environmental conditions. Birds in each experimental unit were placed in a cage (wire floor – 45 × 40 × 30 cm) furnished with an electrical bulb to provide continuous lighting and

age-appropriate supplemental heat controlled by an electrical dimmer. Temperature was maintained at 35ºC at the arrival of chicks for the initial three days and then gradually reduced 2.5ºC per week until a temperature of 22ºC was achieved. The study protocol was conducted in accordance with the Animal Care and Use Review Committee guidelines of Tarbiat Modares University, Tehran, Iran. The probiotic treated groups were offered a water dispersible probiotic (Protexin, Somerset, UK) within 24 h after hatch, continuously or intermittently either in feed or drinking water till the end of the experiment. The duration of the experiment was 35 days. The seven experimental groups were (Table 1).

Table 1

Continuous and intermittent patterns of probiotic administration in feed and drinking water

Treatments	Day																																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
A	–	–	–	–	–	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f	f
C	f	f	-	-	f	f	-	-	f	f	-	-	f	f	-	-	f	f	-	-	f	f	-	-	f	f	-	-	f	f	-	-	f	f	-
D	f	-	-	-	-	f	-	-	-	-	f	-	-	-	-	f	-	-	-	-	f	-	-	-	-	f	-	-	-	-	f	-	-	-	-
E	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w	w
F	w	w	-	-	w	w	-	-	w	w	-	-	w	w	-	-	w	w	-	-	w	w	-	-	w	w	-	-	w	w	-	-	w	w	-
G	w	-	-	-	-	w	-	-	-	-	w	-	-	-	-	w	-	-	-	-	w	-	-	-	-	w	-	-	-	-	w	-	-	-	-

f – probiotics supplemented in feed
w – probiotics supplemented in drinking water
A – no probiotics administered (control group),
B – probiotics continuously in feed through out the experiment (F_1),
C – probiotics in feed in a pattern of 2 d on -2 d off (F_2),
D – probiotics in feed in a pattern of 1 d on -4 d off (F_3),
E – probiotics in drinking water continuously throughout the experiment (W_1),
F – probiotics in drinking water in a pattern of 2 d on -2 d off (W_2),
G – probiotics in drinking water in a pattern of 1 d on -4 d off (W_3).

The in-feed probiotics groups received 100 or 150 g ton^{-1} probiotics during d 1 to 14 and d 15 to 35, respectively. The drinking water groups received half the amount of probiotics which was supplemented in feed because water intake was assumed two-fold higher than feed intake. For in-water groups, drinking water was measured every 12 hours and replaced by fresh supplemented water since viability of microorganisms might lose after 12 hours of addition in the water.

Diets and probiotic preparation

Experimental diets were isocaloric and isonitrogenous, based on corn--soybean meal to meet or exceed NRC (1994) specifications for Japanese quail (Table 2). Each cage was equipped with a nipple drinker and a feeder. All birds had *ad libitum* access to water and feed.

Table 2

Composition of the basal diets

Item	1–35 d
Ingredient [%]	
Yellow corn	42.32
Soybean meal [44% CP]	40.20
Vegetable oil	7.48
Fish meal [65% CP]	7.30
$CaCO_3$	1.21
Di-calcium phosphate	0.01
Sodium chloride	0.28
Mineral and vitamin premix *	0.50
DL-Methionine	0.03
Washed sand	0.67
Total	100
Calculated value**	
ME [kcal kg^{-1}]	3130
CP [%]	25.90
Lys [%]	1.40
Met + Cys [%]	0.81
Calcium [%]	0.86
Nonphytate phosphorus [%]	0.32

* Supplied the following per kilogram of diet: retinyl acetate – 9,000 IU; cholecalciferol – 2,000 IU; DL-*a*-tocopheryl acetate – 12.5 IU; menadione sodium bisulfite – 1.76 mg; biotin – 0.12 mg; thiamine – 1.2 mg; riboflavin – 3.2 mg; calcium D-pantothenate – 6.4 mg; pyridoxine – 1.97 mg; nicotinic acid – 28 mg; cyanocobalamine – 0.01 mg; choline chloride – 320 mg; folic acid – 0.38 mg; $MnSO_4.H_2O$ – 60 mg; $FeSO_4$.$7H_2O$ – 80 mg; ZnO – 51.74 mg; $CuSO_4$.$5H_2O$ – 8 mg; Iodized NaCl – 0.8 mg; Na_2SeO_3 – 0.2 mg.
** Calculated from NRC (1994).

The probiotic supplement, Protexin (Protexin, Somerset, UK) used in this study contained $2 \cdot 10^9$ cfu g^{-1} of *Aspergillus oryzae* PXN 68, *Lactobacillus acidophilus* PXN 35, *L. rhamnosus* PXN 54, *L. plantarum* PXN 47, *L. bulgaricus* PXN *39, Bifidobacterium bifidum* PXN 23, *Enterococcus faecium* PXN 33, *Streptococcus thermophilus* PXN 66 and *Candida pintolope-*

sii PXN 70. Probiotic was supplemented in feed (0.1 g kg^{-1}) or drinking water (0.05 g l^{-1}). Probiotic suspensions were prepared in sterile phosphate buffered saline directly before administration.

Data collection

Performance: body weight (BW) and feed intake (FI) were recorded for d 1–14 and d 15–35 then body weight gain (BWG) and feed conversion ratio (FCR) were calculated.

Small intestinal morphometric assay

The birds were killed by severing the cervical vessels at d 35 and tissues collected accordingly. Segments (approximately two cm) taken from the midpoint of duodenum and ileum were gently flushed twice with phosphate buffer saline and were fixed in fresh 10% formalin. All samples were dehydrated, cleared, and embedded in paraffin. Sections of five µm thickness placed on glass slides were stained using eosin-haematoxylin-alcian blue and periodic acid-Schiff which manifest acidic mucin producer and neutral mucin producer goblet cells, respectively (KIERNAN 2008). Villus height, crypt depth, number of goblet cells (acidic mucin producer and neutral mucin producer) along 100 µm of villus length was determined under light microscope (Carl ZEISS standard 20, Germany). The results of the morphometric determinations were from at least ten well-oriented crypt villus structures from each chick. The measurements were done using DinoCapture software (Dino-lite, Ver. 3.3.0.0, Korea).

Immune responses. Two male birds per cage were immunized by intramuscular injection of 0.2 ml of sheep red blood cells (SRBC) suspension in PBS (5% v/v) on d 11. Blood samples were drawn 7 days following the SRBC injection. Anti-SRBC antibodies were tittered before and after 2-mercaptoethanol (ME) treatment to further assess the total immunoglobulin (IgT), ME sensitive (IgM) and ME resistant (IgY) titres. Antibody titres were reported as $\log_2$ of the reciprocal of the last dilution at which complete agglutination was observed (QURESHI and HAVENSTEIN 1994).

Heterophil to lymphocyte ratio (H:L): Blood smears were prepared from two male birds per cage on d 35 to obtain H:L. Specimens were stained by Wright's stain (LUCAS and JAMROZ 1961). Total of 100 white blood cells including heterophils and lymphocytes were counted differentially and the H:L ratio was calculated by dividing the total number of heterophils by the total number of lymphocytes.

Statistical analysis

Data were analysed by one-way ANOVA using the GLM procedure of Statistical Analysis System (SAS Institute Inc). Statements of statistical significance were based on $P \leq 0.05$ or lower (STEEL and TORRIE 1980). Duncan's multiple range comparison tests were used to examine significant differences between treatment means.

Results and Discussion

The effects of different probiotics administration methods and frequencies on BWG, FI and FCR are presented in Table 3. In d 1–14 only the group F_1 which received probiotics continuously in feed showed higher BWG than control ($P < 0.01$), however the different frequencies in each method (i.e. in-feed or in-water methods) were not different among the relevant method. In d 15–35 the different frequencies of each method did not show significant differences in BWG as well. In the whole period (d 1–35) supplementation of probiotics in feed resulted in higher BWG ($P < 0.01$).

Table 3

Effects of probiotic administration methods and consumption frequency on BW, BW gain and FCR of Japanese quail*

	BWG [g]			FI [g]			FCR		
Days	1–14	15–35	1–35	1–14	15–35	1–35	1–14	15–35	1–35
Control	73.88^{b}	142.54^{abc}	216.42^{c}	110.92^{c}	576.07^{a}	686.99^{a}	1.50^{ab}	4.04^{a}	3.17^{a}
F_1	80.93^{a}	151.85^{a}	232.79^{a}	128.26^{a}	548.22^{bc}	676.49^{ab}	1.58^{a}	3.61^{bc}	2.91^{bc}
F_2	79.37^{ab}	149.92^{ab}	229.29^{ab}	115.79^{bc}	536.92^{c}	652.71^{bc}	1.46^{ab}	3.58^{c}	2.85^{c}
F_3	78.59^{ab}	144.43^{abc}	223.03^{ab}	110.42^{c}	525.68^{cd}	636.11^{cd}	1.40^{b}	3.65^{bc}	2.85^{c}
W_1	73.32^{b}	148.62^{abc}	221.93^{c}	112.41^{bc}	563.93^{ab}	676.35^{ab}	1.54^{ab}	3.80^{abc}	3.05^{ab}
W_2	78.18^{ab}	138.17^{c}	216.35^{c}	120.45^{ab}	537.65^{c}	658.10^{bc}	1.54^{ab}	3.89^{ab}	3.04^{ab}
W_3	74.53^{b}	139.72^{bc}	214.25^{c}	114.96^{bc}	510.81^{d}	625.77^{d}	1.54^{a}	3.66^{bc}	2.92^{bc}
P-value	0.004	0.006	0.0001	0.0001	0.0001	0.0001	0.0107	0.0008	0.0001
SEM	0.711	1.265	1.429	1.333	4.418	4.495	0.015	0.038	0.024

$^{a–c}$ Means with different superscripts in the same column differ ($P < 0.05$ or lower).
BVG – body weight gain, FI – feed intake, FCR – feed conversion ratio.
Control: no probiotics administered; F_1 – probiotics continuously in feed throughout the experiment; F_2 – probiotics in feed in a pattern of 2 d on -2 d off; F_3 – probiotics in feed in a pattern of 1 d on -4 d off; W_1 – probiotics in drinking water continuously throughout the experiment; W_2 – probiotics in drinking water in a pattern of 2 d on -2 d off; W_3 – probiotics in drinking water in a pattern of 1 d on -4 d off.
* Mean represent 4 pens per treatment.

The quail supplemented via feed received less probiotics than those supplemented in drinking water, because half the amount of probiotics in feed was added to water, considering the assumption of water intake is approximately two fold of feed intake. The average water: feed ratio were 3.484, 2.443 or 2.618 for starter (d 1–14), grower (d 15–35) or total period (d 1–35), respectively, showing that a higher dose of probiotics in drinking water was required. Previously, KARIMI-TORSHIZI et al. (2010) had reported higher BWG in broiler chicken consumed probiotics through feed or drinking water than untreated control birds. In another study MASTBAUM et al. (1997) also confirmed that probiotics administration in feed or in water significantly increased live weight gain and feed conversion efficiency in broilers. The higher BWG which observed in the present study through probiotic supplementation in feed is in agreement with CHIMOTE et al. (2009) who found that supplementation of Japanese quails' feed with probiotics improved BWG. However, KARIMI-TORSHIZI et al. (2010) in broilers found out probiotic supplementation in drinking water was better than feed. Meanwhile, in the present study, despite the higher amount of probiotics consumed by probiotic drank birds, frequencies of in-water method showed lower BWG than in-feed counterparts.

Probiotics treatment in feed or in water resulted in significantly lower d 1–35 FI than un-treated control group ($P < 0.01$); however, the groups consumed probiotics in feed or in water continuously (F_1 and W_1) were not significantly different from the control. Findings of d 1–14 and d 15–35 FI looks surprising such that in the former all probiotics-consumed groups consumed more feed than control, whereas in the later, untreated control birds had the highest FI. The more feed intake observed in the probiotics-treated birds than control in the early part of the present study (d 1–14) may be due to the earlier establishment of gut microflora in those birds. Probiotic microorganisms can optimize intestinal flora and hence trigger the symbiotic effect of host animal's enzymes helping to improve the nutrients digestibility. Increased digestibility can lead to faster digest a passage along intestine resulting in more feed intake and eventually improved BWG as observed in the present study. BAI et al. (2013) found improved growth performance in the early stage (d 1–21) of broilers supplemented with a probiotics product composed of $1 \cdot 10^7$ cfu g^{-1} of *Lactobacillus fermentum* and $2 \cdot 10^6$ cfu g^{-1} of *Saccharomyces cerevisiae*. YEO and KIM (1997) and ZULKIFLI et al. (2000) also reported that supplementation of broilers with *Lactobacillus* improved average daily gain and feed efficiency from 1 to 21 d of age, but not from 22 to 42 d. LI et al. (2008) reported improved growth performance in d 1–21 in broiler supplemented with a probiotics mixture, and they observed no significant difference among different

levels (0.2 to 0.6%). However, discrepancies are seen in the results of probiotic supplementation. The discrepancy may be due to the differences in microbial species or strains of microorganisms used, dosage of supplementation or probiotic concentrations.

All frequencies of in-feed method as well as W_3 showed significantly lower 1 to 35 d FCR than control ($P < 0.01$). ARSALN and SAATCI (2004) pointed out that quails consumed probiotics in feed or in water had significantly lower FCR than untreated control quails. In agreement to our findings, they also stated that although probiotics-treated groups consumed less feed than control group, they showed higher live weight gain, leading to improved FCR. It is clear that in the present study probiotics consumption has led to lower FI concomitant with higher live weight gain resulting in improved FCR. This may be due to the fact that probiotics can optimize intestinal flora and hence trigger the symbiotic effects of host animal enzymes leading to improved nutrients digestibility. Over all, Table 3 showed that by decreasing the amount of probiotics consumed, FCR was improved. This means that likely there is no need to continuous or everyday use of probiotics in Japanese quail production.

Table 4

Effects of probiotic administration methods and consumption frequency on small intestinal morphology of Japanese quail*

	Small intestinal length [cm]		Villus height [µm]		Crypt depth [µm]		Villus height: crypt depth	
	D	*I*	*D*	*I*	*D*	*I*	*D*	*I*
Control	10.12	22.00^{bcd}	807.95^{c}	233.23^{d}	22.10^{d}	19.76^{c}	36.58	11.80b^{c}
F_1	10.00	22.25^{bcd}	840.51^{ab}	263.56^{a}	23.26^{ab}	21.26^{ab}	36.14	12.40^{a}
F_2	10.82	23.72^{abc}	833.39^{ab}	244.09^{c}	22.93^{abc}	21.06^{ab}	36.35	11.59^{c}
F_3	10.50	25.12^{a}	827.19^{b}	240.14^{c}	22.44^{dc}	20.93^{ab}	36.87	11.47^{c}
W_1	10.87	21.25^{dc}	846.29^{a}	268.53^{a}	23.45^{a}	21.38^{a}	6.09	12.56^{a}
W_2	9.75	20.25^{d}	831.80^{ab}	254.30^{b}	23.06^{abc}	21.22^{ab}	36.07	11.98^{b}
W_3	10.25	24.32^{ab}	826.75^{b}	242.59^{c}	22.62^{bc}	20.80^{b}	36.54	11.66^{bc}
P-value	0.756	0.010	0.0001	0.0001	0.0001	0.0001	0.399	<.0001
SEM	0.202	0.432	2.455	2.352	0.100	0.106	0.110	0.079

$^{a–c}$Means with different superscripts in the same column differ ($P < 0.05$ or lower).
BVG – body weight gain, FI – feed intake, FCR – feed conversion ratio.
Control: no probiotics administered; F_1 – probiotics continuously in feed throughout the experiment; F_2 – probiotics in feed in a pattern of 2 d on -2 d off; F_3 – probiotics in feed in a pattern of 1 d on -4 d off; W_1 – probiotics in drinking water continuously throughout the experiment; W_2 – probiotics in drinking water in a pattern of 2 d on -2 d off; W_3 – probiotics in drinking water in a pattern of 1 d on -4 d off.
D – duodenum, *I* – ileum
* Mean represent 4 pens per treatment.

The values of small intestinal morphometric study are shown in Table 4. Duodenum length was not affected by probiotics consumption, but there were significant differences in ileum length among the experimental groups ($P < 0.01$) such that ileum was longer in the groups received fewer probiotics in feed or in water through the experiment. Administration of probiotics in feed or in water resulted in longer length of villi and deeper crypts than un-treated control quail ($P < 0.01$). AWAD et al. (2009) reported that supplementing probiotics in broiler feed caused longer duodenum and ileum villi than non-supplemented controls. Increasing the villus height introduced an increased surface area capable of greater absorption of available nutrients (CASPARY 1992). Crypts are considered as the villus factory and deeper crypts indicate fast tissue turnover to permit renewal of the villus as needed in response to normal sloughing or inflammation caused by pathogens (YASON et al. 1987, PAGAN et al. 1999). The intestinal epithelial cells originate form crypts migrating along the villus surface upward to the villus tip and are extruded into the intestinal lumen within 48 to 96 h (IMONDI and BIRD 1966, POTTEN 1998). A shortening of the villi and deeper crypts may lead to poor nutrient absorption, increased secretion in gastrointestinal tract and lower performance of animal (XU et al. 2003). In contrast, increases in the villus height and villus height: crypt depth ratio is directly correlated with increased epithelial cell turnover (FAN et al. 1997), and longer villi are associated with activated cell mitosis (SAMANYA and YAMAUCHI 2002).

The number of neutral and acidic mucin producer goblet cells is shown in Table 5. In duodenum the number of neutral mucin producer goblet cells was not affected by treatments, whereas the numbers of acidic mucin producer goblet cells showed significant differences ($P < 0.05$) as F_1 had the highest number. In the ileum the numbers of both acidic and neutral mucin producer goblet cells showed significant differences ($P < 0.01$). In the whole period, the numbers of any kinds of goblet cells in duodenum as well as in ileum decreased as the total days of probiotic consumption decreased; i.e. the less amount of probiotics consumed the less numbers of goblet cells were observed. Goblet cells produce mucins which possess potential binding sites for both commensal and pathogenic organisms, may performing defensive role during establishment of the intestinal barrier. Formation of the mucus gel is through goblet cell secretion of polymeric mucin glycoprotein (FORSTNER and FORSTNER 1994, KLINKEN et al. 1995). These glycoproteins compete with bacteria for adhering via heterogeneous oligosaccharide chains (BELLEY et al. 1999), thereby preventing noxious agents from coming into contact with the underlying epithelial cells. Mucin provides a desirable environment for proliferation of specific

Table 5

Effects of probiotic administration methods and consumption frequency on small intestinal goblet cells of Japanese quail*

Specification	Number of acidic mucin producer goblet cells/100 μm of villus length		Number of neutral mucin producer goblet cells/100 μm of villus length	
	D	*I*	*D*	*I*
Control	10.62^{bc}	11.02^{bc}	11.02	11.25^{bc}
F_1	11.65^{a}	12.12^{a}	11.67	11.97^{a}
F_2	11.07^{abc}	11.37^{abc}	11.35	11.62^{ab}
F_3	10.24^{c}	10.92^{bc}	10.90	11.05^{c}
W_1	11.55^{ab}	11.87^{ab}	11.37	11.67^{ab}
W_2	11.30^{ab}	11.72^{abc}	11.10	11.47^{abc}
W_3	10.17^{c}	10.82^{c}	10.60	10.92^{c}
P-value	0.0131	0.00405	0.3583	0.0039
SEM	0.1498235	0.1369876	0.1257235	0.0878348

$^{a–c}$Means with different superscripts in the same column differ ($P < 0.05$ or lower).
BVG – body weight gain, FI – feed intake, FCR – feed conversion ratio.
Control: no probiotics administered; F_1 – probiotics continuously in feed throughout the experiment; F_2 – probiotics in feed in a pattern of 2 d on -2 d off; F_3 – probiotics in feed in a pattern of 1 d on -4 d off; W_1 – probiotics in drinking water continuously throughout the experiment; W_2 – probiotics in drinking water in a pattern of 2 d on -2 d off; W_3 – probiotics in drinking water in a pattern of 1 d on -4 d off.
* Mean represent 4 pens per treatment.

microflora due to their high carbohydrate content (DEPLANCKE and GASKINS 2001). Thus, the chemical composition of mucus is essential for establishment of the intestinal barrier.

Findings of immune responses are shown in Table 6. There were no difference among the groups for numbers of heterophil and lymphocyte and H:L. Data analysis did not reveal any significant difference of IgT, IgM and IgY concentration in response to SRBC injection between different frequencies of probiotic administration, however continuous patterns of each method showed significantly higher concentrations than control group ($P < 0.01$).

The immune modulation property of probiotics has already been well addressed (COX and DALLOUL 2015). It is possible that commensal bacteria or their products which interact closely with cells within the chicken gut-associated lymphoid tissue play a role in the development of immune response (HAGHIGHI et al. 2005). Heterophil to lymphocyte ratio is regarded as a traditional stress indicator in birds, showing bird response to environmental stressors (DAWKINS et al. 2004). Probiotic regardless of the way of administration had no significant effect on H:L in the present study. Similar results were reported in probiotic-fed broilers raised in low and high stocking densities (CENGIZ et al. 2015).

Table 6
Effects of probiotic administration methods and consumption frequency on some SRBC-reactive immune responses*

Specification		Heterophil	Lymphocyte	Heterophil: lymphosyte	IgT	IgM	IgY
		[%]			Log_2		
Control	0.0	40.25	59.75	0.68	3.50^{c}	1.33^{b}	2.16^{c}
F_1	1.0	37.13	62.87	0.64	5.33^{ab}	2.00^{ab}	3.33^{ab}
F_2	0.5	35.00	65.00	0.54	4.42^{bc}	1.67^{b}	2.74^{bc}
F_3	0.2	34.00	66.00	0.53	3.79^{bc}	1.54^{b}	2.25^{bc}
W_1	1.0	38.13	61.87	0.70	6.67^{a}	2.37^{a}	4.29^{a}
W_2	0.5	35.50	64.50	0.58	4.87^{bc}	1.56^{ab}	3.31^{ab}
W_3	0.2	26.45	73.55	0.36	4.25^{bc}	1.43^{b}	2.82^{bc}
P-value		0.239	0.259	0.289	0.0002	0.0437	0.0037
SEM		1.462	1.462	0.039	0.229	0.173	0.157

$^{a–c}$Means with different superscripts in the same column differ ($P < 0.05$ or lower).
BVG – body weight gain, FI – feed intake, FCR – feed conversion ratio.
Control: no probiotics administered; F_1 – probiotics continuously in feed throughout the experiment; F_2 – probiotics in feed in a pattern of 2 d on -2 d off; F_3 – probiotics in feed in a pattern of 1 d on -4 d off; W_1 – probiotics in drinking water continuously throughout the experiment; W_2 – probiotics in drinking water in a pattern of 2 d on -2 d off; W_3 – probiotics in drinking water in a pattern of 1 d on -4 d off.
IgT – immunoglobulin T; Ig M – immunoglobulin M; IgY – immunoglobulin Y.
* Mean represent 4 pens per treatment.

Conclusions

Findings of the current study showed that administration of probiotics in feed or in water improved Japanese quail's performance. However, the study illustrated that it was not necessary to supplement Japanese quail with probiotics continuously in rearing period and then not-every-day frequencies are possible.

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RECTAL PROLAPSE (*PROLAPSUS RECTI*) IN SWINE – AS A STILL OPEN PROBLEM

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Key words: *prolapsus recti,* pig, sow, modified treatment, amputation, animal welfare.

Abstract

Rectal prolapse (*prolapsus recti*) in swine is relatively common. Factors influencing the development of this phenomenon are described in literature: infections (*Actinobacillus pleuropneumoniae*, swine flu, salmonellosis, spirochetosis, adenomatosis, colibacteriosis, parasites), environmental factors (too low temperature, excessive number of animals), nutritional (fiber deficiency, hypovitaminosis E, excess of lysine, feed containing mycotoxins), genetic (the gene responsible for rectal prolapse – P), pharmacological (tylosin, lincomycin, florfenicol), techno-logical (grouping of sows, low birth mass of piglets, too short pruning of tails in piglets). The paper describes the advantages and disadvantages of the treatment, including minimally--invasive and surgical procedures. The authors' modification of the treatment is presented too.

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WYPADNIĘCIE PROSTNICY (*PROLAPSUS RECTI*) U ŚWIŃ JAKO NADAL OTWARTY PROBLEM

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Słowa kluczowe: wypadnięcie odbytu, świnie, maciory, modyfikacja leczenia, amputacja, dobrostan zwierząt.

Abstract

Wypadnięcie prostnicy (*prolapsus recti*) u świń występuje stosunkowo często. Opisano czynniki wpływające na powstanie tego zjawiska: choroby (pleuropneumonię świń wywoływaną przez *Actinobacillus pleuropneumoniae*, grypę świń, salmonellozę, spirochetozę, adenomatozę, kolibakteriozę, pasożyty), czynniki środowiskowe (zbyt niską temperaturę w chlewni, nadmierne zagęszczenie), żywieniowe (niedobór włókna i witaminy E, nadmiar lizyny, paszę zawierającą mikotoksyny), genetyczne (gen warunkujący wypadanie odbytu – *P*), farmakologiczne (tylozyna, linkomycyna, florfenikol), technologiczne (grupowe utrzymanie loch, zbyt mała masa urodzeniowa prosiąt, zbyt krótkie przycinanie ogonków u prosiąt). Podano wady i zalety leczenia wypadniętej prostnicy, opisując zabiegi mało inwazyjne i chirurgiczne. Opisano również własną modyfikację leczenia.

Introduction

Rectal prolapse is the eversion the anal mucosa along with a small section of the rectum, and the formation of a bulging roller of reddish, dirt-red, color. This lesion is often covered with scabs, and relatively often ulcerations and extravasations are observed (BAJKOWSKI 2015). The direct cause of the prolapse of the handpiece is increased pressure of the abdominal press and weakening of the anal sphincter as well as anal tissues (BAJKOWSKI 2015, GARDNER et al. 1988, STANLEY 1999, THOMSON and FRIENDSHIP 2012, WHITE 2017).

The discussed malady is observed in many animal species: most often cattle, small ruminants (STANLEY 1999), chinchillas (GRUDZIEŃ 2017), dogs and pigs (ANDERSON and GUY 2012, ANDERSON and MIESNER 2008, BOROBIA-BELSUÉ 2006, FREESE 2017, GARDNER et al. 1998, NJOKU et al. 2014, PABOEUF et al. 2014, PAPATSIROS ET AL. 2012, SMITH 1981, STANLEY 1999, WALTER 2011). Its occurrence is estimated at about 1–2% of the population of these animals (BAJKOWSKI 2015). Sometimes, it can even affect 15% of the herd (WHITE 2017).

In pigs, the rectal prolapse is observed even in 2-day-old piglets (WHITE 2017). Pigs are particularly predestined to this type of disorder at the age of 6–20 weeks (PEJSAK 2007, THOMSON and FRIENDSHIP 2012. It usually affects 8–10 cm of the final part of the large intestine (WHITE 2017). However, the highest percentage of pigs with this change (36.4%) is observed between at age of 77–98 days (PABOEUF et al. 2014). The rectal prolapse also occurs in sows in the perinatal period (BOROBIA-BELSUE 2006, ČECH et al. 2010, GREENWOOD 1989, GRUDZIEŃ 2015, PAPATSIROS 2012). However, it was observed that the size of the sow does not affect the frequency of this change (ANIL et al. 2002). In addition, the occurrence of the vagina, uterus and bladder (GREENWOOD 1989, SCHULZ and BOSTEDT 1995) is relatively common in pigs. Pigs with the discussed change are characterized by significantly lower growth and abdominal distension (SMITH 1981).

Predisposing factors

Diseases

Infectious factors that predispose to the rectal prolapse are diarrheal diseases: colibacteriosis, salmonellosis, dysentery, and spirochetosis (BAJKOWSKI 2015, PEJSAK 2007). Diseases of the respiratory system, including Porcine Respiratory Disease Complex and porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae*, manifest in a strong cough, which leads to an increase in intra-abdominal pressure and increased tissue tension and, as a result, pushes the anus outward (BAJKOWSKI 2015). A significant role in the etiopathogenesis of the disease is played by internal parasites, mainly nematodes (BAJKOWSKI 2015, PEJSAK 2007, THOMSON and FRIENDSHIP 2012). They cause excessive contractions of the intestinal musculature, and consequently weakening the wall of the infested intestine (BAJKOWSKI 2015). Also cystitis, urolithiasis, urethral obstruction, cancer, and prostate and traumatic spine diseases can predispose to *prolapsus ani* (GARDNER et al. 1988).

Environmental factors

Environmental factors include, eg. too sloping and slippery floor. Pigs have increased muscular tonuswhile walking (BAJKOWSKI 2015, PEJSAK 2007). Keeping too low a temperature in the pig house predisposes the

animals to stay in the clusters. Under these conditions, an increase in intra-abdominal pressure in the pigs persists leading to the push of the rectum (Bajkowski 2015). The excessive compaction of animals affects similarly (Bajkowski 2015, Stanley 1999). Too long breaks in the access of animals to water and fodder conduct to constipation, and as a result lead to pressure and prolapse in large intestine (Bajkowski 2015, Stanley 1999, White 2017). In addition, in advanced cases of constipation Hemorrhagic Bowel Syndrome is formed, i.e. due to excessive intestinal fermentation, intestinal *Clostridium sp.* develop, which produce toxins and a significant amount of gas (Bajkowski 2015).

Nutrition

Fibers' deficiency in a food dose predisposes pigs to constipation that may be the cause of the lesions (Grudzień 2015). Excessive crumbling of feed (particles less than 1 mm) and frequent change of feed lead to the prolapse as a result of increased intestinal fermentation.

The handpieces are affected by mycotoxins, in particular zearalenone (Pejsak 2007, Obremski et al. 2003). In such cases, the hyperestrogenic effect of mycotoxins is influencial (Grudzień 2015, Obremski et al. 2003). Pigs are particularly susceptible to zearalenone. The zearalenone dose of 1 mg kg^{-1} of feed induces an estrogenic effect in this species (Obremski et al. 2003). Long-term supply of low doses of mycotoxins in feed also predisposes to the prolapse as well as to certain disorders on the part of the reproductive and immune systems (Gajęcka et al. 2011, Jakimiuk et al. 2010, Zielonka et al. 2010). The discussed illness in pigs is also influenced by too high lysine supply and too low supply of calcium and vitamin E in the ration (Freese 2017). Also, the prior intussusception may result in the falling out of the large intestine (Grudzień 2015, Grudzień 2017, Njoku et al. 2014).

Genetic factors

There is currently a dispute regarding the prolapse on the genetic basis. According to some authors such relationship doesn't exist (Gardner et al. 1988). There is also an opinion that the rectal prolapse could be inherited (Figure 1) (Walter 2011). Genetic predisposition to this change in pigs is highly noticeable in pure reproductive lines (Freese 2017).

x	P	N
P	PP	PN
N	PN	NN

Fig. 1. Inheritance of the gene responsible for rectal prolapse in heterozygotes (WALTER 2011): x – crossing (mating), P – gene responsible for rectal prolapse, N – lack of gene responsible for rectal prolapse (normal), 25% PP – pigs with rectal prolapsus during stress, 50% PN – pigs carrying gene responsible for rectal prolapse, 25% NN – pigs free from the gene responsible for rectal prolapse

Other factors

The use of certain antibiotics, tylosin, lincomycin or florfenicol, is a factor conducive to the prolapse of the rectum in pigs. Tylosin causes diarrhea, anal pruritus and anal mucosal edema, whereas lincomycin and florfenicol induce diarrhea in pigs. All these factors lead to the rectal prolapse (NJOKU et al. 2014, PEJSAK 2007).

Too short trimming of the tails (through nerve damage) predisposes to the rectal prolapse as well as simultaneous genital tract's prolapse (*prolapsus vaginae et uteri*) in sows (BAJKOWSKI 2015). This disease is induced also by inguinal hernia.

The age of the animals is also important. The elder the pig are, the frequent prolapse occur. This is related to the weakening of the tonus of the anal sphincter muscle. Therefore, the prolapse often is diagnosed in older sows (FREESE 2017).

Exceptionally fast growth predisposes pigs to the rectal prolapse (WHITE 2017) and mechanical injuries to this organ (NJOKU et al. 2014). A frequent occurrence in sows is anal damage caused by a back gate of the delivery cage (BOROBIA-BELSUÉ 2006). Period of predilection in sows is estrus and first 2 weeks of lactation (BOROBIA-BELSUÉ 2006). The sows' maintenance system also has a significant impact. Most cases of anal damage occur in grouped sows (MCGLONE et al. 2004).

It was found that piglets, whose body mass at birth was less than 1 000 g, are more likely to be susceptible to the prolapse (PABOEUF et al. 2014). Daily weight gains in the first days after birth are also important. Animals with lower growth rates in this period are more likely to suffer from this disease (PABOEUF et al. 2014). It was also shown that higher than average weight gains over a period of 2 weeks predispose to the *prolapsus ani* (ANDERSON and MIESNER 2008, PABOEUF et al. 2014).

Medical treatment

In any case of the rectal prolapse, the most important is to isolate sick animals from healthy ones in order to avoid cannibalism (GRUDZIEŃ 2015).

If the rectum falls out to 5 cm, self-healing is very common. The addition of a β-lactam antibiotic accelerates the treatment (SMITH 1981).

If the prolapsed fragment is more than 5 cm in length, additional treatmentis usually required. Classical surgical procedure is used, under local anesthesia, including a drainage and retention of the rectum at the place of repositioning using a purse string suture (SMITH 1981). In the case of infection of the fallen intestine, it is amputated (SMITH 1981, VONDERFECHT 1978).

Another surgical method is the insertion of the large intestine to the previous site and the fixation of the anus with 3 stitches made on the left, right and upper side using the classic threads. The advantage of this method is that it can be used in pigs in any age (FILIPOV 1981). Its modification is the use of catgut threads during the procedure (KJAR 1976).

Another surgical method is also to retain the repaired rectum using a double thread strung on the Gerlach needle. The needle is inserted from the center of the fallen anus and a double thread is passed through the entire thickness of the organ and the surrounding skin. Such threads are made on the outside. In this method, the whole prolapsed part is attached with a double thread (ČECH et al. 2010).

The first non-invasive and simple method of treatment was the introduction of a 15–20-cm-long piece of rigid garden hose (used to spread water) to the anus and clamping the structure with a rubber band (BEILAGE and BEILAGE 1994). After a few days (usually 3–4 days), necrosis develops, and the organ itself falls out (autoamputation) along with a rubber hose (BEILAGE and BEILAGE 1994).

There is a similar method of treating a rectified rectum with the rectal ring (DOUGLAS 1985). In this case, a rigid profiled tube similar to the shape of an hourglass is used. A rubber band or tape can also be used, and then the amputation time takes about 7 days (GREENWOOD 1989).

The use of tubes is relatively the best, safe, cheap and without the need for anesthesia for the treatment of this change (DOUGLAS 1985). A helper puts the pig in the right position with the aid of a propulsion plate or a throttle. The procedure is best done within 24 hours of the rectal prolapse (DOUGLAS 1985). The operator, in a gentle circular motion, inserts the flexible tube completely into the fallen intestine and further into the part of the rectum in the pelvic cavity. The tube is positioned in such a way that half is at the level of the anus. With proper care, this can be done with

a pig without fear that the animal will perform defensive moves. Then, after inserting the tube into the dropped rectifier, just next to the anus, two elastic bands should be put on, making as many loops overlapping as possible. The pressure of the elastics will keep the tube in this position (to avoid falling out), and the blood supply will be blocked. In order to prevent infection, a broad-spectrum antibiotic should be given over a relatively long period of time. After 3 days from the procedure, the necrotic part of the intestine shall be cut with a scalpel (about 1 cm from the attached elastics), then the elastics should be removed and the tube pulled out of the anus. Meanwhile, the elastic tube left in the handpiece guarantees the evacuation of gases and feces from the intestine. It is recommended to give the animal laxatives such as bran or linseed, or sodium sulfate (Glauber's salt). An additional advantage is that the tubes are available in three different sizes, depending on the pigs' technological group: piglets – Ø 20 mm, porkers– Ø 25 mm, dungeons – Ø 32 mm.

Authors' own modification of the described method is the additional use of paraffin oil inside the tube, to facilitate the excretion of feces by the pig. This reduces the risk of constipation in the treated animal.

Introduced review of the literature suggests that the rectal prolapse in animals, and especially in pigs, remains an open problem. The presented causes of this phenomena in pigs will allow the use of better prophylaxis to prevent this disorder. On the other hand, the advantages and disadvantages of therapeutic treatment give a base of knowledge for dealing with these difficult cases.

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