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Original Paper

Molecular and pathogenic characterization of Iranian isolates associated with leaf spot disease of potato

Armita Pourarian[1], Mehdi Nasr Esfahani[2], Mehdi Sadravi[1]
[1]*Yasuj University Plant Protection Department, Yasuj, Iran*
[2]*Isfahan Center for Agricultural and Natural Resources Research and Education, (AREEO), Plant Protection Research Department, Isfahan, Iran*

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Alternaria alternata (Fr.) Keissler is one of the main causal agents of leaf spot on potato in Iran and worldwide. In this study, random amplified polymorphic DNA (RAPD) and pathogenicity assay were employed to analyze 28 *A. alternata* isolates obtained from potato plants. The isolates were collected from main potato growing regions of Iran, including Ardebil, Hamedan, Isfahan and Fars provinces. Cluster analysis of genotypes produced by RAPD marker, using UPGMA method indicated that the isolates have been clustered into different groups with no correlation to geographical origins of the isolates. Pathogenicity assay indicated that all *A. alternata* isolates were pathogenic on potato; however, virulence variability was observed among the isolates. The findings revealed that because of extant diversity in pathogenicity and genetics of *A. alternata* isolates, a single isolate should not be used for evaluating resistance of potato.

Keywords: *Alternaria alternata*, pathogenicity, RAPD, *Solanum tuberosum* L.

1 Introduction

Alternaria alternata (Fr.) Keissler has been isolated from a wide range of foods including fresh fruits and vegetables, nuts and cereals (Andersen et al., 2005). *Alternaria alternata* is a common saprobe found on many plants and other substrata worldwide. This species is also an opportunistic pathogen affecting many agricultural crops in field and during postharvest storage of vegetables and fruits. The fungus causes brown necrotic lesions on foliage and black pit of potato (Droby et al., 1984). Previous reports have shown that *A. alternata* could destroy more than 20% of potato production (Rotem, 1994; Van der Waals et al., 2003). In Iran, the two species *A. alternata* and *A. solani* (Ellis & Martin) has been reported as the causal agent of leaf spot on potato, which *A. alternata* is the dominant species (Nasr Esfahani and Ansaripour, 2006).

The efficacy of control strategy on the plant pathogen populations are inhibited by limited information on genetic variability (McDonald and Linde, 2002). The most common adopted effort is the use of fungicides and resistant cultivars. Therefore, understanding genetic variations within the pathogen populations is imperative and should be considered as one of the first steps for the delineation of disease management programs (McDonald and Linde, 2002).

In Iran, no comprehensive study has been done to determine the genetic diversity among *A. alternata* isolates causing leaf spot of potato in different geographical regions. Therefore, the objective of this study was to estimate the genetic and virulence variability among *A. alternata* isolates obtained from potato plants in main potato growing regions of Iran.

2 Materials and methods

2.1 Fungal isolates

Twenty-eight monoclonal isolates of *A. alternata* were obtained from the Plant Pathology Laboratory, Isfahan Research Center for Agriculture and Natural Resources, Isfahan, Iran (Table 1). The isolates were collected from main potato growing regions of Iran, including Ardebil, Hamedan, Isfahan and Fars provinces in 2010. Previous study has confirmed that all 28 isolates belonged to *A. alternata* based on morphological characteristics (Van der Waals et al., 2011). Conidia of all isolates were produced in chains on conidiophores, and presented the shapes of inverted pears with short beaks, dark

***Corresponding Author:** Mehdi Nasr Esfahani, Isfahan Center for Agricultural and Natural Resources Research and Education, (AREEO), Plant Protection Research Department, Isfahan, Iran
e-mail: mne2011@gmail.com

brown, with dimensions of 20–50 × 9–18 µm, and 2–6 (4) transversal and 1–4 (2) longitudinal septa.

2.2 Fungal DNA extraction

Mycelium of each isolate was grown in 50 ml volumes of potato dextrose broth in 250 ml flasks incubated on a rotary shaker (150 rpm) at 25 ±2 °C for 3 days. The mycelium was harvested by filtration and washed with sterile-distilled water. Total genomic DNA was extracted from all isolates using a modification of the CTAB method described by Talbot (2001). The mycelium was ground into a fine powder under liquid nitrogen and suspended in 500 µl extraction buffer. The slurry was incubated for 30 minutes at 65 °C in 1.5 ml micro centrifuge tubes. DNA samples were purified with equal volumes of Chloroform: Iso-amyl Alcohol (24 : 1) mixture, and precipitated with Iso-propanol. The tubes were centrifuged at 13000 rpm (Eppendorf Centrifuge) for 10 minutes and DNA pellets were rinsed with 70% ethanol, air dried, suspended in ddH_2O or TE buffer (pH 8.0) and stored at -20 °C for further use. A NanoDrop ND-1000 spectrophotometer (LMS Co., Ltd., Tokio, Japan) was used to check the quality and concentration of genomic DNA.

Table 1 Origins and virulence variability of *Alternaria alternata* isolates used in this study

No.*	Isolate	Species	Location	State	Year	Percent Disease Severity (*PDS*)**
1	Aa-H1	*Alternaria alternata*	Asad Abad	Hamedan	2010	23.83 ab
2	Aa-H2	*A. alternata*	Shirin Su	Hamedan	2010	48.00 ab
3	Aa-H3	*A. alternata*	Kabodar Ahang	Hamedan	2010	49.50 ab
4	Aa-H4	*A. alternata*	Bahar and Saleh Abad	Hamedan	2010	45.33 ab
5	Aa-H5	*A. alternata*	Hamedan	Hamedan	2010	67.50 ab
6	Aa-H6	*A. alternata*	Qabaq Tappeh	Hamedan	2010	12.50 b
7	Aa-H7	*A. alternata*	Famenin	Hamedan	2010	31.67 ab
8	Aa-A1	*A. alternata*	Agha Bagher Village	Ardebil	2010	16.50 b
9	Aa-A2	*A. alternata*	Khalifeh Lu Village	Ardebil	2010	28.83 ab
10	Aa-A3	*A. alternata*	Yunjalu Village	Ardebil	2010	52.33 ab
11	Aa-A4	*A. alternata*	Tupraghlu Village	Ardebil	2010	17.67 b
12	Aa-A5	*A. alternata*	Samian Village	Ardebil	2010	44.67 ab
13	Aa-A6	*A. alternata*	Soltan Abad	Ardebil	2010	42.83 ab
14	Aa-A7	*A. alternata*	Ardebil	Ardebil	2010	37.67 ab
15	Aa-F1	*A. alternata*	Kushk Mola Village	Fars	2010	48.88 ab
16	Aa-F2	*A. alternata*	Boroj Village	Fars	2010	30.67 ab
17	Aa-F3	*A. alternata*	Dariun Village	Fars	2010	36.50 ab
18	Aa-F4	*A. alternata*	Deh Bid	Fars	2010	69.17 a
19	Aa-F5	*A. alternata*	Shirin Abad Village	Fars	2010	50.00 ab
20	Aa-F6	*A. alternata*	Hasan Abad Village	Fars	2010	38.00 ab
21	Aa-F7	*A. alternata*	Bovanat Village	Fars	2010	36.00 ab
22	Aa-I1	*A. alternata*	Chadegan	Isfahan	2010	46.00 ab
23	Aa-I2	*A. alternata*	Daran	Isfahan	2010	52.50 ab
24	Aa-I3	*A. alternata*	Rozveh	Isfahan	2010	55.00 ab
25	Aa-I4	*A. alternata*	Semirom	Isfahan	2010	27.17 ab
26	Aa-I5	*A. alternata*	Golpayegan	Isfahan	2010	43.33 ab
27	Aa-I6	*A. alternata*	Mehdi Abad	Isfahan	2010	38.00 ab
28	Aa-I7	*A. alternata*	Nisian	Isfahan	2010	49.33 ab

* – *Alternaria alternata* isolates causing leaf spot of potato; ** – values followed by the same letter in the column did not differ significantly (0.05 level) in Duncan's multiple range test

2.3 RAPD analysis

Six primers OPA-16, OPC-06, OPC-08, OPP-16, OPP-19 and OPX-12 (Operon Technologies Inc., Alameda, CA) with high polymorphism and reproductive profiles were chosen among 15 primers to perform RAPD analysis on *A. alternata* isolates based on the results of initial screening against a set of representative isolates (Table 2). PCR amplification of RAPD loci was carried out in a 25 ml containing 0.5 µM primer, 2.5 µl of a 10× buffer (200 mM Tris-HCl, 500 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U Taq DNA polymerase and 2 µl of DNA template (10 ng). RAPD analysis was carried out as described by Nasehi et al. (2014). PCR amplification was conducted in a thermocycler programmed with the following parameters: 45 cycles of 94 °C for 1 min (denaturation), 35 °C for 1.5 min (annealing) and 72 °C for 2 min (extension) with the initial denaturing of 94 °C for 4 min and final extension of 72 °C for 10 min. All PCR were performed in three replications to confirm the consistency of amplification.

Table 2 RAPD primers utilized to identify and assess interspecific genetic diversity among *Alternaria alternata* isolates collected from potato plants

Primera	Sequence (5´-3´)
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-13	CAGCACCCAC
OPA-16*	AGCCAGCGAA
OPB-17	AGGGAACGAG
OPB-18	CCACAGCAGT
OPC-06*	GAACGGACTC
OPC-08*	TGGACCGGTG
OPE-01	CCCAAGGTCC
OPP-16*	CCAAGCTGCC
OPP-17	TGACCCGCCT
OPP-18	GGCTTGGCCT
OPP-19*	GGGAAGGACA
OPX-12*	TCGCCAGCCA
OPX-14	ACAGGTGCTG

a Primers with an asterisk (*) were utilized to identify and assess interspecific genetic diversity among *Alternaria alternata* isolates. These primers with high polymorphism and reproductive profiles were chosen among all primers based on the results of initial screening against a set of representative isolates

2.4 Gel electrophoresis and staining

PCR products of RAPD analysis were size-separated in 1% agarose gel under 1× TAE buffer (40 mM Tris, 20 mM Acetic acid and 1 mM EDTA) at 70 V for 45 min at room temperature. Gels were stained with ethidium bromide, visualized under UV light and photographed using a gel documentation system (GeneSnap Ver 6.03, Syngene Laboratories, Cambridge, United Kingdom). The sizes of amplified and digested DNA fragments were estimated using GeneTools (Ver 3.00.13, Syngene Laboratories) by comparison with a 2-Log DNA Ladder (0.1–10 kb) marker.

2.5 RAPD data analysis

The bands were considered as binary characters, and were scored as 1 for presence and 0 for absence of DNA bands. The scores were then entered into a matrix for analysis by the numerical taxonomy and multivariate analysis system, NTSYS-pc 1.8 program (Applied Biostatistics Inc., Setauket, NY, USA) (Rohlf 1993). The similarity matrix was calculated among the isolates using Jaccard's similarity coefficient. Clustering was performed using the unweighted pair group method using arithmetic averages (UPGMA) to generate the dendrogram. And the RAPD clustered analyses were compared to geographical origins of the isolates for presence of any correlation coefficients.

2.6 Pathogenicity

Pathogenicity test was conducted in a greenhouse using all 28 isolates of *A. alternata*. The experiment was arranged in a completely randomized design in 10 replications (pots) for infected plants as well as control plants. Each replication consisted of 10 leaves of each plant. Conidial suspension (10^3 spores per ml) was used to inoculate one month old potato leaves (var. *Agria*). Seven days after inoculation, disease rating was scored based on a scale of 0–7 points, where: 0 = no disease symptoms, 1 = lesions as pinpoints and non-measurable, 2 = <10% of the leaves with brown necrotic lesions, 4 = 10 ≤ to 25%, 8 = 25≤ to 50%, 16 = 50≤ to 75% of the leaves with brown necrotic lesions, and 32 = 75≤ to ≥100% of the leaves with brown necrotic lesions or completely brown (NIAB 1985). The experiments were repeated twice. Re-isolation of the inoculated fungi was performed to fulfill Koch's postulate. The following formula was used to calculate percent disease severity (*PDS*) in each replication. In this formula, *T* is the total number of leaves in each category; *R* is the disease severity scale; *N* is the total number of leaves tested; *S* is the highest number in the scale:

$$\textit{Percent disease severity } (PDS) = \frac{\sum RT \cdot 100}{S \cdot N}$$

Data were transformed to arcsine square-root and then subjected to the analysis of variance procedure (ANOVA, $P < 0.05$), and the means were compared by Duncan's multiple range test using SAS software version 9.2.

3 Results and discussion

3.1 RAPD analysis

Application of six RAPD primers generated a total of 47 consistently amplified fragments (100–3000 bp), of which all the 47 fragments were polymorphic (100%). The dendrogram produced from UPGMA analysis based on Jaccard's similarity coefficient indicated that the variability was high among isolates of *A. alternata*, and the similarity value was ranged from 0 to 100% with the mean value of the Jaccard's similarity coefficient 0.50 (Fig. 1). The isolates obtained from main potato growing regions of Iran, including Ardebil, Hamedan, Isfahan and Fars provinces were clustered into different groups with no correlation to geographical regions of the isolates.

3.2 Pathogenicity

Symptoms of leaf spot were observed in all the inoculated potato plants seven days after inoculation. Foliar lesions initially were as pinpoints, irregular to circular, brown spots on the lower leaves, and then became circular spots spreading over much of the leaves. Control plants remained healthy without showing any symptom of the disease. *Alternaria alternata* isolates was re-isolated from the inoculated plants and was found to be identical to the original isolates based on morphological characteristics. These results revealed that *A. alternata* was the causal agent of leaf spot of potato plants in Iran; however, virulence variability was observed among the isolates (Table 1). The isolate Aa-F4 (*PDS* of 69.17) was the most virulent isolate and located in a single subgroup in comparison to the others three close virulent isolates, Aa-A3, Aa-I2 and Aa-I17 (Fig. 1). While, the three isolates Aa-H6 (*PDS* of 12.50), Aa-A1 (*PDS* of 16.50) and Aa-A4 (*PDS* of 17.67) were the least virulent isolates. Other isolates had the moderate virulence.

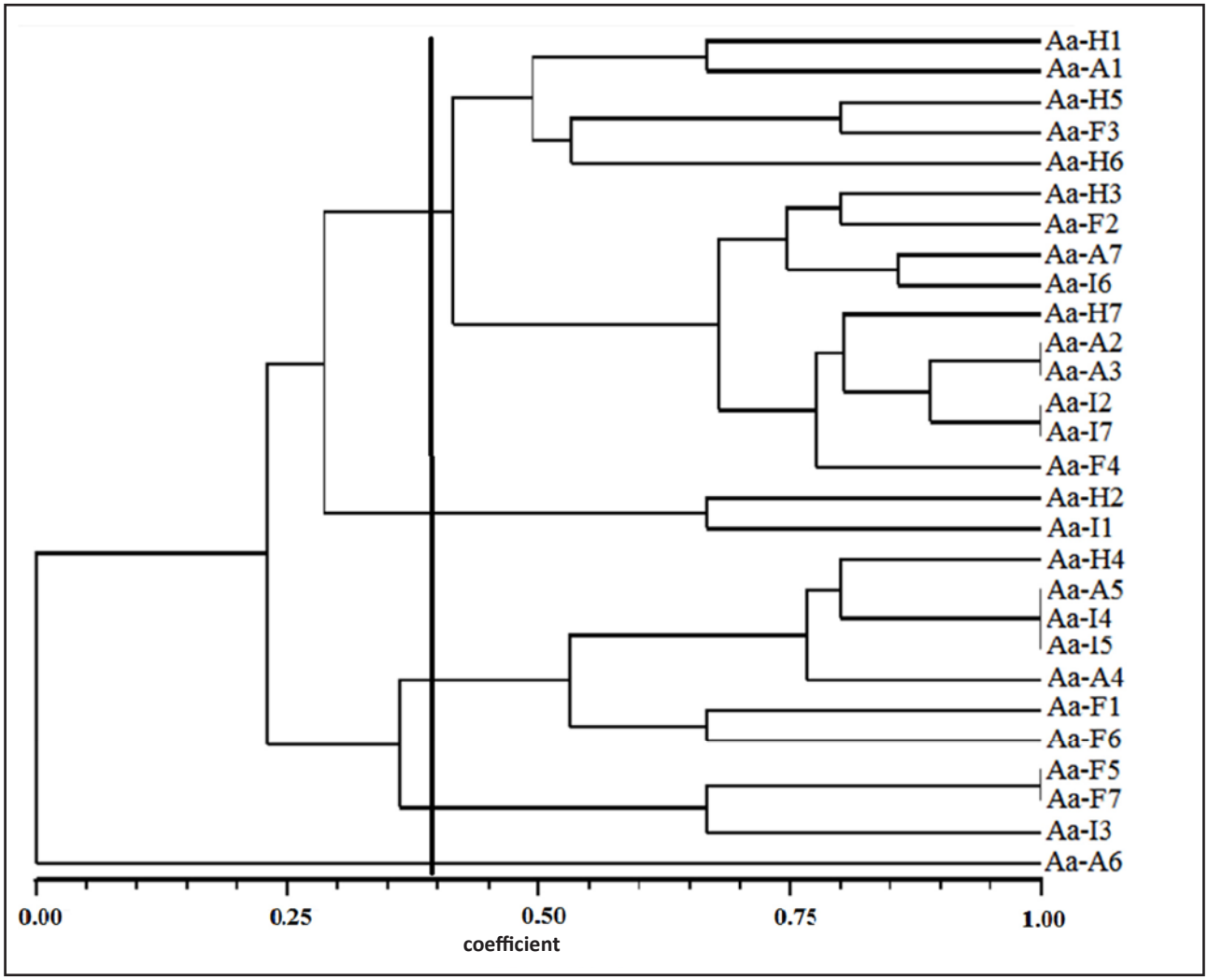

Figure 1 Dendrogram generated by UPGMA showing the genetic variability of 28 *Alternaria alternata* isolates derived from combination of six RAPD primers (OPA-16, OPC-06, OPC-08, OPP-16, OPP-19 and OPX-12). The name of the isolates is based on Table 1

RAPD PCR method is an extremely powerful tool to separate individuals having intraspecific and interspecific variability. This method has been employed to provide comprehensive information regarding the genetic variation in populations of *A. alternaria* (Morris et al., 2000; Pryor and Michailides, 2002; Weir et al., 1998) and other plant pathogenic fungi (Mahmodi et al., 2014; Nasehi et al., 2014). In this study, RAPD analysis indicated that isolates of *A. alternata* have a high diversity in main potato growing regions of Iran with no correlation to geographical origins of the isolates.

The results of pathogenicity indicated that all isolates of *A. alternata* used in this study were pathogenic on potato, but virulence variability was observed among them. Based on the virulence variability, all isolates were clustered into three groups of most virulent (one isolate), least virulent (three isolates) and moderate virulent isolates (25 isolates). These results were in agreement with the previous studies which have confirmed the existence of virulence variability among *A. alternata* isolates obtained from different geographical regions (Kakvan et al., 2012; Meena et al., 2015). In addition, the grouping based on virulence variability was not correlated with the result of RAPD analysis, as well as geographical regions, which is in agreement with previous study on *A. alternata* (Kakvan et al., 2012).

4 Conclusion

In conclusion, this study was conducted to examine the structure of pathogen population by studying of diversity in pathogenicity and genetics of *A. alternata* isolates. The results indicated that because of extant diversity in pathogenicity and genetics of *A. alternata* isolates, a single isolate could not be used for evaluating resistance of potato. This study suggests that the isolate Aa-F4 as the most virulent isolate and a few representative isolates which had moderate virulence could be used for evaluating resistance of potato. The data of RAPD markers could also be expanded for a wider genetic diversity of *A. alternata* on different host plants from different geographical regions.

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Original Paper

The changes of the assimilation pigments content of turf *Festuca* spp. leaves after application of different nutrition forms

Peter Hric*, Ľuboš Vozár, Peter Kovár
Slovak University of Agriculture in Nitra, Slovak Republic

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The aim of this experiment was to compare find out of the changes of assimilation pigments content of turf *Festuca* spp. leaves after application of different nutrition forms under non-irrigated conditions. In period April 2012 – May 2015 (without June 2014 and February 2015) experiment was carried out in warm and dry conditions in area of Nitra (Slovak Republic). Concentration of assimilation pigments (chlorophyll *a*, chlorophyll *b* and total carotenoids) was determined spectrophotometrically. The experiment was included 10 treatments: 1. Without fertilization; 2. Saltpetre with dolomite, superphosphate, potassium salt; 3. Turf fertilizer NPK 15-3-8 (+ 3 MgO + 0.8 Fe + 18S); 4. Slow release fertilizer NPK 14-5-14 (+ 4CaO + 4MgO + 7S); 5. Controlled release fertilizer NPK (S) 13-9-18 (+ 6S); 6. Organic fertilizer NPK 5-1-1,7. Organic fertilizer NPK 3-2-1 and 3 mycorrhizal preparations. The use of inorganic and organic fertilizers resulted in an increase chlorophyll *a*, *b* content and total chlorophyll in leaves *Festuca* spp. More pronounced increase in chlorophyll content was found by the application of the Turf fertilizer. Application of this fertilizer has a statistically significant effect on content of chlorophyll *a* + *b* than in the other evaluated treatments without turfs fertilized by Controlled release fertilizer and Organic fertilizer NPK 5-1-1. A statistically significant increase in the total carotenoids concentration was observed after the use of Saltpetre with dolomite, superphosphate, potassium salt and Turf fertilizer as compared to the non-fertilized control.

Keywords: turf, *Festuca* spp., fertilizers application, chlorophyll, total carotenoids

1 Introduction

Colour, as one of the qualitative indicators of turfs, is closely related to the concentration of green leaf dye-chlorophyll. The most important pigments are chlorophyll *a* (blue-green) and chlorophyll *b* (yellow-green). Carotenoids, together with chlorophyll, are part of photosynthetic organisms and form a reciprocal process (Beard, 1973; Xu et al., 1995; Kovár and Gregorová, 2009). Biosynthesis of assimilation pigments in plants is affected by variety of external and internal factors (Masarovičová et al., 2000). The color of leaves, as the main component for assessing the aesthetic quality of turfs, is often evaluated in field experiments (Karcher and Richardson, 2003; Gregorová and Kovár, 2010; Hric et al., 2016a).

Nitrogen (N) is a component of chlorophyll. N is involved in the conversion of kinetic solar energy into chemical energy. In the absence of nitrogen of plants change their appearance. If the nitrogen content in the leaves of plants is low, then the leaves turn yellow. With a large nitrogen deficiency, the leaf dies (Hrabě et al., 2009; Aldous, 2011).

Nitrogen application significantly improves turf grass color and other turf qualitative parameters (Bell et al., 2004; Bilgili and Acikgoz, 2007; Pessarakli, 2007; Altissimo and Peserico, 2008; Hejduk, 2012). Several studies have been conducted to determine the effects of N application time and rates on turf quality and plant growth (Wehner et al., 1988; Moore et al., 1996; Bilgili and Acikgoz, 2007; Hric et al., 2016b).

The aim of this experiment was to compare the changes of the assimilation pigments content of turf *Festuca* spp. leaves after application of different nutrition forms under non-irrigated conditions. In the contribution compares the effect of fertilization on inorganic, organic and mycorrhizal preparations on the content of chlorophyll *a*, *b* and total carotenoids.

2 Material and methods

In period April 2012 – May 2015 (without June 2014 and February 2015) turf experiment was located in moderate climatic zone of warm and dry area in Nitra (Slovak Republic). In June 2014 and February 2015 samples were not analyzed (decimated turf).

***Corresponding Author:** Peter Hric, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Grassland Ecosytems and Forage Crops, Tr. Andreja Hlinku 2, 979 76 Nitra, Slovak Republic, e-mail: peter.hric@uniag.sk

The experiment was established in early October 2011. We used turf mixture designed for low slowly growing turfs with following composition: *Lolium perenne* L. (30%), *Festuca rubra* L. (50%) and *Festuca ovina* L. (20%). Gradually dominated *Festuca* spp. leaves in turf.

Experimental plots area was 2.4 m^2 and each treatment was in 3 random replications. Experiment was established in the form of a Latin square.

There were used 10 treatments:

1. Without fertilizing (Control).
2. LAD (27% N), P_2O_5 (19% P), K_2O (60% K) (N + P + K).
3. Turf fertilizer NPK 15-3-8 (+ 3 MgO + 0.8 Fe + 18 S) (TF).
4. Slow release fertilizer NPK 14-5-14 (+4 CaO + 4 MgO + 7 S) (SRF).
5. Controlled release fertilizer NPK (S) 13-9-18 (+6S), (CRF).
6. Organic fertilizer NPK 5-1-1 (OF1).
7. Organic fertilizer NPK 3-2-1 (OF2).
8. Mycorrhizal preparation (MP1).
9. Mycorrhizal preparation (MP2).
10. Mycorrhizal preparation (MP3).

For the recommended dose of fertilizer the value 18 g m^{-2} N was taken, which meets the requirements for intensively used turfs (Cagaš et al., 2011). System of fertilizing is presented in Table 1.

LAD contains 27% nitrogen with dolomite. Nitrogen is in the ammonium and nitrate form. Superphosphate is 19% P_2O_5 and potassium salt is 60% K_2O.

Turf fertilizer 15-3-8 (+ 3 MgO + 0.8 Fe + 18 S) is the granulated fertilizer intended for use for turfs throughout the year in the form of multiple fertilizer applications (3–5×) during the growing season. Nitrogen is in the ammonium form.

SRF NPK 14-5-14 (+ 4 CaO + 4 MgO +7 S) is a complex NPK fertilizer containing urea formaldehyde component as a source of nitrogen enriched with micronutrients. Part of major NPK nutrients is founded in fast-dissolving form.

CRF NPK (S) 13-9-18 (+ 6S) is coated controlled release fertilizer nutrient (for 5–6 months).

OF 5-1-1 content is comprising C, H, O, N, P, K, Ca, Mg, S, Fe etc., in the form of organic components of the starch material from the milled cereals (30%), enriched hydrolysate of whey (30%), lignocelluloses raw material from wood processing (30%), by hydrolysis of whey enriched (30%) and in 10% mineral constituent zeolite of sodium aluminium silicate. Philosophy of this fertilizer is unlike mineral fertilizers aimed at improving the carbon balance.

OF 3-2-1 is produced by modern technology from natural materials without the use of chemicals and preservatives. Production procedure at high temperature leads to inactivation of pathogens and weed seeds. This fertilizer is characterized as high-quality organic fertilizer with gradual release of the main nutrients and essential trace elements. Its high biological value is increased due to harmless processing, content balance, easy handling and hygiene applications in practice. Compared with manure it constitutes a modern compensation for of manure.

Mycorrhizal preparation 1 is grass conditioner consisting of natural argillaceous media, 6 kinds of mycorrhizal fungi, natural ingredients that support mycorrhiza (humates, ground minerals, extracts of marine organisms), biodegradable polyacrylamide granules, sapropel (biological sediment). It contains 1.3% N, 0.6% P, 3% K, 1% Mg, 2.8% Ca and 0.8% S.

Mycorrhizal preparation 2 is based on the basis of endomycorrhizal fungi. It contains natural argillaceous media, 6 kinds of mycorrhizal fungi, natural ingredients

Table 1 System of fertilizing of individual treatments (2.4 m^2)

Type of fertilizer (number of applications per year)	Yearly dose (g)	Beginning of vegetation (g)	App.* 5. June (g)	App. 20. July (g)	Half of July (g)	Half of August (g)	Half of September (g)
LAD (4×)	160.00	40.00	40.00		40.00		40.00
P_2O_5 (1×)	130.43	130.43					
K_2O (2×)	69.40	34.70			34.70		
TF (3×)	288.00	96.00		96.00		96.00	
SRF (2×)	288.00	144.00			144.00		
CRF (2×)	332.32	166.16			166.16		
OF1(1×)	864.00	864.00					
OF2 (1×)	1440.00	1440.00					

* app. – approximatelly

that support mycorrhiza (humates, ground minerals, extracts of marine organisms) and biodegradable polyacrylamide granules.

Mycorrhizal preparation 3 contains mycorrhizal fungi, keratin, natural humates, ground minerals (zeolite, serpentine, apatite) a 5% N, 6% P, 4% K, 2% Mg, 2% S and 4% Ca.

Mycorrhizal preparations were applied before sowing turfgrass mixture in dose 360 g per variant.

Concentration of assimilation pigments (chlorophyll *a*, chlorophyll *b* and total carotenoids) was determined spectrophotometrically by Spectrofotometer CAMSPEC M108 UV/VIS. Samples of plant material were taken each time in the last decade of the respective month and were analyzed immediately after collection. Four *Festuca* spp. leaves were analyzed in each replication 4 times. After sawing leaves on underlying glass we removed the protruding parts and measured the width of leaves. The leaf segments thus prepared were homogenized in mortar in 80% acetone-containing blend. The impurities were separated by centrifugation at 2500 rpm for 2 minutes. Absorbance of leaf extract supernatant was measured by spectrophotometer at wavelengths (λ) 663 nm (chl *a*), 647 nm (chl *b*) and 470 nm (total carotenoids). The absorbance of impurities (proteins, tannins) in the supernatant was measured at wavelength of 750 nm. The absorbance value of the solution at λ 750 nm was deducted from absorbancies at λ 663, 647 and 470 nm. The concentration of the individual assimilation pigments (mg l^{-1} extract) was calculated according to the following equations (Lichtentaler, 1987):

$$\text{Chl } a = (12.25A_{663} - 2.79A_{647})D$$

$$\text{Chl } b = (21.50A_{647} - 5.10A_{663})D$$

$$\text{Total carotenoids} = [(1000A_{470} - 1.82\text{chl } a - - 85.02\text{chl } b)/198]D$$

that, A_{663}, A_{647} a A_{470} is the absorbance of the extract at individual wavelengths (663, 647 a 470 nm) and D is the thickness of the spectrophotometrically cuvette (cm).

Total concentration of chl *a*, chl *b* and total carotenoids in leaves of used grasses was calculated on mg m^{-2} leaf area by relationship:

$$\text{mg m}^{-2} = \text{mg l}^{-1} \times K \Longrightarrow K = x$$

where:
K – coefficient of conversion
V – sample volume (ml)
A – leaf area of segments (m^2)

Results were statistically evaluated by the Analysis of Variance (One-way ANOVA, Method: 95.0 percent LSD) using statistical software STATISTICA 7.1 (Stat Soft. Inc. 2007).

3 Results and discussion

The average values of chlorophyll *a* and *b* are given in Table 2. The content of chlorophyll *a* in turfgrass leafs was higher (p = 0.000302) on fertilized treatments (467.77–496.69 mg m^{-2}) compared to non-fertilized control (404.05 mg m^{-2}) and mycorrhizal preparation 2 (422.99 mg m^{-2}). The lowest content of chlorophyll in leaves we measured on control (404.05 mg m^{-2}). Similar results were obtained by Larimi et al. (2014) and Mahmoud et al. (2017). Conversely, the highest content of chlorophyll *a* (496.69 mg m^{-2}) in the leaves of grasses was recorded on the treatment fertilized by turf fertilizer. The highest connect values of chlorophyll *a* were on turfs fertilized by inorganic fertilizers (475.62–496.69 mg m^{-2}) and organic fertilizer 1 (492.16 mg m^{-2}). Treatment fertilized by TF was also characterized by the highest variability in chlorophyll *a* content (δ = 146.5).

The concentration of chlorophyll *b* in grass tissues was in the reference period in from 167.85 mg m^{-2} (control) to 218.67 mg m^{-2} (TF). Also, with this assimilation pigment, its content on treatment with application TF (218.67 mg.m^{-2}) was higher (p = 0.000029) than on control and on turfs with mycorrhizal preparations 1 and 2 (202.92 and 183.17 mg m^{-2}). Similar results were obtained by Larimi et al. (2014). The most variability content of chlorophyll *b* over the whole experiment period was treatment fertilized by turf fertilizer (δ = 59.31).

Total chlorophyll content (chl *a* + chl *b*) is presented in Table 3. Values were found in a range from 571.91 mg m^{-2} (TF) to 757.02 mg m^{-2} (control). Application of TF resulted in the highest (p = 000006) concentration of the monitored parameter (757.02 mg m^{-2}) compared to fertilized treatments N + P + K (680.65 mg m^{-2}), SRF (675.84 mg m^{-2}), OF2 (671.36 mg m^{-2}) and mycorrhizal preparations (606.166–675.29 mg m^{-2}). Control treatment has lowest total chlorophyll content. The use of the mycorrhizal preparation resulted in an increase in the total chlorophyll content as compared to the control. Equally, Vafadar et al. (2013) in their experiment after application of mycorrhizal fungi found an increase in chlorophyll content compared to control. Kuo (2015) found in his experiment with using SRF and fast release fertilizers 3× higher chlorophyll content than the control. Once again, the highest imbalance in chl *a* + chl *b* concentration was represented by TF (δ = 271.72). The most stable connect of chl *a* + chl *b* reached treatments with application mycorrhizal preparations (δ = 31.07–30.67).

Table 2 The average values of chlorophyll *a* and *b* in leaves

Treatment	Chl *a* (mg m^{-2})	δ	Chl *b* (mg m^{-2})	δ
Control	404.05^{b}	119.01	167.85^{d}	53.87
N + P + K	478.32^{a}	125.60	202.34^{abc}	55.89
TF	496.69^{a}	146.50	218.67^{c}	59.31
SRF	475.62^{a}	90.05	200.22^{abc}	40.74
CRF	483.90^{a}	98.85	209.24^{ab}	40.99
OF1	492.16^{a}	91.81	206.86^{ab}	45.12
OF2	467.77^{a}	93.45	203.59^{ab}	46.52
MP1	472.37^{a}	89.23	202.92^{ab}	46.02
MP2	422.99^{b}	88.56	183.17^{cd}	45.33
MP3	468.94^{a}	85.49	196.79^{ac}	42.66

$^{a, b, c, d}$ – statistically significant differences (Fisher LSD test, = 0.05), δ = standard deviation, *n* = 6

Table 3 The average values of chlorophyll *a* + *b* and total carotenoids in leaves

Treatment	Chl *a* + *b* (mg m^{-2})	δ	Total carotenoids (mg m^{-2})	δ
Control	571.91^{bc}	167.97	133.19^{a}	41.11
N + P + K	680.65^{a}	195.33	152.34^{b}	44.57
Turf fertilizer	757.02^{b}	271.72	152.26^{b}	46.55
SRF	675,84^{a}	121.88	141.44^{ab}	36.88
CRF	693.15^{ab}	137.51	148.37^{ab}	40.81
OF1	699.02^{ab}	128.81	147.52^{ab}	34.06
OF2	671.36^{a}	123.74	140.69^{ab}	31.25
MP1	675.29^{a}	126.19	144.80^{ab}	31.07
MP2	606.16^{cd}	128.12	135.89^{a}	31.41
MP3	665.73^{ad}	123.63	144.60^{ab}	30.67

$^{a, b, c, d}$ – statistically significant differences (Fisher LSD test, = 0.05), δ = standard deviation, *n* = 6

The concentration of total carotenoids was higher in fertilized treatments by N + P + K (152.34 mg m^{-2}) and TF (152.26 mg m^{-2}). In previous experiment (Hric et al., 2016a), the effect of use fertilizers on the content of total carotenoids did not notice a positive effect. Statistically significantly highest content of total carotenoids was found on the treatment fertilized with N + P + K and turf fertilizer compared with control and mycorrhizal preparation 2. At the same time, turf fertilized by turf fertilizer was characterized by the highest imbalance of this assimilation pigment (δ = 46.55). Again the most stable content of total carotenoids reached treatments with application mycorrhizal preparations (δ = 30.67–31.41).

4 Conclusions

On the basis of the results, it can be stated that the use of inorganic and organic fertilizers as well as the mycorrhizal preparation resulted in an increase of the assimilation pigments content of *Festuca* spp. leaves. The highest values of assimilation pigments were observed on treatments fertilized by inorganic fertilizers (N + P + K, TF, SRF, CRF and OF1). These fertilizers are likely to provide the greenest colour of turf. The highest increase in assimilation pigments occurred after application of TF. At same, this treatment was characterized by the greatest imbalance in assimilation pigment values. In the next period, is planning to continue in the experiment and spread it on other turfgrass species and fertilizers.

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Review

Effect of biochar on soil structure – review

Martin Juriga*, Vladimír Šimanský
Slovak University of Agriculture in Nitra, Slovak Republic

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Soil structure and organic matter are important indicators of soil quality. In the literature it states that there is a linear relation between soil structure and the organic matter. Mechanisms of formation and stabilization of aggregates have also been described in the literature, but it is evident that not every mechanism is applicable to various soil-climatic conditions. Recently, the modern but not the new term has become a biochar. It is anticipated that biochar is a significant source of C, and its application to the soil will improve the aggregation process in the soil. Lately we have been working in this area and we wanted to provide an overview of this issue through this review. The aim of this review was to collate and synthesize available information on soil structure and SOM. The emphasis of this review is on biochar and its combination with other organic and mineral fertilizers in relation to soil structure.

Keywords: biochar, soil organic matter, aggregation, aggregate stability

1 Introduction

Soil structure is a main soil property since it regulates soil water content, aeration and temperature of soils (Neira et al., 2015). Soil structure also positively influences plant germination and root growth. Therefore, assessing soil structure is an important issue in a determining soil quality (Ball and Munkholm, 2015).

Soil structure is usually defined as the spatial arrangement of soil particles and soil voids (i. e. soil pores), which may also be defined as the spatial distribution of soil properties. Soil structure includes the physical habitat of soil living organisms, and controls many important physical, chemical and biological soil functions and associated ecosystem services (Dexter, 1988). However, soil structure is more than only the physical arrangement of particles and pores and includes structural stability (i. e. the ability to resist endogenous factors or stresses) and structural resilience (i. e. the ability of recover upon stress removal) (Kay and Angers, 2001). Soil aggregation is responsible for soil structure and it is fundamental for soil to function as well as agricultural productivity.

Soil aggregates are secondary particles formed through the combination of mineral particles with organic and inorganic substances. Formation of soil aggregates as basic unit of soil structure is a function of physical forming forces (such as: inter and intramolecular forces, electrostatic, and gravitational forces) between soil particles (Grosbellet et al., 2011; Li and Fan, 2014; Hu et al., 2015) however, its stabilization is influenced by internal and external factors, and their interactions (Chenu and Cosentino, 2011; Paradelo et al., 2013; Šimanský et al., 2013; Jozefaciuk and Czachor, 2014; Šimanský and Bajčan, 2014).

Soil organic matter (SOM) also plays an important part in controlling soil quality and resilience because of the fundamental role it plays in determining a wide range of soil properties including buffering capacity, microbial biodiversity, water retention, and structural stabilization (Szombathová 1999; Šimanský et al., 2013; Šimanský and Polláková, 2016). For instance, humic substances (part of SOM) control buffering, cation exchange and water retention capacity of soils (Šimanský and Polláková, 2014), as well as the formation and stabilization of water-stable aggregates (Wang and Xing, 2005; Šimanský et al., 2013; Polláková et al., 2017). SOM is the major determinant and indicator of soil fertility and quality, and is closely related to soil productivity (Huang et al., 2007). Soil organic carbon (SOC) is an important agent responsible for binding soil mineral particles together creating an aggregate hierarchy (Oades and Waters, 1991). The dynamics of aggregate formation seem to be closely linked with soil organic carbon storage in soils (Golchin et al., 1997). Agro-technical operations and environmental changes modify the content and turnover of SOC. Intensive cultivation practices can stimulate

***Corresponding Author:** Martin Juriga, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Genetics and Animal Breeding Biology, Tr. Andreja Hlinku 2, 979 76 Nitra, Slovak Republic, e-mail: juriga.martin@zoznam.sk

biodegradation of the initially physically protected carbon in soil, and hence it could be responsible for the decrease of SOC (Norton et al., 2012; Khorramdel et al., 2013; Šimanský, 2017). Organic fertilizers, such as farmyard manure, compost, crop residues and others are often the most important sources of organic compounds in systems with continuous removal of organic crop residue. The last two decades have been characterized by a continuous decrease of livestock population, which had effect on decreasing availability of organic fertilizers and mainly production of perennial fodder for livestock (significant source of organic matter). At the same time, environmental and regulatory constraints have driven arable agriculture towards lower-input soil management, highlighting the need to maintain optimal soil function and a favourable balance of organic compounds in the soil. The same situation is in the Slovak republic. At present, from the point of view of the need for organic substances a 30–50% deficit is estimated (Green Report, 2014). From the point of view of sustainable land management, the balanced equilibrium of organic substances is essential and so new resources must be sought. One of the possible and innovative solutions can be the application of biochar.

Under these circumstances, the aim of this review is gather and synthesize available information on soil structure and SOM. The emphasis of this review is on biochar and its combination with other organic and mineral fertilizers in relation in soil structure.

2 Biochar as potential tool for improve agronomic practice

Biochar is a solid, carbon-rich product of thermal decomposition of organic matter, called pyrolysis, at a temperature higher than 400 °C and usually lower than 900 °C under conditions of oxygen deficit (Ahmad et al., 2014; Lehmann and Joseph, 2009). It is produced for environmental or agricultural application. Biochar is composed mainly of aromatic molecules that are not organised in ideally adherent layers (Hussain et al., 2016; Lehmann and Joseph, 2009). The structure and properties of biochar are in close relation to the conditions of pyrolysis (temperature and time of heat action). With increasing temperatures of pyrolysis, the content of carbon in biochar increases, while its content of hydrogen and oxygen decreases. Within the range from 400 to 700 °C an increase in pyrolysis temperature leads to higher aromaticity and hydrophobicity of biochar as well as higher volume of pores and specific surface area (Ahmad et al., 2014; Lehmann and Joseph, 2009). Various types of biomass can be used as feedstock for biochar production, such as wastes from wood processing, municipal wastes, sewage sludge, wastes from animal breeding and agricultural production (Hussain et al., 2016; Inyang et al., 2016; Stefaniuk and Oleszczuk, 2015; Usman et al., 2015; Zielińska et al., 2015).

The kind of matter used as feedstock has also an effect on properties of biochar. Therefore, is of the utmost necessity to consider the choosing of starting feedstock, according to the purpose to witch the biochar is to be used for. For example, biochar produced from animal manures usually has smaller specific surface area, than biochar which has been produced from wood and plant mass (Ahmad et al., 2014; Zielinska et al., 2015). Biochar has become the main focus of research in the recent past. The results of the experiments showed that application of biochar can be a sustainable way of improving physical, chemical, and biological properties. For example, up to now, there have been several studies published (Laghari et al., 2015; Agegnehu et al., 2016; Šimanský, 2016; Šimanský et al., 2016; Šimanský et al., 2017; Šimanský et al., 2017a) where authors concluded that biochar increased SOC in the soils due to its unique properties and structure which can potentially increase carbon sequestration (Lehmann and Joseph 2009; Šimanský et al., 2017a). In addition, the highly porous structure and large surface area of biochar (Figure 1) provide refuge for beneficial soil microorganisms such as mycorrhiza or bacteria (Pietikainen et al., 2000). This would be positive on microbial processes involved in nutrient cycling, decomposition of organic matter, and greenhouse gas emission (Pietikainen et al., 2000; Grossman et al., 2010; Deal et al., 2012). Among other aspects, it allows the carbon dioxide immobilization in soil and, in consequence, a reduction of its emission to the atmosphere (Conte, 2014; Horák and Šimanský, 2017). In experiments with biochars produced from biomass obtained from areas contaminated with heavy metals no elevated levels of any potentially toxic element (e.g. Cd or Pb) were noted (Evangelou et al., 2014). Another positive effect of the addition of biochar to soil to which various pesticides were applied was demonstrated by Oleszczuk et al. (2014). Biochar can also improve nutrient absorption, cation exchange capacity (Zwieten et al., 2010) sorptive parameters, mainly sorpion of soil organic matter (Šimanský et al., 2017) and increase of soil pH mainly in acidic soils (Karami et al., 2011; Horák et al., 2017). Rajkovich et al. (2012) refers that the biochar ash contains nutrients including base cations such as Ca and Mg causing a positive effect on the values of the degree of sorption complex saturation by base cations. Other studies found that abiotic changes after biochar addition take a short-time, with abiotic effects such as enhanced nitrogen or potassium availability disappearing after a single growth season, while the effects on functions such as plant productivity remained enhanced, suggesting that microbial changes rather than abiotic changes could

Table 1 Benefits of biochar application (Review)

Soil organic matter	Biochar is source of stabile organic matter and increase of C sequestration provide refuge for beneficial soil microorganisms such as mycrorrhiza or bacteria	Fischer and Glaser 2012; Pietikainen et al., 2000; Šimanský et al., 2016; Agegnehu et al.,2016; Horák and Šimanský, 2016
Greenhouse gasses	Reduce N_2O and CO_2	Horák et al., 2017; Horák and Šimanský, 2017;
Water retention characteristics	Improve soil matter regimes and available plant water content	Abel et al., 2013; Abrol et al., 2016; Novak et al., 2012
Cation exchange capacity	Improve sorptive properties of soils	Šimanský et al., 2017; Rajkovich et al., 2012
pH	Neutralization of acid soil	Karami et al., 2011; Horák, 2015; Horák et al., 2017
Porosity	Improve pore size distribution and pore continuity	Obia et al., 2016
Bulk density	Increase of bulk density	Ajayi and Horn, 2016; Mukherjee and Lal, 2013
Soil structure	Improve aggregation processes and increase of aggregate stability	Cornelissen et al., 2013; Herath et al., 2013; Šimanský, 2016

have led to the persistent effects of biochar addition (Mukherjee et al., 2014; Oram et al., 2014). Reviews of biochar effects on plant growth reported overall positive effects (Biederman and Harpole, 2013), but also show that there is considerable variability between studies, and that several studies showed negative effects (Liu et al., 2017; Hansen et al., 2017). In Table 1 are summarized some benefits of biochar application to the soils according to our available knowledge. Benefits of biochar as a soil amendment may vary with its properties, time after its application, and in relation to soil texture and mineralogy (Butman et al., 2015).

2.1 Effect of pure biochar on soil structure

Generally, there are several mechanisms of aggregation such as: hierarchical theory of aggregation (Edwards and Bremner, 1967), the concentric theory (Santos et al., 1997), the precipitation of hydroxides, oxides, phosphates and carbonates enhances aggregation (Bronick and Lal, 2005), cations also form bridges between clay and SOM particles resulting in aggregation (Jankowski, 2013) or it is possible that aggregates form through a combination of these processes (Bronick and Lal, 2005). All these mechanisms can be responsible for the formation and stabilization of soil structure after application of biochar to the soil. Applied biochar can be joined with mineral particles in the soil (Figure 2) or can be part of the soil aggregates (Figure 3 A, B). Biochar contains base cations (Rajkovich et al., 2012), which can be joined by the means of cationic bridges with clay and organic particles (Bronicki and Lal, 2005) and thus creating a favorable soil structure condition. Multivalent ions associated with biochar may have a positive effect through interactions with negative charged surface functional groups on SOM (e.g., R-COO–) and soil minerals (e.g., Al-O–, Si-O–) (Mukome et al., 2013). The bridging effects of multivalent ions, such as Fe^{3+} can enhance sorption of SOM to clay minerals (Feng et al., 2005). Other mechanism can be explained also through base cations, which can act as a bond between the mineral particles of the soil and the biochar particles (Lin et al., 2012 Joseph et al., 2013). Biochar in soil occurs not only as free particles, but these particles can also be connected with water-stable aggregates (Brodowski et al., 2006) but the effects on individual fractions of aggregates can differ, as indicated in study of Šimanský et al. (2016). For example, biochar (10 t ha^{-1}) applied without N fertilizer increased content of water-stable macro-aggregates (WSA_{ma}) in size fraction 5–2 mm, but at the same time decreased WSA_{ma} 0.5–0.25 mm content. Application of biochar (20 t ha^{-1}) had no remarkable influence on the content of WSA_{ma}. Adding lower amounts of biochar may thus be more beneficial for soil aggregation than higher rates of biochar addition. Secondly, aggregation effects mainly on lower size of aggregates depend on biochar particles. For example, conversion to WSA_{ma} 0.5–0.25 mm might therefore be difficult and could occur only after some time. According to Piccolo and Mbagwu (1999) hydrophobic components of organic matter contribute more to soil aggregate stability than hydrophilic components. Through, it´s highly aromatic C structure, biochar can improve aggregation by helping to bind native SOM, enhancing the resistance of soil aggregates to water and making aggregates more resistant to physical disturbance (e.g., wet-dry cycles). Biochar can also influence soil aggregation by change of the ionic composition of the soil solution. The surface of biochar particles after oxidation may by rich for hydroxyl and carboxylic groups which are able to adsorb soil particles and clays and form macro-aggregates

(Jien and Wang, 2013) however, this process is tedious and it takes long time. Several mechanisms may be involved in the biochar-induced improvements in soil aggregation. Previous research indicates that biochar can influence soil aggregation by changes of soil pH and enhance aromaticity of soil organic C pool (Chan et al., 2008; Novak et al., 2009). Higher soil pH can increase the flocculation of clay particles (Haynes and Naidu, 1998), it facilitating the formation of water-stable aggregates (Boix-Fayos et al., 2001). A slight increase of soil pH due to biochar amendment can benefit soil aggregation (Kookana et al., 2011). Biochar can also significantly affect on soil microbial communities by providing feedstock for production of extracellular polymeric substances that there are as cementing agents for soil aggregates (Le Guillou et al., 2012). Earthworms can affect aggregate stability in soil (1) mechanical bondings between soil and biochar particles, (2) mechanism in increase of fungi which is induced of excreted casts. The fungal hyphae are considered to be a significant stabilizing factors for soil aggregates because it connects soil particles by production of polysaccharides (Rahman et al., 2017). Soil bacteria are almost activated on joining of smaller

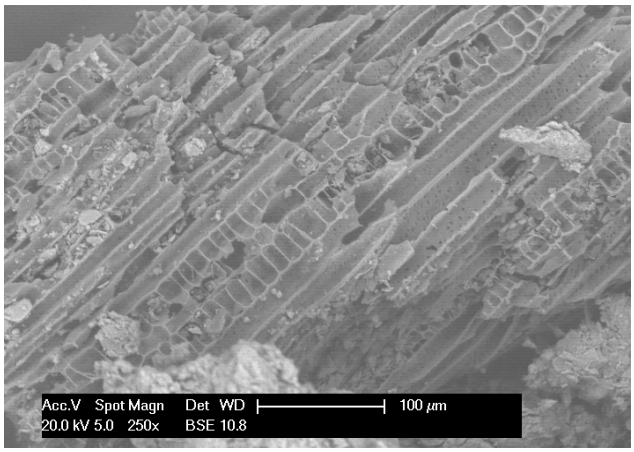

Figure 1 Porous structure and large surface area of biochar (Authors)

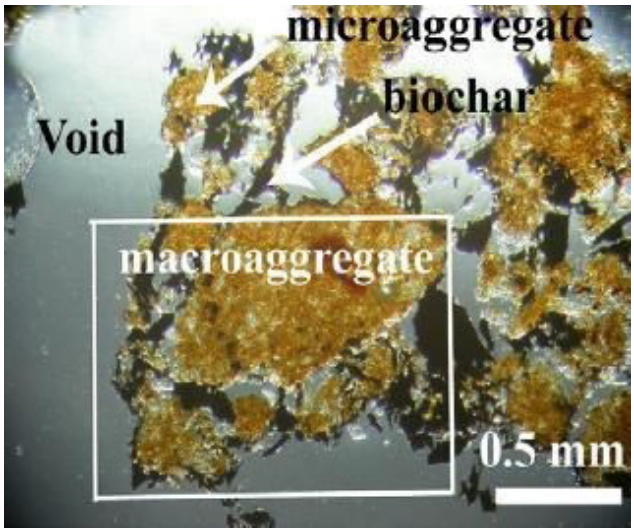

Figure 2 Biochar can join mineral particles in the soil (Jien and Wang, 2013)

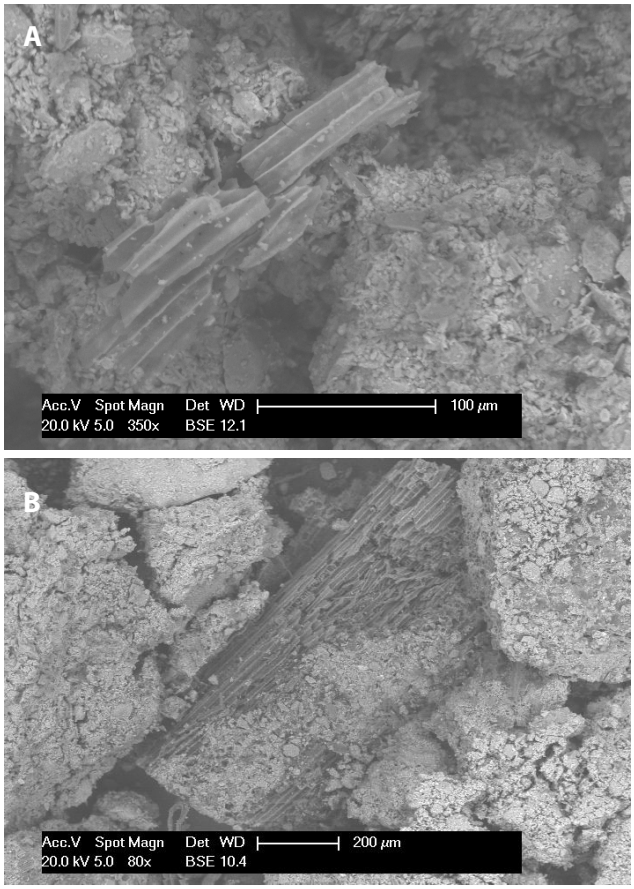

Figure 3 Biochar incorporated into soil aggregate (Authors)

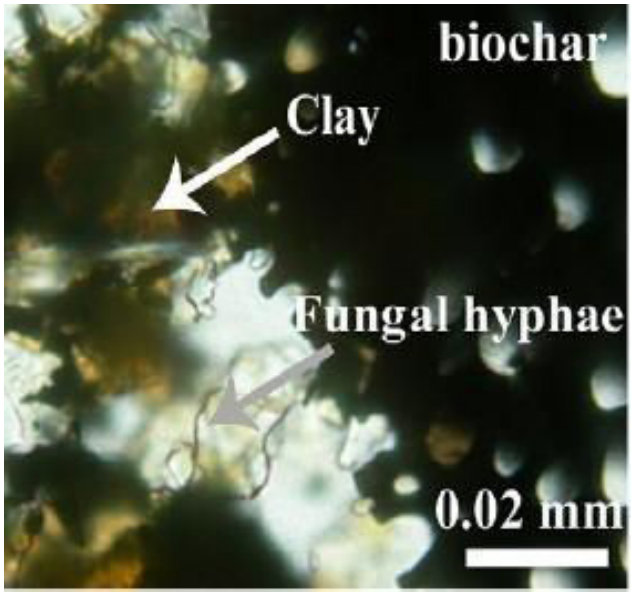

Figure 4 Fungal hyphae improve aggregation between biochar and soil particles (Jien and Wang, 2013)

particles, while soil fungi are active upon connecting to larger particles (Figure 4).

The different effects might be a result of differences in the biochar reactivity (i.e., amount of reactive functional groups) that strongly depends on production conditions and feedstock (Keiluweit et al., 2010) but also a length of time (length of contact between biochar and soil particles). Significant effect of biochar on soil aggregation is associated with applied doses of biochar and size particles of biochar (Šimanský et al., 2016).

2.2 Effect of biochar combined with other organic or mineral fertilizers on soil structure

One of the problems with the use of organic residues such as composts, manures, crop residues, which are added to soil for C sequestration is their relatively fast rate of degradation, leading to the release of carbon-dioxide, thereby becoming a source for greenhouse gas emission. Therefore, there have been increasing interests in the conversion of organic residues into biochars in order to reduce the rate of decomposition, which enhances C sequestration in soils (Kookana et al., 2011). Biochar is emerging as an attractive option to improve the efficiency of fertilizer use (Zhang et al., 2010). Biochar, due to its mostly inert nature, is often applied to soils in conjunction with organic or mineral fertilizers (Asai et al., 2009; Laird et al., 2010). The reasons for higher aggregate stability could be explained by the application of higher rates of biochar together with nitrogen fertilizer. Application of fertilizers generally improve soil aggregation (Munkholm et al., 2002). Added nitrogen to the soil can increase the processes of biochar mineralization and the result can be also a higher aggregation (Bronic and Lal, 2005; Šimanský et al., 2016). Despite beneficial properties of biochar, in most intensively managed agro-ecosystems, biochar is usually applied in combination with chemical fertilizers due to its low N, P, and K contents (Lima and Marshall 2005; Chan et al., 2007). In study of Šimanský et al. (2016), the application of N fertilizer together with biochar had a positive effect on the incorporation of biochar into the larger aggregates. In case of B20N80 treatment (20 t biochar ha^{-1} and 80 kg N ha^{-1}), the values of WSA_{ma} in the size fractions 3–2 mm (75%) and 5–3 mm (149%) were higher, while the size fraction of 0.5–0.25 mm (27%) was lower than in B20N0 (20 t biochar ha^{-1} + no nitrogen). Considerably lower content of WSA_{ma} 5–2 mm was observed in B10N80 (10 t biochar ha^{-1} and 80 kg N ha^{-1}). Adding nitrogen to the soil can improve microbial activity (Lehmann et al., 2011), increase the intensity of the biochar mineralization processes and increase CEC and active surface area (Yeboah et al., 2009), which results in higher aggregation (Bronic and Lal, 2005). The reduction of the addition of the chemical fertilizer rate may depend on the surface oxidation of biochar through changes in pH, oxidative state, or microbial community structure (Liu et al., 2011). According to Ma et al. (2015) both the NPK fertilizer + maize straw and NPK + biochar treatment significantly increased the relative proportion of macro-aggregates (>2 mm) and the mean weight diameter, and reduced the relative proportion of micro-aggregates (<0.25 mm). The higher proportion of macro-aggregates was recorded in the NPK + biochar treatment; the proportion of >2 mm aggregates was 15% higher than in the NPK + straw treatments, respectively. Compared with the NPK treatment, both the NPK + maize straw and NPK + biochar treatments significantly improved the stability of soil aggregates. When biochar is applied together with green manure as e.g. *Tithonia diversifolia*, there is likely a higher amount of microbial community and their activity (Li et al., 2012) and at the same production of metabolites which, through a variety of bonding mechanisms, can contribute to aggregate build. On the other hand, the biochar produced with the combination of wood and straw had no effect on aggregate stability (Annabi et al., 2007). The combined application of biochar and slurry might be a way to increase the biochar reactivity and, consequently, the ability to form macro-aggregates because slurry contains reactive compounds such as organic acids (Provenzano et al., 2014). The combined application of biochar and slurry led to lower aggregate yields than the solitary application of slurry. However, interactions between biochar and mineral soil particles were already found shortly after the application in both the incubations and in the field trial, leading to an increase of aggregate-occluded and thus protected soil organic carbon, almost in combination with a slurry application (Helfrich et al., 2008; Le Guillou et al., 2012).

3 Conclusion

In recent years, research activity on the use of biochars in soils has been increased and this trend is likely to continue over the next years due to the numerous potential benefits which we mentioned in this review and on the other hand risks associated with the use of biochars alone or in combination with other organic or mineral fertilizers. Due to the inherent complexity of biochar, soil properties, and cropping system, the effect of biochar on soil aggregate may be very different and the knowledge gap with respect to biochar follow from various crop residue feedstocks on dynamic of SOC and soil aggregate stability is very large. Soil aggregate stability can be changed based on biochar amendment in some cases depending on soil and biochar type but also a length of time (length of contact between biochar and soil particles) as well as applied doses of biochar and size particles of biochar. It is still unclear how combining

biochar with N fertilization affects soil structure, but the major responsible factors include particle-size distribution of studied soils application rate of biochar, time after biochar application.

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Original Paper

Energy content of hybrid *Rumex patientia* L. × *Rumex tianschanicus* A.Los. (Rumex OK 2) samples from autumn months

Michal Rolinec[1]*, Dzhamal Rakhmetov[2], Daniel Bíro[1], Miroslav Juráček[1], Milan Šimko[1], Branislav Gálik[1], Ondrej Hanušovský[1]
[1]*Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Animal Nutrition, Slovakia*
[2]*M. M. Gryshko National Botanical Garden of Ukraine National Academy of Sciences in Kyiv, Ukraine*

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Aim of this experiment was to determine the gross energy concentration of fresh, wilted and ensiled hybrid of *Rumex patientia* L. × *Rumex tianschanicus* A.Los. (Rumex OK 2). Samples were collected in autumn months of the year 2017. The plant of Rumex OK 2 consist during autumn months only from rosette of leaves. The height of leaves was in autumn months following, September 56.68 ±13.80 cm; October 59.29 ±11.93 cm and November 55.98 ±10.80 cm. Rumex OK 2 silage was made from wilted matter, with or without of addition of dried molasses. Gross energy was determined as the heat released after combustion of a sample (Leco AC 500) in MJ per kilogram of dry matter of the sample. By the autumn months the concentration of dry matter, as well as the concentration of gross energy increased, except Rumex OK 2 silage from November. The highest concentration of gross energy had wilted Rumex OK 2 from November (18.02 MJ kg^{-1} of dry matter). There was no significant effect of addition of dried molasses to wilted Rumex OK 2 before ensiling on gross energy concentration in Rumex OK 2 silages (P >0.05). Gross energy concentration of all types of analysed samples had relative high value (16.98 to 18.02 MJ kg^{-1} of dry matter). Fresh or ensiled Rumex OK 2 can be used as a part of feed ratio for ruminants or can be utilised in biogas station. However, due to the low content of dry mater in fresh or wilted material the production of silage can be in autumn months problematic.

Keywords: Rumex OK 2, silage, gross energy, dry matter

1 Introduction

Human population growth (1 billion in 1800 to 7.6 billion in 2017) together with rising living standards create growing demand of energy. Nowadays all human activities are connected with consumption of various sources of energy (mainly from fossil energy sources). Therefore the changeover to environmental friendly and a renewable energy sources is expected and appreciate. Maga et al. (2008) defined renewable energy sources as a constantly replenishing source of energy, like a solar or wind energy. Also biomass is a renewable source of energy. Biomass have been used for heat production, as well as for electricity production. Agriculture produce biomass in form of a straw (from cereals, maize or rapeseed), wood waste from fruit grove and vineyards. In Slovak republic, these biomass is insufficiently used for energy purpose (Pepich, 2006). Today is farmed only agricultural soil suitable for intensive crop growing. Soil with low production function, bad localisation or bad climatic condition is not suitable for intensive crop growing. However, this soil can be used for production of biomass. Fodder sorrel, also called "Rumex OK 2" (*Rumex patientia* L. × *Rumex tianschanicus* A.Los.) can be used as a feed as well as for biomass production. Depends on season and on processing, Rumex OK 2 provide different types of biomass: green vegetable biomass, silage and dried vegetable biomass (Rakhmetov and Rakhmetova, 2006; 2011). Dried Rumex OK 2 is produced only in summer months (Usťak, 2007). In this article we aimed to determine the energy value of different Rumex OK 2 samples collected in autumn months of the year 2017.

2 Material and methods

Rumex OK 2 (*Rumex patientia* L. x *Rumex tianschanicus* A. Los.) was used for this experiment. Plants of Rumex OK 2 were grown in experimental fields under Institute of Biodiversity Conservation and Biosafety (SUA in Nitra). Samples of fresh matter were collected in the year 2017, during months September, October and November (always around 20th day of the month). During sampling,

***Corresponding Author:** Michal Rolinec, Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Department of Animal Nutrition, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovak Republic, e-mail: michal.rolinec@uniag.sk

the height of leaves was recorded. Fresh samples were wilted for three days. Wilting was realized in indoor conditions, by the open windows and without heating. After wilting, Rumex OK 2 plants were cut to the theoretical length of cut 1.5 centimeter and ensiled. First variant (Rumex OK 2 silage) was ensiled without additives. Second variant (Rumex OK 2 silage + molasses) was ensiled with a 1.0% addition of dried molasses to wilted Rumex OK 2 matter. All samples prepared for ensiling were stored in plastic bags without air (hermetic sealed). During fermentation process, which last for five weeks, plastic bags with silage samples were stored in room without light and at 20 °C. Fresh, wilted and silage samples were prepared for dry matter and energy concentration determination. Dry matter was determined by drying at 103 ±2 °C to constant weight. Gross energy concentration was determined by Calorimeter LECO AC 500 (Leco Corporation, USA). Each sample was analysed in triplicate. Gained results were statistically processed with IBM SPSS v. 20.0. Differences of means between months within type of sample were tested by Tukey HSD test. Differences of means between silages samples (with or without an addition of dried molasses) within month were tested by independent samples *T*-test. $P < 0.05$ was considered as significant.

3 Results and discussion

Rumex OK 2 (*Rumex patientia* L. x *Rumex tianschanicus* A.Los.), which is high productive crop with interesting amount of protein has potential to be used in feed industry, as a food, as a medical herb, as well as a technical plant (Usťak, 2007; Rakhmetov and Rakhmetova, 2011; Bazhay-Zhezherun and Rakhmetov, 2014). Growth and production process of plants is affected by many factors (Hric et al., 2013). Rumex OK 2 is plant with sufficient production of biomass also in autumn months. In autumn months whole Rumex OK 2 plant consist only from different number of leaves which create rosette of leaves.

The average height of leaves from Rumex OK 2 used in this experiment was as follows: in September 56.68 ±13.80 cm; in October 59.29 ±11.93 cm; in November 55.98 ±10.80 cm. The utilization of Rumex OK 2 depends mainly of its dry matter concentration and plant morphology. As shown Table 1, fresh Rumex OK 2 samples from autumn months have very low concentration of dry matter, from 4.89 to 7.70%. After three days of wilting, concentration of dry matter increased, however wilted Rumex OK 2 samples were for direct combustion unusable. During September was unfavourable weather, a lot of rainfall and high humidity, and that was the reason of low dry matter value in fresh, as well as in wilted samples. Wilted Rumex OK 2 matter was used for ensiling. Compared to wilted Rumex OK 2 matter, silage samples contain similar amount of dry matter. In general fresh Rumex OK 2 samples contain very low amount of dry matter. Plants like Rumex OK 2 also contain low amount of dry matter. For example dock (*Rumex obtusifolius* L.) contains 11.4% of dry matter (Derrick et al., 1993); spinach (*Spinacia oleracea* L.) contains 8.40% of dry matter, rhubarb (*Rheum undulatum* L.) contains 6.41% of dry matter (Kováčiková et al., 1997). Leaves from sugar beet (*Beta vulgaris* var. *altissima* Döll) and from turnip (*Beta vulgaris* L.) used as a feed contain 16.5% and 17.0% of dry matter respectively (Petrikovič et al., 2000; Pajtáš et al., 2009; Gálik et al., 2016). Petříková (2009 and 2012) published concentration of dry matter in Rumex OK 2 samples collected between 25th of April to 26th of May with values between 8.89 to 13.01%, bud *Rumex* spp. in these months has different morphology, has besides rosette of leaves the stalk with flowers and seeds. Rumex OK 2 silage from September following the dry matter concentration in wilted matter had very low concentration of dry matter. With such a low concentration of dry matter (7.82%) is ensiling of wilted matter in farm condition unviable and with dry matter of wilted matter around 17% is problematically. Low concentration of dry matter of wilted matter causes outflow of silage effluent during fermentation (Skládanka et al., 2014). Hejduk and Doležal (2008) wilted *Rumex obtusifolius* L. for 24 hour and reached dry matter 16.84%, which is similar to our results. Suitability of Rumex OK 2 for ensiling will be considered better after evaluation of data from results of fermentative process. Petrikovič et al. (2000) published

Table 1 Energy value of different Rumex OK 2 samples from autumn months (MJ kg^{-1} of DM)

Month 2017	Fresh Rumex OK 2		Wilted Rumex OK 2		Rumex OK 2 silage		Rumex OK 2 silage + molasses		SEM
	DM	GE	DM	GE	DM	GE	DM	GE	GE
September	4.89^{a}	17.76	7.82^{a}	17.52^{a}	7.04^{a}	17.00^{a}+	7.37^{a}	16.98^{a}+	0.095
October	n.d.	n.d.	16.91^{b}	17.67^{b}	18.23^{b}	17.75$^{b\circ}$	17.23^{b}	17.53$^{b\circ}$	0.050
November	7.70^{b}	17.99	17.01^{c}	18.02^{c}	17.97^{b}	17.65$^{b\square}$	18.45^{c}	17.66$^{b\square}$	0.053

DM – dry matter concentration of sample in %; GE – gross energy concentration of sample in MJ kg^{-1} of dry matter; n. d. – non defined; SEM – value of standard error of the mean for gross energy in that month; ^{abc} – means within a column bearing different superscript differ significantly at $P < 0.05$; +$^{\circ\square}$ difference of mean values of GE between Rumex OK 2 silage and Rumex OK 2 silage + molasses were within month nonsignificant ($P > 0.05$)

concentration of dry matter in ensiled leaves from *Beta vulgaris* var. *altissima* Döll and *Beta vulgaris* L. on value 17.00% for both, which is less, bud comparable with our results from months October (18.23 and 17.23%) and November (17.97 and 18.45%).

Energy value of samples is expressed as the amount of gross energy in 100% of sample dry matter (Table 1). Highest concentration of gross energy contains wilted Rumex OK 2 from November.

The addition of 1% of dried molasses to wilted Rumex OK 2 matter before ensiling did not affect the concentration of gross energy in silages (P >0.05 for all three months). Statistical differences of means between months within type of sample (column) is shown in Table 1. Due to low concentration of dry matter of samples from September, the concentration of gross energy in samples from September was significantly lower, compared to October and November samples. *Spinacia oleracea* L. contains 8.81 MJ kg^{-1} of dry matter, *Rheum undulatum* L. contains 3.74 MJ kg^{-1} of dry matter (Kováčiková et al., 1997), which is much less than by the Rumex OK 2 fresh matter. Gross energy concentration of leaves of *Beta vulgaris* var. *altissima* Döll is 14.75 MJ kg^{-1} of dry matter, and leaves of *Beta vulgaris* L. contains 14.83 MJ kg^{-1} of dry matter. Rumex OK 2 was cultivate for high production of biomass with sufficient amount of crude protein, therefore is concentration of gross energy higher compared to spinach, rhubarb, turnip or sugar beet leaves. By months increase the concentration of gross energy in Rumex OK 2 fresh and wilted matter, similar it is with silage samples, except Rumex OK 2 silage from November (Table 1). Petříková (2011) stated, that dry matter concentration of young *Rumex* spp. plant is for ensiling low. But *Rumex* spp. plant with stalks from spring months can be ensiled together with grass with high dry matter concentration. Such a silage can be used as a feed or for biogas production. *Rumex* spp. from autumn months consist only from rosette of leaves, which mean without stalks with flowers and seeds. In this experiment, only wilted and cut Rumex OK 2 leaves were ensiled, with or without an addition of dried molasses. In the past, connection of words *Rumex* spp. and silage was only in articles describing the effect of broadleaved dock (*Rumex obtusifolius* L.) on grass silage quality (Hejduk and Doležal, 2004 and 2008); abundance of broadleaved dock (*Rumex obtusifolius* L.) in silage (Humphreys et al., 1999); as a part of silage for reindeer (Wallsten, 2003), or the survival of broadleaved dock (*Rumex obtusifolius* L.) in an unmanaged grassland (Martinkova et al., 2009) was researched. These researches were aimed on *Rumex obtusifolius* L., which is considered as one of the most troublesome weeds in intensively managed permanent grassland (Holm et al., 1977). It seems that silage only from Rumex was not neither made nor researched and if, than the information about gross energy concentration is missing. Only possible comparison can be with silage from leaves of *Beta vulgaris* var. *altissima* Döll, or leaves of *Beta vulgaris* L., which contain 14.77 and 15.00 MJ kg^{-1} of dry matter respectively (Petrikovič et al., 2000). These values of gross energy concentration are lower than those of Rumex OK 2 silages (Table 1). Compared to gross energy concentration in maize silages (18.50 to 19.18 MJ kg^{-1} of dry matter) used for bioenergy utilization published by Juráček et al. (2010) the values of gross energy determined for Rumex OK 2 samples were lower. Reason for this is that maize silage was made from maize with corn, whereas Rumex Ok 2 from autumn months used for silage production in this study did not contain seeds. As published Bíro et al. (2007) the nutritive value as well as the energy concentration of maize silage is in positive correlation to amount of corn on a plant.

4 Conclusions

Rumex OK 2 produced during the autumn months attractive amounts of biomass with low concentration of dry matter, bud with relative high concentration of gross energy in kg of dry matter. Biomass produced by Rumex OK 2 during autumn months is in farm condition hardly usable for ensiling (due to low dry matter concentration), however this fresh biomass or silage can be used as a pasture for ruminants or used as a part of feed ratio or can be utilized in biogas station.

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Original Paper

Egg production, quality parameters and sensory attributes of Japanese quails (*Coturnix japonica*) fed low crude protein diet supplemented with lysine

Taiwo Ojediran*, Sandra Azubuzor, Isiaq kolawole, Motunrayo Afolabi, Damilola Adeola
Ladoke Akintola University of Technology, Department of Animal Nutrition and Biotechnology, Ogbomoso, Nigeria

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Egg production, quality parameters and sensory attributes were assessed using two hundred and eight (208) uniform female Japanese quails aged six weeks fed low-protein diet supplemented with lysine. The birds were raised on a conventional diet before being allocated into 4 dietary groups of 4 replicates of 13 birds each in a completely randomized design in a six week feeding trial. Diet T1 had a crude protein (CP) content of 21% and lysine inclusion of 0.10% while diets T2, T3 and T4 contained 19%, 17% and 15% CP with lysine inclusion of 0.15%, 0.20% and 0.25% respectively. Birds fed T2 had significantly higher (p <0.05) egg weight. External egg parameters including egg length, egg width, eggshell thickness, eggshell percentage, egg shape index and eggshell surface area were significantly influenced (p <0.05). Internal egg quality characteristics including average yolk weight, yolk height, yolk length, yolk colour, albumen length, albumen weight and yolk index were significantly different (p <0.05). The panelist response on egg sensory properties showed that ease of eggshell peeling, taste and overall acceptability were also significantly influenced (p <0.05). Quails fed T1 and T2 compared favourably interms of egg weight, egg length, eggshell index, eggshell surface area, yolk weight, yolk height, yolk length, yolk colour, yolk index, albumen weight, egg taste and overall acceptability unlike those fed T3–T4. Quails fed T4 had the overall least egg weight, quality parameters and acceptance because they were not easily peeled and tastes unusual. Therefore, a 19% CP diet with 0.20% lysine is adequate for laying quails.

Keywords: amino acid, egg, external parameters, internal qualities, organoleptic properties

1 Introduction

Japanese quails (*Coturnix japonica*), a micro-livestock specie could be used to bridge the animal protein intake (Ani et al., 2009) among the increasing human population especially in the developing countries because of their short generation intervals, early attainment of sexual maturity, onset of lay between 5–6 weeks of age and more than 200 eggs in the first year of lay (NRC, 1991; Hemid et al., 2010). Most often than not, quails are usually reared for their eggs which is known for its high quality protein and biological value. As with other livestock species, nutrition plays an important role in the performance of quails. Diets are usually formulated to meet the NRC recommendations but the optimum least cost feed formulation and profit maximization has always been the target of livestock producers.

The protein content of a feed among other things is important but the attendant cost of protein sources has necessitated searches into alternative ways of staying competitive, decrease cost of production and still produce high quality product for consumers (Teguia and Beynen, 2004). Proteins are made up of amino acids, thus, an overall inadequate consumption of protein, a protein deficiency, caused by either one or more limiting amino acids resulted in decreases in parameters such as nitrogen retention and feed utilization in broilers (Corzo et al., 2005), while Sklan and Plavnik (2002) had earlier reported that an over-consumption of protein results in the catabolism of amino acids through deamination and excretion as uric acid which is both energetically and economically inefficient. It is essential to try to meet the requirement of the birds as closely as possible in order to maximize production and profitability.

The commercial availability of synthetic amino acids provides an avenue to decrease crude protein but has also necessitated the need for the appraisal of the limiting amino acids in low crude protein diets. Amino acids have been known to play an important role in animal performance (Ojediran et al., 2017). Thus, combining available feed ingredients in the best possible way becomes paramount because feeding birds with poor quality feed due to high cost of feeding will result in physiological imbalance and yield poor products.

***Corresponding Author:** Taiwo Ojediran, Ladoke Akintola University of Technology, Department of Animal Nutrition and Biotechnology, Ogbomoso, Nigeria. E-mail: ojedirantaiwo@gmail.com or tkojediran@lautech.edu.ng

2 Material and methods

2.1 Site of the Experiment

The experiment was carried out at the Poultry unit of the Teaching and Research Farm, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.

2.2 Experimental Birds and Management

The total of two hundred and eight (208) uniform female Japanese quails were selected (on the basis of weight and fitness) from a six week old flock. The birds were acclimatized on a conventional diet having 21% crude protein before being allocated into 4 dietary group of 4 replicate of 13 birds each. The experiment lasted for six weeks. The feed ingredients were purchased from FMG feedmill. L-Lysine HCl 99% feed grade manufactured by Ajinomoto animal nutrition group was used.

2.3 Data collection

Data were recorded on the egg production performance indices such as feed intake, number of egg produced, egg weight, hen-day-production, daily feed consumption per crate of egg produced and feed cost per crate of egg produced; external egg quality parameters includes average egg weight, length, width, thickness, shell percentage, egg shape index and shell surface area; internal egg quality parameters: average yolk weight, height, length and colour, average albumen weight, height, length and colour, average Haugh unit, yolk index, albumen index and egg shape index; sensory egg parameters: ease of peeling boiled egg, albumen, yolk colour, smell, taste, texture and overall acceptability. Egg production parameters, external and internal parameters were recorded and measured on daily basis. Eggs collected on two consecutive days are weighted (egg weight (g), yolk weight (g), albumen weight (g) and shell weight (g) using a digital sensitive scale. Yolk index was calculated as the ratio of yolk height to yolk width while the colour was scored using Roche colour fan. Haugh unit was also calculated. To get accurate recording of parameters, each egg was carefully broken and the shell was removed. The eggshell was dried using tissue paper and weighed. The internal content which includes albumen and egg yolk was carefully separated using a spoon. Eggshell thickness (mm) was measured

Table 1 Gross composition of experimental diet

Ingredients (%)	T1 (21%)	T2 (19%)	T3 (17%)	T4 (15%)
Maize	45.02	46.04	51.64	56.64
Soybean meal	11.76	9.75	7.89	5.89
Groundnut meal	23.52	19.51	15.77	12.77
Corn bran	10.00	14.95	14.90	14.85
Bone meal	3.00	3.00	3.00	3.00
Limestone	6.00	6.00	6.00	6.00
Salt	0.20	0.20	0.20	0.20
Methionine	0.15	0.15	0.15	0.15
Lysine	0.10	0.15	0.20	0.25
Premix	0.25	0.25	0.25	0.25
Total	100.00	100.00	100.00	100.00
Calculate composition				
ME (Kcal kg-1)	2680.91	2668.00	2714.00	2755.00
Crude protein	20.81	18.71	16.71	14.92
Ether extract	3.84	3.72	3.67	3.62
Crude fibre	3.56	3.73	3.56	3.40
Calcium	2.96	2.94	2.93	2.92
Available P	0.52	0.51	0.50	0.50
Lysine	0.98	0.93	0.88	0.84
Methionine	0.43	0.41	0.39	0.37
Cost/Kg (₦)	108.38	104.69	104.98	105.00

ME – metabolizable energy, P – phosphorus

from three part of the egg (top, middle, bottom) together with shell membranes at the using a micrometer screw guage.

The sensory evaluation involved 10 untrained panelists but usual egg consumers. Eggs were collected three days before each test and stored at 5 °C. The collected egg samples were placed in 150 ml of water to a egg, heated at 97 *C for 15 min after which they were transferred into a cool water and served to the panelist as coded samples. The descriptor were quantified on a 9-point hedonic scale (1 = dislike extremely, 9 = like extremely).

2.4 Experimental design and statistical analysis

All data collected were subjected to analysis of variance in a completely randomized design using SAS (2000) software package and means were separated using Duncan (Duncan, 1955) multiple range test of the same package.

3 Results and discussion

Table 2 shows the egg production performance of layer quails fed low crude protein diet supplemented with lysine. Average egg weight was significantly influenced (p <0.05) by the dietary treatments unlike the feed intake, average egg produced, % hen day production, daily feed consumption per crate of egg and feed cost per crate of egg. Birds fed T2 had the highest egg weight; birds on the control was comparable to those fed T3 while quails on T4 had the least value. Observations on the egg production performance of layer Japanese quails fed low crude protein diet supplemented with lysine are similar to the findings of Demuner et al. (2009a, b) who evaluated the nutritional requirements of digestible lysine for Japanese quails at the laying phase. Contrary to this, Garcia et al. (2005) and Manju et al. (2015) obtained lower egg production with 16% CP which may be as result of inadequate quantity of amino acid in the diet while Bunchasak et al. (2005) also reported poor egg production, egg weight and egg mass from birds that received 14% CP. Similar to the result of this study, Murakami et al. (1993) had earlier observed a non-significant effect of dietary protein level on feed intake of Japanese quails. This is similar to the report of Costa et al. (2004) that feed intake is not necessarily controlled by protein levels in the diet, although (Dumont et al., 2017) reported an increase in feed intake among birds fed low crude protein level.

Below 17% CP and 0.20% lysine inclusion, average egg weight was below that observed for the control. Egg weight climaxed at 19% CP and 0.15% lysine and decreased linearly such that reducing the CP and fortifying with lysine did not remedy the decrease in weight. Garcia et al. (2005) recorded significant influence of dietary lysine on egg weight but Abd El-Maksoud et al. (2011) attributed the increase to increase in CP levels from 12 to 16% for layers. Although, Bamgbose and Biobaku (2003), reported that methionine influence egg production and egg size on laying hens. But from this study, low crude protein and lysine supplementation caused insignificant setback to feed intake, rate of egg production, hen day production, feed consumption per crate of egg produced and cost of feed per crate of egg produced. This may not be on the long term as explained by Khajali et al. (2008) and Alagawany and Mahrose (2014), however, lysine supplementation may have played a role.

External egg quality of layer quail fed varying level of low crude protein supplemented with lysine is shown on Table 3. The average egg weight, length, width, shell thickness, % shell, egg shape index and shell surface area

Table 2 Egg production performance of layer quails fed low crude protein diet supplemented with lysine

Parameter	T1	T2	T3	T4	SEM
TFI (kg)	1.49 ±1.29	1.53 ±0.10	1.57 ±0.26	1.42 ±1.42	0.26
ADFI (g\d)	353.88 ±30.90	364.82 ±2.45	374.46 ±6.16	337.42 ±33.88	6.28
ADFI (g\b\d)	27.22 ±2.37	28.07 ±0.19	28.80 ±0.47	25.96 ±2.60	0.48
TEP	476.00 ±23.09	458.00 ±28.87	473.00 ±40.41	439.00 ±2.31	7.21
AEP/rep	11.34 ±0.55	10.91 ±0.69	11.27 ±0.96	10.45 ±0.06	0.17
AEM (g)	10.15 ±0.18b	11.18 ±1.07a	9.42 ±0.09bc	8.87 ±0.03c	0.25
% HDP	87.18 ±4.23	83.88 ±5.29	86.63 ±7.40	80.41 ±0.42	1.32
DFC/30 (kg)	0.94 ±0.04	1.01 ±0.07	1.01 ±0.09	0.97 ±0.10	0.02
CF/30 (₦kg)	101.38 ±3.94	105.42 ±7.35	105.40 ±10.72	101.73 ±10.75	2.00

[abc] – mean in the same row not sharing a common superscript are significantly different (p <0.05); T1: 21% CP supplemented with 0.10% lysine; T2: 19% CP supplemented with 0.15% lysine; T3: 17% CP supplemented with 0.20% lysine; T4: 15% CP supplemented with 0.25% lysine; TFI: total feed intake, ADFI g/b/d: average daily feed intake gram/bird/day, TEP: total egg production, AEP: average egg production, AEM : average egg mass, % HDP: percentage hen day production; DFC/30: daily feed consumption per crate produced, CF/30: cost of feed per crate of egg produced

were significantly influenced (p <0.05) while the shell weight was not significantly different (p >0.05). A linear decrease (p <0.05) was observed in egg weight as the crude protein level of the diet decreases. The egg weight was highest in birds fed T1 while those fed T4 had the least, although birds on T1 and T2 compare favourably. This trend is similar for the egg width, egg length, egg shape index (ESI) and the shell surface area. Bawa et al. (2011) reported that a good quail egg should weigh about 9.3 g but birds fed T4 (15% CP supplemented with 0.25% lysine) fell short of this. This suggests that there is a limit to lysine supplementation. Although,Garcia et al. (2005) recorded significant influence of dietary lysine on egg weight. The decrease in the egg weight is as a result of the decrease in the dietary protein level of the diet. This shows that the weight of the egg is dependent on the protein content of the diet (Bawa et al., 2011). The statistical pattern of egg length, egg width, eggshell thickness, eggshell index and eggshell surface area suggested that egg weight may depend on these parameters. The percentage of the egg shell indicates that lysine may not influence calcium deposition or shell formation in quails. The eggs with the thickest eggshell was observed in quails fed 21% and 19% CP, although no specific reason could be suggested to this trend as dietary protein has no direct effect on egg calcification during egg formation. Alagawany et al. (2014) had similar report at 20% CP. Nevertheless, it was observed that the eggshells were thicker at the middle for the said CP. The increase in lysine level could be a contributing factor to the thinness of the eggshell according to Pinto et al. (2003) and Panda et al. (2010). The decrease in the ESI and SSA can also be attributed to the increase in the lysine level of the diet not the protein level as stated earlier. The most important quality traits of the eggshell are its strength and thickness.

Table 4 shows the internal egg quality parameters of layer quail fed varying levels of low crude protein diets supplemented with lysine. Significant differences (P< 0.05) were observed in the average yolk weight, yolk height, yolk length, yolk colour, average albumen length, yolk weight and yolk index unlike (p >0.05) the average albumen height, average Haugh unit, yolk percentage, albumen percentage and yolk:albumen ratio. Linear decreases were observed in the yolk weight from T1–T4, yolk length, average albumen length, albumen weight while quadratic responses were observed in average yolk height, yolk colour, and yolk index with birds fed T2 having the highest value. Wu et al. (2005) explained that the decrease of egg weight due to feeding 14% and 2600 kcal ME is attributable to a decrease of yolk weight, which is affected to a greater extent rather than albumen weight by the nutrients in the diets. Furthermore, the significant linear decreases in yolk weight, yolk height and yolk length; albumen length and albumen weight can be attributed to the size of the egg as shown on Table 4. Result of this study conforms with the observation of Gunawardana et al. (2008) who indicated that dietary protein had a significant effect on egg yolk colour, however, egg specific gravity decreased, when dietary energy level increased in the diet of laying chickens, which contrast Novak et al. (2008) report that albumin percentage decreased, whereas yolk percentage and colour increased when fed laying chickens on low protein

Table 3 External egg quality parameters of layer quails fed varying level of low crude protein supplemented with lysine

Parameters	T1	T2	T3	T4	SEM
Av egg wt (g)	10.59 ±0.55^{a}	10.23 ±0.20^{a}	9.69 ±0.60^{b}	9.21 ±0.35^{c}	0.12
Av Egg length (cm)	3.13 ±0.07^{a}	3.08 ±0.04^{ab}	3.00 ±0.09^{b}	3.04 ±0.09^{b}	0.02
Av Egg width (g)	2.46 ±0.04^{a}	2.43 ±0.02^{a}	2.42 ±0.06^{ab}	2.38 ±0.06^{b}	0.01
Av shell tk top	0.09 ±0.01^{a}	0.08 ±0.01^{a}	0.08 ±0.01^{a}	0.07 ±0.01^{b}	0.002
Av shell tk middle	0.09 ±0.01^{ab}	0.11 ±0.06^{a}	0.08 ±0.01^{ab}	0.07 ±0.01^{b}	0.007
Av shell tk bottom	0.09 ±0.01^{a}	0.08 ±0.01^{ab}	0.07 ±0.01^{bc}	0.07 ±0.01^{c}	0.002
Av shell tk (mm)	0.09 ±0.01^{a}	0.09 ±0.02^{a}	0.08 ±0.01^{b}	0.07 ±0.01^{b}	0.003
Av shell wt (g)	1.00 ±0.00	0.99 ±0.01	1.00 ±0.00	1.00 ±0.00	0.001
SPER (%)	10.87 ±0.48^{a}	10.36 ±0.27^{b}	9.74 ±0.65^{c}	9.47 ±0.41^{c}	0.13
ESI	3.38 ±0.12^{a}	3.32 ±0.07^{ab}	3.23 ±0.12^{b}	3.03 ±0.11^{c}	0.03
SSA	23.06 ±0.81^{a}	20.54 ±0.29^{a}	21.72 ±0.91^{b}	20.99 ±0.54^{c}	0.18

^{abc} – mean in the same row not sharing a common superscript are significantly different (p <0.05); Av: average, tk: thickness, wt: weight, SPER: eggshell percentage, ESI: egg shape index, SSA: eggshell surface area; T1 (control) = 21% crude protein diet supplemented with 0.10% lysine, T2 = 19% crude protein diet supplemented with 0.15% lysine, T3 = 17% crude protein diet supplemented with 0.20% lysine, T4 = 15% crude protein diet supplemented with 0.25% lysine, SEM = standard error mean

Table 4 Internal egg quality of layer quail fed varying level of low crude protein supplemented with lysine

Parameter	T1	T2	T3	T4	SEM
Av. yolk weight (g)	3.17 ±0.20^{a}	3.06 ±0.13^{ab}	2.85 ±0.21^{bc}	2.67 ±0.30^{c}	0.05
Av. yolk height (cm)	0.97 ±0.03^{b}	1.05 ±0.07^{a}	0.98 ±0.05^{b}	0.95 ±0.04^{b}	0.01
Av. yolk length (cm)	2.22 ±0.13^{a}	2.19 ±0.05^{ab}	2.12 ±0.08^{b}	2.12 ±0.05^{b}	0.02
Av. yolk colour	4.30 ±0.21^{ab}	4.33 ±0.21^{a}	4.130.10^{b}	4.23 ±0.17^{ab}	0.03
Av. alb length (cm)	4.83 ±0.21^{a}	4.58 ±0.36^{b}	4.05 ±0.18^{c}	3.77 ±0.13^{d}	0.09
Av. alb height (cm)	2.89 ±0.28	3.04 ±0.20	2.84 ±0.16	2.92 ±0.27	0.04
Av. alb weight (g)	5.31 ±0.30^{a}	5.13 ±0.17^{ab}	5.00 ±0.33^{b}	4.58 ±0.20^{c}	0.07
Av. Haugh unit	79.96 ±1.87	81.37 ±1.39	80.37 ±1.14	80.87 ±2.65	0.33
yolk index	0.44 ±0.04^{b}	0.48 ±0.03^{a}	0.46 ±0.04^{ab}	0.45 ±0.02^{ab}	0.01
% yolk (%)	29.97 ±2.18	29.95 ±1.43	29.51 ±2.10	29.22 ±3.44	0.41
% albumen (%)	50.19 ±0.92	50.10 ±1.41	51.64 ±2.13	49.82 ±2.50	0.34
Yolk: albumen	0.60 ±0.01	0.60 ±0.01	0.57 ±0.00	0.59 ±0.01	0.71

^{abcd} – means with superscript are significantly (p <0.05) different; T1 (control) = 21% crude protein diet supplemented with 0.10% lysine; T2 = 19% crude protein diet supplemented with 0.15% lysine; T3 = 17% crude protein diet supplemented with 0.20% lysine; T4 = 15% crude protein diet supplemented with 0.25% lysine; SEM = standard error mean; Av. = average

Table 5 Organoleptic/ sensory (egg) properties of quails fed varying levels of low crude protein supplemented with lysine

Parameters	T1	T2	T3	T4	SEM
Peeling Ease	4.38 ±2.48^{a}	3.67 ±1.86^{ab}	5.33 ±3.08^{a}	1.50 ±0.84^{b}	0.52
Albumen strenght	5.67 ±2.66	7.50 ±0.83	6.33 ±0.51	5.33 ±2.50	0.40
Yolk colour	3.33 ±0.82	3.83 ±0.75	4.00 ±1.90	3.33 ±2.07	0.29
Egg Smell	3.83 ±2.40	3.50 ±0.55	2.83 ±0.75	3.00 ±1.26	0.29
Egg Taste	6.83 ±0.75^{ab}	7.67 ±0.52^{a}	7.17 ±1.47^{ab}	6.33 ±1.03^{b}	0.22
Egg Texture	6.67 ±2.33	6.00 ±1.79	6.67 ±1.51	6.50 ±2.88	0.42
Overall	7.83 ±0.75^{ab}	8.00 ±0.63^{a}	7.50 ±1.04^{ab}	6.17 ±2.22^{b}	0.29

^{abc} – mean in the same row not sharing a common superscript are significantly different (p <0.05); T1 (control) = 21% crude protein diet supplemented with 0.10% lysine, T2 = 19% crude protein diet supplemented with 0.15% lysine, T3 = 17% crude protein diet supplemented with 0.20% lysine, T4 = 15% crude protein diet supplemented with 0.25% lysine, SEM = standard error mean

diet. The yolk colour may be attributed to the nutritional composition of the experimental diets which involve an equivalent inclusion corn amongst the treatment. Corn is rich in pigments which directly affect the colouring of yolks. Moreover, Tuleun et al. (2013) demonstrated that egg composition could be influenced at varying protein levels of 17–21% CP.

The analysis of the panelist response (Table 5) showed the organoleptic/ sensory (egg) properties of quails fed varying levels of low crude protein supplemented with lysine. The ease of eggshell peeling, taste and overall acceptability were significantly influenced (p <0.05) by the dietary treatments while the albumen strength, yolk colour, egg smell and egg texture were not significantly influenced (p >0.05). Quadratic responses were observed across the treatments with eggs from birds fed T2 and T3 compared favourably with those fed the control diet (T1). Birds fed T3 had the highest ease of peeling, followed by T1 and T2 respectively while those fed T4 had the least. The taste and overall acceptance followed similar trend with eggs from birds fed T4 having the least; Taste score from T1–T4 was 6.83, 7.67, 1.17 and 6.33 respectively while the overall acceptability score was 7.83, 8.00, 7.50 and 6.17 respectively from T1–T4. The significant (ease of peeling, taste and overall acceptability) sensory analysis shows that consumer preference was adversely influenced. Birds fed T4 was not easy to peel, the taste and acceptability was slightly liked. Ease of peeling boiled eggs is an important consumption quality because eggs easily peeled gives a smoother surface and visual appeal to consumers unlike ones with rough surface which gives and impression that they are spoilt (Shittu and Ogunjimi, 2011). It is believed that the albumen of just laid egg contains a store of dissolved carbon dioxide which exits

the egg over time through the tiny pores in the shell thus increasing the pH making the albumen less acidic causing the membrane tougher. At a lower pH, the proteins in the albumen binds tightly to the membrane during cooking but upon the alkalinity, the membrane soften leading to a looser bond.

4 Conclusions

Quails fed 21% and 19% CP with 0.10 and 0.15% lysine respectively compared favourably: feeding quails with a 19% CP diet with 0.20% lysine inclusion does not adversely affect egg weight, egg length, egg shell index, eggshell egg surface area, yolk weight, yolk height, yolk length, yolk colour, yolk index, albumen weight, egg taste and overall acceptability unlike those feed lower CP diets. Quails fed 15% CP diet with 0.25% lysine inclusion had the overall least egg weight, quality parameters and acceptance. Therefore, a 19% CP diet with 0.20% lysine is adequate for laying quails.

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