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MOLECULAR CHARACTERIZATION OF ESBL GENE IN CITROBACTER SPP AND ANTIBACTERIAL ACTIVITY OF OMEGA-3 AGAINST RESISTANT ISOLATES

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Abstract

This study aimed to investigated the prevalence and resistance pattern of different *Citrobacter* species phenotypically and genotypically to β-lactam and some most common antibiotics then evaluate the antibacterial activity of omega-3 extracted from flaxseed against isolates that harboring resistance genes. 19 *Citrobacter* isolates were isolated from100 stool and urine samples taken from patients attended to AL-Sadar Hospital during June-December 2016. Clinical samples were cultured on specific media, thereafter isolates were identified depending on morphological, biochemical characteristics and VITK-2. The results showed that the *Citrobacter* comprise 24% of isolated bacteria which divided into 11 (14.1%) were *C. freundii*, 5 (6.41%) *C. kosaeri* and *C. farmeri* were 3 (3.8%). The antagonistic activity was evaluated by observing a clear zone of inhibition growth, the results showed that all *Citrobacter* (100%) isolates were resistant to Ampicillin, cefoxitin and sensitive to Imipinim, also the isolates showed different degrees of resistant to β-lactam antibiotics initially. By confirmatory test the results observed 17/19 (89.4%) isolated were ESBL producers finally using PCR technique to detect bla– genes (blaCTX-M, OXA, SHV and Z). The results revealed that 14/17 (82.3) of potential ESBL producing *Citrobacter* were harbor one or more of ESBL genes they included 10 of *C. freundii* and 4 of *C. koseri*. The extraction of essential fatty acid semicarbazide (omega-3) from *Linum usitatissium* (Flax seed) were tested to evaluate their activity against resistant isolates, results explained broad spectrum antibacterial property of EFASC compounds against resistant bacteria. In conclusion, this study found increase prevalence of MDR *Citrobacter* spp as causative agents in clinical cases. Considering antibacterial activity of EFASC, it was observed highly activity against resistant pathogens deservedly, therefore attention must be paid to development their used as alternative antibiotics.

Key words: Citrobacter infection, ESBL-genes, omega-3 antibacterial activity

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INTRODUCTION

Citrobacter is a gram negative rods motile bacteria, one members of Enterobacteriaceae. It name derived from its ability to use citrate as a sole carbon source (JANDA et al. 1994). Infections with

Citrobacter spp. have been increasing importancy as a cause of serious nosocomial outbreaks and difficult to treated by most common antibiotic (NADA et al. 2004; SHIH et al. 1996). Locally, several studies referred to the prevalence levels of *Citrobacter* infections (AL-HASNAWI 2014; AL-HISSNAWY et al. 2012; TUWAIJ 2016). Extended spectrum B-lactam (ESBL) a members of β-lactamases enzymes hydrolyzes the β-lactams ring lead to loss of bactericidal activity of wide variety of antibiotics including third generation cephalosporins, penicillins and monobactam (HARVEY & CHAMPE 2012). The increase prevalence of ESBL producing gram negative bacteria is a significant problem in treating bacterial infection, in addition to different side effects like allergy to some antibiotic, nephrotoxicity, ototoxicity, and alteration of normal gut flora. For this reason seeking to a new alternative medicine to control pathogens with reduced side effects has become a crucial part of drug development research. On the other hand, green medicine has been used for the medication of different bacterial disease (FUAD et al. 2012).

Linum usitatissimum L. (Flaxseed) the annual plant. Its' seeds containing about 36 to 40% of oil is a rich source of the following unsaturated essential fatty acids: Omega 3 (linolenic acid), Omega 6 (linoleic acid) and oleic acid content (WANG et al. 2017). Linolenic acid and other compound of (EFAs) used as possible new agents to treat skin infections caused by *P. acnes* and *S. aureus* (DESBOIS & LAWLOR 2013). Several study confirmed the successful treatment of USFA against *S. aureus, P. aerogenosa, L. monocytogenes* (SHIN et al. 2007) semicarbazides are the raw material of semicarbazones that possess a wide spectrum of antibacterial activities (SINGHAL & PAUL 2011).

METHODOLOGICALLY BACTERIAL CHARACTERIZATION

A total of 100 clinical specimens from Stool and Urine were collected under aseptic condition. These specimens were collected from patients attending to Al-Sadar Medical City in AL-Najaf province inoculated on MacConkey agar and XLD agar (Oxoid Cambridge, UK) and incubated at 37 °C for 24 h. The morphological characteristics of the colonies including size, shape, color, were recorded, the suspected Citrobacter were relevant by biochemical test (MACFADDIN 2000), then finally confirmed by using Vitek-2 Compact (Bio Mérieux, France).

Antibiogram test: Antibiotics susceptibility was carried out on all isolate using Kirby Bauer disc diffusion method. Results were inter operated by measuring the zone of inhibition in mm. Then using results of cefotaxime, ceftazidime, ceftriaxone, and aztreonam (30 µg of each one) as initially screened to detect β-lactam resistant isolates (ESBL Production) according to (CLSI 2014). Then confirmed by the disk approximation test according to (BATCHOUN et al. 2009). Any augmentation (increase in diameter of inhibition zone) between the central Amoxiclav disk and any of the cefotaxime, ceftazidime, ceftriaxone, and aztreonam (surrounded the plate around the Amoxiclav). Disks that showing resistance or intermediate susceptibility was recorded.

Genomic DNA extraction: The cell pellets from all resistant isolates were used to extract genomic DNA by Genomic DNA Mini extraction kit (Geneaid, USA) following the manufacturer's instructions. Extracted DNA was kept in sterile eppendorf tubes and stored at -20 °C prior to PCR.

Detection of resistance genes: PCR amplification for detection the four bla genes, bla-CTX-M, bla-TEM, bla-SHV and bla-OXA were carried out according to information of Bioneer corporation, Korea) as shown in Table 1. PCR mixtures (25 µL) contained 5 µL of DNA template, 12.5 µL master mix (Promega, USA) and 1.25 µL of each primer and 5 µL of sterilized distilled water was used. PCR amplifications were performed in Agilent, USA Thermo Cycler according to condition (HASSAN et al. 2013; COLOM et al. 2003; SVÄRD 2007). PCR products were electrophoresized on 1.5% agarose gels, stained with ethidium bromide (Biobasic, Canada) and visualized by UV illumination and were photographed by a Cleaver gel documentation system (Biometer/Germany).

Tab. 1: The sequnces of synthesized oligonucleatoide

Primer name		-)	Molecular weight of mplicon (bp)	Ref.
CTX-M	**F**	CGCTGTTGTTAGGAAGTGTG	754	16
	R	GGCTGGGTGAAGTAAGTGAC		
SHV	**F**	AGGATTGACTGCCTTTTTG	392	17
	R	ATTTGCTGATTTCGCTCG		
OXA	**F**	ATATCTCTACTGTTGCATCTCC	619	18
	R	AAACCCTTCAAACCATCC		
TEM	**C**	ATCAGCAATAAACCAGC	516	18
	H	CCCCGAAGAACGTTTTC		

Plant collection: Flaxseed were obtained from seller of herbal and medicinal plants in Al-Najaf City. The plants were washed with distilled water, then air dried, powdered, and stored in refrigerator at 4 °C for further processes (AHMAD et al. 2017).

Preparation of oil: The oil of Flaxseed were extracted with hexan solvent (1:4 w:v) by continous extraction in a soxhlet apparatus (Preciso, England) for 12 hours. Then isolation of EFA from oil using cleavenger (Shepreth, England) according to (AHMAD et al. 2017). Purity and Identification of EFA-omega3 compounds by TLC was carried out according to (HARBORNE 1984).

Preparation of EFA – Semicarbazide (EFASC): One gram of EFA (omega-3) were dissolved in 4 ml of methanol and 1:1 H2SO4, then 4gm of thiosemicarbazide in methanol were added to this solution with constant stirring at room temperature for 4 hr and then added NH4OH till alkaline stir for about 15 min and kept it overnight. Crystals was filtered, dried and recrystallized (BORHADE 2014). Determination of antibacterial activity Agar well diffusion method was used to determine the antibacterial activity of EFASC of Flaxseed compounds according to (EGHAREVBA et al. 2010).

Statistical analysis: Analysis of data was performed by using Statistical Package for Social Science (SPSS) system/ version 17 and Microsoft Office Excel 2007. Results expressed as mean ±S.D. P-value was considered significant when it is less than 0.05. The analysis of variance (ANOVA) were used.

RESULTS

Identification of Citrobacter

Nineteen of bacterial isolated were identified as *Citrobacter* spp from 78 positive bacterial growth on MacConkey agar recovered from the 100 clinical specimens collected with a frequency (24.3%). The isolates were represented by 11 (14.1%) isolates of *C. freuendii*, 5 (6.41%) *C. kosori* and 3 isolates (3.8%) were identify as *C. farmeri* (Fig. 1), while 59 isolates (75.65%) showed growth of other gram negative bacteria which included *Klebsiella* species, *E. coli*, *Pseudomonase* and *Proteus* species.

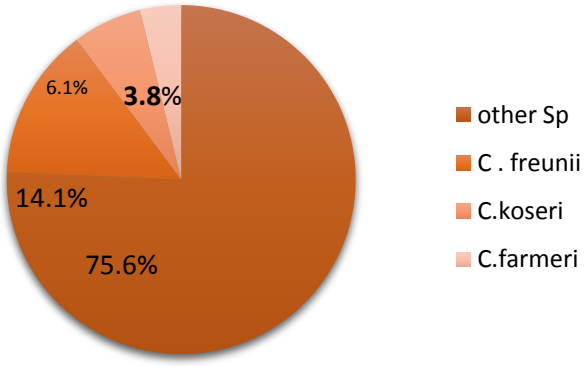

Fig. 1: Pie chart showing the distribution of *Citrobacter* spp.

Antibiogram test

The results of antibiogram tests for all *Citrobacter* species to 11 antibiotics were summarized in Figure 2. The results revealed that all isolates were multidrug resistant and all of them were 100% resistance to Ampicillin and Cefoxitin, while 100% sensitive to Imipenem antibiotic. Among the third-generation cephalosporins tested, *C. freundii* appear highly resistance against amox-clave and cefotaxime in 90.1% of isolates, while resistance to ceftazidime and cefriaxon was recorded in 81.8% of isolates, and various levels of resistance was observed towards oxacillin 72.7%, Ciprofloxacin 54.5% and Gentamycin 63.6%. The resistance pattern of *C. koseri* and *C. farmeri* were shown in Figure 2.

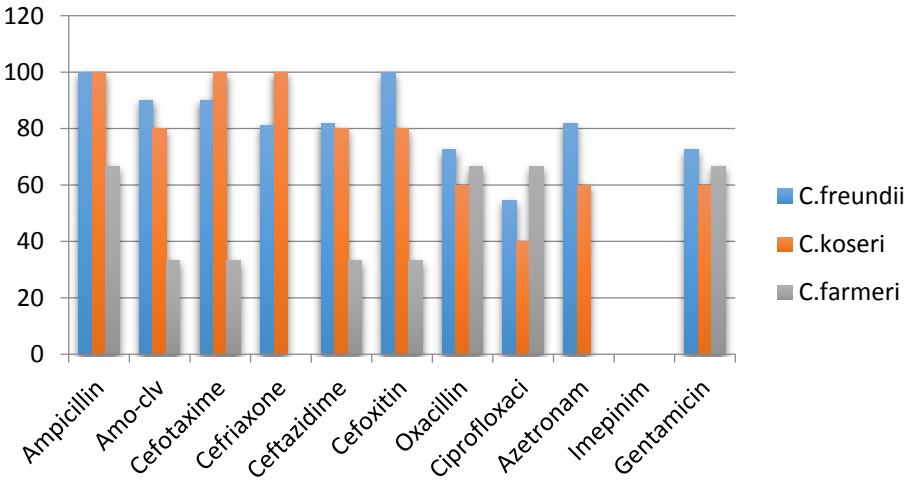

Fig. 2: Antibiotic resistance pattern of *Citrobacter* spp.

Initially, Confirmatory and Detection of resistance genes

As shown in Table 2 the results of initially and confirmatory test revealed that all *Citrobacter* isolates were gave a potential ESBL-producers initially, while the confirmed results showed that only 17/19 (89.4%) of the isolates were ESBL producers. The results for detection bla – genes (blaCTX-M , bla-TEM, bla-SHV and bla-OXA) by PCR revealed that 14/17 of potential ESBL producing *Citrobacter* were carried at least one of ESBL genes they included 10 of *C. freundii* and 4 of *C. koseri*. The results illustrated that 12 isolates were contained only one type of ESBL-genes as following 5 bla-CTX-M, 3 bla-TEM, 2 bla-SHV and 2 bla-OXA genes. While, 2 isolates of *C. freundii* had the combination of two genes: one blaSHV genes combination with bla-CTX-M genes and one bla-TEM genes combination with blaCTX-M genes Table 2 and Figure 3.

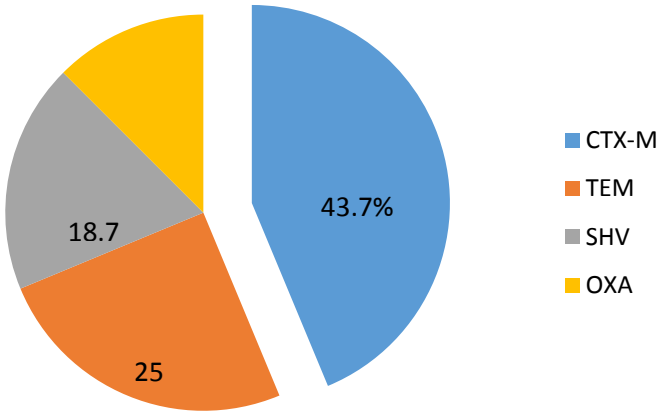

Fig. 3: ESBL-gene distribution in *Citrobacter* spp

Tab. 2: The frequency of phenotypic, genotypic to antibiotic resistance in *Citrobacter* spp.

Name & No. of isolate	Phenotypic		Genotypic			
	Initial	Confirmatory	CTX-M	TEM	SHV	OXA
C. f. 1			-		-	-
C. f. 2			-		-	-
C. f. 3				-		-
C. f. 4					-	-
C. f. 5				-	-	-
C. f. 6			-	-	-	
C. f. 7			-	-	-	
C. f. 8			-	-		-
C. f. 9				-	-	-
C. f. 10				-	-	-
C. f. 11			-	-	-	-
C. k. 1			-	-		-
C. k. 2			-		-	-
C. k. 3				-	-	-
C. k. 4				-	-	-
C. k. 5			-	-	-	-
C. far. 1			-	-	-	-
C. far. 2		-	-	-	-	-
C. far. 3		-	-	-	-	-
Total	**19**	**17**	**7**	**4**	**3**	**2**

Frequency of ESBL-genes in *Citrobacter* spp

The results of detection ESBL-gene distribution revealed that CTX-M β-lactamase was the most prevalent (43.75%) among the ESBL producing isolates; followed by TEM -β-lactamase (25%) and SHV β-lactamases were (18.75%) while, OXA β-lactamase gave (12.5%) Figure (3, 4 A, B, C, D).

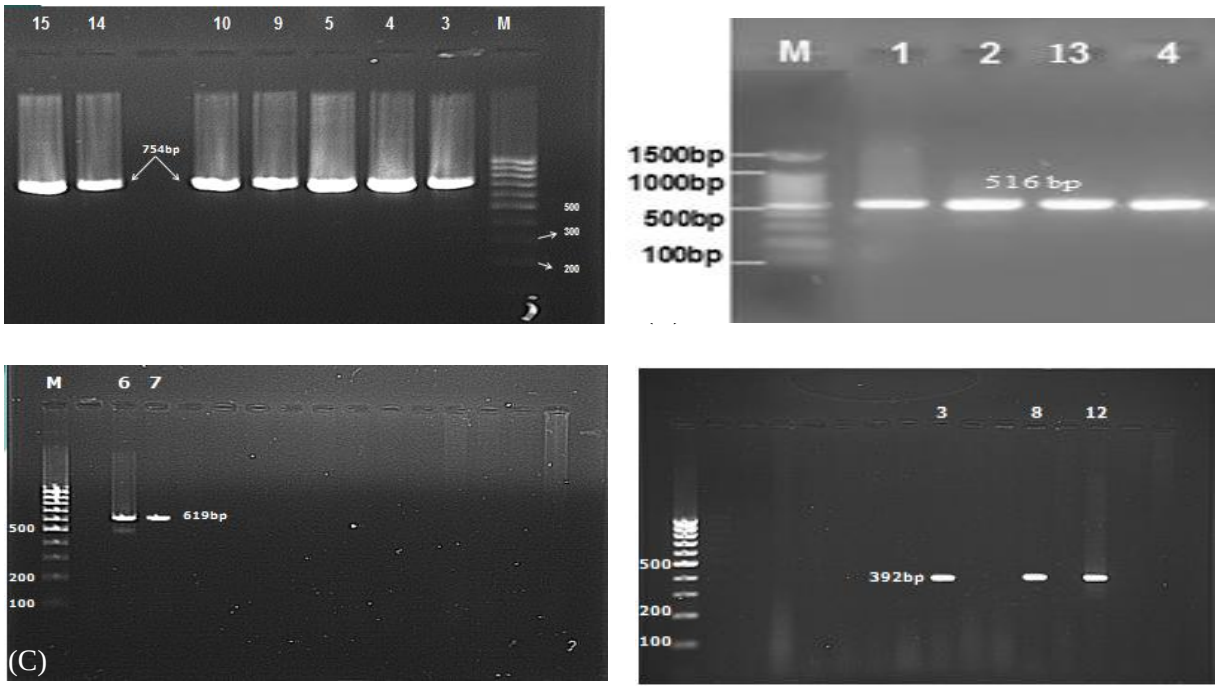

Fig. 4: Ethidium bromide stained agarose gel (1.5% agrose gel, 75 V, 1.25 hours) showing PCR amplification products with (A) CTX-M gene. Lane L: Ladder (100-1517 bp). Lane (3, 4, 5, 9, 10) C.f. No. 3, 4, 5, 9, 10 respectively. Lane (14, 15) C. koseri No. 3, 4. (B) TEM gene. Lane (1, 2, 4) C. f. No. 1, 2, 4. Lane (13) *C. koseri* No. 2. (C) OXA gene. Lane (6, 7) C. f. No. 6, 7 (D) SHV gene. Lane (3, 8) C. f. No 3, 8, respectively. Lane (12) *C. koseri* No. 1

Thin Layer Chromatography

The analysis of TLC chromatography of EFASC –omega3 as show in Table 3 revealed the presence of light brown spot on day light and light green by using UV light deeper after spraying with iodine spray, has R*f* equal to 0.37 that similar to the stander (EFASC-Omega3) which has R*f* value 0.37 appear as dark brown at day light and dark green under UV-light.

Tab. 3: Thin Layer Chromatography Essential Fatty Acid Semicarbazide of flaxseed (oil) compounds

Properties	EFA (Omega 3)	EFASC
Rf	0.37	0.37
Color by day-light	Dark Brown	Light Brown
Color under UV-Light	Dark Green	Light Green

Evaluation the antibacterial activity of EFASC against resistant isolates

The result of antibacterial activity illustrated that highest inhibition zone of extracts in 500mg/ml concentration was demonstrated on the growth of *C. koseri* 31 ± 0.93 and *C. freundii* 29 ± 0.93. The results revealed highly effect with significant different in concentrations (62.5, 125, 250 and 500 mg/ml) of EFASC against isolates as explain in Table 4.

Tab. 4: Antibacterial activity of EFA SC against *Citrobacter* spp isolates

Conc. of EFASC	*C. freundii*	*C. koseri*
500 mg/ml	29 ± 0.93	31 ± 0.93
250 mg/ml	27 ± 1.20	28 ± 1.20
125 mg/ml	22 ± 0.79	24 ± 0.99
62.5 mg/ml	20 ± 0.75	22 ± 0.75
Cefreaxone	19 ± 0.5	18 ± 0.48

DISCUSSION

Citrobacter spp was gram negative colonies appear pink small convex on MacConkey agar and yellow, smooth, flat and round on XLD agar. Regarding to biochemical tests, all the 19 isolates of *Citrobacter spp* were lactose fermenting, motile and given positive test for catalase, methyl-red, citrate, and negative results for Indole (except *C. koseri*), oxidase, Voges-Proskuaer, also have ability to ferment glucose on kliglerś iron agar gave (A/A). The results demonstrate with ID message confidence level excellent by VITEK-2 compact system.

This study revealed that *C. freundii* was the most infectional agents recovered from different clinical specimens. In the same line, (AL-MUSLEMAWI TH. A. 2007) founded that *C. freundii* is the most common pathogen in frequency 6.6% of diarreatic patients, 2% of UTI patient , and 2% from wound followed by *C. farmeri*. This result online with study done by (SALIH et al.2016) state that *Citrobacter* spp isolated from UTI with 6% percent. According to (STEWART et al. 2017) they recognized that *C. koseri* were causative agents of UTI while (WARREN et al. 2000) isolated *C. farmeri* from UTI and wound infection.

Antimicrobial resistance is a major clinical problem on treating bacterial infection worldwide. However, Most of these isolates are considered multidrug resistant this results agree with study done locally by (TUWAIJ 2016) who found *C. freundii* isolates were 100% resistance to cefoxitin and revealed varying degree of resistance to ceftazidime, aztreonam, ciprofloxacin and gentamicin. The results were not different widely from the results of (HASSAN et al. 2014) they isolate *C. freundii* from UTI and founded that all *Citrobacter* isolates were resistance to cefotaxime and ceftriaxone and considered as MDR-bacteria. AL-MUSLEMAWI (2007) who observed that *C. freundii* were 100%

resistance to β-lactam antibiotics while (METRI et al. 2013) They founded that *C. koseri* were predominant urine pathogen and recorded high rate of resistant to Cefexime, Amox-cla and Cephaloxin. Some *Citrobacter* isolate contain chromosomally mediated β–lactamases like Cephalosporinase and Penicillinase that lead to emergence of drug resistance and treatment failure despite initial susceptibility (SAMI et al. 2017). The resistance to Cephalosporins may be due to the phenomena minimizing membrane permeability based on membrane proteins purine (Porin-mediated permeability) this processes have a great impact of resistance to Cephalosporins (DANCER 2001).

In this study only 17 (89.4%) of the isolates were ESBL producers (Table 2). The rates of resistance to Cephalosporins and monobactam might be as markers for the production of ESBL by these isolates which may be by producing the common group of class A β-lactamases, consisting of TEM, SHV and CTX-M β-lactamases that has extended hydrolytic spectrum activity on Cephalosporins (BUSH et al. 1995).

Ten of *C. freundii* and 4 of *C. koseri* were carried at least one of ESBL genes they included 12 isolates were contained only one type of ESBL-genes. While, 2 isolates had the combination of two genes. This finding in accordance with 27 they reported that *C. freundii* were ESBL-producers and they founded that isolates possess CTX_M 1 and 2 genes. Also, AL-HASNAWI (2014) who revealed that *C. freundii* were ESBL-producers and possess 100 % CTX-M gene. The prevalence of bacteria which produced more than one type of ESBL enzymes is considered more dangerous for human hygiene (ERLANDSSON 2007).

This study show CTX-M β-lactamase was the most prevalent (43.75%) among the ESBL producing isolates Figure 3. Several studies improved this results such as (AL-MUHANNAK 2010) in Najaf, who found that CTX-M β-lactamase was the most prevalent (38.7%) among the ESBL producing G-ve isolates; followed by SHV (33.9%); while, TEM and OXA β-lactamases were the less (27.4% for each). Also, SHAHID (2010) who noticed in all the *Citrobacter spp* harboring *bla* genes and the prevalence of these genes as the following blaCTX-M, blaTEM, blaSHV, and bla ampC, respectively. Finally, PERILLI et al. (2005) revealed that *C.koseri* isolated from UTI patients were multidrug resistance and harporing TEM, SHV–ESBL genes. SHV β-lactamases enzymes are mainly found in G-ve bacteria (HUANG et al. 2004).

The analysis of TLC revealed the presence of brown spot on thin layer chromatography in the same local with standard (EFA omega 3) and both of them gave Rf = 0.37 these results in accordance with PANDYA et al. (2013) who explained that EFA – omega 3 by use hexan solvent gave Rf value 0.34 and with AHMAD et al. (2017) who founded that Rf value = 0.36.

The fourteen isolates that revealed β-lactam resistance antibiotics were choosen to examine the impact of EFASC extracts. The result of antibacterial activity illustrated that highly effect of different concentrations (62.5, 125, 250 and 500 mg/ml) of EFASC which illustrated the susceptibility of resistance isolate to EFASC.

The inhibition zone of EFASC of flaxseed was **29 ± 0.93** mm against *C. freundii* and **31 ± 0.93** mm against *C. koseri* in concentration 500 mg/ml, Table 4. In general 12 of 14 (85.7%) resistant bacteria were inhibit their growth by using different concentration of EFASC. Results of AHMAD et al. (2017) also show that EFASC of seed oil possess good antibacterial activity against nosocomial infection bacteria. In the same line (BORHADE 2014) indicated the strong effect of EFASC against *E. coli* and *S. aureus* at varied level and compatible with study by(SEIDEL & TAYLOR (2004) they found that the antibacterial action of fatty acids is usually attributed as being a property of the long-chain unsaturated fatty acids, including oleic acid, linoleic acid, and linolenic acid. MOGENSEN (2009) mention that polyunsaturated essential fatty acid play role in inhibition the growth of bacteria that containing a penicillinase plasmid. In this regard (IBARGUREN et al.2014) explain that fatty acid modulate the fluid

permeability of cell membranes which can greatly affect membrane property.

Therefore EFASC consider useful approach in treatment wide range of antibiotic resistant bacteria because they are safe and dependable with less harmful than antibiotic which more cost, have side effect and most bacteria became resist against it.

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APPLICATION OF FUZZY RAYLEIGH DISTRIBUTION IN THE NONISOTHERMAL PYROLYSIS OF LOOSE BIOMASS

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Abstract

This paper studied the implementation of fuzzy logic on the relevant parameter of biomass pyrolysis. Frequency factor, the upper limit of 'dE', and the scale parameter of Rayleigh distribution are fuzzified in order to estimate the randomness in estimating the parametric values. Distribution function, $f(E)$, of activation energies is assumed to follow the Rayleigh distribution. Thermo-analytical data has been found experimentally with the help of TGA/DTG analysis. The approximated solution of distributed activation energy model (DAEM) is obtained by using asymptotic approach.

Key words: Fuzzy number, Rayleigh distribution, distributed activation energy model, kinetic parameters, biomass pyrolysis

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INTRODUCTION

One of the primal tenets of modern science is that a phenomenon cannot be claimed to be well understood until it can be characterized in quantitative terms. Considering the same perspective, the constituting elements of the core scientific knowledge may be regarded as a reservoir of concepts and techniques which can be drawn upon to construct mathematical models of various types of systems and thereby yield quantitative information concerning their behaviour.

When dealing with a mathematical model, one has to pay special attention towards imprecision in data. Zadeh's (ZADEH 1978) principle of incompatibility stated that when the complexity of a system increases, our aptitude to formulate precision and meaningful statements decreases up to a threshold beyond which precision and significance became mutually exclusive characteristics. The real question is whether replacing stubbornly imprecise data by fixed ones will influence our investigation or not. If you replace arbitrarily imprecise data by the fixed values in a model, you will leave no other chance to

the model but to churn out meaningless outcomes sometime. False certainty is a wrong practice in field of science and it can be untoward if it stunts articulation of critical choices. Although the probability theory manifests the model decision making imprecision (TSOUKALAS & UHRIG 1997), yet there are various qualitative aspects of indeterminacy which are not under purview of probabilistic tools. Considering the same fact, fuzzy concept came into existence which has been implemented in many engineering problem to tackle the shortcomings of probabilistic theory.

Based on the nature of fuzzy human thinking, Lofti Zadeh propounded the "fuzzy logic" or "fuzzy set theory" in 1965. In fuzzy set theory based on fuzzy logic a particular parametric value has a degree of membership in a given set that may be anywhere in the range of 0 (completely not in the set) to 1 (completely in the set) (KERF 1975). For the same reason fuzzy logic is often defines as multi valued logic (0 to 1), compared to bi-valued Boolean logic.

In this article we propose to study fuzzification of the relevant parameters of biomass pyrolysis by using fuzzy Rayleigh distribution. The asymptotic approximation is used to estimate the kinetic parameters with the help of distributed activation energy model (DAEM). It is to be noted that the results of this paper can be used to measure randomness in the thermo-analytical data and to estimate the realistic values of parameters which in turn are used to know the kinetic mechanism of biomass.

MATERIAL AND METHODS

Distributed Activation Energy Model

The Distributed Activation Energy Model (DAEM) or Multiple Reaction Model (MRM) assumes that many decomposition reactions take place. It can also be comprehended as a summation of an unlimited number of parallel single step decomposition reactions, where each reaction has the following form:

$$\frac{dv_i}{dt} = A_{0i} \exp\left(-\frac{E_i}{RT}\right)(v^*{}_i - v_i) \quad (1)$$

where subscript i means one of several constituents, v_i is the total release mass of i^{th} constituent, t is time, A_{0i} is the frequency factor, E_i is the activation energy, R is the gas constant and T is the absolute temperature.

If the number of decomposition reactions involve is numerous, it can be assumed that activation energies of these reactions are distributed, and the reactions can be expressed as a function of the activation energy.

$$dv^* = v^* f(E)\, dE \quad (2)$$

The right-hand side of equation (2) expresses the fraction of maximum mass loss v^* in the given interval of activation energy. Usually, $f(E)$ is taken to be a Gaussian distribution. However, with the selection of an appropriate distribution function for the molecular activation energies, it is advantageous to consider the Rayleigh distribution function over symmetric one, i.e. Gaussian. More, the Rayleigh is mathematically flexible.

DAEM equation for nonisothermal time dependent temperature regime can be derived by combination of equations (1) and (2) using the Rayleigh distribution and it is given as:

$$v = \int_0^\infty \left[exp\left(\int_{T_0}^{T} \frac{-A}{\theta} exp\left(\frac{-E}{RT}\right) dT\right)\right] f(E) dE \quad (3)$$

where θ (°C/min) is heating rate, E is activation energy (kJmol^{-1}) and A is frequency factor (s^{-1}). Constant value of the frequency factor (A) for every decomposition reaction with various activation energies is assumed (CAI & LIU 2008). The value of the frequency factor can also be expressed as a function of activation energy or temperature.

Let $f(E)$ is chosen to be Rayleigh Distribution with mean E_0 and variance σ and expressed as

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$$f(E) = \frac{E}{\beta^2}\, exp\left(\frac{-E^2}{2\beta^2}\right) \quad (4)$$

Mean and the variance of Rayleigh distribution can be expressed as

$$E_0 = \beta\sqrt{\frac{\pi}{2}} \qquad \sigma^2 = \left(\frac{4-\pi}{2}\right)\beta^2$$

Asymptotic expansion

One of the main problems with solution of the equation (3) is the evaluation of the double integral, since it requires the large computing resources, especially when it needs to evaluate many times. In the previous approximations to the solution of equation (3), especially those seeking to convert the DAEM back into an equivalent SFOR (Single first order reaction), there is complication in extrapolating to other heating regimes and hence arise the problem in finding the volatile distribution *f*(*E*) from the thermo-analytical data. The relationship between the DAEM and the SFOR model has been explored by Niksa and Lau, which is based on approach of holding the activation energy fixed, and defining an effective or nominal rate constant <A> (NIKSA & LAU 1993).

The characteristic of rapidly-varying double exponential function (DExp) is defined by a piece-wise linear function, which has three distinct regions: DExp is zero, DExp is equal to unity, and DExp rises linearly from zero to one.

Approximation to the double exponential is, represented as

$$DExp = exp\left(-A\int_0^t e^{-\frac{E}{RT}}\, dt\right) \quad (5)$$

In order to carry out the stepwise simplification of this integrand, it is very important to consider the typical values of the parameters and functions on which it depends.

The frequency factors or pre exponential term is mainly in the range of $A{\sim}10^{10} - 10^{13}s^{-1}$, whereas the activation energies of interest are in the range 100- 300 kJ/mol. The typical values of temperatures depend on the particular experiments, but 1000-2000 K are usually used. To demonstrate the simplification method, the temperature is considered to ramp linearly with time (*t*) can be expressed as $T = \theta t$. Then DExp becomes,

$DExp = exp\left(-\int_0^t Ae^{-\frac{E}{R\theta u}}\, du\right)$. where θ(°C/min) is heating rate.

The interal in the exponent can be approximated by using the Laplace approach where the parameter $\frac{E}{R\theta t}$ is assume to be large, hence the major contribution from the integral is when *u* is near *t*, or the temperature is near its maximum. This provides the well-known asymptotic approximation to DExp function:

$$DExp = exp\left(-\int_0^t Ae^{-\frac{E}{R\theta u}}\, du\right) \sim exp\left(\frac{-AR\theta t^2}{E}\right)\ as\ \frac{E}{R\theta t} \to \infty \quad (6)$$

or

Approximation of equation (5) can also be expressed in the form

$$Dexp = exp\left(-exp\left(\frac{E_s - E}{E_w}\right)\right),$$

where the function varies from zero to one as *E* increases, over a range of step size E_w around the central value E_s.

Let $h(E) = \frac{E_s - E}{E_w}$ then equation (5) can be written as

$Dexp = exp\left(-exp\big(h(E)\big)\right)$, where $h(E) = \frac{-E}{R\theta t} + ln\left(\frac{AR\theta t^2}{E}\right)$.

The behaviour of E_s is of interest, so *h*(*E*) is expanded by using Taylor series

$h(E) \sim h(E_s) + (E - E_s)h'(E_s) + \cdots$

Now $h(E)$, E_s and E_w are chosen in such a manner, that

$h(E_s) = 0$ and $h(E_s) = \frac{-1}{E_w}$.

After solving, we have

$E_s = R\theta tY(At)$ and $E_w = \frac{R\theta tE_s}{R\theta t + E_s}$

where $Y(x)$ represents Lambert W function and defined to be one of roots of the equation.

As it has been observed that DExp varies like a smooth step-function, which rises rapidly for large values of tA from zero to one in a range of activation energies of step size width E_w around the value $E=E_s$, where both E_s and E_w are function of time.

There are two different limits, which are applied to the distribution function $f(E)$. One which is relatively wide initial distribution as compared with the width of DExp, known as the wide distribution. If the initial distribution function is relatively narrow as compared with the width of DExp, defines the narrow initial distribution. The significance of the distribution type defines the shape of the total integrand, which changes with time and applied limit. When the initial distribution is relatively wide compared to E_w, the total integrand initially behaves similar to the distribution $f(E)$. As time proceeds, it is truncated from the left by the step- like DExp. Moreover, the location of the maximum of the total integrand varies significantly and hence the shape becomes quite skewed.

In order to apply the approach, we considered the Rayleigh distribution, centered at E_0 with standard deviation σ.

Approximations are found to the integral $v = \int_0^\infty \frac{E}{\beta^2} exp\big(g(E)\big)dE,$

where $g(E) = -exp\left(\frac{E_s - E}{E_w}\right) - \left(\frac{E}{\beta}\right)^2$ and E_s and E_w are functions of time (t).

Energy is now rescaled by $y = \frac{E}{E_0}$. So the problem becomes

$$v = \frac{\pi}{2}\left(\int_0^\infty y\, exp\left(-exp\left(\frac{y_s - y}{y_w}\right) - \frac{\pi}{2}y^2\right)dy\right) \quad (7)$$

and $$g(y) = -exp\left(\frac{y_s - y}{y_w}\right) - \frac{\pi}{2}y^2 \quad (8)$$

For linear ramp temperature $T = \theta t$,

$y_s = \frac{R\theta tY(At)}{E_0}$, $y_w = \frac{y_s}{1 + Y(At)}$

In the wide distribution, the initial distribution is much wider than DExp. To tackle this, the limit $\sigma y_w \ll 1$ is considered. In this limit, DExp jumps from zero to one near $y = y_s$, as given previously (HOWARD 1981; PITT 1962; SUUBERG 1983; VAND 1943) and been approximated by the step-function.

$$H(y - y_s) = \begin{cases} 1, & y \geq y_s \\ 0, & y < y_s \end{cases}$$

The equation (10) is rewritten in the form

$$v = \frac{\pi}{2}\int_0^\infty y\left[exp\left(\left(-exp\left(\frac{y_s - y}{y_w}\right)\right)\right) - H(y - y_s)\right]exp\left(-\frac{\pi}{2}y^2\right)dy + \frac{\pi}{2}\int_{y_s}^\infty yexp\left(-\frac{\pi}{2}y^2\right)dy$$

or $$v = \frac{\pi}{2}\int_0^\infty y\left[exp\left(\left(-exp\left(\frac{y_s - y}{y_w}\right)\right)\right) - H(y - y_s)\right]exp\left(-\frac{\pi}{2}y^2\right)dy + \big(1 - C(y_s)\big)$$

where $C(y_s) = 1 - \exp\left(-\frac{\pi}{2}{y_s}^2\right)$

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Note that in the first integral, the integrand is the initital distribution multiplied by function which is very small everywhere but in a neighbourhood of size y_w around the point $y=y_s$. Therefore, this integrand can be approximated by expanding the initial distribution

Expand the term $D(y) = y\left(exp\left(-\frac{\pi}{2}y^2\right)\right)$ with the help of Taylor series about $y = y_s$,

$D(y)\sim \mathrm{D}(y_s) + (y - y_s)D'(y_s) + \frac{(y-y_s)^2}{2!}D''(y_s) + \frac{(y-y_s)^3}{3!}D'''(y_s)$ or

$D(y)\sim y_s\left(exp\left(-\frac{\pi}{2}{y_s}^2\right)\right)\left(1 - \frac{(y-y_s)}{y_s}(\pi {y_s}^2 - 1) + \pi\frac{(y-y_s)^2}{2!}(\pi {y_s}^2 - 3) - \frac{(y-y_s)^3}{y_s 3!}\pi((\pi {y_s}^2)^2 - 6\pi {y_s}^2 + 3)\right)$

Substituting $x = \frac{y-y_s}{y_w}$, we get

$v = \frac{\pi}{2}\int_0^\infty y_w\left[exp\left(\left(-exp(-x)\right)\right) - H(x)\right]y_s\left(exp\left(-\frac{\pi}{2}{y_s}^2\right)\right)\left(1 - \frac{y_w}{y_s}x(\pi {y_s}^2 - 1) + \pi\frac{{y_w}^2}{2!}x^2(\pi {y_s}^2 - 3) - \frac{{y_w}^3x^3}{3!y_s}\pi((\pi {y_s}^2)^2 - 6\pi {y_s}^2 + 3) + \cdots\right)dx + \left(1 - C(y_s)\right)$

Each of the integrals arising from a term in the Taylor series can be integrated separately to get the result for first order reaction (n=1) as

$v\sim\frac{\pi}{2}\left(exp\left(-\frac{\pi}{2}{y_s}^2\right)\right)\left(y_w y_s M_0 - {y_w}^2 M_1(\pi {y_s}^2 - 1) + \pi\frac{{y_w}^3 y_s}{2}M_2(\pi {y_s}^2 - 3) - \frac{{y_w}^4}{6}\pi M_3((\pi {y_s}^2)^2 - 6\pi {y_s}^2 + 3) + \cdots\right) + \left(1 - C(y_s)\right)$ (9)

The values of coefficient M_n need only to be evaluated once, since they are independent of any parameters. The first few values are

$M_0 \approx -0.5772, M_1 \approx -0.98906, M_2 \approx -1.81496, M_3 \approx -5.89037.$

The values of remaining coefficients is evaluated by integral

$M_n \equiv \int_{-\infty}^{\infty} x^i\left(e^{-e^{-x}} - H(x)\right)$

Fuzzy sets

Primarily, the fuzzy set theory was introduced by Zadeh in 1965 (ZADEH 1965), which is based on the assumption that the membership degree is equal to one minus non membership degree. In real life situation an object may or may not be member of set A to a certain degree. In other words, some hesitation about the degree of belongingness may exist. The idea of fuzzy set is in tune with human representation of reality that is more nuances than clear cut. Some philosophical related issues ranging from ontological level to application level via epistemological level may be found elsewhere (ZADEH 1978).

In a fuzzy set, the membership degree of an element is expressed by any real number from 0 to 1 rather than the limiting extremes. More formally, a fuzzy set of a set $A \neq \phi$ is characterized by a membership function

ξ :A→[0,1].

Membership and non-membership functions

Now we will consider that the activation energy, frequency factor and scale parameter (β) are not clearly defined and are fuzzfied, so we will replace them with trapezoidal fuzzy number as

$\tilde{E} = \{a_1 a_2 a_3 a_4 {a_1}' {a_2} {a_3} {a_4}'\}$

$\tilde{A} = \{b_1 b_2 b_3 b_4 {b_1}' {b_2} {b_3} {b_4}'\}$

$\tilde{\beta} = \{c_1 c_2 c_3 c_4 {c_1}' {c_2} {c_3} {c_4}'\}$

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and define its membership $\xi_{\tilde{E}}, \xi_{\tilde{A}}, \xi_{\tilde{\beta}}$ and non-membership $\phi_{\tilde{E}}$, $\phi_{\tilde{A}}, \phi_{\tilde{\beta}}$ in the following manner:

$$\xi_{\tilde{E}} = \begin{cases} \dfrac{E-a_1}{a_2-a_1}, a_1 \le E \le a_2 \\ 1, a_2 \le E \le a_3 \\ \dfrac{a_4-E}{a_4-a_3}, a_3 \le E \le a_4 \\ 0, \quad \text{otherwise} \end{cases} \qquad \xi_{\tilde{A}} = \begin{cases} \dfrac{A-b_1}{b_2-b_1}, \quad b_1 \le A \le b_2 \\ 1, b_2 \le A \le b_3 \\ \dfrac{b_4-A}{b_4-b_3}, b_3 \le A \le b_4 \\ 0, \quad \text{otherwise} \end{cases}$$

$$\xi_{\tilde{\beta}} = \begin{cases} \frac{\beta-c_1}{c_2-c_1}, c_1 \le \beta \le c_2 \\ 1, c_2 \le \beta \le c_3 \\ \frac{c_4-\beta}{c_4-c_3}, c_3 \le \beta \le c_4 \\ 1, \quad \text{otherwise} \end{cases} \qquad \phi_{\tilde{\beta}} = \begin{cases} \frac{\beta-c_1{}'}{c_2-c_1}, c_1{}' \le \beta \le c_2 \\ 0, c_2 \le \beta \le c_3 \\ \frac{c_4{}'-\beta}{c_4{}'-c_3}, c_3 \le \beta \le c_4{}' \\ 1, \quad \text{otherwise} \end{cases}$$

$$\phi_{\tilde{E}} = \begin{cases} \dfrac{E-a_1{}'}{a_2-a_1{}'}, a_1{}' \le E \le a_2 \\ 0, a_2 \le E \le a_3 \\ \dfrac{a_4{}'-E}{a_4{}'-a_3}, a_3 \le E \le a_4{}' \\ 1, \quad \text{otherwise} \end{cases} \qquad \phi_{\tilde{A}} = \begin{cases} \dfrac{A-b_1{}'}{b_2-b_1{}'}, b_1{}' \le k_0 \le b_2 \\ 0, b_2 \le k_0 \le b_3 \\ \dfrac{b_4{}'-A}{b_4{}'-b_3}, b_3 \le k_0 \le b_4{}' \\ 1, \quad \text{otherwise} \end{cases}$$

The α-cut of above functions is obtained as follow:

$\tilde{E}\,[\alpha] = \{[a_1 + \alpha(a_2-a_1), a_4 - \alpha(a_4-a_3)\,][a_1{}' + \alpha(a_2-a_1), a_4{}' - \alpha(a_4{}'-a_3)\,]\}$

$\tilde{A}\,[\alpha] = \{[b_1 + \alpha(b_2-b_1), b_4 - \alpha(b_4-b_3)\,][b_1{}' + \alpha(b_2-b_1{}'), b_4{}' - \alpha(b_4{}'-b_3)\,]\}$

$\tilde{\beta}\,[\alpha] = \{[c_1 + \alpha(c_2-c_1), c_4 - \alpha(c_4-c_3)\,][c_1{}' + \alpha(c_2-c_1{}'), c_4{}' - \alpha(c_4{}'-c_3)\,]\}$

Fuzzy Rayleigh distribution function

If the remaining mass fraction of biomass sample is modeled by the Rayleigh distribution, then

$$f(E) = \frac{\tilde{E}}{\tilde{\beta}^2}\, exp\left(\frac{-\tilde{E}^2}{2\tilde{\beta}^2}\right), E > 0, A > 0, \beta > 0,$$

where E (activation energy), A (frequency factor), and β (scale parameter) are crisp in nature. Let fuzzy numbers $\tilde{E}$, $\tilde{A}$, $\tilde{\beta}$ replace E, A and β. Then, the fuzzy probability of obtaining a value in the interval $[a, b]$, is as $\tilde{P}(m \le X \le n)$ and compute its α-cut as follows.

$$\tilde{P}(m < v < n)[\alpha] = \left\{ \int_m^n \frac{\tilde{E}}{\tilde{\beta}^2} e^{\left[-exp\left(\frac{E_S-\tilde{E}}{E_W}\right)-\left(\frac{\tilde{E}}{\tilde{\beta}}\right)^2\right]} dE | E \in \tilde{E}\,[\alpha] \right\} = \left[P^L[\alpha], P^U[\alpha]\right],$$

$$\tilde{P}(m < v < n)[\alpha] = \left\{ \int_m^n \frac{\tilde{E}}{\tilde{\beta}^2} e^{\left[-exp\left(\frac{E_S-\tilde{E}}{E_W}\right)-\left(\frac{\tilde{E}}{\tilde{\beta}}\right)^2\right]} dE | A \in \tilde{A}\,[\alpha] \right\} = \left[P^L[\alpha], P^U[\alpha]\right],$$

$$\tilde{P}(m < v < n)[\alpha] = \left\{ \int_m^n \frac{\tilde{E}}{\tilde{\beta}^2} e^{\left[-exp\left(\frac{E_S-\tilde{E}}{E_W}\right)-\left(\frac{\tilde{E}}{\tilde{\beta}}\right)^2\right]} dE | \beta \in \tilde{\beta}\,[\alpha] \right\} = \left[P^L[\alpha], P^U[\alpha]\right],$$

for all α, where

$$P^L = min\left\{ \frac{\tilde{E}}{\tilde{\beta}^2} e^{\left[-exp\left(\frac{E_S-\tilde{E}}{E_W}\right)-\left(\frac{\tilde{E}}{\tilde{\beta}}\right)^2\right]} \Big| E \in \tilde{E}\,[\alpha] \right\},\ P^U = max\left\{ \frac{\tilde{E}}{\tilde{\beta}^2} e^{\left[-exp\left(\frac{E_S-\tilde{E}}{E_W}\right)-\left(\frac{\tilde{E}}{\tilde{\beta}}\right)^2\right]} \Big| E \in \tilde{E}\,[\alpha] \right\}$$

$$P^L = min\left\{\frac{\tilde{E}}{\tilde{\beta}^2}e^{\left[-exp\left(\frac{E_S-\tilde{E}}{E_w}\right)-\left(\frac{\tilde{E}}{\tilde{\beta}}\right)^2\right]}\Big|A \in \tilde{A}\,[\alpha]\right\},\ P^U = max\left\{\frac{\tilde{E}}{\tilde{\beta}^2}e^{\left[-exp\left(\frac{E_S-\tilde{E}}{E_w}\right)-\left(\frac{\tilde{E}}{\tilde{\beta}}\right)^2\right]}\Big|A \in \tilde{A}\,[\alpha]\right\}$$

$$P^L = min\left\{\frac{\tilde{E}}{\tilde{\beta}^2}e^{\left[-exp\left(\frac{E_S-\tilde{E}}{E_w}\right)-\left(\frac{\tilde{E}}{\tilde{\beta}}\right)^2\right]}\Big|\beta \in \tilde{\beta}\,[\alpha]\right\},\ P^U = max\left\{\frac{\tilde{E}}{\tilde{\beta}^2}e^{\left[-exp\left(\frac{E_S-\tilde{E}}{E_w}\right)-\left(\frac{\tilde{E}}{\tilde{\beta}}\right)^2\right]}\Big|\beta \in \tilde{\beta}\,[\alpha]\right\}$$

APPLICATION

For applications point of view, the experiment has been conducted for the non-isothermal pyrolysis of pine needles. It is to be noted that the result of this paper has been used in the fuzzification process to obtain the randomness and authentic values of parameters. Table1 indicates the chemical composition which is also obtained with the help of CHNO-S analysis of pine needle samples.

Table 1: Chemical composition of pine needles

Biomass Type	C	H	N	O	V.M*	H.H.V**	S	Ash
Pine Needle	53.64	5.36	0.62	33.92	68.4	20.8	0.20	2.1

*Volatile matter **High heating value

RESULTS AND DISCUSSION

Numerical illustration

Supposing the initial distribution of activation energy follows the Rayleigh distribution with fuzzy scale parameters $\tilde{E}, \tilde{A}$ and $\tilde{\beta}$ taken as the trapezoidal fuzzy number. The values of parameters obtained after fuzzification are taken at α= 0.

Let $\tilde{E}(kJmol^{-1}) = [(119.66,129.6,149.6,159.66)]$, $\tilde{A}\ (s^{-1}) = [(15e+11, 18e+11, 42e+11, 24e+10)]$ and $\tilde{\beta}(kJmol^{-1}) = [(47,45,40,30)]$. The effect of fuzzified parameters with temperature is illustrated by means of fuzzified bands Re_1, Re_2, Re_3, Re_4.The Re_1 *and* Re_2 represent the fuzzified bands corresponding to membership function; whereas Re_3 *and* Re_4 represent those corresponding to non-membership function. After the fuzzy analysis of outer limit of "d*E*" integral, $\tilde{E}$, we have evaluated four fuzzified bands which are depicted in Figure 1. If the nature of the outer limit of "d*E*" integral, *E* had been crisp, all the four fuzzifies bands would have been converged to a single band. In the beginning of pyrolysis process, the remaining mass proportion must be close to one. From the fuzzified bands obtained in Figure1, it has been observed that the remaining mass fraction curves is less than one for fuzzy set interval (149.6, 159.66), whereas the remaining mass fraction agrees to thermo analytical data for the narrow fuzzy set interval (119.66, 129.6). The effect of the fuzzified frequency factor on the numerical results is shown in Figure 2. According to the fuzzified bands, increase in fuzzy interval causes the remaining mass fraction curves to shift up. However, the membership function for an interval $(15e+11, 18e+11)$ provides the good fit with experimental data. Influence of scale parameter of fuzzy Rayleigh distribution on the fuzzy analysis is depicted in Figure 3, where it is observed that the inflexion point of curves approach to zero, and the mass fraction curves is shift down the temperature scale by increase in the fuzzy band size of non-membership function.

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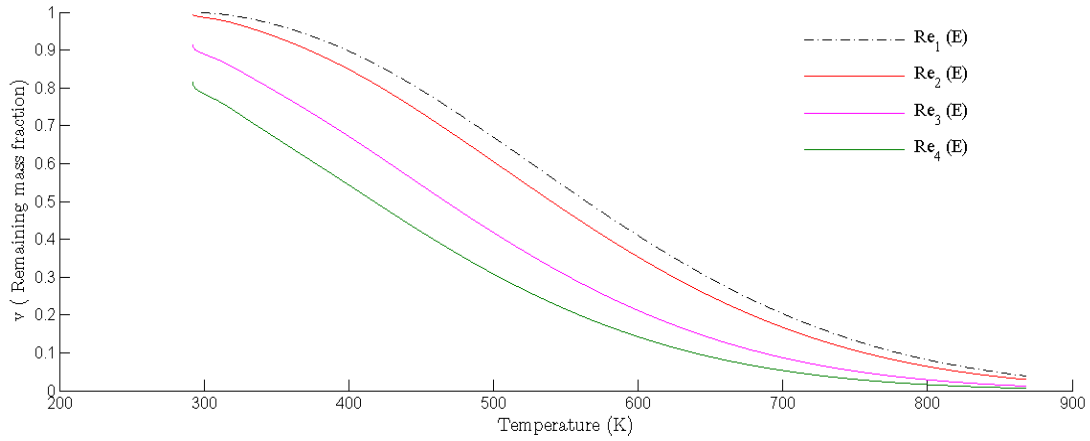

Fig. 1: The effect of fuzzified upper limit of "dE" integral on the numerical solution of DAEM

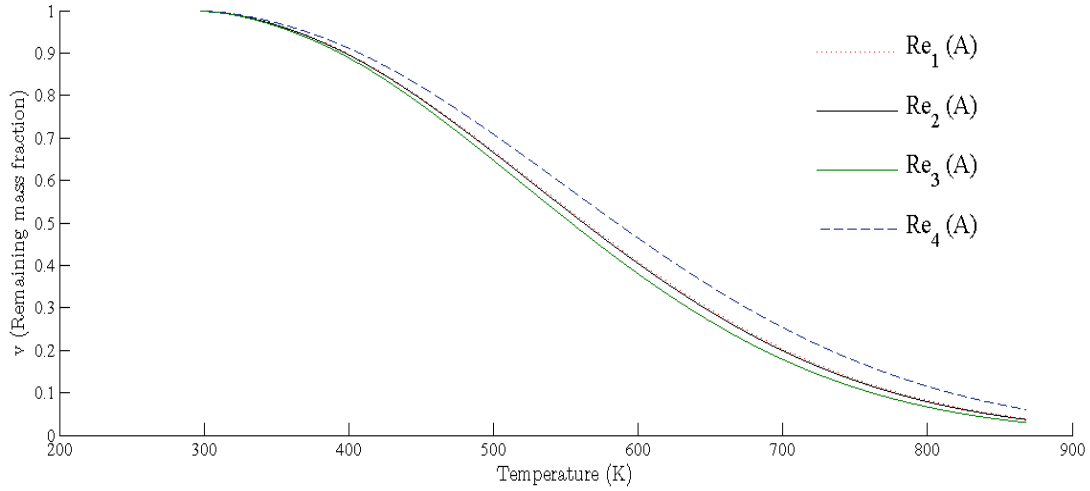

Fig. 2: The effect of fuzzified frequency factor (A) (s-1) on the numerical solution of DAEM

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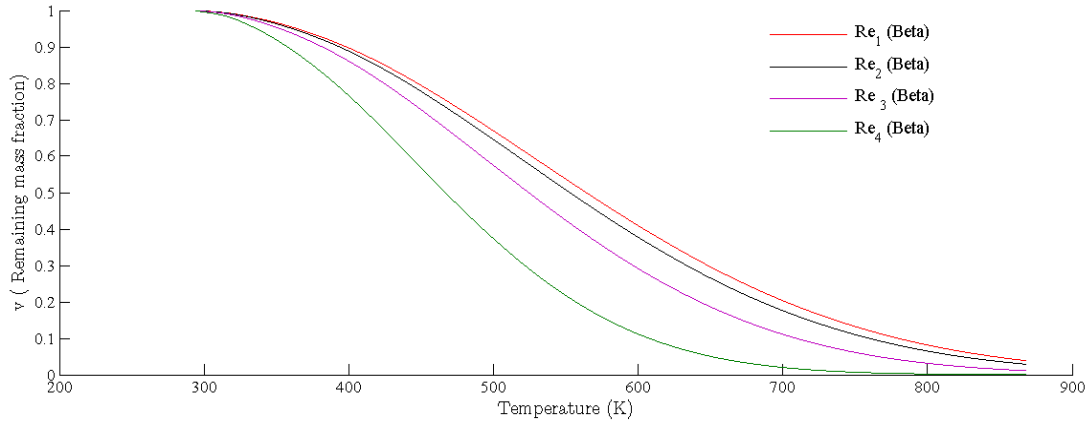

Fig. 3: The effect of fuzzified scale parameter (β) (kJmol^{-1}) of Rayleigh Distribution on the numerical solution of DAEM

CONCLUSION

In this article the kinetic parameters of DAEM have considered in fuzzy form. The fuzzy Rayleigh distribution has been successfully applied to the DAEM. Whenever, the distribution of activation energies and the parameters of distribution function contain randomness and fuzziness respectively, the conventional system is found to be infeasible. Thus, in order to overcome this complication, we have successfully implemented the fuzzy logic. The use of fuzzified kinetic parameters in the DAEM has overshadowed the demerits of the crisp-kinetic parameters, as the crisp data doesn't provide the membership interval for which the model can be approximated to some other realistic values. The fuzzy sets have handled the randomness or fuzziness to a certain desired level of accuracy, which in turn helped in making our analysis more precise and authentic.

The membership and the non-membership function of fuzzy set have been computed. Using this method, the fuzzified relevant parameters of biomass pyrolysis have been evaluated. It has been observed that the membership function provides good simulation with thermo analytical data for relatively small interval rather than large size of fuzzy set.

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ANTIOXIDATIVE RESPONSES OF MICROALGAE TO HEAVY METALS

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Abstract

Microalgae are unicellular free living entities and therefore their responses to excess of heavy metals must be faster and more efficient than those in vascular plants protected by various types of tissues. Up to date, numerous studies reported metal bioaccumulation potential of algae but metabolic responses have relatively rarely been monitored. Here I provide basic overview of quantitative changes of ascorbic acid (AA), reduced glutathione (GSH), phytochelatins (PCs) and selected related enzymes (ascorbate peroxidase and glutathione reductase) in some common microalgae exposed to various metals (cadmium mainly). Despite various culture and exposure conditions, some common signs of metal toxicity (including e.g. enhancement of phytochelatin biosynthesis) are clearly identifiable in algae. Other metal chelators such as organic acids are also briefly mentioned. Comparison with macroalgae, mosses and vascular plants is discussed in terms of basal values and evolutionary similarities.

Key words: antioxidants, heavy metals, oxidative stress

Abbreviations: APX – ascorbate peroxidase; AA – ascorbic acid; Cys – cysteine; DW – dry weight; FW – fresh weight; Glu – glutamic acid; Gly – glycine; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione; HPLC – high performance liquid chromatography; LC-MS/MS – liquid chromatography tandem-mass spectrometry; PCs – phytochelatins; PCS – phytochelatin synthase; ROS – reactive oxygen species

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INTRODUCTION

Excess of metals becomes a global problem owing to increasing anthropogenic activities leading to higher toxicity for biota including plants (FARGAŠOVÁ 2012). Among them, cadmium (Cd) is one of the most important contaminants as it accumulates in food chains and has negative impact on cell biochemistry (KOVÁČIK et al. 2015, 2016, 2017a-c). Other common metallic contaminants (limited or no physiological functions in plants) with relatively lower toxicity include nickel (Ni) and lead (Pb) among others (ŠMELKOVÁ et al. 2013; PIOTROWSKA-NICZYPORUK et al. 2015). Metals such as Cu and Zn are essential micronutrients but they are toxic if present in excess both for vascular plants and algae

(TÓTHOVÁ et al. 2011; HAMED et al. 2017).

Excess of metals typically stimulates formation of reactive oxygen species (ROS) which may damage cellular biochemistry if not effectively removed. Plants have developed an array of mechanisms to protect against ROS excess including synthesis of antioxidative molecules such as ascorbic acid (AA) and glutathione (GSH) or metal chelators such as phytochelatins and organic acids (SIMMONS et al. 2009; BRAÜTIGAM et al. 2011; DRESLER et al., 2014). Quantitative accumulation of these metabolites may differ between vascular and non-vascular plants (KOVÁČIK et al. 2017c) and certainly differs among algal species. These metabolites were not frequently reported in the literature owing to low absolute amount in algae requiring precise detection. On the other hand, assay of antioxidative enzymes including ascorbate peroxidase and glutathione reductase is more common owing to inexpensive detection by spectrophotometry.

AA and GSH are not only efficient antioxidants for ROS removal but GSH also serves as a substrate for the synthesis of phytochelatins (PCs, SIMMONS et al. 2009). Recent research indicates that cross-talk between AA and GSH is more complex in algae (LIN et al. 2016). Manipulation of AA biosynthesis in alga *Chlamydomonas reinhardtii* also revealed that algae possess an efficient system for a manifold increase in ascorbate content under stress conditions which is distinct from land plants (VIDAL-MEIRELES et al. 2017). This is fully in agreement with the assumption that microalgae may not rely on tissue structure present in vascular plants and thus regulation of metabolism must be faster and more efficient.

Despite the wide use of algae for biosorption of metals, their metabolic responses to metallic stress have only rarely been reported. Quantitative changes of common antioxidants including ascorbic acid, GSH and related enzymes (ascorbate peroxidase and glutathione reductase) as well as metal chelators phytochelatins (PCs) and organic acids in selected algal species and their evolutionary comparison with macroalgae, mosses and vascular plants are briefly discussed.

ROLE OF NON-ENZYMATIC ANTIOXIDANTS

ASCORBIC ACID. Typical AA amount in Bryophytes and algae is ca. 0.1 – 0.6 μmol g^{-1} FW but mostly >5 μmol g^{-1} FW (2 – 20 μmol g^{-1} FW) in the leaves of higher/vascular plants (GEST et al. 2013). It should be noted that water content of mosses (ca. 75%) is lower if compared with algae or vascular species (ca. 90 – 95%, J. Kováčik, personal observation) then comparison of the data expressed per g DW and g FW must be done with caution (and g DW is certainly better formulation in this case). This fact is mainly visible if chamomile (1.751 μmol g^{-1} DW) and *S. quadricauda* (0.185 μmol g^{-1} DW, Table 1) control AA values are compared: we see almost 10-fold difference indicating evolutionary differences. On the other hand, even chamomile value (using water content ca. 90% in this species) would be ~0.1751 μmol AA g^{-1} FW which falls within above-mentioned range for algae (0.1 – 0.6 μmol AA g^{-1} FW) and it is clear that this range is not universal. Basal AA content, at least in some algal species, must be lower as also shown by our earlier data if *Coccomyxa* and *Scenedesmus* (assayed by LC-MS/MS) are compared (Table 1). Spectrophotometric determination of AA (see control of *S. acutiformis*, Table 1) is also within mentioned range 0.1 – 0.6 μmol g^{-1} FW. Data from other algal species such as often studied *Chlorella vulgaris* show that AA content may differ in relation to culture and/or analytical detection: values 0.11 – 0.39 mg AA g^{-1} DW (= 0.63 – 2.21 μmol AA g^{-1} DW, YUSOF et al. 2011) and ~500 μg AA g^{-1} DW (= 2.83 μmol AA g^{-1} DW, GOIRIS et al. 2015) were reported. In terms of evolutionary similarities, moss *Taxiphyllum* (control) contained similar amount of AA as found in *S. quadricauda* while chamomile and mainly *Ceratophyllum* values are far higher and confirms differences between non-vascular and vascular species (Table 1). Another strong indication of such variation is visible between *Taxiphyllum* (moss) and *Ceratophyllum* (vascular

plant, Table 1) and this difference was confirmed by two methods (spectrophotometry and HPLC). Surprisingly macroalga *Ulva* contains higher amount of AA than microalgal species and even chamomile leaves (Table 1), urging for further research in this algal group.

Metal excess certainly affects main antioxidants due to altered ROS formation as recently confirmed in *S. quadricauda* exposed to 100 μM Cd over 1 h where increase in AA and ROS generation was observed: application of AA biosynthetic inhibitor (lycorine) depleted Cd-induced AA accumulation and enhanced ROS formation, indicating that AA prevents the appearance of ROS under Cd excess (KOVÁČIK et al. 2017b). Unfortunately algae are mainly studied in terms of their eventual usefulness for biofuel production and responses of AA to metallic stress have only relatively rarely been studied. Few available studies reported an increase in AA under Zn excess in *C. sorokiniana* or *S. acuminatus* after prolonged (7 days) exposure (HAMED et al. 2017) or in *S. acutiformis* after 30 days of exposure to Cd (KOVÁČIK et al. 2017a), reinforcing the assumption that AA synthesis is one of the protective mechanisms under metal excess. Additionally, short-term exposure (24 h) to relatively low Cd and Ni doses (1 or 10 μM) evoked increase in AA not only at 1 μM Cd but also at 10 μM Ni more intensively in young than in old culture (KOVÁČIK et al. 2016). Vascular plants exposed (mainly) to Cd revealed increase in AA or unaltered content under higher metal doses (Table 1). On the other hand, redox-active metals such as Cu evoked rapid depletion of AA in macroalga *Ulva compressa* despite elevated activities of main AA biosynthetic enzymes (see MELLADO et al. 2012 for details). Protective effect of AA in algae has only rarely been studied through exogenous application of AA and it was shown that it may affect Cd uptake but maximum depletion (by 15.6%) required 150 μM AA against 25 μM Cd (EL-NAGGAR & EL-SHEEKH 1998). Indirect manipulation of AA level was also observed in microalgae, e.g. nitric oxide donor (sodium nitroprusside) showed a correlation between NO appearance and AA content in combination with Cd excess in *Coccomyxa subellipsoidea* (KOVÁČIK et al. 2015). Manipulation of AA biosynthesis has only recently been published in algae and *Chlamydomonas reinhardtii* with altered *VTC2* gene (encoding GDP-L-galactose phosphorylase) had AA content lower by 90% and was more susceptible to stress including rapid induction of *VTC2* gene by ROS (VIDAL-MEIRELES et al. 2017). It appears to be evident that algae possess an efficient system for a manifold increase in ascorbate content under stress conditions distinct from land plants (VIDAL-MEIRELES et al. 2017) and further studies could highlight this aspect also under metal excess.

GLUTATHIONE (exactly its reduced form GSH) is an essential component of the ROS removal through AA-GSH cycle and a precursor for phytochelatins (see below). GSH is therefore usually more abundant than AA as confirmed also by our data (cf. control values of *Coccomyxa*, *S. quadricauda* or chamomile leaves in Tables 1 and 2). Evolutionary comparison between algae and mosses or vascular plants indicates lower differences than those for AA (less than 5-fold considering water content ~95%; see controls in Table 2).

In terms of metal excess, comparison of Cd and Ni in *S. quadricauda* (1 or 10 μM over 24 h) revealed depletion in Cd treatments and slower response in Ni treatments (KOVÁČIK et al. 2016) but 1 h of 100 μM Cd had no impact on GSH. Longer maintenance of algae *ex vitro* (under non-sterile conditions) affected response to Cd (see KOVÁČIK et al. 2017b for details). In agreement, low Cd doses (0.12 – 1.9 μM) had no impact on GSH after 6 h of exposure in *Pseudokirchneriella subcapitata* (MACHADO & SOARES 2016) but more extensive GSH changes were observed during short-term (0.5 – 14 h) in *Desmodesmus armatus* under 93 μM Cd (POKORA et al. 2014). On the contrary, in vascular plant, moss or macroalga, Cd and Cu evoked rather elevation of GSH amount (Table 2) which correlated with enhancement of GSH-related biosynthetic enzymes in Cu-treated macroalga *Ulva* (MELLADO et al. 2012). In microalgae, even high Zn doses (600 or 1000 μM) had relatively negligible impact on GSH increase (HAMED et al. 2017). *Coccomyxa subellipsoidea* revealed both decrease and

increase in relation to applied Cd concentration and co-application of nitric oxide donor indicated that GSH and Cd accumulation could be correlated (KOVÁČIK et al. 2015). Dinoflagellate *Lingulodinium polyedrum* exposed to 18 μM Cd showed time-independent (24 and 48 h) depletion (ROMANO et al. 2017) and GSH also decreased in Cd-exposed (24 h) *Chlamydomonas* (BRAÜTIGAM et al. 2011). In *Acutodesmus* (formerly *Scenedesmus*) *obliquus*, Pb doses over 10 μM depleted GSH in all time points investigated (PIOTROWSKA-NICZYPORUK et al. 2015) though absolute GSH amount (per g of biomass) was not quantified and cannot be compared with other species. Impact of metals on GSH accumulation depends on the exposure time and applied metal/concentration in various microalgae and the action of Cd seems to be more prominent than that of Ni or Zn (Table 2). Of course, GSH quantitative changes are affected not only by its synthesis but also by consumption for phytochelatin synthesis and/or GSSG-GSH enzymatic conversion through glutathione reductase as mentioned below.

PHYTOCHELATINS (PCs) are oligopeptides synthesized from GSH by plants including algae (hence the prefix phyto-) which are able to bind various metals. They usually contain amino acids $(\gamma\text{-Glu-Cys})_n$-Gly where n = 2-11 (for their synthesis and identification see e.g. PERALES-VELA et al. 2006; SIMMONS et al. 2009). Microalgae typically contains short PCs such as PC2 and PC3 but longer chains (PC4) or their derivatives (CysPCs) were detected in *Chlamydomonas* (BRAÜTIGAM et al. 2011), dinoflagellate *Lingulodinium polyedrum* (ROMANO et al. 2017) or macroalga *Ulva* (MELLADO et al. 2012). In terms of basal (control) values, their accumulation in unstressed algae is lower than that of GSH, often even 100- or 1000-fold differences were observed (e.g. *Coccomyxa* and *Scenedesmus* as detected by LC-MS/MS, cf. Tables 2 and 3). Surprisingly, macroalga *Ulva* contained almost identical amount of PC2 and GSH in control thalli (considering water content ~90%, cf. Tables 2 and 3) while chamomile, a vascular plant, shows amount of PCs (PC2+PC3) ca. 15-fold lower in comparison with GSH (cf. Tables 2 and 3).

Accumulation of PCs is mainly enhanced by excess of Cd, including the activation of enzyme phytochelatin synthase (PCS, SIMMONS et al. 2009). In agreement, microalga *Coccomyxa subellipsoidea* revealed almost 150-fold increase in PC2 in response to 10 and 100 μM Cd over 24 h while GSH amount rather decreased (KOVÁČIK et al. 2015). In *S. quadricauda*, 1 and 10 μM Cd treatment over 24 h evoked over 25-fold elevation of PC2 and GSH also decreased Cd concentration-dependently (KOVÁČIK et al. 2016). At the same time, (though it was argued that Ni is the 6th most efficient PCS inducer), Ni had no impact on PC2 accumulation in *S. quadricauda* (KOVÁČIK et al. 2016). Elevation of PC2 was even detected after 1 h of 100 μM Cd excess in *S. quadricauda* (KOVÁČIK et al. 2017b). We did not observe PC3 or longer chains in *Scenedesmus* but *Chlamydomonas reinhardtii* exposed to 70 μM Cd up to 48 h produced not only PC3 and PC4 but also Cys(PCs) derivatives which increased more pronouncedly in responses to Cd than respective PCs (SIMMONS et al. 2009). *Acutodesmus armatus* produced PC2 and PC3 mainly but also small amount of PC4 in response to Cd was detected (POKORA et al. 2014). Also dinoflagellate *Lingulodinium polyedrum* produced PC3 and PC4 strongly during short-term (24 h) exposure to relatively low Cd doses 9 – 27 μM (ROMANO et al. 2017) and marine macroalga *Ulva* produced rather PC2 than PC3 or PC4 in response to Cu (MELLADO et al. 2012). In vascular plant chamomile, the same method as used for *Scenedesmus* (LC-MS/MS, KOVÁČIK et al. 2016) showed strong induction of PC2 and PC3 in response to Cd (over 10-fold) with higher absolute values of PC3 (see Table 3 and KOVÁČIK et al. 2014). As in the case of GSH mentioned above, also PC2 changes in *Coccomyxa subellipsoidea* were affected by Cd and co-application of nitric oxide donor, indicating that accumulation of Cd and thiols could be correlated (KOVÁČIK et al. 2015). It is clear that some algal species produced PCs similar to those in vascular plants (PC2-3) while inter-specific differences among algae and production of CysPCs derivatives indicate evolutionary differences requiring further research.

Tab. 1: Metal-induced quantitative changes of ascorbic acid (AA) in selected microalgae (macroalga [x], moss [xx] and vascular plant [xxx] for comparison). Values shown as graphs in the cited papers were deducted as exactly as possible.

Species	Applied metal (µM)	Duration	AA content (µmol g^{-1} FW or DW) (control value in brackets)	Reference
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	0.0058 and 0.0026 FW (0.006 FW)	Kováčik et al. 2015
Scenedesmus quadricauda	1 and 10 Cd	24 h	0.817 and 3.139 DW (0.185 DW)*	Kováčik et al. 2016
Scenedesmus quadricauda	1 and 10 Ni	24 h	0.192 and 1.391 DW (0.185 DW)*	Kováčik et al. 2016
Scenedesmus acutiformis	1 and 10 Cd	30 days	0.132 and 0.178 FW (0.117 FW)	Kováčik et al. 2017a
Scenedesmus acuminatus	600 or 1000 Zn**	7 days	0.406 FW (0.247 FW)	Hamed et al. 2017
Chlorella sorokiniana	600 or 1000 Zn**	7 days	0.441 FW (0.229 FW)	Hamed et al. 2017
Scenedesmus quadricauda	100 Cd	1 h	0.597 (0.242 DW)	Kováčik et al. 2017b
Ulva compressa [x]	10 Cu	1 – 7 days	<0.5 DW (4.5 DW)	Mellado et al. 2012
Taxiphyllum barbieri [xx]	10 and 100 Cd	24 h	0.249 and 0.254 DW (0.163 DW)	Kováčik et al. 2017c
Ceratophyllum demersum [xxx]	10 and 100 Cd	24 h	18.23 and 23.66 DW (15.16 DW)	Kováčik et al. 2017c
chamomile leaves [xxx]	60 Cd	48 h	1.582 DW (1.751 DW)	Kováčik et al. 2014

* marked as "young" culture in the cited paper, ** applied dose unclear from the cited paper

Tab. 2: Metal-induced quantitative changes of reduced glutathione (GSH) in selected microalgae (macroalga [x], moss [xx] and vascular plant [xxx] for comparison). Values shown as graphs in the cited papers were deducted as exactly as possible.

Species	Applied metal (µM)	Duration	GSH content (µmol g^{-1} FW or DW) (control value in brackets)	Reference
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	0.091 and 0.036 (0.053 FW)	Kováčik et al. 2015
Scenedesmus quadricauda	1 and 10 Cd	24 h	0.618 and 0.348 DW (0.934 DW)*	Kováčik et al. 2016
Scenedesmus quadricauda	1 and 10 Ni	24 h	0.514 and 0.859 DW (0.934 DW)*	Kováčik et al. 2016
Scenedesmus acuminatus	600 or 1000 Zn**	7 days	0.10 FW (0.05 FW)	Hamed et al. 2017
Chlorella sorokiniana	600 or 1000 Zn**	7 days	0.14 FW (0.09 FW)	Hamed et al. 2017
Lingulodinium polyedrum	18 Cd	24 and 48 h	0.5 DW (1.5 DW)	Romano et al. 2017
Chlamydomonas reinhardtii	70 Cd	24 h	0.05 FW (0.1 FW)	Braütigam et al. 2011
Scenedesmus quadricauda	100 Cd	1 h	0.620 (0.569 DW)	Kováčik et al. 2017b
Ulva compressa [x]	10 Cu	1 – 7 days	1.5 – 6.0 DW (1.5 DW)	Mellado et al. 2012
Physcomitrella patens [xx]	10 Cd	3 days	0.88 FW (0.3 FW)	Hermsen et al. 2010
chamomile leaves [xxx]	60 Cd	48 h	21.7 DW (3.79 DW)	Kováčik et al. 2014

* marked as "young" culture in the cited paper, ** applied dose unclear from the cited paper

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Tab. 3: Metal-induced quantitative changes of phytochelatins of various chain length in selected microalgae (macroalga [x] and vascular plant [xx] for comparison). Values shown as graphs in the cited papers were deducted as exactly as possible.

Species	Applied metal (µM)	Duration	PC content (nmol g^{-1} FW or DW) (control value in brackets)	Reference
phytochelatin 2				
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	2.171 and 4.378 FW (0.029 FW)	Kováčik et al. 2015
Scenedesmus quadricauda	1 and 10 Cd	24 h	163.52 and 213.46 DW (8.047 DW)*	Kováčik et al. 2016
Scenedesmus quadricauda	1 and 10 Ni	24 h	6.863 and 7.214 DW (8.047 DW)*	Kováčik et al. 2016
Chlamydomonas reinhardtii	70 Cd	24 h	19.04 FW (33.3 FW)	Braütigam et al. 2011
Scenedesmus quadricauda	100 Cd	1 h	18.49 (6.165 DW)	Kováčik et al. 2017b
Ulva compressa [x]	10 Cu	1 – 7 days	250 – 600 FW (100 FW)	Mellado et al. 2012
chamomile l eaves [xx]	60 Cd	48 h	794 DW (53.8 DW)	Kováčik et al. 2014
phytochelatin 3				
Chlamydomonas reinhardtii	70 Cd	24 h	19.04 FW (9.52 FW)	Braütigam et al. 2011
Lingulodinium polyedrum	9 – 27 Cd	24 h	64.9 – 31.3 DW (3.1 DW)	Romano et al. 2017
Ulva compressa [x]	10 Cu	1 – 7 days	5 – 35 FW (5 FW)	Mellado et al. 2012
chamomile leaves [xx]	60 Cd	48 h	2435 DW (206 DW)	Kováčik et al. 2014
phytochelatin 4				
Lingulodinium polyedrum	9 – 27 Cd	24 h	118.7 – 89.1 DW (1.2 DW)	Romano et al. 2017
Ulva compressa [x]	10 Cu	1 – 7 days	35 – 53 FW (30 FW)	Mellado et al. 2012

* marked as "young" culture in the cited paper

INVOLVEMENT OF ENZYMATIC ANTIOXIDANTS

Plant cell possesses an array of enzymes to scavenge ROS formations due to metal excess (and other stresses) which were reviewed many times before. Here I will mention mainly ascorbate peroxidase (APX) as the main AA-decomposing enzyme and glutathione reductase (GR) regenerating GSH from its oxidized state (GSSG). Both these enzymes are essential for hydrogen peroxide removal through ascorbate-glutathione cycle (see e.g. GEST et al. 2013 for details).

Comparison of APX activity in selected microalgal species revealed roughly similar control values (at the level of hundreds of nmol min^{-1} mg^{-1} protein, see Table 4) and lower value in comparative vascular plant chamomile (assayed by identical method as algae). Also *A. obliquus* data are lower compared to other microalgae presented in Table 4. Exceptionally high basal APX activity in *Coccomyxa subellipsoidea* could account for low AA content in this species (cf. Table 1 and 4) and such high APX activity (3.76 U mg^{-1} protein = 3760 nmol min^{-1} mg^{-1} protein) has also been reported in other *Coccomyxa* species (RUIZ-DOMÍNGUEZ et al. 2015). Depletion of APX activity after very short (1 h, KOVÁČIK et al. 2017b) or short (24 h) impact of Cd (KOVÁČIK et al. 2015) was reported in microalgae or water moss *Taxiphyllum* (KOVÁČIK et al. 2017c). Longer studies with microalgae showed either elevation (7 days, HAMED et al. 2017) or unaltered activity (30 days, KOVÁČIK et al.

2017a) in response to various metals. One of few combined studies revealed interesting responses in microalga *C. subellipsoidea* where combination of Cd with nitric oxide donor elevated AA content and APX activity (see KOVÁČIK et al. 2015 for details), indicating direct relation between these parameters. In agreement, APX not only use AA as a cofactor for hydrogen peroxide removal but is also inactivated by low AA content (GEST et al. 2013, see also evolution and significance of APX in this work). At the gene expression level, APX expression was variously affected by metals in microalga *C. reinhardtii* (after 2 weeks of exposure): Cd doses 10 – 40 μM evoked dose-dependent increase while Cu or Hg doses over 3 or 5 μM had strongly negative impact (NOWICKA et al. 2016), indicating metal-specific action even in the given species.

Glutathione reductase (GR) is important e.g. for providing reduced glutathione (GSH from GSSG) for regeneration of oxidized ascorbate generated through ascorbate-glutathione cycle (see e.g. GEST et al. 2013 for details). Basal activities of this enzyme appear to be more variable among various algal species than APX mentioned above. I mainly note that activity at the level of 0.5 nmol min^{-1} mg^{-1} protein (PIOTROWSKA-NICZYPORUK et al. 2015) is far lower than in other species from the (former) genus *Scenedesmus* or others, arising the question about quantitative assay: however, low impact of Pb on GR activity in the given species indicates tolerance of *Acutodesmus* to even 100 μM Pb (Table 4). Unaltered or elevated activity of this enzyme was also reported in various plants and microalgae and only short-term (1 h) exposure to Cd evoked depletion (KOVÁČIK et al. 2017b). The complexity of interaction between AA and GSH is further visible in a recent study using *Chlamydomonas reinhardtii* where overexpression of dehydroascorbate reductase (converting oxidized to reduced AA at the expense of GSH) increased GSH/GSSG ratio, glutathione pool and APX or GR activities and protected algae against photoxidative stress (LIN et al. 2016). Algae with increased antioxidative protection could provide more tolerant strains for metal absorption in the future.

Tab. 4: Metal-induced quantitative changes of AA- and GSH-related enzyme activities (nmol min^{-1} mg^{-1} protein) in selected microalgae (moss [x] and vascular plant [xx] for comparison). Values shown as graphs in the cited papers were deducted as exactly as possible.

Species	Applied metal (μM)	Duration	Enzyme activity (control value in brackets)	Reference
ascorbate peroxidase				
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	615 and 459 (1291)	Kováčik et al. 2015
Scenedesmus acuminatus	600 or 1000 Zn*	7 days	266 (133)	Hamed et al. 2017
Chlorella sorokiniana	600 or 1000 Zn*	7 days	720 (400)	Hamed et al. 2017
Acutodesmus obliquus	1 – 10 – 100 Pb	24 h	24 – 21.5 – 18.9 (24)	Piotrowska-Niczyporuk et al. 2015
Scenedesmus acutiformis	1 and 10 Cd	30 days	192 and 205 (204)	Kováčik et al. 2017a
Scenedesmus quadricauda	100 Cd	1 h	287 (412)	Kováčik et al. 2017b
Taxiphyllum barbieri [x]	10 an 100 Cd	24 h	196 and 185 (256)	Kováčik et al. 2017c
chamomile leaves [xx]	60 Cd	48 h	51.4 (53)	Kováčik et al. 2014
glutathione reductase				
Coccomyxa subellipsoidea	10 and 100 Cd	24 h	173 and 119 (141)	Kováčik et al. 2015
Scenedesmus acuminatus	600 or 1000 Zn*	7 days	62 (43)	Hamed et al. 2017
Chlorella sorokiniana	600 or 1000 Zn*	7 days	155 (80)	Hamed et al. 2017
Acutodesmus obliquus	1 – 10 – 100 Pb	24 h	0.55 – 0.48 – 0.45 (0.51)	Piotrowska-Niczyporuk et al. 2015
Scenedesmus acutiformis	1 and 10 Cd	30 days	292 and 375 (304)	Kováčik et al. 2017a
Scenedesmus quadricauda	100 Cd	1 h	322 (536)	Kováčik et al. 2017b
chamomile leaves [xx]	60 Cd	48 h	25.8 (27.5)	Kováčik et al. 2014

* applied dose unclear from the cited paper

ORGANIC ACIDS

Aliphatic organic acids are potential chelators of metals in various plants (DRESLER et al. 2014). Also water moss *Taxiphyllum* and vascular aquatic plant *Ceratophyllum* revealed elevated production of citrate and malate in response to Cd with molar ratio 1.2:1 and 4:1, indicating potential involvement in Cd chelation (KOVÁČIK et al. 2017c). In microalga *C. subellipsoidea*, it was observed that quantitative changes of malate evoked by Cd and co-application of nitric oxide donor showed potential correlation with Cd accumulation (KOVÁČIK et al. 2015). Comparison of *Scenedesmus* cultures of various age exposed to Cd or Ni revealed that organic acids including citrate and malate decreased or increased in relation to age of the culture and the impact of Ni was more pronounced compared to Cd (KOVÁČIK et al. 2016). Also short-term (1 h) of Cd presence affected often considerably accumulation of Krebs cycle acids (KOVÁČIK et al. 2017b). Accumulation of organic acids in microalgae has only rarely been studied probably owing to low quantitative levels (up to ten of $\mu g\ g^{-1}$ FW in comparison with vascular plants) requiring sensitive detection techniques. Further studies focused on inter-specific comparison among microalgae and macroalgae could certainly reveal the impact of metals on organic acids and their eventual role in metal chelation.

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HOW THE MANAGEMENT MAY AFFECT DISPERSAL OF SLENDER SPEEDWELL (*VERONICA FILIFORMIS* SMITH) IN MEADOWS AND PASTURES

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Abstract

Slender speedwell (*Veronica filiformis* Smith, family Plantaginaceae) is a non-native and invasive species of grassland in the Europe. The aim of the study was to test the ability of the growth and spreading in nine differently managed grasslands (mowing, mulching, grazing). The best survival was found in two-years mowed lots with fertilisation and in all lots with not permanent cattle pasture crops. There were found the highest number of survived plants and plants with big "rosette" diameters. Number of survived plant for two seasons was 18 and total number of vegetative peaks per plant was 688. The potential for the spreading of such plant population is great. On the other hand, plants did not survived in lots with mulch treatment and in fenced lots with permanent cattle grazing.

Key words: agricultural management, grazing, invasive plants, mesophilic grasslands, mowing

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INTRODUCTION

Structure of grass cover can be influenced by invasive and expansive plant species. Mainly invasive species (PERGL et al. 2016) are in the centre of attention. Traits predetermining the ability to invade are neither easily nor unambiguously set. It is often the case that successful invasive plant species can show very opposing traits (PYŠEK 2001). The success can be supported by high production of seeds and fruits on one hand, and on the other hand by total absence of reproductive propagation, substituted with intense vegetative spread. Generally speaking, conditions for invasion are competition ability, fertility, seed germination, fast growth and a large production of biomass (PYŠEK 2001).

Growth and absence of generative reproduction are characteristic for Slender speedwell (*Veronica filiformis* Smith, family *Plantaginaceae*). This species comes from the western Caucasus, classified as

domesticated neophyte in the Czech Republic (PYŠEK et al. 2012). Currently it is common in central, northern and north-western Europe (SCALONE & ALBACH 2012), in North America (USDA, NRCS 2016) and New Zealand (WEBB et al. 1988).

Slender speedwell is a low dicotyledonous herbaceous plant with a procumbent stem, delicate round leaves and fine blue-white blossoms. Stems can root by adventive way in nodes, create other vegetative apexes, and thus enable quick vegetative regeneration (HARRIS & LOWELL 1980a; ŠERÁ 2012). The plants have a low genetic variability created by a limited number of clones. The production of pollen and seeds is also low, seeds either have low germination, or they are not developed at all (SCALONE & ALBACH 2012).

In our conditions Slender speedwell always preferred and prefers grasslands and mowing lawns (SOJÁK & ŠOUREK 1959; JEHLÍK 1961, 1998; JEHLÍK & SLAVÍK 1967; PENIAŠTEKOVÁ & ZLINSKÁ 1995; KAPLAN et al. 2016). It is ranked among synanthropic species spreading by planting or by swiping of agricultural and small municipal engineering (MÜLLER & SUKOPP 1993). Particularly colder and wetter conditions suit him (HARRIS & LOWELL 1980b; SCALONE & ALBACH 2012).

Slender speedwell propagates mainly in grass covers, and is able to regenerate from vegetative parts. However, little is known about the influence of various types of management on the species propagation. This paper presents the results of an experiment focused on the influence of various frequency grass moving and grazing on mesophilic meadows and pastures with regard to the propagation possibilities of this species. We assume that the Slender speedwell will prosper only under the some type of the tested managements. This kind of management will be the "risk" for grassland, because it may encourage the spread of this invasive species.

MATERIAL AND METHODS

Experimental location and lots

The experiment was run on a grass location called Velký Chuchelec, which are experimental grounds of the Department of Plant Production and Agroecology, the Faculty of Agriculture at the University of South Bohemia in České Budějovice. The grounds are located at the foothills of the Šumava on the western part of the Kaplice basin. The climate has a highland character of moderate to lower humidity, slightly influenced by the rain shadow of the Šumava and the Alps. Average monthly temperatures and precipitation are shown in Table 1. Other characteristics: altitude of 650 m a. s. l., slope of 11°, ESE exposure, soil deep cambisol, N 48°45'26" E 14°44'76").

The experimental grounds of Velký Chuchelec is divided into lots of 3x10 metres which are managed by mowing (harvesting), mulching and cattle grazing in the long-term run (Fig. 1).

Tab. 1: The average monthly air temperature and average monthly precipitation at the location Velký Chuchelec. Data come from the local university hydrometeorological station

Meteorological characteristics	Year/month	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Mean
Temperature (°C)	2010	-4.6	-2.2	+3.0	+9.5	+11.9	+16.5	+19.7	+17.5	+12.1	+8.2	+2.8	-4.6	+7.5
	2011	-1.4	-0.9	+4.5	+9.8	+14.1	+16.8	+16.5	+17.7	+14.4	+7.1	+3.1	+1.1	+8.5
	2012	-0.9	-2.9	+4.4	+7.7	+12.8	+16.0	+17.3	+18.1	+14.1	+7.5	+3.1	-0.7	+8.0
Precipitation (mm)	2010	30.0	26.5	29.5	45.8	118.6	106.3	171.6	111.6	49.9	17.4	28.0	29.4	764.6
	2011	33.7	10.6	47.9	26.5	116.3	65.2	114.6	50.0	94.9	62.4	0.0	39.2	661.3
	2012	54.4	19.5	11.3	58.5	67.2	125.2	160.2	101.6	80.6	39.4	22.4	56.5	797.2

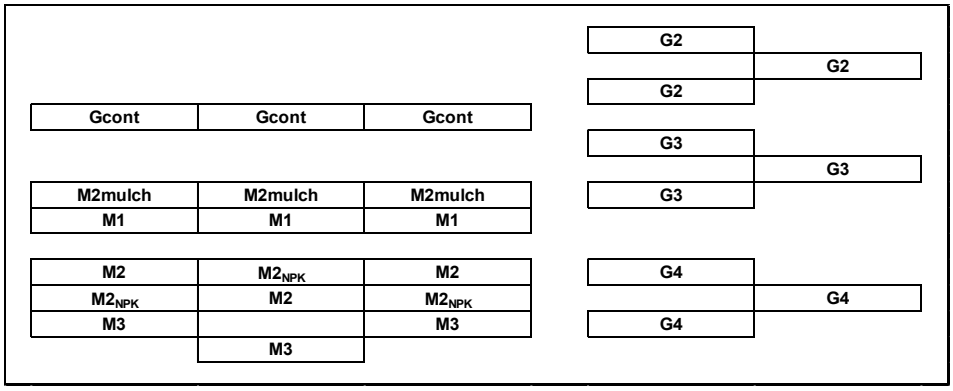

Fig. 1: Spatial distribution of plots (3x10 m) in the location Velký Chuchelec in 2010 – 2012. Experimental treatments are marked according to: M1 – 1 one-mowing per a year, M2 – two mowings per a year, M3 – three mowings per a year, M2NPK – two mowings and a fertilisation per a year, M2mulch – two mowings and mulch per a year, G2 – two grazings per a year, G3 – three grazings per a year, G4 – four grazings per a year, Gcont – continual grazing.

Long-term management (mowing, mulching, grazing)

Experimental mowing lots are cut from 1 to 3 times a year with an MF-70 grass cutter, the length of grass set to 4-5 cm. The harvested biomass is immediately removed. A Vari-Tajfun mulcher is used in case of mulching management, combined mulching and mowing, or mulching and grazing option. The mulch is kept on the location. One of the two-harvest lots in manually fertilized with NPK fertilizers, in the annual amount of 100 kg N, 30 kg P and 50 kg K per 1 ha (fertilizers: ammonium nitrate with lime 27.5% N, granulated triple superphosphate 45% P_2O_5 and potassium nitrate 60% K_2O). A doze of nitrogen was applied separately 70 kg N per 1 ha in the spring and 30 kg after the first harvest. Fenced grazing is 2-4 times a year, and/or continuous grazing. The cattle herd consisted of two breeds, Charolais and Simental. Further details on the lot management are in KOBES et al. (2013).

Our experiment was run during 2010 – 2012 on experimental lots having three basic managements (mowing, mulching, grazing) with more options. We had 9 management options (all together) and each management option in three repetitions (see Fig. 1):

Mowing (M): M1 – 1 one-mowing per a year, M2 – two mowings per a year, M3 – three mowings per a year, M2NPK – two mowings and a fertilisation per a year,

Mulching (Mmulch): M2mulch – two mowings and mulch per a year,

Grazing (G): G2 – two grazings per a year, G3 – three grazings per a year, G4 – four grazings per a year, Gcont – continual grazing.

Plant material

The source of the plant material was a population of Slender speedwell (*Veronica filiformis*) taken in the spring of 2009 from the region of Velké Skaliny (foothills of the Novohradské hory Mountains, N 48°44'49.2" E 14°34'43.4"). Individual plants were generated vegetatively from that material and grown in separate pots (3 x 3 x 6 cm), using a standard substrate (AGRO with active humus). 270 healthy plants of minimum stem length 4.5 cm were used in the experiment.

Slender speedwell growing

The plants were planted in the lots (see above, Fig. 1) managed in various ways in the spring of 2010 in a way avoiding a marginal effect and potential inner-species influences (at minimum 0.5 m distance). 10 plants were used on each lot, 3 lots for each type of management (30 plants per one

treatment). After the plants rooted, standard management continued.

During vegetation, the following aspects were measured 2-3 times a year: number of living individuals, diameter of the "rosette" (maximum length of the plant laid on the ground) and a total number of apexes on the plant. The length of the plants at the beginning of the experiment was used as the basis (100%) for evaluation of growth characteristics ("rosette" diameter). Growth development and differences in growth characteristics among lots with different management were evaluated using MS Excel.

RESULTS

Majority of speedwell creeping plants survived the first winter on all lots, regardless the type of management (Figs. 2, 3). After two years of management, the numbers of plants started to differ. The biggest number of plants survived on grazed lots (except for permanently grazed lots) and on two-harvest lots with fertilizing (Fig. 2). On the other hand, a quick and big mortality was recorded on mulched lots, one-harvest lots, and lots under permanent grazing. Slender speedwell disappeared on those lots totally (Fig. 3).

Concerning the diameter of the "rosette", the fastest growing plants were on two and three-harvested lots (130% and 120%), on harvested lots with fertilizing (116%) and on 4-times-a-year in fenced grazing (112%) (Figs. 4, 5). The smallest expansion shortly after planting was recorded on mulched lots (30%) and on lots with permanent grazing (55%) (Figs. 4, 5).

At the end of the experiment, the biggest diameters were recorded on two-harvest lots with fertilizing (62%) and on 4-times-a-year in fenced grazing (59%) (Figs. 4, 5). Slender speedwell survived on two-harvested lots but the plants were small and had the smallest diameters of "rosette" (5%) (Fig. 4).

In total, 18 plants out of 270 survived (Table 2). The biggest individuals were plants with the "rosette" diameter of 1.01 m and 156 terminals (fenced grazing 4 times a year), diameter of 0.87 m and 155 terminals (grazing 2 times a year), and a plant with an diameter of 0.67 m and 131 terminals (fenced grazing 3 times a year). The most successful lots were harvested lots with fertilizing and in fenced grazing 4 times a year, on which 5 and 4 individuals of plants survived two years of the experiment (Table 2).

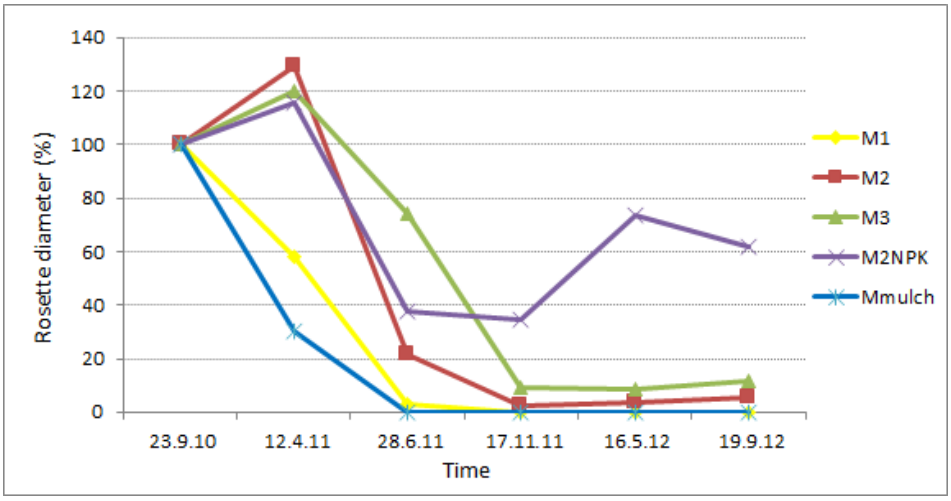

Fig. 2: Number of Sledner speedwell individuals in experimental mowing grassland. Marking of treatments corresponds with Figure 1.

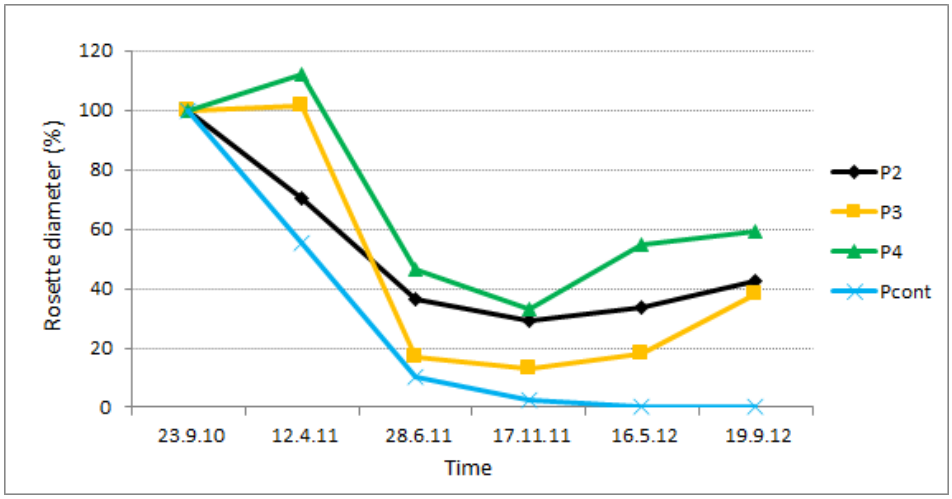

Fig. 3: Number of Sledner speedwell individuals in experimental grazing grassland. Marking of treatments corresponds with Figure 1.

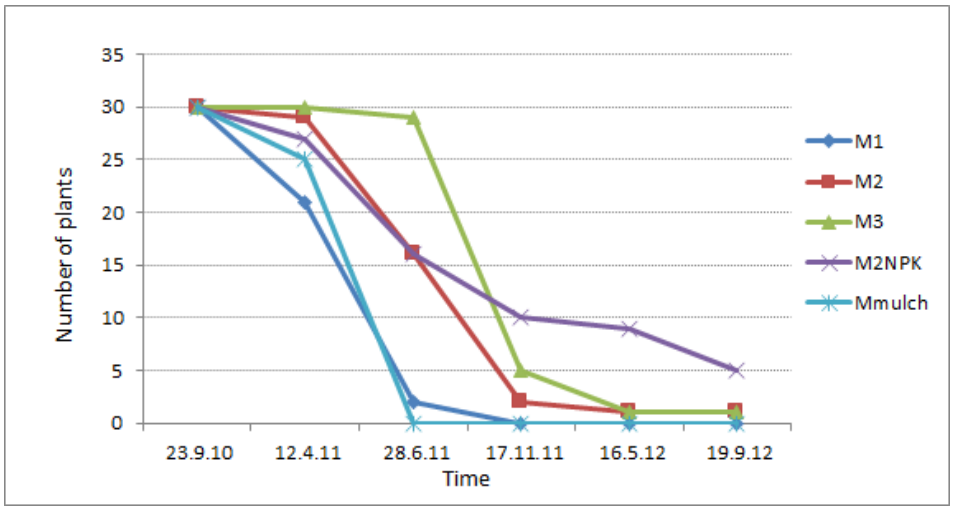

Fig. 4: Percentage of "rosette" diameter of Sledner speedwell in experimental mowing grassland. Marking of treatments corresponds with Figure 1.

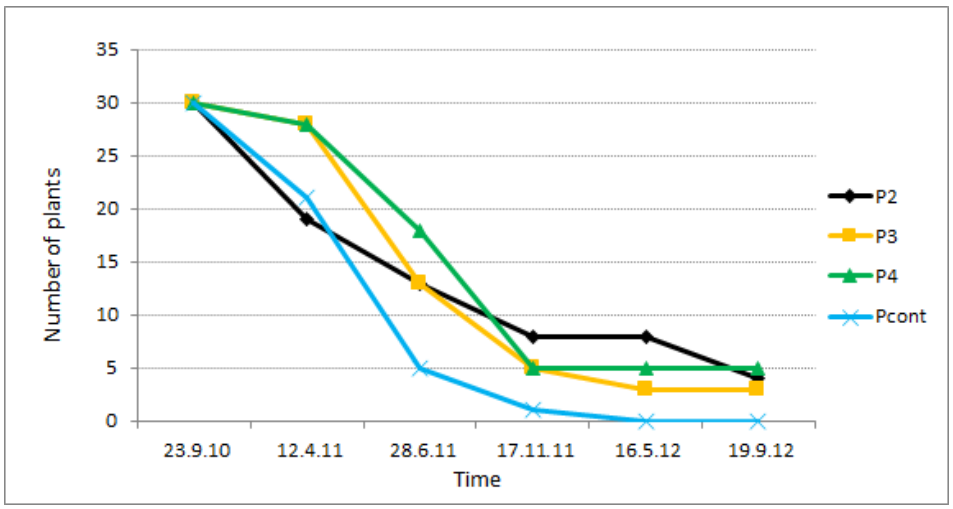

Fig. 5: Percentage of "rosette" diameter of Sledner speedwell in experimental grazing grassland. Marking of treatments corresponds with Figure 1.

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DISCUSSION

The experiment found that Slender speedwell was able to survive two winters on the lots, and for a short period of time also under certain grass cover managements. The ability of expansion was confirmed for some maternal plants (ŠERÁ 2012). The number and size of the plants depended on the particular management. A new finding of this experiment proves that Slender speedwell can prosper in grazed covers. For hilly regions, HEJCMAN et al. (2005) recommend grazing at least once a year, but in our experiment Slender speedwell better prospered in covers grazed 3 and 4 times a year, with a lower and more stable height.

Tab. 2: Characteristics of surviving plants of Slender speedwell in research lots with various management. Marking of treatments corresponds with Figure 1.

Order of plant	Treatment	Rossete diameter			Number of peaks (1)		
		Start of experiment (cm)	Finish of experiment (cm)	Finish of experiment (%)	Start of experiment (1)	Finish of experiment (1)	Finish of experiment (%)
1	M3	9	32	356	1	13	1300
2	M2	11	17	160	3	3	100
3	$M2_{NPK}$	10	46	474	2	31	1550
4	$M2_{NPK}$	6	37	587	1	10	1000
5	$M2_{NPK}$	9	38	427	3	23	767
6	$M2_{NPK}$	11	25	238	1	12	1200
7	$M2_{NPK}$	13	18	135	3	9	300
8	G2	16	36	220	3	11	367
9	G2	11	24	212	2	7	350
10	G2	14	87	613	3	155	5167
11	G2	15	35	240	1	7	700
12	G3	11	34	298	2	8	400
13	G3	10	25	255	2	13	650
14	G3	11	67	588	2	131	6550
15	G4	10	5	51	1	2	200
16	G4	11	23	207	2	12	600
17	G4	10	102	1010	2	156	7800
18	G4	8	39	513	1	85	8500

As was previously proved, Slender speedwell prospers in the vicinity of some dicotyledonous species (e.g. *Lotus corniculatus*) and it usually does not grow near monocotyledonous plants (eg. *Agrostis tenuis*, *Dactylis glomerata*, *Festuca rubra*, *Lolium perenne*) (ŠERÁ 2012). The presented experiment shows that Slender speedwell prospered better on grazed lots (except for permanent grazing) than on harvested covers. Within harvested covers, the most suitable were two-harvest lots with fertilizing (Figs. 2, 4). Dominant species on grazed lots were: *Taraxacum* sect. *Ruderalia*, *Trifolium pratense*, *Plantago lanceolata*, *Cynosurus cristatus*, *Poa pratensis* and *Lolium perenne*. Harvested lots, where Slender speedwell did not prosper (1 x harvested, 2 x mulched) were dominated by wide-leaf grasses: *Arrhenatherum elatius*, *Festuca pratensis* and *Holcus lanatus*. These discussed the dominant plant species were taken from previous work (KOBES et al. 2013).

Intensity of harvesting and removing phytomass reduces the number of grass species and supports dicotyledonous plants which are less competitive (KLAUDISOVÁ & MUDRA 2004; ŠERÁ 2004). On pastures, speedwell can be supported when grazing animals step on the plant and press it to the soil (rooting possibility). Grazing animals might as well transport the creeper plants on short distances (plants fall out of their moths before they swallow them). The amount of above-ground biomass is more balanced on grazed covers during vegetation comparing to harvested covers, there are also fewer old plants and more grass species (HAZI et al. 2012; KOHLER et al. 2004), particularly in fertilized covers (LANTA et al. 2014).

A similar structure can be found in wetter, medium harvested or mulched grasslands on mildly wet soils, where Slender speedwell often propagates. "Leftovers" after grazing, particularly around solid excrements, providing big amounts of nitrogen (HEJCMAN et al. 2004), can also have a positive influence on the development of Slender speedwell.

It seems obvious that survival and growth of Slender speedwell is connected with the structure of the cover in terms of species, height and microclimate in the bottom layer of the cover (because of the delicate size of Slender speedwell). Slender speedwell seems to prosper better in medium high covers (see above) with enough light and humidity. Also changes in microclimatic conditions after harvesting the biomass are probably less distinct. Slender speedwell prospers better on localities rich in nutrients (see results about fertilizing and grazing). WEBER & GUT (2004) describe the risk of non-indigenous species planted as ornamental plants and appearing recently on new locations. They are able to survive in more diverse conditions and covers, they are not categorized as weeds and killed with herbicides. Despite its low growth, Slender speedwell is in the risk category III, like Japanese knotweed or giant hogweed.

Survival and growth of plants might have been influenced by climatic conditions as well. Slender speedwell prospers better in colder and humid climate (HARRIS & LOWELL 1980b). In 2011, the locality of Velký Chuchelec provided good conditions for the growth of Slender speedwell at the beginning of the vegetation period. Later that year, in August, after harvesting and removing the biomass, total rainfall was 50 mm (Table 1) and temperatures were above average. Grass localities were thus exposed to temporary dry conditions, which might have caused mortality or growth ceasing of the tested plats at summer and autumn evaluation (Figs. 2, 4).

Surviving plants were only on some lots and in low numbers (1-5 plants per a treatment, total number of plants 18). The spereading potencial of those plants was enormous, because average number of vegetative peaks per shoot was 38 and summary of the peaks was 688 (Table 2). Those plants were large and richly ramified, usually with adventitious roots and fertile blossoms (seed germination was not tested, but seeds were probably barren). There is an assumption that apexes are a source of vegetative propagation. Our experiment was run on harvested lots with immediate phytomass removal. Cut parts of Slender speedwell could not have been used. Such experiment brings results which are strictly applied to survival and growth possibilities of the planted individual. Above-ground parts of Slender speedwell regenerate best from apexes (ŠERÁ 2012), and can root more easily (in three days, SCALONE & ALBACH 2012). Taking into account the fact that phytomass is usually left on commonly harvested meadows, invasive potential of Slender speedwell is relatively bigger.

Generally, mesophilic meadows are not largely invaded by neophytes, and have thus a relatively low invasibility (CHYTRÝ et al. 2008). Slender speedwell is an invasive species, naturalised in harvested grass covers (KAPLAN et al. 2016). The results of the presented experiment show that not only harvested meadows but also grazed pastures belong to possible biotopes of Slender speedwell. The presented results are of partial character, being a part of a more complex research which includes meadows of various altitudes. All acquired data are being processed and will be gradually published.

SUMMARY

The presented data are results from an experimental location in Velky Chuchelec. The study confirms that Slender speedwell (*Veronica filiformis*) may grow fast in grassland with differend management. The best growth of plants was found in two-years mowed lots with fertilisation and in lots with cattle pasture crops (not permanent cattle grazing). Only small number of individuals survived at plots during two-years experiment, but the potential for the spreading of the species population is great.

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