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Original scientific paper

Nutritive value responses to biological products in alfalfa forage (*Medicago sativa* l.)

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SUMMARY

The effects of the biological insecticides Madex and Agricolle, applied alone or in combination with the biological fertilizers Lithovit and Nagro, on chemical composition and enzyme *in vitro* digestibility, as well as energy feeding value of alfalfa dry mass were studied. Treatments were carried out at the beginning of the flowering stage during the second cutting for control of economically important pests, testing a method of organic pest control. Independent and combined applications of several biological products were found to influence positively the chemical composition, enzyme *in vitro* digestibility, and energy and protein nutritional value of alfalfa feed. An optimal combination of decrease in the content of plant cell wall fiber components and a significant increase in forage enzyme *in vitro* digestibility was revealed after the application of Agricolle and a combination of Agricolle and Nagro. Digestibility reached 60.4 and 59.9%, respectively, which is an increase of 12.0 and 11.0%. Alfalfa low fiber, high protein content, and digestibility made a very good complement to grains and other forages in dairy rations after treatment with biological products.

Keywords: Biopesticides; Biological fertilizers; Forage quality; Alfalfa

INTRODUCTION

Pests in alfalfa are a major challenge. They can inhibit successful stand establishment, reduce yields, lower forage quality, and reduce stand life. That is why most forage crops are grown using pesticides. Chemical control of weeds, insects and other pests has increased agricultural productivity. However, these economic benefits are not without risks in terms of animal health and environmental damage. Feed and fodder offered to animals are often contaminated with pesticide residues (Kumar et al., 2013) and the residues are assimilated by feeding into animal organisms (Prassad & Chhabra, 2001).

Pesticide residues in milk of ruminants is a matter of public health concern since milk and dairy products are widely consumed by people around the world. Also, toxins are transferred from feed to animal products, and then the contaminated products are eaten by humans (Ashraf et al., 2010), which may harm their health in the future.

Biopesticides can be excellent alternatives to chemical pesticides, which are toxic to livestock. They have an increasingly important role in the plant protection

sector (Copping & Menn, 2000), i.e. they are highly efficient, safe and environmentally acceptable (Nathan et al., 2004).

Assessing the quality of resulting forage is seen as an essential step in feed production. Thus, using biopesticides for pest control will help to produce forage with increased nutritional values and digestibility. Studies of nutritional values of feed in organic production are still limited in Bulgaria (Gerdzhikova et al., 2012).

Studies evaluating the energy and nutritional value of fodder crops treated with biopesticides have been insufficient (Georgieva et al., 2014; Nikolova et al., 2015 a,b). It is essential therefore to continue and expand research in that direction.

The aim of this study was to track the changes in the chemical composition, energy and protein nutritional value of alfalfa forage after treatment with organic products.

MATERIAL AND METHODS

A field experiment was conducted at the Institute of Forage Crops, Pleven, Bulgaria from 2015 to 2017. The effects of the biological insecticides Madex and Agricolle, applied alone or in combination with the biological fertilizers Lithovit and Nagro, on chemical composition and enzyme *in vitro* digestibility, as well as energy feeding value of alfalfa dry mass, were studied (test variants are shown in Table 1). Treatments were carried out at the beginning of the flowering stage during the second cutting (BBCH 59-60) for control of economically important pests as a method of organic pest control.

A split-plot design with three replications was used in soil without a pesticide treatment history that is rich in macronutrients.

The main feeding characteristics, i.e. chemical composition and *in vitro* degradability of dry matter, were studied for forage quality evaluation.

The main components of alfalfa forage were determined: crude protein (CP) by Keldahl (N x 6.25), and crude fiber (CF) by Weende system (AOAC, 2001). The content of plant cell wall fiber component, or neutral-detergent fiber (NDF), acid-detergent fiber (ADF) and acid-detergent lignin (ADL), were determined following a systematic detergent analysis of Goering & Van Soest (1970), and degree of lignification-coefficient =ADL/NDFx100. Enzyme *in vitro* dry matter degradability/digestibility (IVDMD) was determined as a percentage by a two-stage pepsin-cellulase method of Aufrere (1982, cited by Todorov, 2010). In a comparative analysis of forage quality between variants of forage treatment, the individual and mean values were evaluated according to their significance for forage quality.

The energy feeding value estimation was performed as: 1. Evaluation of feeding value based on fiber components: relative feeding value, RFV; potential intake of digestible dry matter (Linn & Martin, 1991), digestible dry matter (DDM% = 88.9 - (0.779 x ADF%); dry matter intake (DMI / % body weight / = 120 / NDF%) and relative feeding value (RFV= DDM x DMI / 1.29). 2. Energy feeding value was calculated by the French system UFL-UFV (INRA, 1988), recalculated in Bulgarian; feed units for milk and feed units for growth (FUM-FUG) by coefficients, according to Todorov (1997) and Dutch (VEM-VEVI) systems. The protein feeding value (PDIN=PDIA+PDIMN and PDIE=PDIA+PDIMN in g kg^{-1} dry matter were established by the French system (INRA, 1988) using the parameters: TDP/PBD - total digestible protein/protéines brute digestible, and truly digestible protein in ruminant small intestine, PDIN (protein digestible in intestines, depending on nitrogen) and PDIE (protein digestible in intestines depending on energy).

The results were summarized and presented for a three-year period.

Nine variants were tested:

1. Control (untreated)
2. Madex (600 ml ha^{-1})
3. Agricolle (300 ml ha^{-1})
4. Nagro (500 ml ha^{-1})
5. Lithovit (2000 g ha^{-1})
6. Madex + Nagro (600 ml ha^{-1}+ 500 ml ha^{-1})
7. Agricolle + Nagro (300 ml ha^{-1}+500 ml ha^{-1})
8. Madex + Lithovit (600 ml ha^{-1}+2000 g ha^{-1})
9. Agricolle + Lithovit (300 ml ha^{-1}+2000 g ha^{-1})

RESULTS

The application of the biological products Madex and Agricolle, as well as Nagro and Lithovit (alone and in combination, shown in Table 1), had high positive effects on forage quality in alfalfa. The results showed an increase in crude protein content and a decrease in fiber component (Table 2). Crude protein (CP) data were 11.5% higher, and crude fiber (CF) 6.9% lower on average, relative to the control. The application of Madex and Madex+Lithovit was associated with the highest increase in CP of 15.6 and 15.1%, respectively. The lowest fiber content was found after treatment with Madex (10.0%) and Agricole (9.9%), and the differences compared to control data were statistically significant.

Table 1. Characteristics of products

Commercial product	Dose	Active substance	Producer
Madex, bioinsecticide	600 ml ha^{-1}	granulous virus 3X10 ^13	Switzerland
Agricole, bioinsecticide	300 ml / 100 l water	natural polysaccharides for sticking small insects	Cal-Agri producta LLC, USA
Nagro, bio-organic nano-fertilizer	500 ml ha^{-1}	contains micro and macroelements (molybdenum, magnesium, cobalt, manganese, zinc, iron, copper, boron, nitrogen and phosphorus), mesoelements, microhumans, vitamins, polyacids, amino acids, phytohormones, organic solvents, organic calcium, antioxidants, adoptogens, metabolites, nitrogen fixators	Scientific production association „Bioplant" Russian Federation
Lithovit, natural leaf nano-fertilizer	2000 g ha^{-1}	contains calcium carbonate from natural deposits with micronutrients: 79.19% CaCO3; 4.62% MgCO3; 1.31% Fe	Ctheo Vita Ltd., Germany

Table 2. Principal composition, content of fiber components, and digestibility of dry alfalfa biomass after treatment with biological products

Variants	CP	CF	NDF	ADF	ADL	HEMI	SELLU	LIGNIF	IVDMD	IVTMD
1	155.9 a	344.7 b	469.5 a	410.5 bcd	100.6 b	64.1 a	309.9 abc	21.3 a	53.94 a	53.44 ab
2	180.2 b	310.2 a	499.5 b	424.4 d	94.0 ab	75.1 abc	330.4 d	19.0 a	52.57 a	52.32 a
3	171.0 ab	310.5 a	483.4 ab	401.1 abc	93.6 ab	82.3 bc	307.6 abc	19.3 a	60.41 e	59.72 d
4	176.9 b	318.5 a	490.0 ab	416.7 cd	93.8 ab	73.3 abc	323.0 cd	19.2 a	53.49 a	52.89 ab
5	176.4 b	328.2 ab	468.3 a	383.1 a	87.7 ab	85.2 c	295.5 a	18.7 a	57.95 cd	56.98 cd
6	174.7 b	325.1 ab	467.5 a	395.3 abc	88.4 ab	72.3 abc	306.4 abc	19.0 a	56.64 bc	54.52 abc
7	175.4 b	315.1 a	487.6 ab	416.1 cd	95.5 ab	70.6 ab	320.7 bcd	19.6 a	59.90 de	59.40 d
8	179.5 b	316.0 a	488.6 ab	411.0 bcd	79.1 a	77.7 bc	331.9 d	19.0 a	54.33 ab	53.43 ab
9	174.6 b	318.7 a	478.7 ab	393.8 ab	91.9 ab	84.9 c	301.9 ab	19.2 a	56.63 bc	56.00 bc
$LSD_{0.05\%}$	1.579	2.494	27.006	21.779	19.399	13.217	18.998	2.842	2.381	3.391
Average	173.8	320.8	481.4	405.8	91.6	76.1	314.1	19.4	56.20	55.41

CP - Crude protein, g kg^{-1}; CF - Crude fiber; NDF - Neutral-detergent fiber; ADF - Acid-detergent fiber; ADL -Acid-detergent lignin; % dry matter; HEMI - Hemicellulose; CELLU - Cellulose; LIGNIF - Degree of lignification = ADL/NDFx100; IVDMD - *In vitro* dry matter digestibility; %, IVTMD - *In vitro* total matter digestibility; %, 1 - Control; 2 - Madex; 3 - Agricole; 4 - Nagro; 5 - Lithovit; 6 - Madex+Nagro; 7 - Agricole+Nagro; 8 - Madex+Lithovit; 9 - Agricole+Lithovit; *Means in each column marked by the same letter are not significantly different ($P > 0.05$)

The content of the fraction of structural fibers (polyosides) of plant cell walls had a prevailing trend of decrease relative to the untreated control. An exception was found in the neutral detergent fiber (NDF) content, where a prevailing increase of 2.5% on average was observed. However, no significant differences were found, so that it was insignificant. Only treatment with Madex, Lithovit, and the combination of Madex and Nagro were noted as causing significantly higher NDF contents relative to the control.

The content of acid-detergent fibers (ADF) was 68.4-75.1 g kg^{-1} lower than the content of NDF, while in the course of the plant growing process the trend was different. A statistically considerable decrease in ADF, relative to the control, was found only after using the nano-fertilizer Lithovit (by 6.7%), while its decrease after treatment with Agricolle, Lithovit, and the combinations Agricolle + Lithovit and Madex + Nagro was insignificant. In all other variants, there was a slight increase in ADF values.

The influence of the products on ADL content was entirely positive, associated with a decrease in values from 5.1 (Agricolle + Nagro) to 21.4% (Madex+ Lithovit), and a significant difference was found only for the combination of Madex + Lithovit.

A trend of low total digestible animal polyoside hemicellulose in alfalfa dry mass - from 64.1 to 85.2 g kg^{-1}, was confirmed, as well as a trend of high content of non-digestible cellulose - from 295.5 to 331.9 g kg^{-1}. Lithovit had the most significant influence on hemicellulose content, increasing it 21.1 g kg^{-1} (32.9%) when it was applied alone, and 20.8 g kg^{-1} (32.4%) in combination with Agricolle, while the treatment with Agricolle resulted in 18.2 g kg^{-1} (28.4%) increase.

Table 3. Potential energy and protein feeding value of dry alfalfa biomass after treatment with biological products

Variant	DDM	DMI	RFV	UFL	UFV	FUM	FUG	VEM	VEVI	PBD	PDIN	PDIE
1	56.31 ab	2.46 ab	107.5 ab	0.618 a	0.500 a	0.513 a	0.409 a	790 a	1.714 a	112 a	98 a	81 a
2	55.84 a	2.41 a	104.5 a	0.595 a	0.474 a	0.494 a	0.387 a	782 a	1.701 a	136 b	114 b	84 a
3	57.66 abc	2.49 ab	111.5 abc	0.656 a	0.544 a	0.544 a	0.443 a	826 a	1.768 a	128 b	108 b	86 ab
4	56.44 ab	2.45 ab	107.0 ab	0.616 a	0.497 a	0.510 a	0.406 a	797 a	1.724 a	131 b	110 b	85 a
5	59.07 c	2.57 ab	118.0 c	0.617 a	0.498 a	0.512 a	0.407 a	798 a	1.726 a	132 b	110 b	85 a
6	57.73 abc	2.60 b	116.0 bc	0.625 a	0.507 a	0.518 a	0.414 a	804 a	1.734 a	131 b	110 b	86 ab
7	56.49 ab	2.47 ab	108.5 abc	0.746 b	0.645 b	0.619 b	0.527 b	897 b	1.880 b	132 b	110 b	94 b
8	56.89 abc	2.46 ab	108.5 abc	0.621 a	0.502 a	0.515 a	0.410 a	802 a	1.732 a	135 b	112 b	86 ab
9	58.23 bc	2.51 ab	113.0 abc	0.645 a	0.528 a	0.534 a	0.431 a	819 a	1.758 a	130 b	110 b	86 ab
$LSD_{0.05\%}$	2.326	0.166	10.462	0.077	0.087	0.063	0.067	112.602	104.468	11.171	7.708	8.260
Average	57.18	2.49	110.50	0.638	0.522	0.529	0.426	813	1.749	130	109	86

DDM - Digestible dry matter, %; DMI - Dry matter intake, % of body weight; RFV - Relative feeding value, relative %; UFL (Fr) FUM, VEM (Dutch) - Feed units for milk; UFV (Fr) FUG, VEVI (Dutch) - Feed units for growth, g kg^{-1}; PBD (TDP) - Protéines brute digestible (Total digestive protein), PDIN - Proteins digestible in intestines, depending on nitrogen, g kg^{-1}; PDIE - Proteins digestible in intestines depending on energy, g kg^{-1}; 1 - Control; 2 - Madex; 3 - Agricole; 4 - Nagro; 5 - Lithovit; 6 - Madex+Nagro; 7 - Agricole+Nagro; 8 - Madex+Lithovit; 9 - Agricole+Lithovit; *Means in each column marked by the same letter are not significantly different ($P > 0.05$)

The increased contents of these parameters against the control were statistically established (for the combination Madex + Lithovit as well). The most significant decrease in cellulose content was effected by Lithovit (4.7%) and its combination with Agricolle (2.6%).

Lignification degree was not affected by the action of the tested preparations, and there were no significant differences compared to control data.

Digestibility *in vitro* of alfalfa dry matter after treatment tended to increase, except in the case of Madex and Nagro bio-insecticides. Regarding the other products, whether applied alone or in combination, an increase in digestibility was noted from 0.7 to 12.0% (except Madex + Lithovit). The highest values were found after the use of Agricolle (12.0% increase) and Agricolle + Nagro (11.0% increase). Similar results were obtained for the digestibility of total matter.

Digestible dry matter content (DDM) was lower, compared to the untreated control, only after using Madex (Table 3). A similar trend was observed in relation to the DMI and RFV values after treatment with Madex and Nagro. After treatment with the other products, there was an increase, and it was only significant after using Lithovit (4.9% for DDM and 4.5% for RFV).

The energy and protein nutritional values of alfalfa, the UFL (FUM, VEM) feed units for milk, and UFV (FUG, VEVI) growth units, had the highest values after treatment with the combination of Agricolle and Nagro, as the differences relative to control data were statistically significant. The respective increase was 20.7 (UFL) and 29.0% (UFV). Relative increase in energy nutrition was observed after the application of Agricolle (6.1% for UFL and 8.8% for UFV), and Agricolle + Lithovit (4.4% for UFL and 5.6% for UFV), but the differences were not significant. Madex in combination with the nano-fertilizers Lithovit and Nagro also caused insignificant increase.

Protein nutritional values, measured as total digestible protein (PBD), digestible in the intestine, depending on nitrogen (PDIN) and protein digestible in intestine depending on energy (PDIE), were highest after treatment with Agricolle + Nagro. The increase reached 17.9; 12.2 and 16.0%, respectively, with significant differences relative to the control for the three indicators.

The use of all other products and combinations, regardless of their origin, also had positive effects on the feed protein nutritional value and led to an increase that ranged from 16.1-21.4% (PBD), 10.2-16.3% (PDIN), and 3.7-16.0% (PDIE).

DISCUSSION

Factors that affect forage quality include different plant species, leaf-to-stem ratio, stage of growth, soil microorganisms, climate, harvesting, diseases, and pests (Lascano et al., 2001; Dumont et al., 2014). Insect pests, as one of the factors, can reduce considerably forage yield and quality (NAFA, n.d.; Dellinger et al., 2006). Quesnel (2012) reported that the leafhopper (*Empoasca fabae* Harris) injected a toxin during feeding, which caused a chemical change in plants. They produced less proteins

and more sugars, resulting in a major reduction in feed value. According to the author, protein was the most serious loss of alfalfa due to potato leafhopper feeding. Studies on these problems had also been conducted much earlier. For example, Godfrey et al. (1987) reported that larval feeding of *Sitona hispidulus* (F.) reduced first-harvest crude protein content by ≤1.07%. Crude protein (CP) values also decreased in second and third harvests, in response to earlier presence of *Sitona* weevils.

This study focused on changes in fibrous and protein fractions, changes in fiber digestibility and content of indigestible fiber components, as well as the energy and protein potential feeding value of dry alfalfa biomass as a consequence of treatment with biological products against several key insect pests: leaf aphids, plant bugs, and cicadas. The results of the study showed that crude protein content was lowest in the untreated control (155.9 g kg^{-1}), while crude fiber content was highest (344.7 g kg^{-1}). Biological treatment had an impact on the quality of forage, resulting in an increase in crude protein content and a decrease in fiber content. Similar results were reported by Sulc et al. (2015), showing that crude protein in summer alfalfa averaged 10-22 g kg^{-1} lower ($P < 0.05$) in untreated plants than in those treated early with an insecticide against *E. fabae*. In addition, feeding of that species was found responsible not only for reduced quality (especially lower protein content) but for reduced yield as well, and led to reduced longevity of stands (Kouskolekas & Decker, 1968). According to several studies, the use of biologically active substances has a positive effect on protein content (Petkova, 2006; Stakhova et al., 2000) and leads to increase in crude protein productivity and feed units (Zhelyazkova, 2007; Zhelyazkova et al., 2007).

In the present study, positive influence of the products showed varying degrees, depending on their origin. The optimal matching of increased content of crude protein and reduced content of crude fiber was found in treatments with the bioinsecticides Madex and Agricolle. That was due to the protective seed productivity effect of those products against some key insect pests, such as the seed chalcid *Bruchophagus roddi* Guss., alfalfa plant bug, *Adelphocoris lineolatus* Goeze and pea aphid, *Acyrthosiphon pisum* Harr. (Nikolova and Georgieva, 2018).

The fiber components NDF and ADF increased in most variants, probably due to an increase in parameters such as height, i.e. the productivity of biomass after the use of preparations. A similar trend was observed regarding cellulose and hemicellulose (i.e. increase in content).

Fiber digestibility is an important component of forage quality, having an impact on its intake and digestibility by dairy cows. Lignin is a phenolic compound found in most plant secondary cell walls, and it is indigestible and cross-links with other cell wall components, resulting in decreased cellulose digestibility (Martin, 2007). That leads to reduced digestibility of cellulose, which was also observed in this study.

The degree of lignification decreased under the influence of preparations but varied to different degrees depending on their origin and mechanism of action. The most pronounced decrease was found for Lithovit (12.2%), probably due to its better effect compared to Nagro (unpublished data).

According to Bamualim et al. (1980), lignin content is the best index for preliminary evaluation of digestibility of dry matter in forage legume crops. In that study, reduced lignin content after alfalfa treatment was mainly associated with increased digestibility of dry matter because a strong negative relation was revealed between them. Similar results were reported by other authors (Bamualim et al., 1980; Nikolova et al., 2015a). Absorption is expected to be higher in variations with lower lignin content. That tendency was shown after treatment with the biological foliar fertilizer Lithovit and the bio-insecticide Agricolle.

Contemporary assessment of biomass quality is based mainly on energy feeding values and determined by feed units for milk and growth (UFL (Fr) FUM, VEM and UFV (Fr) FUG, VEVI) (Petkova & Pavlov, 2008).

High digestibility results in higher net energy feeding value after treatment with Agricolle and the combination Agricolle+Nagro. The use of biological products alone reduced the potential net energy feeding value (0.2-3.7% for UFL and 0.4-5.2% for UFV), but combination of the two products was associated with a significant increase, reaching 20.7 and 29.0% for UFL and UFV, respectively. Energy feeding value estimated by Dutch (VEM-VEVI) and Bulgarian (FUM-FUG) systems reflected the same relationships under the influence of the investigated preparations.

Total digestible protein (PBD) after treatment with the test products increased from 16.1 to 21.4%, while PDIN and PDIE values were highest for Agricolle + Nagro, Lithovit and Agricolle. Similar trends were found in other studies (Nikolova et al., 2015a; Georgieva et al., 2017).

High-quality alfalfa is palatable and often maximizes intake and production by dairy cows. Low alfalfa fiber, high protein content, and digestibility after treatment with biological products made a very good complement to grains and other forages in dairy rations.

CONCLUSION

Independent and combined application of biological products had a positive influence on the chemical composition, enzyme *in vitro* digestibility, and energy and protein nutritional values of alfalfa feed.

After applying Agricolle and the combination Agricolle + Nagro, the optimal combination of decrease in the content of plant cell wall fiber components and a significant increase in forage enzyme *in vitro* digestibility was established. Digestibility reached 60.4 and 59.9%, making an increase of 12.0 and 11.0%, respectively.

Low fiber, high protein content and digestibility of alfalfa after treatment with the biological products made a very good complement to grains and other forages in dairy rations.

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Delovanje bioloških preparata na hranljivu vrednost lucerke (*Medicago sativa* l.) kao stočne hrane

REZIME

Proučavano je delovanje bioloških insekticida Madex i Agricolle, primenjenih samostalno ili u kombinaciji sa biološkim đubrivima Lithovit i Nagro, na hemijski sastav i svarljivost enzima *in vitro*, kao i na energetsku vrednost suve mase lucerke kao stočne hrane. Tretmani su izvedeni na početku faze cvetanja u drugom otkosu radi suzbijanja ekonomski značajnih štetočina, a testiran je sistem za suzbijanje štetočina u organskoj proizvodnji. Samostalna i kombinovana primena bioloških proizvoda imala je pozitivno delovanje na hemiski sastav, enzimsku *in vitro* svarljivost, kao i energetsku i proteinsku nutritivnu vrednost lucerke kao stočne hrane. Pokazalo se da primena preparata Agricolle, kao i kombinacije Agricolle i Nagro predstavljaja optimalnu kombinaciju u pogledu smanjenja sadržaja komponenti biljnih vlakana i značajnog uvećanja *in vitro* enzimske svarljivosti. Svarljivost je dostigla 60,4 i 59,9%, respektivno, što predstavlja povećanje od 12.0 and 11.0%. Nizak nivo biljnih vlakana, visok sadržaj proteina i svarljivost lucerke nakon tretmana biološkim proizvodima predstavljaju dragocen doprinos žitaricama i drugoj hrani za ishranu krava.

Ključne reči: Biopesticidi; Biološka đubriva; Kvalitet krme; Lucerka