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Influence of variable mixing rates and nitrogen fertilization levels on the fodder quality of Egyptian clover (*Trifolium alexandrinum* L.) and annual ryegrass (*Lolium multiflorum* Lam.)

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Mixing grasses with legumes for forage production has been a common cropping system, especially in the Mediterranean countries. However, the nutritive value of the end product is greatly dependent on adopting the suitable cultural practices to achieve maximum benefit from the forage mixture, especially the nitrogen (N) fertilizer level and the mixing rate. Thus, the current study was carried out on the winter season of 2012 and 2013 in the experimental station of the Faculty of Agriculture, Alexandria University, Egypt. The main aim was to investigate the variations in 1st cut dry matter (DM) content and nutritive value of Egyptian clover (EC) and annual ryegrass (RG) grown in pure stands and with three mixing rates, under three N fertilization levels (72, 107, and 143 kg N ha^{-1}). Nutritive value was judged upon investigating the crude protein (CP), carbohydrate content, and fiber fractions [neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL)]. Results revealed significant variations among the five forage treatments for all the tested parameters (*P* < 0.001). In addition, significant variations among the three N-levels (*P* < 0.05) and significant two-way interaction among forage treatment and N-level (*P* < 0.01) were declared only in case of 1st cut CP content. In general, pure RG was superior in DM (155.08 g kg^{-1}), carbohydrate (273.00 g kg^{-1}), NDF (505.74 g kg^{-1}), and ADF (279.60 g kg^{-1}) contents. While, pure EC produced the highest-significant CP (155.52 g kg^{-1}) and ADL (23.86 g kg^{-1}) contents. Increasing the applied N-fertilizer level significantly increased the CP content of the sole EC (156.63 g kg^{-1}) and the mixture of 70% EC + 30% RG (132.70 g kg^{-1}). Grass-legume mixtures produced fodder of more balanced nutritive value when compared to the pure stands.

Key words: Forage mixture, Egyptian clover, annual ryegrass, nitrogen fertilization, fodder quality.

INTRODUCTION

Mixture cropping is an important element of self-sustaining, low-input agricultural systems, traditionally adopted in the Mediterranean region (Adesogan et al. 2002). The components of mixtures have the ability to

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utilize the limited resources more efficiently than when grown in pure stands, thus, showing resource complementarity (Atis et al., 2012). Grasses and legumes are considered as important forage crops because of their nutritional value, especially protein content in legumes and crude fiber in grasses (Rakeih et al., 2008). However, monocultures of legumes or grasses do not provide satisfactory results for forage production and nutritive value (Osman and Nersoyan, 1986). Therefore, intercropping of grasses with legumes for forage production has been a common cropping system especially in the Mediterranean countries (Lithourgidis et al., 2006). In mixtures, companion grasses are expected to provide structural support for legumes' growth, improve light interception, and facilitate mechanical harvest, whereas legumes improve the quality of forage (Thompson et al., 1992).

Other benefits of mixtures include increased total dry matter content (Holland and Brummer, 1999), greater uptake of water and nutrients, enhanced weed suppression, providing better lodging resistance, and increased soil conservation (Vasilakoglou et al., 2005), yield stability (Lithourgidis et al., 2006), better hay curing, and forage preservation over pure legumes and may increase crude protein percentage, protein yield, and length of optimum harvest period over pure grasses (Carr et al., 1998). Thus, mixing legume forages with grass forages can be an effective way to improve forage quality and nutritive value of the end product (Ross et al., 2004). Literature also revealed that intake of fodder is low when fed as pure fodders, either of legumes or grasses, compared with their grass-legume mixtures (Ansar et al., 2010)

The successful mixture need to be selected from these forage crops that possess compatible maturity and harvesting schemes, complement each other in growth distribution and ecological niche, and do not, severely, compete with each other for growth and life requirements (Holland and Brummer, 1999; Al-Khateeb et al., 2001). Berseem or Egyptian clover is an annual legume that is a vine with climbing growth habit, great productivity due to its high growth rate and good fodder recovery after cutting, and high levels of crude protein (Vasilakoglou and Dhima, 2008). The mixture of annual ryegrass (*Lolium multiflorum* L.) with Egyptian clover was highly recommended by Rammah and Radwan (1977). It was reported that the mixture of ryegrass and Egyptian clover produced the most superior yield and quality than each of them individually (Nor El-Din, 1978; Said and Sharief, 1993). Thus, Egyptian clover and annual ryegrass may have a potential as winter annual mixtures under the Egyptian conditions.

Despite the advantages associated with mixed cropping, its management is rather difficult than sole cropping due to differences in the agronomic practices of the component crops of the mixture. Differences in sowing time, fertilizer and water requirements, growth behavior, phenology and harvesting time of the associated crops pose many problems to the farmers in managing the mixtures. Hence, devising the suitable agronomic practices for mixtures under different ecological zones had been the aim of many researcher (Tuna and Orak, 2007; Nadeem et al., 2010).

Amongst the most important cultural practices that need to be carefully adjusted to achieve maximum benefit from the forage grass-legume mixtures, are the nitrogen (N) fertilizer level and the appropriate mixing rate. Although, the grass-clover relationship is highly affected by N fertilization (Caradus et al., 1993; Shareif et al., 1996), inconsistent results have been reported on the effects of N fertilization on crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) concentrations of forages (Balabanli et al., 2010). While Min et al. (2002) reported that N application at high rates increased CP concentration compared with the control treatment, but ADF and NDF were not affected, Johnson et al. (2001) reported that NDF concentration of grasses decreased linearly with increasing N fertilization.

Similarly, Adeli et al. (2005) reported that NDF peaked at the low fertilization rate and then decreased with increasing fertilizer rates. On the contrary, Belanger and McQueen (1998) observed an increase in NDF concentration with increasing N fertilization, with a quadratic nature of the response. Other researchers reported that increased N fertilization had almost no effect on NDF concentrations (Anderson et al., 1993; Rogers et al., 1996; Cuomo and Anderson, 1996). These inconsistent results highlight the importance of testing the proposed forage mixtures under varying N-levels in the different agricultural systems. Furthermore, in order to have a balanced nutritional diet for animal, optimum mixing rate should be used (Wiersma et al., 1999). Rammah and Radwan (1977) indicated that when proper mixing rate was used, the mixture significantly out yielded Egyptian clover in pure stands.

The main aim of the current study was to assess the dry matter content and prominent quality measures of Egyptian clover-annual ryegrass mixtures grown with variable grass-legume mixing rates and N fertilization levels, under the Egyptian agricultural system.

MATERIALS AND METHODS

Site description

Field experiments were conducted during the winter seasons of two successive years (2012 and 2013) at the experimental farm of the Faculty of Agriculture, Alexandria University in Alexandria, Egypt. Mechanical and chemical analyses of the experimental soil are presented in Tables 1 and 2, respectively.

Experimental design and treatments

A split plot experimental design, with three replicates, was used to evaluate 2 cultivars and 3 mixtures (seeds were mixed before

Table 1. Mechanical analysis of the experimental soil.

Soil texture	Sand %	Silt %	Clay %	pH	E.C. dS m^{-1}	$CaCO_3$ %
Sandy loam	57.50	30.00	12.50	8.44	1.38	8.20

Table 2. Macro- and micro-nutrients availability in the experimental soil.

Organic matter %	Available N %	Available P ppm	Available K ppm	Ca^{+2} mEq L^{-1}	Mg^{+2} mEq L^{-1}	Na^{+} mEq L^{-1}	Cl^{-} mEq L^{-1}	CO_3^{-2} mEq L^{-1}	HCO_3^{-} mEq L^{-1}
1.50	0.02	4.80	0.89	4.50	8.00	15.16	7.50	0.00	4.60

cultivation) under three nitrogen fertilizer levels. Main plots were assigned to test the nitrogen fertilizer applications, amounting to 72, 107, and 143 kg N ha^{-1}. Nitrogen was applied in the form of ammonium nitrate (33.5% N). The five forage treatments, tested in the subplots, were:

1. 100% sole Egyptian clover (*Trifolium alexandrinum* L.).
2. 100% sole annual ryegrass (*Lolium multiflorum* Lam.).
3. 50% Egyptian clover + 50% annual ryegrass.
4. 70% Egyptian clover + 30% annual ryegrass.
5. 30% Egyptian clover + 70% annual ryegrass.

All the forage treatments were drilled with the recommended seeding rates by the Egyptian ministry of agriculture, amounting to 40 kg ha^{-1} for both the berseem clover and annual ryegrass.

Management and sampling

The experimental plots were sown on the 25th and 29th of October in 2012 and 2013, respectively. The plot size was 3 × 2.4 m. All plots were treated similarly, that is fertilized and cut at the same interval in each growing season. Fertilizer applications were split into equal applications and applied before each cut. Broadleaf and grass weeds were hand-removed from plots and no serious incidence of insects or diseases was observed. Egyptian clover seeds were inoculated by *Rhizobium trifolii*, commercially produced by the Ministry of Agriculture, Egypt, to encourage biological N_2 fixation. First cut was taken at 60 days after sowing (DAS), with 30 days interval between each two successive cuts. Plots were manually harvested with a garden cheers to a 5 cm stubble height. A representative sub-sample of approximately 300 g fresh matter per plot was dried at 60°C until constant weight to determine the dry matter (DM) content.

Analytical procedures

The dried sub-samples were uniformly ground to a particle size of 1 mm. The concentrations of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined sequentially using the semiautomatic ANKOM^{220} Fiber Analyzer (ANKOM Technology, Macedon, NY, USA) as described by Van Soest et al. (1991). The NDF and ADF were analyzed without a heat stable amylase and expressed inclusive of residual ash, while ADL content was corrected after the residual ash content. Ash was determined by combusting the sub-sample in a muffle oven at 550°C for 3 h (Association of analytical communities (AOAC, 1990). Before total nitrogen analysis, the dried samples were ground again to a particle size of 10 µm. The nitrogen content was traditionally analyzed by the Kjeldahl procedure (AOAC, 1990), and crude protein (CP) content was calculated from the N content (CP = N × 6.25). Carbohydrate content was determined using the phenol-sulfuric acid method as described by DuBois et al. (1956).

Statistical analysis

The forage treatments, fertilizer applications as well as their interactions were tested for significance using Proc Mixed of SAS 9.1 (SAS Institute, Inc., 2000). Data from 2012 and 2013 growing seasons were presented in a combined analysis, because the test of homogeneity of variance (Winer, 1971), when performed, revealed that the error of the variance between the two experimental seasons was not significantly different. Only replicates were considered random. The quality data (*Q*) then were analyzed according to the following model:

$$Q_{ijk} = \mu + F_i + N_j + R_k + e_{ijk} + (F\ x\ N)_{ij} + s_{ijk}$$

Where µ is the overall mean, F_i is the forage treatment effect (i = 1,2,3,4,5), N_j is the nitrogen fertilizer level effect (j = 1,2,3), R_k is the replication (k = 1,2,3), e_{ijk} is the effect of main plot, $(F \times N)_{ij}$ is the effect of the interaction between the forage treatment and nitrogen level, and s_{ijk} is the effect of sub-plot.

First cut dry matter content and quality data were presented and discussed. Significance was declared at $P < 0.05$ and means were compared with the least-significant difference (LSD) procedure and probabilities were adjusted using Bonferroni-Holm test (Holm, 1979).

RESULTS AND DISCUSSION

The combined analysis of variance, presented in Table 3, revealed significant variations among the five forage treatments for all the tested parameters ($P < 0.001$). In addition, significant variations among the three N-levels ($P < 0.05$) and significant two-way interaction among forage treatments and N-levels ($P < 0.01$) were declared only in case of first cut protein content.

Effect of forage mixing rates

Dry matter (DM) content

Means presented in Table 4 indicated that the sole RG

Table 3. Mean squares and levels of significance of the 1st cut dry matter DM, CP, carbohydrates, NDF, ADF, and ADL contents (g kg^{-1}) as influenced by the three nitrogen levels (N) and five forage treatments (F) and their interaction.

Effect	DF	Mean squares					
		DM	CP	Carbohydrates	NDF	ADF	ADL
Nitrogen (N)	2	20.19^{ns}	595.69*	432.27^{ns}	43.36^{ns}	59.97^{ns}	4.63^{ns}
Forage (F)	4	1742.46***	2857.12***	29940.39***	35707.93 ***	5371.29***	60.99***
N × F	8	63.09^{ns}	74.61**	600.74^{ns}	111.44ns	17.48^{ns}	0.66^{ns}
C.V.		4.13	3.04	9.92	4.24	3.17	9.76

*Significant at 0.05 level of probability - **Significant at 0.01 level of probability - ***Significant at 0.001 level of probability ns: Non-significant - C.V.: Coefficient of variability.

Table 4. Means of 1st cut dry matter DM, CP, carbohydrates, NDF, ADF, and ADL contents (g kg^{-1}) of the five forage treatments.

Parameter	DM	CP	Carbohydrates	NDF	ADF	ADL
100% Egyptian clover (EC)	120.59^{e}	155.52^{a}	170.89^{b}	389.23^{c}	217.58^{d}	23.86^{a}
100% Annual ryegrass (RG)	155.08^{a}	116.94^{c}	273.00^{a}	505.74^{a}	279.60^{a}	18.22^{b}
50% EC: 50% RG	139.29^{c}	117.53^{c}	167.33^{b}	406.12^{bc}	229.11^{c}	20.10^{b}
70% EC: 50% RG	128.02^{d}	132.82^{b}	157.00^{b}	408.30^{b}	228.98^{c}	23.55^{a}
30% EC: 70% RG	146.84^{b}	111.78^{d}	266.78^{a}	523.90^{a}	248.96^{b}	19.02^{b}
L.S.D.$_{0.05}$	5.55	3.76	19.97	18.45	7.42	1.99

*Means followed by different letter(s) within the same column are significantly different according to the LSD test at 0.05 level of probability.

produced the highest-significant DM content amounting to 155.08 g kg^{-1}. Oppositely, the sole EC produced the lowest-significant DM content (120.59 g kg^{-1}). The difference between the two crops grown in pure stands was 3.5% DM in favour of the RG. Concerning the forage mixtures, it was observed that, increasing the RG proportion in the mixture significantly increased the DM content, therefore, the mixture 30% EC + 70% RG was superior to the other two mixtures in DM production (146.84 g kg^{-1}). The achieved results confirm that mixing forage grasses with EC improves the 1st cut DM content compared to the pure EC stands. Similar results were reported for clover-barley and clover-triticale mixtures (El-Karamany et al., 2012, 2014), also for clover-ryegrass mixtures grown under the organic farming conditions (Salama, 2015).

Crude protein (CP) content

Crude protein content is one of the very important criteria in forage quality evaluation (Assefa and Ledin, 2001; Lithourgidis et al., 2006). Expectedly, the sole EC produced the highest-significant CP content (155.52 g kg^{-1}), followed by the mixture 70% EC + 30% RG (132.82 g kg^{-1}), while CP content of the sole RG was comparable to that produced from the other two mixing rates (Table 4). Low protein levels in cereal forage is not uncommon. Caballero et al. (1995) and Giacomini et al. (2003) mentioned that the forage grass monocultures always produced the lowest-significant CP content compared to pure legume and legume-grass stands. Results of the current study showed that mixing EC with RG produced CP content comparable to that produced by the sole EC and more than that produced by the sole RG. These results were in line with the results of Carr et al. (1998) and Berdahl et al. (2004). Similarly, Lynch et al. (2004) suggested that the legume component (equal or more than 30%) of the binary legume-grass mixture acted as an effective "N-buffer" maintaining forage yield and protein content consistently higher, and within a narrower range across all treatments. Shareif et al. (1996) and Al-Khateeb et al. (2001) came to similar observations. They found that planting of pure EC and its mixtures with the highest seeding rates recorded the highest protein percentages.

Carbohydrate content

Table 4 illustrated that the sole RG as well as the mixture 30% EC + 70% RG were characterized by the highest-significant carbohydrate contents, amounting to 273.00 and 266.78 g kg^{-1}, respectively. On the other hand, the carbohydrate contents produced from the sole EC and the other two mixtures were not significantly different,

Table 5. Means of 1st cut dry matter DM, CP, carbohydrates, NDF, ADF, and ADL contents (g kg^{-1}) as affected by the three nitrogen (N) levels.

Parameter	DM	CP	Carbohydrates	NDF	ADF	ADL
72 kg N ha^{-1}	139.08[a]	121.73b	205.80[a]	444.72[a]	238.76[a]	21.33[a]
107 kg N ha^{-1}	136.77[a]	125.09b	202.33[a]	447.91[a]	241.03[a]	21.20[a]
143 kg N ha^{-1}	138.04[a]	133.93[a]	212.87[a]	447.34[a]	242.75[a]	20.31[a]
$L.S.D._{0.05}$	8.04	7.71	30.68	12.52	4.12	2.08

*Means followed by different letter(s) within the same column are significantly different according to the LSD test at 0.05 level of probability.

with 157 g kg^{-1} as lowest value. Forage legumes are known to have higher protein and cell wall fractions, but lower carbohydrate contents than grasses (Waldo and Jorgensen, 1981). Nor El-Din (1978) indicated that ash and nitrogen free extract were higher and crude protein lower in mixture in comparison with clover monoculture, while the reverse was true in ryegrass in pure stand. Haggag et al. (1995) stated that grasses in monoculture or in mixtures with EC, particularly with the lowest seeding rates of EC (25 %), had the superiority for nitrogen free extract contents. Al-Khateeb et al. (2001) observed low nitrogen free extract content with the highest seeding rate (75%) of EC with several evaluated grasses, particularly for the first cut, which they attributed to the vigorous growth of the tested grasses early in the season.

Fiber fractions

Other important quality characteristics for forages are the concentrations of NDF and ADF (Caballero et al., 1995; Assefa and Ledin, 2001). In the current study, means of the three fiber fractions (NDF, ADF, and ADL), presented in Table 4, revealed that sole RG as well as the mixture 30% EC + 70% RG produced the highest-significant NDF contents, 505.74 and 523.90 g kg^{-1}, respectively. Similarly, the sole ryegrass produced the highest-significant ADF content (279.60 g kg^{-1}), followed by the mixture with the largest RG portion (278.96 g kg^{-1}). Expectedly, the sole forage legume produced the lowest-significant amount from the two fiber fractions, 389.23 and 217.58 g kg^{-1} for NDF and ADF, respectively. Comparing legumes to grasses in the dairy studies is usually confounded by the NDF differences between the two species.

Grasses generally contain more NDF and therefore, when diets are formulated to contain an equal amount of forage DM, the total dietary NDF concentration will be higher for diets containing grasses compared to legumes. Increasing dietary NDF concentration most often has a negative impact on the amount of DM consumed by lactating dairy cows (Allen, 2000) which generally translates into reduced milk production. However, Jung and Allen (1995) reported that the impact of NDF concentration on rumen fill is not always consistent, as it is also influenced by the chemical composition and digestibility of the NDF fraction and particle size.

The study of Cherney et al. (2004) supported this claim, as the 1st cut of both orchard grass and fescue had a significantly higher (20 to 25% units) NDF digestibility than the 1st cut of alfalfa (48% NDF digestibility), but milk production and DM intake were not different between diets containing orchard grass, fescue or alfalfa. Similarly, the study of Weiss and Shockey (1991) reported no difference in milk production or DM intake when comparing orchard grass with 52% NDF to alfalfa with 40% NDF, but NDF digestibility of the orchard grass was 75% compared to 49% for the alfalfa.

Unlike the NDF and ADF, the ADL content reached its maximum values in case of the sole EC and the mixture 70% EC + 30% RG, amounting to 23.86 and 23.55 g kg^{-1}, respectively, while the sole RG and the other two clover-ryegrass mixtures were significantly inferior in their ADL contents. Caballero et al. (1995) and Laidlaw and Teuber (2001) reported that forage legume monocultures are generally higher in lignin content than forage grass monocultures.

This trend in the lignin content is most probably because the cell wall of grasses contains less lignin than the cell wall of dicots (Buchanan et al., 2000; Carpita and McCann, 2000).

Effect of N fertilization levels

Generally, only the CP content varied significantly among the three N-fertilizer levels (Table 5). Increasing the N-fertilizer level from 72 to 143 kg N ha^{-1} significantly increased the CP content from 121.73 to 133.93 g kg^{-1}. The higher crude protein at higher nitrogen levels was mainly due to structural role of nitrogen in building up amino acids. The progressive increase in crude protein contents with increasing nitrogen rates were also reported by many researchers, for example Wolf and Opitz von Boberfeld (2003) and Carpici and Tunali (2012). No significant variations due to the N fertilization levels were detected in case of the DM content, carbohydrates and fiber fractions, their means are presented in Table 5. The NDF and ADF as measures of the energy value of plants, and do not change significantly under the influence of N fertilization (Knežević et al.,

Table 6. Means of the 1st cut CP content (g kg^{-1}) as affected by the interaction between the forage treatments and nitrogen levels.

Forage treatment	Nitrogen fertilizer level (kg ha^{-1})		
	72	107	143
100% Egyptian clover (EC)	142.87^{aB}	156.63^{aAB}	167.07^{aA}
100% Annual ryegrass (RG)	115.70^{cA}	110.77^{cA}	124.37^{cA}
50% EC: 50% RG	114.33^{cA}	114.13^{cA}	124.13^{cA}
70% EC: 50% RG	124.53^{bB}	132.70^{bAB}	141.23^{bA}
30% EC: 70% RG	111.23^{cA}	111.33^{cA}	112.87^{dA}

*Means followed by different small letter(s) within the same column or different capital letter(s) within the same row are significantly different according to the LSD test at 0.05 level of probability. $L.S.D._{row}$ = 15.10, $L.S.D._{column}$ = 5.80

2007; Salis and Vargiu, 2008; Carpici and Tunali, 2012).

Effect of the interaction of forage treatment and N fertilization level

The two-way interaction between the two studied factors was significant only in case of CP content (Table 6). The sole EC stands, followed by the forage mixture 70% EC + 30% RG were significantly superior compared to the other forage mixtures. Especially, under the highest N-level (143 kg N ha^{-1}), the sole EC produced the highest-significant CP content, amounting to 167.07 g kg^{-1}. The second highest CP content was produced by the mixture 70% EC + 30% RG, under the same N-level, and amounted to 141.23 g kg^{-1}. On the other hand, the lowest-significant protein content was a character of the sole RG and the mixtures of 50% EC + 50% RG and 30% EC + 70% RG with no significant variations in their responses to the three N-fertilizer levels.

In general, the response of the investigated forage treatments to the N applications was in the same direction, however, with different magnitude.

Conclusion

The results of the current study clearly indicated that intercropping Egyptian clover with annual ryegrass at different mixing rates resulted in improving the nutritive value and dry matter content of the produced herbage. Pure Egyptian clover stands were superior in the CP content and pure ryegrass stands were superior in the dry matter, carbohydrate and fiber fractions.

However, under the increased N-level application, the forage mixtures gave comparably high CP, carbohydrate and fiber fractions. Conducting animal feeding trials to examine the response of the ruminants to the forage mixtures is, thus, highly recommended.

Conflict of interest

The authors have not declared any conflict of interests.

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