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# CHANGES IN SOME PARAMETERS IN MIXTURES OF SAINFOIN WITH SUBTERRANEAN CLOVER

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## ABSTRACT

Changes in some morphological and physiological parameters (leafs/stems ratio, total plastid pigments content, amount of fixed nitrogen) of sainfoin (*Onobrychis* Adans.) and subterranean clover (*Trifolium subterraneum ssp*.), pure grown and in mixtures in ratio 50 : 50 per cent were studied in filed experiment in the Institute of Forage Crops, Pleven (2011-2015). Local population of sainfoin and three subspecies of subterranean clover, i. e. *Trifolium subterraneum ssp. brachycalicinum* (cv. Antas), *Trifolium subterraneum ssp. yaninicum* (cv. Trikkala) and *Trifolium subterraneum ssp. subterraneum* (cv. Denmark) were used. Leafs/stems ratio of sainfoin in pure stands was 0.35 and it was found be enhanced in mixtures with *Trifolium subterraneum ssp. brachycalicinum* (0.39) by 12.8 per cent, and with *Trifolium subterraneum ssp. yaninicum* (0.38) by 9.5 per cent. Total plastid pigments in mixtures increased by 8.3 per cent for *Trifolium subterraneum ssp. yaninicum ssp. brachycalicinum* and *Trifolium subterraneum ssp. yaninicum,* and by 9.8 per cent for *Trifolium subterraneum ssp. subterraneum,* and decreased in sainfoin by 17.2 per cent for mixture with *Trifolium subterraneum ssp. yaninicum.* There were from 2.20 kg N/da (sainfoin+*Trifolium subterraneum ssp. subterraneum*) to 2.88 kg N/da (sainfoin+*Trifolium subterraneum ssp. brachycalicinum*) more fixed nitrogen in comparison to the amount fixed from pure grown sainfoin. Sainfoin and subterranean clover in mixtures showed good mutual tolerance, morphological and physiological status.

**Key words :** leafs/stems ratio, plastid pigments, sainfoin, subterranean clover, mixtures

Mixed crops between legumes and grasses have an essential role in building a system of sustainable and ecologically friendly farming (Luscher *et al.,* 2014; Kusvuran *et al.,* 2014). They are more effective than pure crop cultivation in using environmental resources, better withstand adverse conditions and are more productive (Porqueddu *et al.,* 2003). Mixtures involved several components, relationships between which are complex and depend on many factors. On the one hand, species are competitors, on the other, interact positively in which many morphological and physiological parameters changed (Baytulin, 2007). The productivity and quality of forage from mixtures depends on the physiological status of the components involved.

One of the most important factors determining productivity, is the photosynthesis process by which green plants accumulate organic matter and energy (Nichiporovich, 1971; Smirnova *et al.,* 2013). Ingestion and transformation of solar energy is achieved by photosynthetic pigments-chlorophyll a and b, and carotenoids. A major chlorophyll is chlorophyll a and it provides higher efficiency of the conversion of carbon dioxide and of water in the organic compounds. Carotenoids also have a protective function-prevent destructive photooxidation of organic compounds of protoplasm in the presence of free oxygen.

Sainfoin is perennial legume crops with high protein content, palatability and high nutritional value properties (Vuèkoviæ, 2004; Delgadom *et al.,* 2008; Turk *et al.,* 2011). It is suitable for pasture use as it does not cause swelling of animals due to the condensed tannins content (Mowrey and Matches, 1991; Rumball and Claydon, 2005; Jacobs and Siddoway, 2007). It can be grown both - pure and in mixtures. Leaves are richer in mineral nutrients than stems (Turk *et al.,* 2011) and the proportion of leaves declines to maturity because of senescence of the lower leaves (Albrecht and Marvin, 1995). Leaf/stem ratio is an important factor in determining quality, diet selection, and forage intake of sainfoin (Arzani *et al.,* 2001).

Sainfoin as a legume crop has nitrogen fixing ability and nitrogen from biological nitrogen fixation is

used directly by plants (Peeters *et al.,* 2006). Many authors (Hume, 1985; Carlson and Huss-Danell, 2003; Provorov and Tikhonovich, 2003, Hardarson and Atkins, 2003, Campillo *åt al.,* 2005; Prosser *åt al.,* 2006) found lower amount of fixed nitrogen compared to other legumes (white and red clover, and alfalfa). As a possible reason, they point to the fact that sainfoin needs twice larger amount of $CO_2$ for 1 mol $N_2$ compared to other legumes included in the study. In addition, sainfoin has less ability to absorb carbon, as well smaller leaf surface. The root system of sainfoin has little major branches and more fine and numerous lateral roots, where the most nodules are located (Tamas and Savatti, 2006). In addition to this. It has small nodule number, low specific nodulating activity and lower effectiveness of the process of nitrogen fixation (Vasileva and Ilieva, 2009; Shakirov *et al.,* 2010).

Subterranean clover (*Trifolium subterraneum* L.) is widespread component in pastures of temperate areas of middle and northern Europe, and America (Nichols *et al.,* 2012). Studies with subterranean clover during last years as a component of pasture mixtures showed that this crop is suitable for the climatic conditions of Bulgaria (Vasilev, 2006; Vasileva and Vasilev, 2012; Ilieva *et al.,* 2015).

Having in mind its positive qualities as a crop it is highly urgent to introduce it in practice. In order to accomplish this goal, it is necessary to study its growth and development in intercropped species with traditional pasture type species as sainfoin.

The purpose of this work was to study changes in some morphological and physiological parameters, i. e. leaf/stem ratio, chlorophyll a and b, carotenoids, total plastid pigments and amount of fixed nitrogen in plants of sainfoin and subterranean clover grown pure and in mixtures with direction of use for forage.

## MATERIALS AND METHODS

The trial was conducted at the experimental field (43° 23'N, 24° 34'E, 230 m altitude) of the Institute of Forage Crops, Pleven, Bulgaria (2011-2015) on podzolized soil sub-type without irrigation. Long plot method was used with size of plots 70 $m^2$ four replicated. Sainfoin (*Onobrychis Adans*.) (local population) and three subclover subspecies, i.e. *Trifolium subterraneum ssp. brachycalicinum* (cv. Antas), *Trifolium subterraneum ssp. yaninicum* (cv. Trikkala) and *Trifolium subterraneum ssp. subterraneum* (cv. Denmark) were the object of study. They were pure grown (100%) and in mixtures sainfoin:subterranean clovers (50 : 50%) as follows: sainfoin+*Trifolium subterraneum ssp. brachycalicinum* (50 : 50%); sainfoin+*Trifoilium subterraneum ssp. yaninicum* (50 : 50%); sainfoin+*Trifolium subterraneum ssp. subterraneum* (50 : 50%).

The sowing was done in autumn 2011 and sowing rates were for sainfoin 12 kg/da and for subterranean clover 2.5 kg/da, between rows space 11.5 cm. The sowing rate in mixtures was half of that in pure stands. No fertilizers and pesticides were applied during the vegetation. One cut was obtained during the first year after sowing (2012), two cuts during the second and third year (2013 & 2014) and one cut during the fourth year (2015). Samples were taken before the cutting from all replications using 0.25 $m^2$ quadrate, separated into leaves and stems and weighted (g fresh weight). Leaf/stem ratio based on fresh weight, was calculated.

In fresh plant samples, plastid pigments content (chlorophyll a, chlorophyll b and carotenoids) was determined according to Zelenskii and Mogileva (1980). For the mixtures samples were taken from every component. Ratios chlorophyll a/chlorophyll b, chlorophyll a+b, chlorophyll a+b/carotenoids and total plastid pigments content were calculated.

A formula of Carlsson and Huss-Danell (2003) was used for roughly estimation of fixed amount of nitrogen in sainfoin (pure grown and in mixtures with subterranean clover). Data were averaged and statistically processed using SPSS (2012).

## RESULTS

The period of study was characterized as an unfavorable in regard to the agro meteorological conditions. Data for mean air temperature and cumulative rainfall was shown on the Table 1.

After the sowing long dry period occur. Small quantities and unevenly distributed rainfall in May of the second year marked the beginning of early spring drought lasts one month. The period is accompanied by average monthly temperatures with values above the norm for the area. After the first ten days of July lasting drought occur and continued in August and September. The average annual temperature in the third year had higher values (12.3 °C), there are no extremely high temperatures and prolonged dry periods, and rainfall (856.8 l/m2) were greatest. These conditions impacted

TABLE 1
Agro meteorological conditions for the period of study

| Months | 2012 | | 2013 | | 2014 | | 2015 | |
|---|---|---|---|---|---|---|---|---|
| | °C | l/m2 | °C | l/m2 | °C | l/m2 | °C | l/m2 |
| January | -5.2 | 17.8 | 0.5 | 19.0 | 0.9 | 41.8 | 2.0 | 14.4 |
| February | 8.5 | 7.6 | 3.9 | 60.9 | 2.6 | 3.2 | 2.3 | 39.2 |
| March | 14.8 | 46.3 | 6.3 | 39.6 | 9.7 | 76.9 | 6.8 | 68.4 |
| April | 17.4 | 85.2 | 14.0 | 50.8 | 12.2 | 139.8 | 12.1 | 43.6 |
| May | 24.1 | 40.3 | 19.5 | 63.7 | 16.7 | 83.0 | 18.7 | 30.4 |
| June | 27.8 | 1.4 | 21.3 | 112.4 | 20.6 | 54.3 | 20.8 | 95.7 |
| July | 25.8 | 35.6 | 22.7 | 105.8 | 23.1 | 71.8 | 24.9 | 21.5 |
| August | 21.1 | 21.0 | 24.9 | 20.2 | 23.7 | 23.9 | 24.4 | 30.2 |
| September | 15.0 | 56.0 | 18.4 | 15.8 | 17.9 | 142.6 | 20.0 | 130.1 |
| October | 8.1 | 4.0 | 12.2 | 59.2 | 12.1 | 79.1 | 10.9 | 92.7 |
| November | -1.0 | 56.7 | 8.7 | 29.1 | 5.6 | 48.1 | 10.1 | 50.1 |
| December | -5.2 | 17.8 | 0.2 | 2.3 | 2.0 | 92.3 | 5.5 | 0.9 |
| Avd/summ | 13.0 | 416.1 | 12.7 | 578.8 | 12.3 | 856.8 | 13.2 | 617.2 |

on the overall development of sainfoin and subterranean clover.

Leaf/stem ratio in subterranean clover in mixtures increased according to the size of the leaves of subspecies. Thus, the values of the leaf/stem ratio were higher than those in pure stands namely, *Trifolium subterraneum ssp. brachycalicinum* (1.05 vs. 0.94), *Trifolium subterraneum ssp. yaninicum* (0.94 vs. 0.86) and *Trifolium subterraneum ssp. subterraneum* (0.84 vs. 0.79).

The leaf/stem ratio in sainfoin retain unchanged in the first year after sowing only, when the agrometeorological conditions were very unfavorable. In subsequent years of study the leaf/stem ratio had higher values in the both components of mixtures as compared to pure stands. In the second year of study, the leaf/stem ratio was found higher - to 16.6 per cent in sainfoin and to 12.9 per cent in subterranean clover. The tendency for the highest leaf/stem ratio in *Trifolium subterraneum ssp. brachycalicinum* retained.

In mixtures with *Trifolium subterraneum ssp. brachycalicinum* leafs/stems ratio in sainfoin (0.39) was found significantly increased (by 12.8%), as well in these with *Trifolium subterraneum ssp. yaninicum* (0.38) (by 9.5%).

Values of the chlorophyll a+b/carotenoids ratio

TABLE 2
Leaf/stem ratio in sainfoin and subterranean clover (pure and in mixtures)

| Treatments | 2012 | | 2013 | | 2014 | | 2015 | |
|---|---|---|---|---|---|---|---|---|
| Sainfoin (100%) | 0.34 | | 0.37 | | 0.35 | | 0.35 | |
| Sainfoin + | 0.36 | +5.9 | 0.43 | +16.6 | 0.39 | +11.1 | 0.40 | +14.1 |
| *Trs brach* (50:50) | 1.05 | +11.7 | 1.12 | +12.9 | 1.17 | +9.7 | 1.12 | +10.9 |
| Sainfoin + | 0.35 | +2.9 | 0.41 | +10.8 | 0.38 | +9.7 | 0.39 | +11.0 |
| *Trs yanin* (50:50) | 0.94 | +9.3 | 0.97 | +7.9 | 1.02 | +9.5 | 1.03 | +8.3 |
| Sainfoin + | 0.35 | +2.9 | 0.40 | +8.4 | 0.36 | +3.3 | 0.37 | +4.4 |
| *Trs subter* (50:50) | 0.84 | +6.3 | 0.93 | +8.9 | 0.97 | +0.8 | 1.00 | +0.8 |
| *Trs brach* (100%) | 0.94 | - | 0.99 | - | 1.07 | - | 1.01 | - |
| *Trs yanin* (100%) | 0.86 | -8.5 | 0.90 | -9.1 | 0.93 | -13.1 | 0.95 | -5.9 |
| *Trs subter* (100%) | 0.79 | -16.0 | 0.85 | -14.1 | 0.96 | -10.3 | 0.99 | -2.0 |
| SE (P=0.05) sainfoin | 0.03 | | 0.02 | | 0.03 | | 0.02 | |
| SE (P=0.05) subclover | 0.02 | | 0.02 | | 0.02 | | 0.02 | |
| ±SD sainfoin | 0.01 | | 0.03 | | 0.02 | | 0.02 | |
| ±SD subclover | 0.09 | | 0.09 | | 0.09 | | 0.06 | |

in mixtures were lower for sainfoin (4.73-5.08) and in subterranean clovers were closed to its pure grown (5.28-5.41). The lower values of chlorophyll a+b/carotenoids of sainfoin in mixtures with *Trifolium subterraneum ssp. brachycalicinum* and *Trifolium subterraneum ssp. subterraneum* associated with the increasing content of carotenoids and in mixture with *Trifolium subterraneum ssp. yaninicum* - by reducing the contents both, chlorophylls and carotenoids.

Total plastid pigments content in sainfoin pure grown was 282.94 mg/100 mg FW (Figure 2). The values of total plastid pigments content in subterranean clover were closed -*Trifolium subterraneum ssp. subterraneum* (296.66 mg/100 mg FW), *Trifolium subterraneum ssp. yaninicum* (305.85 mg/100 mg FW) and *Trifolium subterraneum ssp. brachycalicinum* (307.95 mg/100 mg FW).

The amount of fixed nitrogen in mixtures was changed and depends on the competition between the components. It is evident that amount of fixed nitrogen obtained from mixtures on average for the period was from 2.20 kg N/da (sainfoin + *Trifolium subterraneum ssp. subterraneum*) to 2.88 kg N/da (sainfoin + *Trifolium subterraneum ssp. brachycalicinum*) more compared to the amount from pure grown sainfoin (Figure 3).

## DISCUSSION

According Mohajer *et al.* (2012) the variation in the morphological parameters is significant. Leafs/stems ratio is an important morphological characteristic. It is associated with quality and forage intake (Cohenm, 2001). In our study, leafs/stems ratio in sainfoin has the lowest values in the first year after sowing in pure stands (0.34) as well in mixtures (0.35 - 0.36) with little variation (Table 2). Slightly higher values of this ratio in mixtures with subterranean clover were not statistically proven. This is understandable because of the unfavourable meteorological conditions. Similar results obtained Kallenbach *et al.* (1996). The age of the crop had effect on the value of the leafs/stems ratio, too.

The leafs/stems ratio in subterranean clover in pure stands strongly varied, i.e. *Trifolium subterraneum ssp. brachycalicinum* (0.94) and *Trifolium subterraneum ssp. subterraneum* (0.79). Morphological feature of *Trifolium subterraneum ssp. subterraneum* are small in size leaves.

Many authors found higher leafs/stems ratio led to higher both, quality and intake of forage (Kallenbach *et al.,* 1996; Smart *et al.,* 1998; Arzani *et al.,* 2001).

Given that leaves of sainfoin are more palatable and retain higher digestibility over time than stems (Mohajer *et al.,* 2013) it is desirable high and balanced leafs/stems ratio (Kirilov, 2010). Improvement of leafs/stems ratio of sainfoin could therefore have a practical effect on animal preference. Improvement of leafs/stems ratio of sainfoin could therefore have a practical effect on animal preference. It has an even greater significance in view of the less leaves surface in this crop (Hume *et al.,* 1985).

TABLE 3
Total plastid pigments content in sainfoin and subterranean clover, grown pure and in mixtures

| Treatments | Chl. a | Chl. b | Chl. a/b | Chl. a+b | Carote noids | Chl. a+b/ carotenoids |
|---|---|---|---|---|---|---|
| | | | **mg/100 mg FW** | | | |
| Sainfoin (100%) | 145.74 | 93.31 | 1.56 | 239.05 | 43.89 | 5.45 |
| Sainfoin + | 139.77 | 92.61 | 1.51 | 232.38 | 45.76 | 5.08 |
| *Trs brach* (50:50) | 165.81 | 114.51 | 1.45 | 280.32 | 53.05 | 5.28 |
| Sainfoin + | 118.62 | 74.74 | 1.59 | 193.36 | 40.92 | 4.73 |
| *Trs yanin* (50:50) | 161.35 | 117.88 | 1.37 | 279.23 | 51.98 | 5.37 |
| Sainfoin + | 146.32 | 94.36 | 1.55 | 240.68 | 49.57 | 4.86 |
| *Trs subter* (50:50) | 159.14 | 115.84 | 1.37 | 274.98 | 50.81 | 5.41 |
| *Trs brach* (100%) | 149.65 | 112.13 | 1.33 | 261.78 | 46.17 | 5.67 |
| *Trs yanin* (100%) | 153.65 | 104.24 | 1.47 | 257.89 | 47.96 | 5.38 |
| *Trs subter* (100%) | 145.78 | 105.79 | 1.38 | 251.57 | 45.09 | 5.58 |
| SE (P=0.05) sainfoin | 6.50 | 4.68 | 0.02 | 11.14 | 1.81 | 0.15 |
| SE (P=0.05) subclover | 2.84 | 2.36 | 0.03 | 4.62 | 1.27 | 0.06 |
| ±SD sainfoin | 13.00 | 9.37 | 0.03 | 22.29 | 3.62 | 0.31 |
| ±SD subclover | 6.36 | 5.28 | 0.06 | 10.33 | 2.85 | 0.14 |

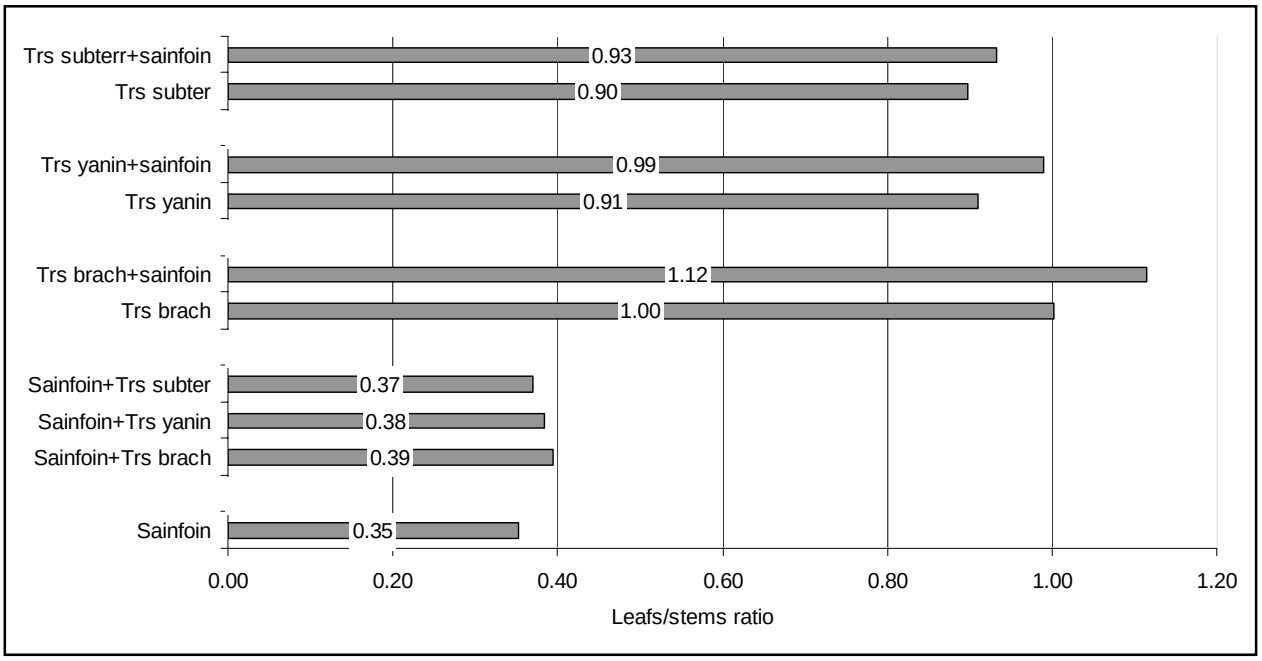


Fig. 1. Leaf/stem ratio in sainfoin and subterranean clover – pure and in mixtures (on averaged for period) (for sainfoin, ±SD=0.02, SE (P=0.05)=0.03; for subterranean clover, ±SD=0.08, SE (P=0.05)=0.04 Plastid pigments content Chlorophyll a and b and carotenoids are the main photosynthetic pigments.

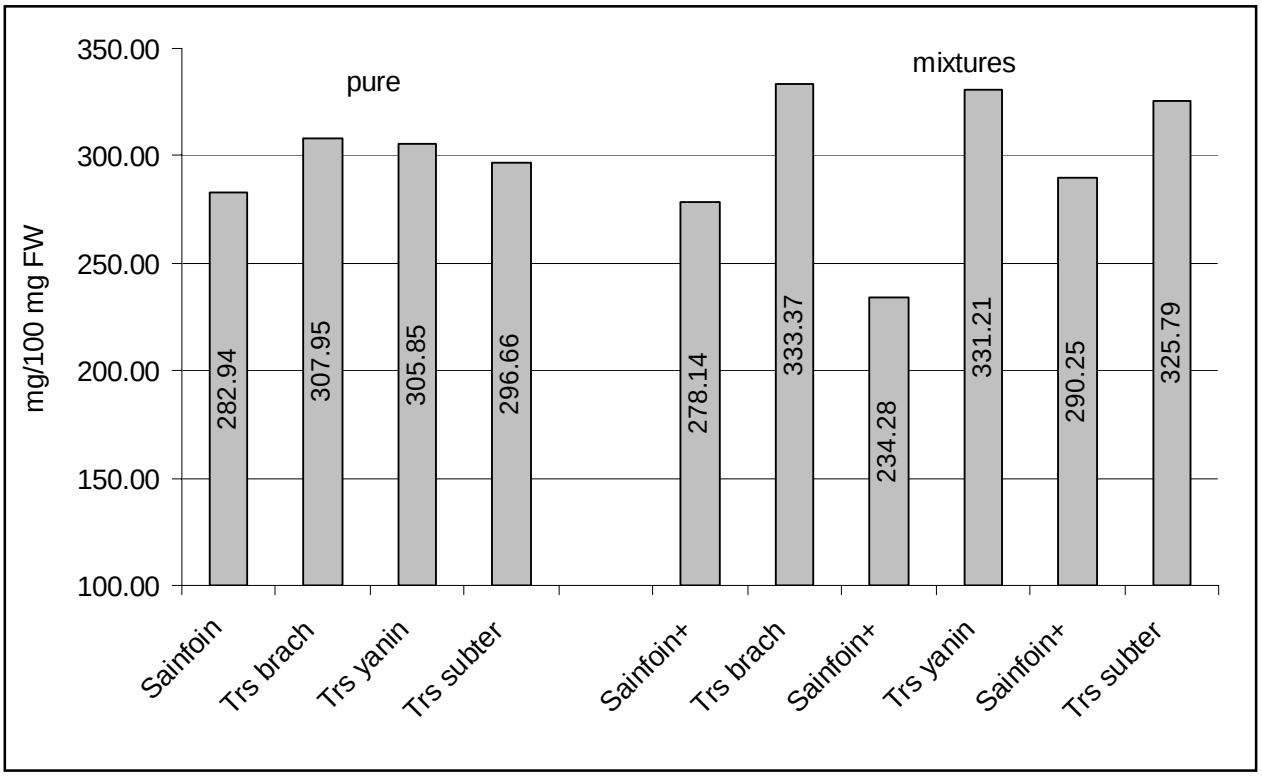


Fig. 2. Total plastid pigments content in sainfoin and subterranean clover, grown pure and in mixtures (for sainfoin, ±SD=25.24, SE (P=0.05)= 12.62; for subterranean clover, ±SD=5.83, SE (P=0.05)= 13.03).

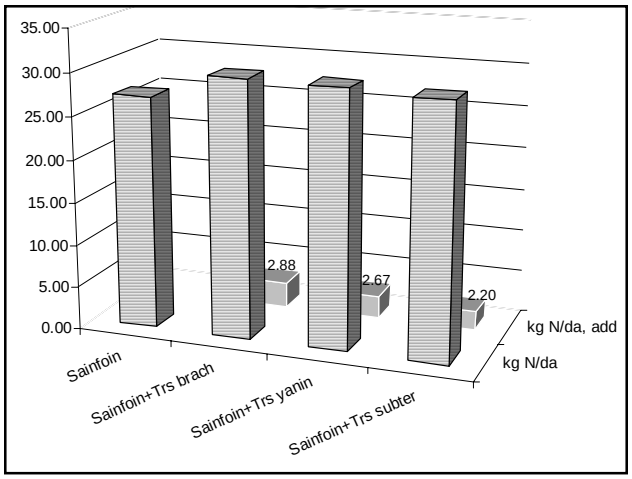


Fig. 3. Amount nitrogen fixed in sainfoin grown pure and in mixtures with subterranean clover for four-year period (±SD=1.32, SE (P=0.05)=0.66).

In subterranean clover, leaf size contributes to plant competitivity in mixtures (Pecetti and Piano, 1998). Competition for major nutritive elements, as well as environmental factors such as light, heat ect. in mixture with *Trifolium subterraneum ssp. subterraneum* was less and it was the reason for higher variation in values of that ratio. Cohen (2001) found strong relationship between leafs/stems ratio and crude protein content in subterranean clover.

Data for leafs/stems ratio on averaged for the period (Fig. 1) showed that in pure grown sainfoin the value was 0.35. Our results correspond to those of Mohajer *et al.* (2012), who found 0.39 (min-max, 0.16-0.88). Compared with other legume crop (birdsfoot trefoil) leafs/stems ratio was significantly lower (Soster et al., 2004; Ayres *et al.,* 2008; Ducati *et al.,* 2015).

Leafs/stems ratio in subterranean clover was found be higher as compared to the same in pure stands, i. e. in *Trifolium subterraneum ssp. brachycalicinum* (1.12 vs. 1.00) by 11.2% and in *Trifolium subterraneum ssp. yaninicum* (0.99 vs. 0.91) by 8.7%, respectively.

For the degree of formation of the photosynthetic apparatus is judged by the ratio of chlorophyll a/chlorophyll b. This is related to the basic activity of chlorophyll a. It is relatively constant and is considered genetically determined value (Titova, 2010). Obtained in our study value for chlorophyll a/chlorophyll b ratio in pure grown sainfoin was 1.56, and in mixtures with subterranean clover ranged from 1.51 to 1.59 (Table 3). The chlorophyll a/chlorophyll b ratio in pure stands of three subterranean clover subspecies ranged in the limits from 1.33 to 1.47 and in the mixtures with sainfoin from 1.37 to 1.45.

Chlorophyll a+b/carotenoids ratio is also important as a characteristic of the photosynthetic apparatus and respond to changes in environmental factors (Titova, 2010). In our study values for pure grown crops are 5.45 for that ratio in sainfoin and from 5.38 to 5.67 in subterranean clover.

Total plastid pigments content in mixtures of sainfoin with *Trifolium subterraneum ssp. brachycalicinum* and *Trifolium subterraneum ssp. subterraneum* retain almost unchanged in sainfoin and increased by 8.3% in subclovers compared to pure stands (Figure 2). Total plastid pigments content in sainfoin in mixture with *Trifolium subterraneum ssp. yaninicum* decreased by 17.2%.

Photosynthetic pigments increased in subterranean clover in three mixtures studied, i.e. by 8.3% for *Trifolium subterraneum ssp. brachycalicinum* and *Trifolium subterraneum ssp. yaninicum*, and by 9.8% for *Trifolium subterraneum ssp. subterraneum*.

Changes in the total plastid pigments content in leaves of sainfoin and subterranean clover subspecies are also related to the leafs/stems ratio. The coefficient of correlation between leafs/stems ratio and total plastid pigments content was found r=0.81.

Competition for soil nitrogen in mixtures can have a beneficial effect on the nitrogen fixing process, as in the case sainfoin and subterranean clover have the same type nitrogen metabolizm. They fixed nitrogen from the atmosphere through nitrogenase in the nodules. According Sebastia *et al.* (2004) legumes effectively regulate processes nodulation and nitrogen fixation in mixtures.

Changes in morphological and physiological parameters of crops studied are related to the metabolic role of nodules which represent a powerful acceptor for carbon assimilates from leaves, mainly in the form of transport amino acids and amides (Atkins *et al.,* 1986).

Based on the morphological and physiological parameters studied could be summarized that sainfoin and subterranean clover grown in mixtures in ratio 50:50% showed good mutual tolerance, morphological and physiological status.

## CONCLUSIONS

Leafs/stems ratio of sainfoin pure grown was 0.35 (0.34-0.37) and increased by 9.5% in mixtures with *Trifolium subterraneum ssp. yaninicum* (0.38) and by

12.8% in mixtures with *Trifolium subterraneum ssp. brachycalicinum* (0.39).

There were from 2.20 kg N/da (sainfoin + *Trifolium subterraneum ssp. subterraneum*) to 2.88 kg N/da (sainfoin + *Trifolium subterraneum ssp. brachycalicinum*) more fixed nitrogen obtained in comparison to the amount from pure grown sainfoin.

Total plastid pigments in mixtures increased by 8.3% for *Trifolium subterraneum ssp. yaninicum ssp. brachycalicinum* and *Trifolium subterraneum ssp. yaninicum,* and by 9.8% for *Trifolium subterraneum ssp. subterraneum,* and decreased in sainfoin by 17.2% for mixture with *Trifolium subterraneum ssp. yaninicum.*

Coefficient of correlation between leafs/stems ratio and total plastid pigments was found be 0.81.

Sainfoin and subterranean clover in mixtures showed good mutual tolerance, morphological and physiological status.

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