Enhancement of total lipid production in vegetative tissues of alfalfa and sainfoin using chemical mutagenesis

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Abstract

Alfalfa (*Medicago sativa* L.) and sainfoin (*Onobrychis viciifoila* Scop.) are two key forage legumes for the western Canadian cattle industry. Despite the high protein content, drawbacks to their use exists, including inefficient protein digestibility and energy use efficiency in the ruminant system leading to economic losses and negative environmental impacts. Increasing the proportion of lipids in the diet of cattle is known to mitigate greenhouse gas emissions; however, the above two forage legumes possess only trace quantities of lipids in the shoot tissues used by the ruminants. In the current study, chemical mutagenesis was used as a conventional breeding approach to enhance lipid levels in the vegetative tissues of alfalfa and sainfoin. The mutagenesis procedures for these two forages need to be firmly established. We developed protocols for ethyl methanesulfonate (EMS)-mediated mutagenesis by optimizing mutagen concentration and seed soaking duration. EMS-treated populations were assessed for morphological variants and total shoot lipid content (TSLC). Fatty acid composition was examined in a subset of plants with increased TSLC. Within 24 months, the screening process identified mutagenized plants with significant increases in TSLC (3 - 5% on a dry weight basis) and a subset of these also displayed alterations in fatty acid composition in both species. These genotypes provide a novel source of germplasm for the future improvement of these two forage species.

Key words: Legume forages; *Medicago sativa* L., *Onobrychis viciifoila* Scop., ethyl methanesulfonate, shoot tissue, fatty acids

Abbreviations

EMS	Ethyl methane sulfonate
FAMEs	Fatty acid methyl esters
GC	Gas chromatography
HCl	Hydrochloric acid
Hrs	Hours
LD-50	Median lethal dose

LSD	Least significant difference
Mbp	Mega base pair
MS	Mass spectrometry
NIR	Near infrared resonance
PROC GLM	General linear model procedure
PROC MIXED	Mixed model procedure
PUFA	Poly unsaturated fatty acids
MUFA	Mono unsaturated fatty acids
SAS	Statistical analysis software
TAG	Triacylglycerol
TSLC	Total shoot lipid content

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important forage crops worldwide due to a plethora of beneficial characteristics (Radovic et al., 2009). Although it provides superior protein content and relatively high yields, alfalfa use is limited by the fact that it causes pasture bloat, which can be lethal for ruminants (Berg et al., 2000). Sainfoin (*Onobrychis viciifolia* Scop.) is another member of the Fabaceae family that is rich in proteins. Unlike alfalfa, however, it contains favourable secondary plant metabolites such as condensed tannins (Koivisto and Lane, 2001), which are responsible for its status as a bloat-free forage legume. Unfortunately, the widespread cultivation of sainfoin is limited due to a lack of adapted varieties with good performance for a wide range of environments.

Alfalfa is an autotetraploid (2n=4x=32) with a genome size of 800-1000 Mbp (Blondon et al., 1994; Biazzi et al., 2017). Sainfoin is another tetraploid (2n = 4x = 28). New sainfoin populations developed in western Canada are now recommended to be used as a mixed stand with alfalfa due to their bloat-free nature and ability to survive and regrow with alfalfa (Acharya et al., 2015).

However, little is known about its genetics with the exception of a limited number of genetic markers and some transcriptomics data (Kempf et al., 2016; Mora-Ortiz et al., 2016) as until recently this crop was considered less productive than alfalfa.

Induced mutagenesis is one of the most widely used methods in the development of desirable traits in various crop species, and has been used to introduce genetic variation in legumes for decades (Tadege et al., 2009). Unlike transgenic methods, chemical mutagenesis can be applied to most species, including those with limited genomic information such as sainfoin. Ethyl methanesulphonate (EMS) is a chemical mutagen that is commonly used to generate mutant populations containing large numbers of point mutations, and has been used successfully to carry out phenotypic and genotypic screens of legumes such as *Medicago trucatula*, birdsfoot trefoil (*Lotus japonicas*), soybean (*Glycine max*), peanut (*Arachis hypogaea*), chick pea (*Cicer arietinum*), common bean (*Phaseolus vulgaris*), field pea (*Pisum sativum*) and fenugreek (*Trigonella foenum-graecum*) (Carrol et al., 1985; Hoffman et al., 1999; Perry et al., 2003; Basu et al., 2007; Cooper et al., 2008; Dalmais et al., 2008; Muehlbauer and Rajesh, 2008; Le Signor et al. 2009; Porch et al. 2009; Ramos et al. 2009) EMS-mediated mutagenesis, however, has yet to be firmly established in either alfalfa or sainfoin.

Livestock production, and particularly that of ruminants, has been suggested to be responsible for approximately 37% of global anthropogenic methane emissions (FAO) and it has been found that supplementing feed with moderate amounts of oil reduces methane production by ruminants (Bayat et al., 2018; Singer et al., 2018). Indeed, it has been predicted that for every 1% increase in feed lipid content, methane emissions could be reduced by up to 5.6% (Beauchemin et al., 2008). This phenomenon likely occurs, at least in part, because the amount of methane generated is correlated with the quantity a ruminant eats (Cosgrove et al., 2004), and increasing the proportion of lipids in feed would augment its caloric density and reduce intake. Dietary lipids have also been suggested to reduce the activity of methanogens and protozoal numbers (Johnson and Johnson, 1995). Unfortunately, the majority of plants produce only very low levels of lipids in their vegetative tissues, and alfalfa has been found to have the lowest total fatty acid content of a range of forage grasses and legumes tested previously (Boufaïed et al., 2003a,b). Since the cost of supplementation with exogenous lipids can be prohibitive and under extensive grazing situations impractical, increasing total shoot lipid content (TSLC) in forages would provide a

beneficial alternative. Enhancing TSLC would also have the added benefit of improving cattle productivity, and it has been estimated that increasing leaf lipid content to between 7-8% DW would yield a 10% increase in energy without impairing rumen function or causing milk fat depression (Flowers et al., 2008).

Mutagenesis has previously been used as a means of increasing lipid content in oil crops (Cvejić et al., 2011; Khan and Tyagi, 2013; Davis, 2015) and microalgae (Kawaroe et al., 2015; Nojima et al., 2017). In addition, mutagenesis has been used to alter the fatty acid composition of acyl lipid in both seed tissue (Wilcox et al., 1984; Green, 1986; James and Dooner, 1990; Rowland, 1991; Osorio et al., 1995; Rahman et al., 2013) and vegetative tissue (Browse et al., 1993; Cantisán et al., 1999) of oleaginous plants, and in microalgae (Kawaroe et al., 2015). Information on modification of lipid metabolism in legume vegetative tissue through mutagenesis, however, is lacking. Recent progress has been made in terms of increasing lipid levels in vegetative tissues through the introduction of foreign transgenes, and in at least one case, oilseed-like amounts of TAG (30-33%) were achieved in tobacco leaf tissues through the metabolic engineering of multiple genes (Vanhercke et al., 2017). While such extreme elevations in leaf oil contents will no doubt be of use in many other crop species, increases above approximately 8% dry weight in forages are not desirable due to possible negative effects on livestock performance and quality (Palmquist and Conrad, 1978).

Given the controversy surrounding genetically engineered crops and regulatory hurdles associated with the introduction of a new genetically engineered forage crop, it seemed reasonable that a chemical mutagenesis approach widely accepted in breeding programs may be useful in developing perennial forage legumes with enhanced lipid content in vegetative tissue. In addition, the mutagenesis approach has the added advantage in that it may reveal new gene targets for increasing the lipid content of vegetative tissue.

In the current study, we started with optimization of EMS treatment parameters for alfalfa and sainfoin, and produced mutagenized population followed by selection of plants with increased total shoot lipid content (TSLC) in both species.

Materials and methods

Plant materials

AC Blue J alfalfa and AAC Mountainview sainfoin seeds (both developed at the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre) were used throughout this study. These cultivars were selected for their adaptation to Western Canadian conditions in addition to their high biomass yield, tolerance to diseases and longevity. AAC Mountainview sainfoin also has the ability to grow with alfalfa in mixed stands to prevent bloat in grazing cattle (Mora-Ortiz et al.. 2016).

Optimization of chemical mutagenesis of alfalfa and sainfoin

Initially, dry seeds were treated with up to 2% (v/v) EMS, but as there were no signs of any morphological variations, all subsequent assessments were carried out using pre-soaked seeds. To determine an optimum EMS concentration for each species, alfalfa and sainfoin seeds were pre-soaked in distilled water for 6 hrs at room temperature. Excess water was then drained from the seeds, which were left moist overnight to imbibe. Subsequently, the seeds were separated into batches of 100 seeds and treated with freshly prepared EMS at four different concentrations (0, 0.5, 1.0 and 2% v/v) for three different seed treatment periods (6, 12 and 24 hr). Each of the twelve treatments (4 concentrations x 3 treatment periods) was repeated three times. Following EMS treatment, the seeds were rinsed 15-20 times with tap water and were kept moist until radicle emergence.

To measure LD-50 values, sainfoin and alfalfa seeds were subjected to narrower EMS concentrations based on the preliminary germination data. EMS treatments from 0.5-1.0 % (0, 0.6, 0.7, 0.8 and 0.9% (v/v) with a 12 hr time interval were selected for alfalfa. For sainfoin, EMS concentrations between 1.0-2.0 % (0, 1.2, 1.4, 1.6 and 1.8% (v/v) with a 12 hr time interval were selected. Following EMS treatments, the seeds were rinsed 15-20 times with tap water. Seeds with radicles were planted in trays containing soil-free mix and kept in the greenhouse.

Radicle lengths were measured on 25 random seeds for each treatment and replication for both species 72 hrs following EMS treatment, before the seeds were transplanted into trays. Seed germination counts were based on seedling emergence, and were determined 7 and 14 days after planting. All the above experiments were repeated three times. The control (0 % EMS) treatment was considered 100% germination and other treatments were adjusted accordingly to determine their percentage germination. In the case of alfalfa, seedling height was measured two weeks

after sowing. For sainfoin, leaf numbers were assessed on each plant three weeks after seeding since EMS-treated plants showed variability for this trait, but not height, at this stage. Plants showing albino or xantha phenotypes were counted and were excluded from further examinations.

Selection of plants containing high total shoot lipid content

Alfalfa and sainfoin plants were allowed to grow for 6-8 weeks until they were at the 10% bloom stage. The plants were then cut back to approximately five centimeters from the surface of the growing media, and were allowed to regrow. Initially, in order to determine the most suitable plant growth stage at which to sample plants for TSLC, whole plants were harvested at immature (completely vegetative stage, with shoots comprising leaves and stems) and mature (10% bloom, with shoots comprising leaves, stems and flowers) growth stages, respectively. For this test, four replicates of 12 alfalfa plants each for each treatment (12x4=48 total) were used. All subsequent experiments were carried out using whole shoots (with bloom, stems and leaves) harvested from plants at the 10% bloom stage for the determination of TSLC.

Since triacylglycerols (TAG) stored in guard cells are broken down to provide ATP required during light-induced stomatal opening and can be used at night as a source of energy when plants cannot photosynthesize (McLachlan et al.. 2016), whole shoots from alfalfa and sainfoin were also collected at three different times of the day (7.00 a.m., 11.00 a.m. and 3.00 p.m.) for lipid analysis to observe the effect of harvesting time on TSLC content.

Out of 6000- 7000 plants of alfalfa and 4000-5000 plants of sainfoin, morphologically normal plants were selected after 6-8 months for further analysis. Total of selected 1300 alfalfa and total of 700 sainfoin shoot samples from all the treatments were oven dried (45ºC) and ground to pass through a 0.5mm sieve prior to the determination of TSLC using near-infrared (NIR) spectrometry (Foss NIR systems 6500) programmed for measuring forage quality parameters including protein, fat, dry matter and crude fibre. Near infrared spectroscopy has been used previously for screening plants in breeding projects as a cost effective method in general, and has been specifically utilized to reveal foliar oil characteristics in a *Melaleuca cajuputi* breeding population (Schimleck et al.. 2003). In our study, all plant samples included 3-4 biological samples and three technical replicates, check and blank cell values were utilized as controls.

Forage plants with higher levels of TSLC were then subjected to NIR again, using 3-4 biological replicates and three technical replicates per plant. The selection of plants were continued for the next generation using the same process after crossing the selected parent population by introducing bees (www.biobestgroup.com).

Lipid extraction and fatty acid composition analysis

In order to determine shoot fatty acid composition in selected high lipid genotypes, total lipid extractions were carried out as described previously (Pan et al. 2013; Xu et al. 2018) with slight modifications. In this study, all plant samples included 3-4 biological samples and three technical replicates, Samples were freeze dried and ground in a small coffee grinder, and approximately 100 mg of dried tissue was used for subsequent lipid extraction. One hundred micrograms triheptadecanoin (C17:0 TAG) was used as an internal standard, and tissue samples were homogenized twice in 1.5 ml of 2:1 chloroform: methanol (v/v) through vigorous shaking with glass beads (0.5mm) in a mini bead beater (Biospec Products, Inc. Bartlesville, OK USA) for 2 min. After brief centrifugation, the phase containing the extracted lipids was collected and ¼ volume of 0.9% NaCl was added. Following centrifugation at 3000 rpm for 5 min, the chloroform phase (lower phase) was collected and dried under a stream of nitrogen gas. Trans-methylation was carried out by incubating in 1 ml of 3N methanolic HCl (Supelco, Sigma-Aldrich, Oakville, Ontario) at 80°C for 1 hr. The resulting fatty acid methyl esters (FAMEs) were extracted twice using hexane and were once again dried under nitrogen gas. Dried samples were then re-dissolved in 0.5 ml of iso-octane containing C21:0 methyl ester (0.1 mg/ml) for analysis using gas chromatography (GC)-mass spectrometry (MS). For GC analysis (Agilent Technologies 7890A GC system, Agilent Technologies Canada Inc., Mississauga, ON), a split/splitless inlet was used and the injection volume was 1 µl in the ten-to-one split mode. A DB-23 capillary column (Agilent Technologies: 30 m × 250 µm × 0.25 µm) was used for FAME separation with helium as the carrier gas (1.2 ml/min). The temperature program was as follows: 165℃ for 4 min, 165-180℃ for 5 min, and 180-230℃ for 5 min. Constituents were determined using MS (Agilent Technology 5977A Mass Selective Detector, Agilent Technologies Canada Inc., Mississauga, ON) and peaks were identified using the software NIST MS Search 2.0 from the National Institute of Standards and Technology (NIST, Gaithersburg, MD).

Statistical analysis

Data were analyzed using PROC MIXED and PROC GLM procedures of SAS (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was carried out to observe if there were any significant ($P < 0.05$) interactions between EMS concentrations and treatment periods on total lipid content and composition. When the interaction effect was found to be significant, the least significant difference (LSD) ($P < 0.05$) test was used to separate treatment means.

Results

Effect of chemical mutagenesis on seed germination and seedling development

In initial experiments, testing of EMS concentrations up to 2% (v/v) on the germination of alfalfa and sainfoin seeds indicated that seed treatment without pre-soaking resulted in 100% germination and no morphological variants in mutagen-treated plants. Conversely, pre-soaking seeds in water and then treating with EMS in general progressively delayed seed germination and seedling emergence as the concentration of the mutagen, and length of treatment, increased. While the percent germination of sainfoin was not significantly different at 0% and 0.5% (v/v) EMS concentrations, regardless of the treatment period, the percent germination of alfalfa seeds treated with 0.5% EMS (v/v) for 24 hr was significantly reduced compared to 6 hr and 12 hr treatment periods, as well as the 0% EMS control (Figures 1a and b). The percent germination of alfalfa at 2% (v/v) EMS was < 10% for all treatment periods, while that of sainfoin was less than 25% for 12 hr and 24 hr treatment periods, but above 40% with a 6 hr treatment (Figures 1a and b). As such, sainfoin seeds displayed higher levels of germination compared to alfalfa at higher EMS concentrations at all three treatment periods (6, 12 and 24 hr). Indeed, the 50% survival level (LD-50) for alfalfa was found to occur with a 0.95% (v/v) EMS concentration and a 12 hr treatment, while that of sainfoin required a 1.5% (v/v) EMS concentration with 12 hr treatment (Figure 1).

The effect of EMS concentration and length of treatment was further assessed by measuring radicle length two days and one week after treatment for alfalfa and sainfoin, respectively. Mean radicle length was significantly reduced with increased EMS concentration in most cases among 0%, 0.5% and 1% (v/v) EMS concentrations, but not between 1% and 2% EMS concentrations, for both alfalfa and sainfoin (Table 1). In addition, there was a significant decrease in radicle length in alfalfa as the time of EMS exposure increased for 0% and 0.5% EMS concentrations,

while there was a significant increase in radicle length in sainfoin with a 24 hr treatment at 0% and 0.5% concentrations (Table 1).

Morphological phenotypes of mutagenized alfalfa and sainfoin

The effectiveness of each EMS treatment was also assessed by measuring seedling height for alfalfa, while in sainfoin, the number of true leaves were counted instead as this species showed more variability for this trait. In alfalfa, progressive stunting of plants was noted at increased EMS concentration assessed regardless of the treatment period, while the number of true leaves produced in sainfoin was reduced between 0.5% and 1% EMS concentrations regardless of the treatment period (Table 2). Other morphological phenotypes (xantha, albino or dwarf) (Figure 2) were also observed in mutagenized alfalfa and sainfoin plants. While alfalfa plants exposed to EMS for 12 hr and 24 hr both exhibited 1% albino and 2% xantha phenotypes after transplanting, the majority of sainfoin plants displaying either of these phenotypes during initial growth stages did not survive long and were therefore not assessed further. In addition, approximately 5% of transplanted sainfoin plants exhibited a dwarf phenotype, with EMS treatment compared to no treatment whereas alfalfa plants with such a phenotype were very rare (approximately 0.04%) (Figures 2a and c). After transplanting seedlings into pots with soil-free mix, approximately 20% of total alfalfa plants and 30% of total sainfoin plants died within 7-10 months regardless of the treatment.

Effect of maturity and sampling time on total shoot lipid content

The TSLC was significantly higher in mature alfalfa shoots (10 % bloom) compared to immature shoots (completely vegetative before flowering) in untreated alfalfa plants (Supplementary figure 1). In terms of the effect of time of day on TSLC, although immature shoots sampled at 11.00 am showed significantly lower TSLC compared with 7.00 am, harvesting time in the greenhouse did not have any other significant effect on TSLC. Based on these results, forage samples were collected in the afternoon (between 1.00 - 3.30 pm) at the mature stage (10% bloom) for all subsequent experiments in order to maximize lipid content in samples and identify plants with higher TSLC.

Selection of alfalfa and sainfoin plants with increased total shoot lipid content

The TSLC on shoot samples from EMS-mutagenized alfalfa 1300 plants and sainfoin 700 plants (including 0% EMS controls) collected from greenhouse ranged from 1.7 to 5.3% and 1.0 to 3.3% DW, respectively. Overall, mean TSLC values for sainfoin were slightly lower (1.8 - 2.1 % DW) than those obtained for alfalfa (2.9 - 3.5 % DW) (Table 3). Only a few means among the four EMS concentrations and three treatment durations were found to be significantly different in either forage legume. Overall, there appeared to be little correlation between TSLC and either EMS concentration or length of treatment (Table 3). Total of ~1,300 alfalfa and 700 sainfoin plants that had been treated with various concentrations of EMS (0% - 2% (v/v)) were analyzed for TSLC, only about 5% of plants showed higher TSLC than the overall mean for the species. While open pollinated alfalfa and sainfoin plants treated with 0 % EMS exhibited average TSLC of approximately 3.2% DW (range of 1.71% to 4.05%) and 1.89% DW (range of 1.75% to 2.6%), respectively, TSLC above 5% and 3% DW were observed in alfalfa and sainfoin selected plants from the EMS treated population (16 alfalfa plants and 9 sainfoin plants showing the highest TSLC), respectively (Figure 3a and b). None of the alfalfa and sainfoin plants showing high TSLC exhibited any other obvious morphological or developmental changes compared to plants containing average TSLC levels. Following the initial assessments, the plants showing high TSLC were cut back and allowed to regrow to the 10% bloom stage, and were then sampled for re-assessment of TSLC using NIR. The protein content ranged between 25.52% to 31.75% DW in alfalfa and 22.87% to 30.01% in sainfoin without showing significant changes compared with controls (24.91% to 32.02% DW in alfalfa and 22.51 to 30.12% DW in sainfoin) (Supplementary table 1). Plants of high TSLC (about 30-50 alfalfa plants showing TSLC above 4% and 20-30 sainfoin plants showing TSLC above 2.7%) were selected and shoot cuttings were used for clonal propagation. Assessment was continued for the next generation after crossing the selected plants by introducing bees. Seeds of those sainfoin and alfalfa selected plants were collected and planted. The TSLC and protein content of the next generation plants were assessed as the same as their parents (Table 4). The average TSLC in both sainfoin and alfalfa from the next generation population (400 alfalfa and 300 sainfoin plants) were significantly higher than the controls (3.85% TSLC in alfalfa treated versus 3.04% TSLC in alfalfa controls and 3.41% TSLC in sainfoin treated versus 1.96% TSLC in sainfoin controls). 10 each alfalfa and sainfoin plants were selected for further fatty acid composition analysis based on the highest TSLC (over 5% and 3% DW respectively) showing no morphological deficiencies. There were no significant

changes in protein content for the selected plants with high TSLC compared with controls similar to their parents.

Fatty acid composition of total shoot lipids from selected plants

Out of EMS-mutagenized alfalfa 1300 plants and sainfoin 700 plants, sixteen alfalfa and 9 sainfoin plants were selected based on the highest TSLC over 5% and 3%, respectively (Figure 3). Ten alfalfa plants and 8 sainfoin plants showing the highest TSLC without any morphological deficiencies were further subjected to fatty acid composition analysis and compared with control plants treated with 0% EMS. In control plants, the major fatty acids extracted were palmitic acid (16:0), palmitoleic acid (16:1Δ^{9cis}; hereafter 16:1), stearic acid (18:0), oleic acid (18:1Δ^{9cis}; hereafter 18:1), linoleic acid (18:2$\Delta^{9cis,11cis}$; hereafter 18:2), α-linolenic acid (18:3$\Delta^{9cis,11cis,15cis}$; hereafter 18:3) and arachidic acid (20:0), which correlated with results from previous studies (Boufaïed et al., 2003; Toral et al., 2016). Margaric acid (17:0) and heneicosylic acid (21:0) were used as standards for normalization. The relative abundance of each fatty acid was calculated as a percentage of the total absolute content of the aforementioned seven fatty acids (Figure 4). A sample GC-MS chromatogram showing resolved fatty acid methyl esters prepared from total lipid extracted from alfalfa is shown in Supplementary figure 2. The most abundant fatty acid in control plants was 18:3, which represented approximately 50% of the TSLC. Linoleic acid and 16:0 each accounted for approximately 20% of the TSLC, whereas oleic and arachidic acid each represented the lowest proportions of total fatty acids.

Interestingly, most of the selected plants with high TSLC also showed significant alterations in fatty acid composition compared to the controls (0% EMS; Figure 4). For example, changes in the proportions of poly- and mono-unsaturated fatty acids (PUFAs and MUFAs) such as 18:1, 18:2 and 18:3 were observed in many cases, as well as signficant changes in the percentages of 16:1 and 18:0. For example, 7 of the 9 high TSLC alfalfa plants showed significantly higher proportions of 18:3 compared to controls (Figure 4 a), and two of these also displayed a concomitant significant reductions in the proportions of 18:1 and18:2 (Figure 4a). Only one of the selected alfalfa plants showed a significant reduction in the proportion of 18:3 and a concomitant increase in 18:2 compared to the control treatment. Conversely, 6 of 8 high TSLC sainfoin plants exhibited significant elevations in 18:2 content, with two of these also displaying concomitant decreases in 18:3 and one displaying a concomitant increase in 18:3 (Figure 4b).

Only one high TSLC sainfoin plant showed a significant decrease in the proportion of 18:2 and a significant increase in the proportion of 18:3 (Figure 4b). From the next generation, 10 each alfalfa and sainfoin selected plants showing the highest TSLC over 5% and 3%, respectively, were subjected to fatty acid composition analysis and compared with control plants of no treatment (Figure 5). Fatty acid composition changes were significant in the selected plants with TSLC and apparently transferred to the next generation. Interestingly, changes in MUFAs and PUFAs such as 18:1, 18:2 and 18:3 in the next generation of alfalfa were similar to their parents (Figure 5a). In the next generation of sainfoin however, 18:2 level changes were significantly lower in all of the selected plants with high TSLC and significantly higher levels of 16:0, 18:1 and 20:0 (Figure 5b). Only one sselected sainfoin plant from the next generation showed a significant increase in 18:3 content. The clonal propagation and the selection process is in progress.

Discussion

Alfalfa has long been domesticated as a forage legume and genetic research with this crop species is accumulating as of late. As such, genomic resources are beginning to become available for this species (https://plantgrn.noble.org/AGED/). Conversely, as sainfoin's popularity as a forage species has only begun in recent years, only very limited genomic information is available due to paucity in genetic research activities. In any case, both crops would benefit substantially from improvement of their nutritive value in order to reduce drawbacks in terms of ruminant productivity, energy use efficiency and environmental sustainability. Forage lipids, which can vary with harvesting season and type of forage (Dewhurst, 2010), are known to improve ruminant productivity by positively influencing the fatty acid compositions of milk and meat (Cosgrove et al., 2004; Flowers et al., 2008). In addition, lipids provide a higher caloric value than the other main energy sources such as carbohydrates and protein in vegetative tissues of forages, and thus lipid supplementation tends to increase energy use efficiency and decrease methane emissions due to reductions in intake (Beauchemin et al., 2008; Bayat et al., 2018). Since alfalfa and sainfoin have low concentration of PUFAs compared to other legumes and do not have substantial variability in TSLC (Boufaïed et al., 2003a,b), improvement of these traits using a conventional breeding approach such as chemical mutagenesis would be highly valuable.

EMS-mutagenesis has been carried out successfully for a variety of non-TSLC-related traits in other legume species previously (Carrol et al., 1985; Hoffman et al., 1999; Perry et al., 2003; Basu et al., 2007; Cooper et al., 2008; Dalmais et al., 2008; Muehlbauer and Rajesh, 2008; Le Signor et al., 2009; Porch et al., 2009; Ramos et al., 2009) and irradiation-based mutagenesis has been conducted on alfalfa and sainfoin (Ehsanpour and Razavizadeh, 2005; Mohajer et al., 2014; Beyaz et al., 2016). In addition, although EMS-mutagenesis for crop improvement has been attempted previously in alfalfa and sainfoin (More, 1992; Yun et al., 2014; Hendon et al., 2018), precise protocols have yet to be established in either case. As such, it was necessary to develop optimized protocols for each species. As is the case in other crops, phenotypic variations in alfalfa and sainfoin populations treated with EMS can be used to suggest the presence of possible genetic variations within the mutagenized population (Porch et al., 2009). Therefore, the mutagenic effect of EMS on alfalfa and sainfoin was ascertained in this study through the observation of higher proportions of seedling death, stunted plants, and albino or xantha phenotypes compared to control plants treated with 0% EMS (Table 1 and Figures 1 and 2). Although the radicle length decreased with higher mutagen concentrations, and plant height (alfalfa) or the number of true leaves (sainfoin) were also inversely correlated with EMS concentration (Tables 2 and 3), after approximately seven weeks of growth, the majority of plants recovered fully and very few plants exhibited visual differences or morphological traits. It is therefore important to observe the plants at an early growth stage to estimate the effectiveness of the EMS treatment.

Both tetraploid legumes displayed the highest survival levels with a 6 hr EMS soaking time, and showed more variability in TSLC than 12 and 24 hr treatments. This suggests that a 6 hr treatment in EMS is ideal to generate mutants at a higher frequency than 12 or 24 hr in both legumes (Figures 1 and Table 3). If a 6 hr treatment period were used, the ideal EMS concentration (yielding an LD50) would be 0.5 and 1% for alfalfa and sainfoin, respectively (Figure 1), suggesting that the seeds of sainfoin can tolerate a higher exposure to EMS than alfalfa based on initial assessments of plant survival.

Interestingly, different species and even varieties of the same species often show diverse responses to mutagenic treatments (Wu et al., 2005). For example, high rates of lethality and low mutation frequencies have been obtained in certain species such as rice (*Oryza sativa*) at higher

mutagen concentrations (Till et al., 2007). In contrast, it has been suggested that EMS-mediated mutation frequency could be increased without adverse effects in paleopolyploid soybean because of the genetic redundancy provided by the largely duplicated gene set (Cooper et al. 2008). Therefore, it is clear that one of the key factors in the efficiency of mutagenesis is mutagen concentration, which can be challenging to optimize in terms of balancing plant survival and mutation frequency. As both alfalfa and sainfoin are both tetraploid species, ploidy level was not considered responsible for sainfoin's increased tolerance to EMS in this case. Previous studies also suggested that EMS-mediated mutation frequency is independent of genome size and the mutation sites are distributed randomly (McCallum et al., 2000; Penmetsa and Cook, 2000; Henikoff and Comai, 2003).

For the selection process we identified alfalfa and sainfoin plants with significant increases in TSLC at all three EMS concentrations (0.5%, 1% and 2%) and all three seed soaking times (6 hr, 12 hr and 24 hr). Indeed, the soaking time and mutagen concentrations for EMS treatment had no significant effect in terms of increasing the average TSLC in these forage crops (Table 3). From optimization stand point it was important to use different EMS concentrations and treatment durations to determine an appropriate treatment for mutagenesis in the two species. However, for crop improvement it was important to identify a number of mutants from each of these two open-pollinated species with high TSLC regardless of the mutagenic treatment to ensure seed production through intercrossing the selected plants. In this study, the highest TSLC contents (above 5% in alfalfa and above 3% in sainfoin) were found from the EMS mutagenized population. Thus, the higher TSLC levels of the forage plants in this study may be mostly due to the mutagenic effect combined with natural genetic variation. Further research is necessary to confirm the mutations of corresponding biosynthetic genes in plant lipid metabolism. Both natural and EMS variation may have led to a selection gain in the next generation. Both alfalfa and sainfoin have partial self-incompatibility and show severe inbreeding depression if forced to self-pollinate. It is also important to note that TSLC levels upwards of approximately 8% are not desirable in forage crops due to potential negative effects on rumen function and an increase in the incidence of milk fat depression (Flowers et al. 2008). The moderate increases obtained in this study are ideal for this purpose and could be further increased in the future through the crossing of high TSLC mutagenized genotypes and directional selection for higher TSLC (Tadege et al., 2009).

In terms of fatty acid composition, the most substantial changes in high TSLC genotypes observed were alterations in the levels of C18:1, C18:2 and C18:3 PUFAs and MUFAs, which are known to be important in reducing cardiovascular disease and hyperlipidemia (Henikoff and Comai, 2003; Singh et al., 2019). Previous evidence has indicated that the C18:3 content of milk fat in dairy cows is directly influenced by C18:3 concentrations in forage (Hebeisen et al., 1993; Penmetsa and Cook, 2000). Furthermore, increased proportions of PUFAs in beef are associated with grazing or feeding forages enriched with PUFA-rich oil (Van Nevel and Demeyer, 1996; LaBrune et al., 2008). Similarly, increased levels of PUFAs such as linoleic acid in forage and/or feed have also been shown to increase levels of conjugated linoleic acids, which have a wide range of health benefits, in ruminant products (Mir et al., 2014). The evidence on beneficial effects of high oleic acid content or MUFA content in forage on beef and dairy industry is limited. However, oleic acid (18:1) have anti-inflammatory and antioxidant properties with beneficial effects on heart health by decreasing both inflammation and oxidative stress (Singh et al., 2019). Therefore, increasing PUFAs and MUFAs in forage may positively influence the animal productivity, quality of milk and meat from ruminants, which would promote human health and nutrition. However, fatty acid composition differences in high TSLC plants compared to controls may not demonstrate that this effect is only due to EMS mutagenesis. While several of the high TSLC alfalfa and sainfoin plants with increases in 18:2 exhibited this trait at the expense of 18:3, and vice versa, five alfalfa plants were identified with significant increases in 18:3 and no concomitant reduction in 18:2, and two high TSLC sainfoin plants were identified with increases in 18:2 and no concomitant decrease in 18:3 (Figure 4), which suggests an overall increase in PUFA concentration. Therefore increasing PUFA in forage may influence the milk and meat production of ruminants that benefits human health and nutrition. Further selection for 18:3 plants will be necessary in each species if improvement in these traits is important in these two open pollinated species. Studies are currently being carried out to confirm the stability of increased TSLC and PUFA content in subsequent generations, and whether directed selection can increase the TSLC further (Tadege et al., 2009). The mutagenized populations developed in this study (with or without high TSLC) also have the potential to be used for the genetic improvement of other traits, such as biomass, seed yield, and other quality traits, in alfalfa and sainfoin in the future.

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Authors' contributions

SNA and RJW planned, executed and supervised the research; CPW, SNA and SDS designed and analyzed the experimental results; KNJ, GC and SS provided assistance in development of the GC/MS protocol and lipid analysis. CPW performed the experiments and wrote the article with contributions from all the authors. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Acharya, S.N. 2015. AAC Mountainview Sainfoin (*Onobrychis viciifoila* subsp. *Viciifolia*). Can J Plant Sci 95:603-607.

Basu, S.K., Acharya, S.N. and J.E.Thomas, 2007. Genetic improvement of fenugreek (*Trigonella foenum-graecum* L.) through EMS induced mutation breeding for higher seed yield under western Canada prairie conditions. Euphytica. DOI 10.1007/s10681-007-9545-9.

Bayat, A. R., Tapio, I., Vikki, J., Shingfield, K. J. and H. Leskinen, 2018. Plant oil supplements reduce methane emissions and improve milk fatty acid composition in dairy cows fed grass silage-based diets without affecting milk yield. J Dairy Sci. 101, 1136-1151.

Beauchemin, K. A., Kreuzer, M., O'Mara, F. and T.A. McAllister, 2008. Nutritional management for enteric methane abatement: a review. Aust J Exp Agric. 48, 21-27.

Berg, B. P., Majak, W., McAllister, T. A., Hall, J. W., McCartney, D., Coulman, B. E., et al. 2000. Cheng KJ. Bloat in cattle grazing alfalfa cultivars selected for a low initial rate of digestion: A review. Can J Plant Sci. 80,493-502.

Beyaz, R., Sancak, C., Yildiz, Ç., Kuşvuran, Ş. And M. Yildiz, 2016. Physiological responses of the M1 sainfoin (*Onobrychis viciifolia* Scop) plants to gamma radiation. Appl Radiat Isot. 118, 73-79.

Biazzi, E., Nazzicari, N., Pecetti, L., Brummer, E. C., Palmonari, A., Tava, A., et al. (2017). Genome-wide association mapping and genomic selection for alfalfa (*Medicago sativa*) forage quality traits. PLoS ONE. 12, e0169234.

Blondon, F., Marie, D., Brown, S. and A. Kondorosi, 1994. Genome size and base composition in *Medicago sativa* and *M. truncatula* species. Genome. 37, 264-270.

Boufaïed, H., Chouinard, P. Y., Tremblay, G. F., Petit, H. V., Michaud, R. and G. Bélanger, 2003a. Fatty acids in forages. I. Factors affecting concentrations. Can J Anim Sci. 83, 501-511.

497 Boufaïed, H., Chouinard, P. Y., Tremblay, G. F., Petit, H. V., Michaud, R. and G. Bélanger,
498 2003b. Fatty acids in forages. II. In vitro ruminal biohydrogenation of linolenic and linoleic
499 acids from timothy. Can J Anim Sci. 83, 513-522.

500 Browse, J., McConn, M., James, Jr. D. and M. Miquel, 1993. Mutants of Arabidopsis
501 deficient in the synthesis of α-linolenate. J Biol Chem. 268, 16345-16351.

502 Cantisán, Martínez-Force E., Álvarez-Ortega, R. and R. Garcés, 1999. Lipid characterization
503 in vegetative tissues of high saturated fatty acid sunflower mutants. J Agric Food Chem. 47,
504 78-82.

505 Carrol, B. J., McNeil, D. L. and P.M. Gresshoff, 1985. Isolation and properties of soybean
506 (*Glycine max* (L.) Merr.) mutants that nodulate in the presence of high nitrate concentrations.
507 Proc Natl Acad Sci USA. 82, 4162-4166.

508 Cooper, J., Till, B. J., Laport, R. G., Darlow, M. C., Kleffner, J. M., Jamai, A., et al. 2008.
509 TILLING to detect induced mutations in soybean. BMC Plant Biol. 8, 9.

510 Cosgrove, G. P., Anderson, C. B., Knight, T. W., Roberts, N. J. and G.C. Waghorn, 2004.
511 Forage lipid concentration, fatty acid profile and lamb productivity. Proc. New Zealand
512 Grassland Assoc. 66, 251-256.

513 Cvejić, S., Jocić, S., Prodanović, S., Terzić, S., Miladinović, D. and I. Balalić, 2011. Creating
514 new genetic variability in sunflower using induced mutations. HELIA. 34, 7-54.

515 Dalmais, M., Schmidt, J., Le Signor, C., Moussy, F., Burstin, J., Savois, V., et al. 2008.
516 UTILLdb, a *Pisum sativum in silico* forward and reverse genetics tool. Genome Biol. 9, R43.
517 DOI 10.1186/gb-2008-9-2-r43.

518 Davis, L. C. 2015. Modification of oil content in cottonseed using chemical mutagenesis.
519 M.Sc. thesis. Texas Tech University.

520 Dewhurst, R. J. 2010. Forage lipids and effects on ruminant productivity. In, Timmis KN
521 (ed), Handbook of hydrocarbon and lipid microbiology. DOI 10.1007/978-3-540-77587-
522 4_238.

523 Ehsanpour, A. A. and R. Razavizadeh, 2005. Effect of UV-C on drought tolerance of alfalfa
524 (*Medicago sativa*) callus. Amer J Biochem Biotechnol. 1, 107-110.

525 Flowers, G., Ibrahim, S. A. and A.A. AbuGhazaleh, 2008. Milk fatty acid composition of
526 grazing dairy cows when supplemented with linseed oil. J. Dairy Sci. 91, 722-730.

527 Food and Agriculture Organization (FAO). 2009. The state of food and agriculture: livestock
528 in the balance. 2009; http://www.fao.org/docrep/012/i0680e/i0680e.pdf (accessed 14 July
529 2017).

530 Green, A. G. 1986. Genetic control of polyunsaturated fatty acid biosynthesis in flax (*Linum*
531 *usitatissimum*) seed oil. Theor Appl Genet. 72, 654-661.

532 Hebeisen, D. R., Hoeflin, F, Reusch, H. P., Junker, E. and B.H Lauterburg, 1993. Increased
533 concentrations of omega-3 fatty acids in milk and platelet rich plasma of grass-fed cows.
534 Internat. J. Vit. Nutr. Res. 63, 229-233.

535 Hendon, B. R., Lowery, C. C., Auld, D. L., Burrow, M., Mendu, V., Xu, W., et al. 2018.The
536 Mutation Creation Station.
537 https://dl.sciencesocieties.org/publications/meetings/download/pdf/2014am/88888 (Accessed
538 29 06 2018).

539 Henikoff, S. and L. Comai, 2003. Single-nucleotide mutations for plant functional genomics.
540 Annu Rev Plant Biol. 54, 375-401.

541 Hoffman, T,, Schmidt, J. S., Zheng, X. and A.F. Bent, 1999. Isolation of ethylene-insensitive
542 soybean mutants that are altered in pathogen susceptibility and gene-for-gene disease
543 resistance. Plant Physiol. 119, 935-950.

544 James, Jr. D. W. and H.K. Dooner, 1990. Isolation of EMS-induced mutants in Arabidopsis
545 altered in seed fatty acid composition. 80, 241-245.

546 Johnson, K. A. and D.E. Johnson, 1995. Methane emissions from cattle. J Anim Sci. 73,
547 2483-2492.

548 Kawaroe, M., Sudrajat, A. O., Hwangbo, J. and D. Augustine, 2015. Chemical mutagenesis
549 of microalgae Nannochloropsis sp. Using EMS (ethyl methanesulfonate). Br J Appl Sci
550 Technol 8, 494-505.

551 Kempf, K., Mora-Ortiz, M., Smith, L.M.J., Kolliker, R. and L. Skot, 2016. Characterization
552 of novel SSR markers in diverse sainfoin (*Onobrychis viciifolia*) germplasm. BMC Genet.
553 17, 124.

554 Khan, M. H. and S.D. Tyagi, 2013. A review on induced mutagenesis in soybean. J Cereals
555 and Oilseeds. 4, 19-25.

556 Koivisto, J. M. and G.P.F Lane, 2001. Sainfoin - worth another look. In: College RA, editor.
557 Sainfoin: worth another look. Royal Agricultural College, Cirencester, on behalf of the BGS
558 Forage Legumes Special Interest Group, UK. Cirencester.

559 LaBrune, H., Reinhardt, C., Dikeman, M. and J. Drouillard, 2008. Effects of grain processing
560 and dietary lipid source on performance, carcass characteristics, plasma fatty acids, and
561 sensory properties of steaks from finishing cattle. J Anim Sci. 86, 167-172.

562 Le Signor, C., Savois ,V., Aubert, G., Verdier, J., Nicolas, M., Pagny, G., et al. 2009.
563 Optimizing TILLING populations for reverse genetics in *Medicago truncatula*. Plant
564 Biotechnol J. 7, 430-441.

565 McCallum, C. M., Comai, L., Greene, E. A. and S. Henikoff, 2000. Targeted screening for
566 induced mutations. Nat Biotechnol. 18, 455-457.

567 McLachlan, D. H., Lan, J., Geilfus, C. M., Dodd, A. N., Larson, T., Baker, A., et al. 2016.
568 The breakdown of stored triacylglycerols is required during light-induced stomatal opening.
569 Curr. Biol. 26, 707-712.

570 Mir, P. S., McAllister, T. A., Scott, S., Aalhus, J., Baron, V., McCartney, D., et al. 2004.
571 Conjugated linoleic acid - enriched beef production. Amer J Clin Nutr. 79, 1207S-1211S.

572 Mohajer, S., Taha, R. M., Lay, M. M., Esmaeili, A. K. and M. Khalili, 2014. Stimulatory
573 effects of gamma irradiation on phytochemical properties, mitotic behaviour, and nutritional
574 composition of sainfoin (*Onobrychis viciifolia* Scop.). Sci World J. 854093.

575 Mora-Ortiz, M., Swain, M. T., Vickers, M. J., Hegarty, M. J., Kelly, R., Lydia, M., et al.
576 2016. De-novo transcriptome assembly for gene identification, analysis, annotation, and
577 molecular marker discovery in *Onobrychis viciifolia*. BMC Genomics. 17, 756. DOI:
578 10.1186/s12864-016-3083-6.

579 More, A. D. 1992. Cytogenetical studies in Medicago sativa L, Ph.D. Thesis. BAM
580 University, Aurangabad.

581 Muehlbauer. F. J. and P.N. Rajesh, 2008. Chickpea, a common source of protein and starch
582 in the semi-arid tropics. In PH Moore, R Ming, eds, Genomics of Tropical Crop Plants. 2008;
583 Springer, New York, pp 171-186.

584 Nojima, D., Ishizuka, Y., Muto, M., Ujiro, A., Kodama, F., Yoshino, T., et al. 2017.
585 Enhancement of biomass and lipid productivities of water surface-floating microalgae by
586 chemical mutagenesis. Mar Drugs. 15, 151.

587 Osorio, J., Fernández-Martínez, J. M., Mancha, M. and R. Garcés, 1995. Mutant sunflower
588 with high concentration of saturated fatty acids in the oil. Crop Sci. 35, 739-742.

589 Palmquist, D. L. and H.R.Conrad, 1978. High fat rations for dairy cows. Effects on feed
590 intake, milk and fat production, and plasma metabolites. J Dairy Sci. 61, 890-901.

591 Pan, X., Siloto, R. M. P., Wickramarathna, A. D., Mietkiewska, E. and R.J. Weselake, 2013.
592 Identification of a pair of phospholipid: Diacylglycerol acyltransferases from developing flax
593 (*Linum usitatissimum* L.) seed catalyzing the selective production of trilinolenin. J Biol
594 Chem. 288, 24173-24188.

595 Penmetsa, R. V. and D.R. Cook, 2000. Production and characterization of diverse
596 developmental mutants of *Medicago truncatula*. Plant Phys. 123, 1387-1398.

597 Perry, J. A., Wang, T. L., Welham, T. J., Gardner, S., Pike, J. M., Yoshida, S. and M.A.
598 Parniske, 2003. TILLING reverse genetics tool and a Web-accessible collection of mutants
599 of the legume *Lotus japonicus*. Plant Physiol. 131, 866-871.

600 Porch, T. G., Blair, M. W., Lariguet, P., Galeano, C., Pankhurst, C. E. and W.J. Broughton,
601 2009. Generation of a mutant population for TILLING common bean genotype BAT 93. J
602 Am Hortic Soc. 134, 348-355.

603 Radovic, J., Sokolovic, D. and J. Markovic, 2009. Alfalfa-most important perennial forage
604 legume in animal husbandry. Biotech Anim Husbandry. 25, 465-475.

605 Rahman, H., Singer, S. D. and R.J. Weselake, 2013. Development of low-linolenic acid
606 *Brassica oleracea* lines through seed mutagenesis and molecular characterization of mutants.
607 Theor Appl Genet. 126, 1587-1598.

608 Ramos, M. L., Huntley, J. J., Maleki, S. J. and P. Ozias-Akins, 2009. Identification and
609 characterization of a hypoallergenic ortholog of Ara h 2.01. Plant Mol Biol. 69, 325-335.

610 Rowland, G. G. 1991. An EMS-induced low-linolenic-acid mutant in McGregor flax (*Linum*
611 *usitatissimum* L.). Can J Plant Sci. 71, 393-396.

612 Schimleck, L. R., Doran, J. C. and A. Rimbawanto, 2003. Near infrared spectroscopy for
613 cost-effective screening of foliar oil characteristics in a *Melaleuca cajuputi* breeding
614 population. J Agric Food Chem. 51, 2433-2437.

615 Singer, S. D., Weselake, R. J. and A. Acharya, 2018. Molecular enhancement of alfalfa:
616 improving quality traits for superior livestock performance and reduced environmental
617 impact. Crop Science. 58, 55-71.

618 Singh, P.K., Gari, M., Choudhury, S., Shukla, A., Gangwar, N. and S.K.Garg, 2019. Oleic
619 acid prevents isoprenaline-induced cardiac injury: Effects on cellular oxidative stress,
620 inflammation and histopathological alterations. Cardiovasc Toxicol. doi: 10.1007/s12012-
621 019-09531-y.

622 Tadege, M., Wang, T. L., Wen, J., Ratet, P. and K.S. Mysore, 2016. Mutagenesis and
623 beyond; Tools for understanding legume biology. Plant Physiol. 151, 978-984.

624 Till, B. J., Cooper, J., Tai, T. H., Colowit, P., Greene, E. A., Henikoff, S., et al. 2007.
625 Discovery of chemically induced mutations in rice by TILLING. BMC Plant Biol. 7, 19.

626 Toral, P. G., Hervás, G, Missaoui, H., Andrés, S., Giráldez, F. J., Jellali, S., et al. 2016.
627 Effects of a tannin-rich legume (*Onobrychis viciifolia*) on *in vitro* ruminal biohydrogenation
628 and fermentation. Span. J Agric Res. 14, e0602.

629 Van Nevel, C. and D. Demeyer, 1996. Influence of pH on lipolysis and biohydrogenation of
630 soybean oil by rumen contents *in vitro*. Reprod Nutr Dev. 36, 53-63.

631 Vanhercke, T., Divi, U. K., El-Tahchy, A, Liu, Q., Mitchell, M., Taylor, M. C., et al. 2017.
632 Step changes in leaf oil accumulation via iterative metabolic engineering. Metabol Engin. 39,
633 237-246.

634 Wilcox, J. R., Cavins, J. F. and N.C. Nielsen, 1984. Genetic alteration of soybean oil
635 composition by a chemical mutagen. JAOCS. 61, 97-100.

636 Wu, J. L., Wu, C., Lei, C., Baraoidan, M., Bordeos, A., Madamba, M. R., et al. 2005.
637 Chemical- and irradiation-induced mutants of Indica rice IR64 for forward and reverse
638 genetics. Plant Mol Biol. 59, 85–97.

639 Xu, Y., Holic, R., Li, D., Pan, X., Mietkiewska, E., Chen, G., et al. 2018. Substrate
640 preferences of long-chain acyl-CoA synthetase and diacylglycerol acyltransferase contribute
641 to enrichment of flax seed oil with α -linolenic acid. Biochem J. BCJ20170910.

642 Yun, N., Shi, F, L., Wang, X. L., Zhang, Y. and F.Y.Yi, 2014. Effects of EMS mutagenesis
643 on seed emergence and seedling growth of Mengnong sainfoin. Chinese J Grassland. 36, 5-9.

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Tables and figures

Table 1. Mean radicle lengths ± standard error of four (0, 0.5, 1 and 2 % v/v) ethyl methanesulfonate (EMS) treatments and three soaking times (6, 12 and 24 hrs) on AC Blue J alfalfa and AAC Mountainview sainfoin.

EMS % V/V	AC Blue J Alfalfa			AAC Mountainview Sainfoin		
	6hr	12hr	24hr	6hr	12hr	24hr
0	11.27^{a} ± 0.37	9.09^{d} ± 1.95	3.80^{f} ± 0.70	16.06^{d} ± 2.89	17.52^{d} ± 1.94	20.45^{a} ± 1.36
0.5	7.12^{b} ± 0.81	2.58^{e} ± 0.44	1.58^{c} ± 0.50	10.12^{b} ± 1.45	10.52^{b} ± 0.90	13.42^{f} ± 0.35
1	1.97^{c} ± 0.28	1.14^{c} ± 0.11	1.01^{c} ± 0.001	7.14^{e} ± 0.39	7.82^{e} ± 0.26	7.07^{e} ± 1.48
2	1.11^{c} ± 0.09	1.11^{c} ± 0.10	1.03^{c} ± 0.03	4.90^{e} ± 0.47	4.86^{e} ± 0.14	7.85^{e} ± 1.39

Means followed by different lower case letters are significantly different according to LSD ($p<0.05$). Each mean radicle length represents the observation on three replicated experiments of 50 individual seeds.

652 **Table 2.** Mean seedling height ± standard error for AC Blue J alfalfa and number of true leaves
653 ± standard error for AAC Mountainview sainfoin plants subjected to four ethane
654 methanesulfonate (EMS) (0, 0.5, 1.0, 2.0% v/v) concentrations and three (6, 12 and 24 hr)
655 treatment periods.

AC Blue J alfalfa			
EMS % V/V	6hr	12hr	24hr
0	$6.76^{b} \pm 0.89$	$7.05^{b} \pm 0.69$	$10.77^{a} \pm 1.09$
0.5	$5.06^{d} \pm 0.84$	$5.15^{d} \pm 0.19$	$7.48^{c} \pm 1.47$
1.0	$3.23^{e} \pm 0.43$	$3.57^{e} \pm 0.39$	$1.39^{f} \pm 0.26$
AAC Mountainview sainfoin			
0	2^{a}	2^{a}	2^{a}
0.5	2^{a}	1^{b}	2^{a}
1.0	1^{b}	1^{b}	1^{b}
2.0	1^{b}	1^{b}	1^{b}

656 Means followed by different lower case letters are significantly different at LSD $P < 0.05$. Each
657 mean represents three replicated experiments of 50 individual plants.

658 AC Blue J alfalfa at EMS 2% v/v survival was very low and did not include in analysis.

Table 3. Mean % total shoot lipid content ± standard error for AC Blue J alfalfa and AAC Mountainview sainfoin treated with four ethyl methanesulfonate (EMS) (0, 0.5, 1.0 and 2.0 % v/v) concentrations and three (6, 12 and 24 hr) treatment periods.

EMS v/v	AC Blue J alfalfa			AAC Mountainview sainfoin		
	6hr	12hr	24hr	6hr	12hr	24hr
0	$3.50^{a} \pm 0.11$	$3.04^{bc} \pm 0.15$	$3.19^{abc} \pm 0.12$	$1.79^{a} \pm 0.12$	$1.91^{ab} \pm 0.14$	$1.96^{ab} \pm 0.17$
0.5	$3.00^{c} \pm 0.09$	$3.18^{b} \pm 0.07$	$3.34^{ab} \pm 0.10$	$2.12^{b} \pm 0.12$	$1.92^{ab} \pm 0.13$	$2.02^{ab} \pm 0.17$
1	$3.30^{ab} \pm 0.08$	$3.13^{abc} \pm 0.16$	$3.27^{abc} \pm 0.13$	$1.77^{a} \pm 0.13$	$2.08^{ab} \pm 0.11$	$2.09^{ab} \pm 0.18$
2	$2.93^{bc} \pm 0.20$	$3.35^{abc} \pm 0.18$	$3.43^{abc} \pm 0.32$	$2.03^{ab} \pm 0.16$	$2.00^{ab} \pm 0.17$	$1.96^{ab} \pm 0.20$

Means with different lower case letters are significantly different according to LSD at $P < 0.05$.Each mean represents three replicated experiments performed on 100-200 individual plants.

665 **Table 4**. Total shoot lipid content and protein content (% dry weight (DW) of selected alfalfa
666 and sainfoin plants of the next generation population.

Forage crop	Plant ID	Lipid content (%DW)	Protein content (%DW)
Alfalfa	A163	5.21±0.09	32.64±2.12
	A195	4.85±0.11 [b]	32.48±2.31
	A205	4.88±0.18 [b]	31.37±1.23
	A309	5.00±0.12 [b]	32.35±1.55
	A260	5.31±0.09 [b]	31.98±1.02
	A467	5.00±0.21 [b]	30.40±2.21
	A463	4.95±0.17 [b]	32.66±1.21
	A456	5.00±0.11 [b]	30.45±2.14
	A453	5.14±0.12 [b]	29.45±2.21
	A341	5.13±0.11 [b]	31.63±3.02
	Control	3.04±0.25 [a]	29.31±2.12
Sainfoin	S515	3.18±0.22 [d]	29.76±1.68
	S504	3.79±0.11 [d]	31.71±1.22
	S478	3.88±0.13 [d]	31.69±1.18
	S445	3.20±0.12 [d]	31.33±1.23
	S444	3.91±0.15 [d]	30.24±2.18
	S437	3.96±0.16 [d]	31.66±1.13
	S431	2.98±0.17 [d]	31.43±2.11
	S379	3.61±0.11 [d]	30.02±2.02
	S353	2.96±0.18 [d]	31.73±1.28
	S1	3.98±0.16 [d]	30.87±2.23
	Control	1.96±0.14 [c]	28.66±2.53

667 Values represent three biological and technical replicates each. No treatment (NT)) denote the
668 control values as Table 4. Differences in total shoot lipid content and proteins in selected plants
669 were compared with plants without EMS treatment (control). Means with different lower case
670 letters are significantly different according to LSD at $P < 0.05$.

671 **Figure legends**

672 **Figure 1.** Mean % germination of AC Blue J alfalfa (a) and AAC Mountainview sainfoin (b)
673 seeds 14 days after seeding when subjected to four (0, 0.5, 1.0 and 2, % v/v) ethyl
674 methanesulfonate (EMS) concentrations and three (6, 12 and 24 hrs) soaking treatments. Each
675 point and bar represents the mean ± standard error of three replicates of 100-200 individual
676 seeds.

677 **Figure 2.** Alfalfa and sainfoin plants showing phenotypic variations following ethyl
678 methanesulfonate (EMS) treatment. a: Alfalfa seedlings displaying normal growth 2-3 weeks
679 after 0% EMS application (left) and stunted growth following treatment with 0.5% (v/v) EMS
680 (right). b. Plants showing xantha (left) and albino (right) phenotypes after application of EMS. c.
681 Normal sainfoin plants (left) and a stunted sainfoin plants (right) of the same age following EMS
682 treatment.

683 **Figure 3.** Total shoot lipid content (% dry weight (DW)) of selected alfalfa (a) and sainfoin (b)
684 plants. Ethyl methanesulfonate (EMS)-treated plants are indicated by blue columns and represent
685 three biological and technical replicates each. Red columns (no treatment (NT)) denote the mean
686 values of approximately 100-200 control plants treated with 0% EMS for 6 h, 12 h and 24 h,
687 respectively. Bars represent standard errors. Asterisks indicate significant differences in total
688 shoot lipid content in selected plants compared with plants without EMS treatment (NT).

689 **Figure 4.** Relative abundance of fatty acid species in selected high total shoot lipid content ethyl
690 methanesulfonate (EMS)-treated alfalfa (a) and sainfoin (b) plants. Columns represent the mean
691 values of three technical replicates per each sample with 3-4 biological replicates. Red columns
692 denote the mean values of 3 control plants treated with 0% EMS. Bars represent standard errors.

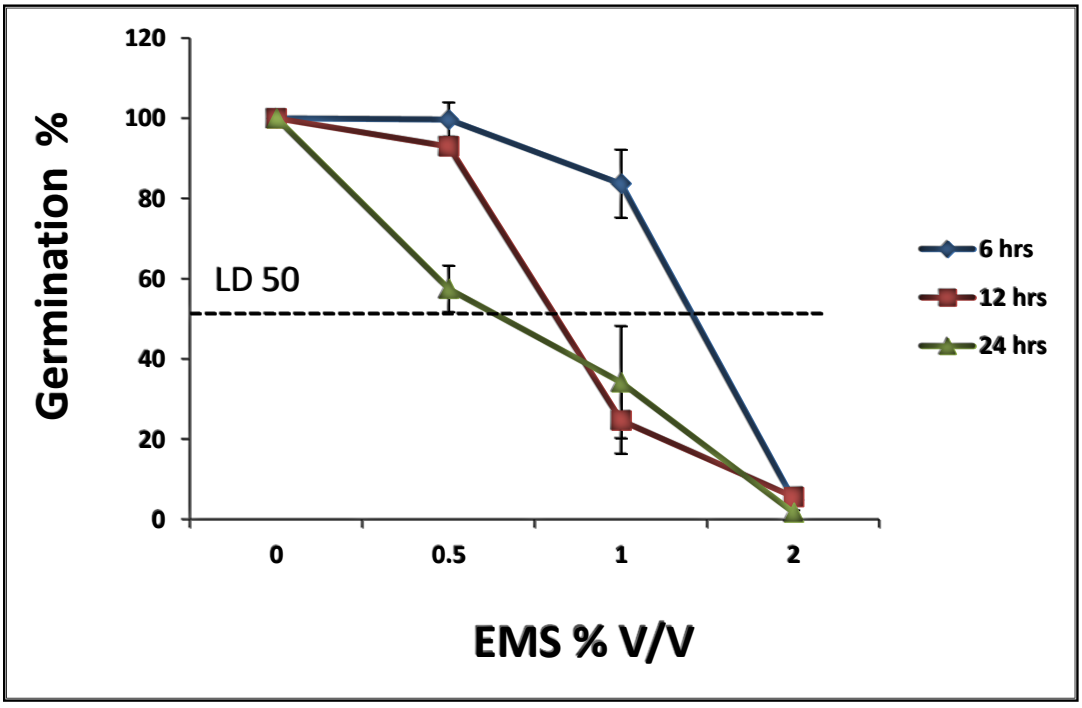
693 **Figure 5.** Relative abundance of fatty acid species in selected high total shoot lipid content in the
694 next generation of alfalfa (a) and sainfoin (b) plants. Columns represent the mean values of three
695 technical replicates per each sample with 3-4 biological replicates. Red columns denote the mean
696 values of 3 control plants treated with 0% EMS. Bars represent standard errors.

697 **Supplementary figure 1.** Total leaf lipid content (% dry weight (DW)) in immature and mature
698 alfalfa plant shoots harvested at different times of the day. Each column represents the mean ±

699 standard errors (bars) of four technical replicates performed on 12 individual plants. Means with
700 different lower case letters are significantly different at LSD $P < 0.05$.

701 **Supplementary figure 2.** A sample Gas chromatography-mass spectrometry (GC-MS)
702 chromatogram showing resolved fatty acid methyl esters prepared from total lipid extracts of
703 alfalfa. The major peaks represent palmitic acid (16:0), palmitoleic acid ($16:1\Delta^{9cis}$), stearic acid
704 (18:0), oleic acid ($18:1\Delta^{9cis}$), linoleic acid ($18:2\Delta^{9cis,11cis}$), α-linolenic acid ($18:3\Delta9^{cis,11cis,15cis}$), and
705 arachidic acid (20:0). 17:0 and 21:0 were utilized as internal controls.

706 **a**



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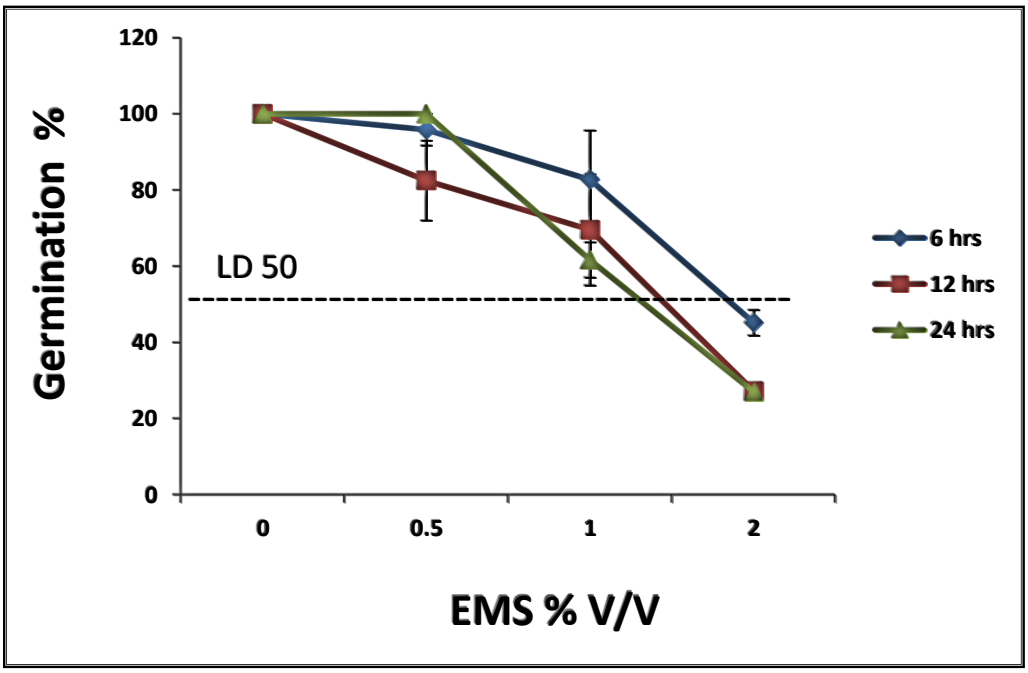
708 **b**



709

710 Figure 1

711

a

712

713 **b**

714

715

716

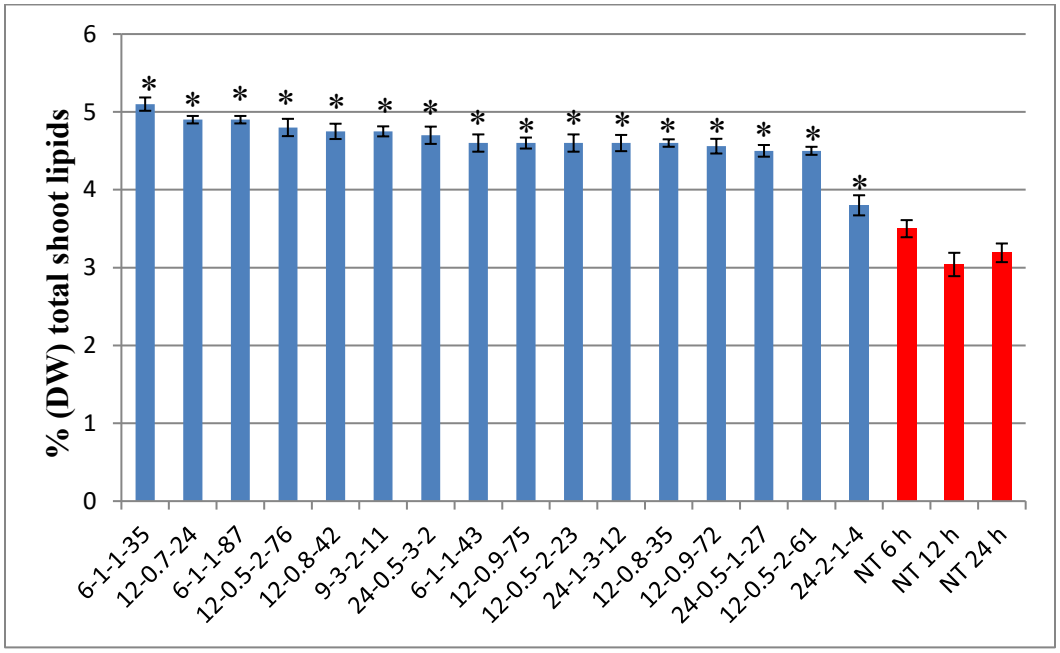
717 **c**

718

719

720 Figure 2

a


% (DW) total shoot lipids
0
1
2
3
4
5
6
6-1-1-35
12-0.7-24
6-1-1-87
12-0.5-2-76
12-0.8-42
9-3-2-11
24-0.5-3-2
6-1-1-43
12-0.9-75
12-0.5-2-23
24-1-3-12
12-0.8-35
12-0.9-72
24-0.5-1-27
12-0.5-2-61
24-2-1-4
NT 6 h
NT 12 h
NT 24 h

b

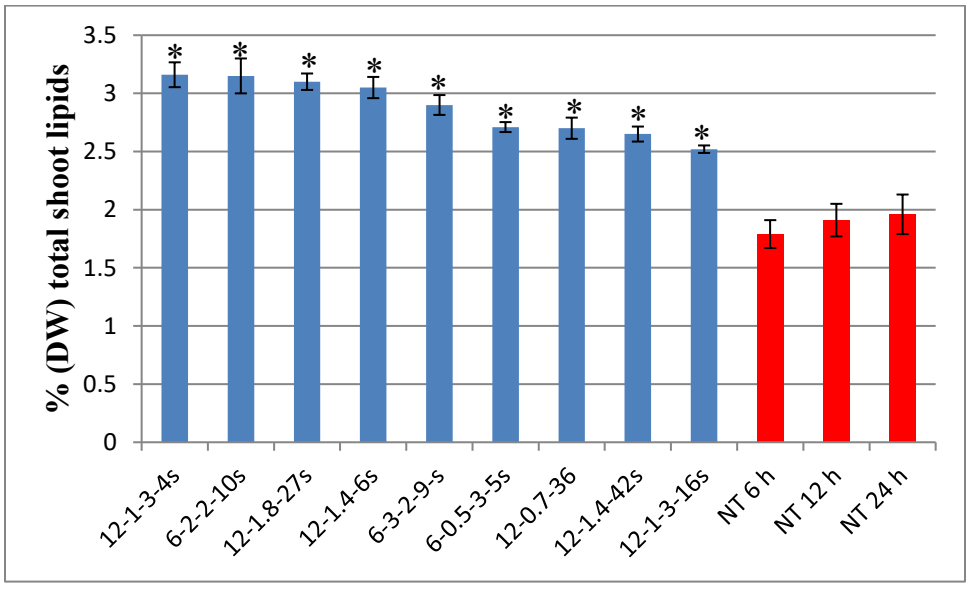
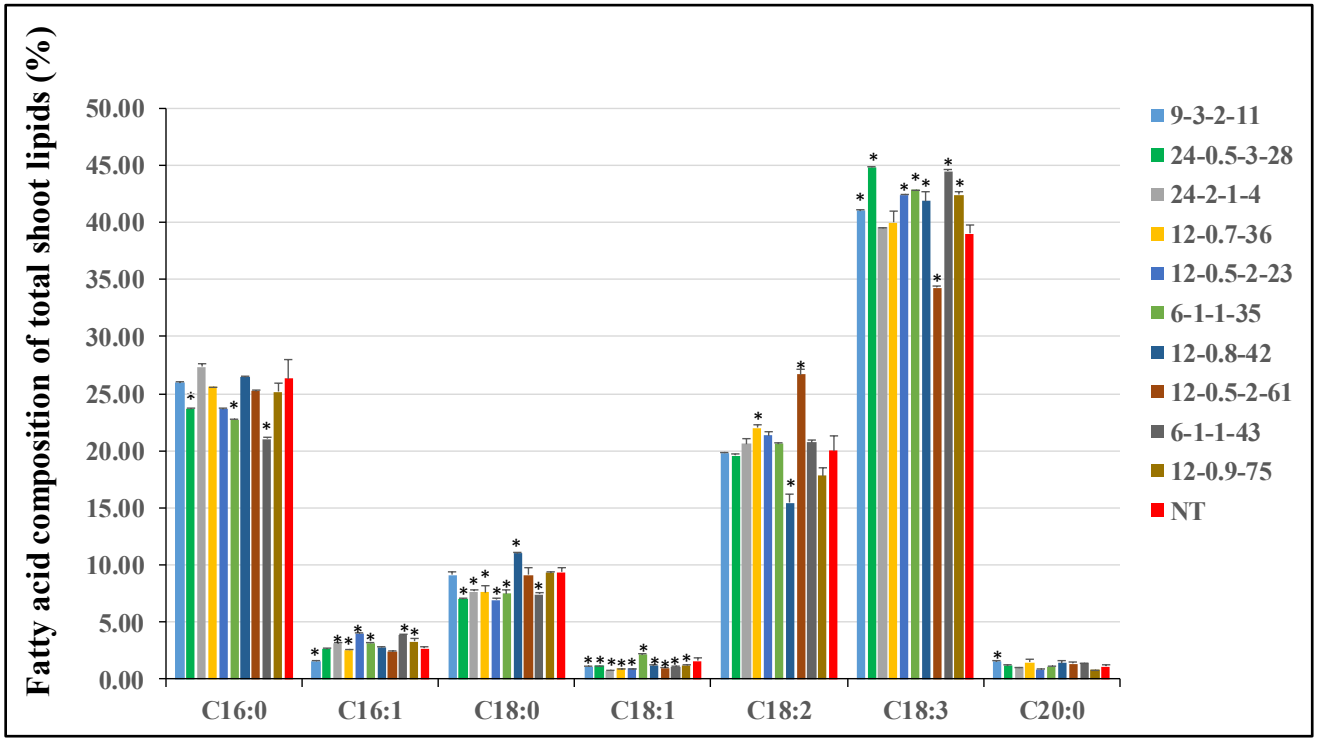

% (DW) total shoot lipids
0
0.5
1
1.5
2
2.5
3
3.5
12-1-3-4s
6-2-2-10s
12-1.8-27s
12-1.4-6s
6-3-2-9-s
6-0.5-3-5s
12-0.7-36
12-1.4-42s
12-1-3-16s
NT 6 h
NT 12 h
NT 24 h

Figure 3

a


Fatty acid composition of total shoot lipids (%)
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
C16:0
C16:1
C18:0
C18:1
C18:2
C18:3
C20:0
9-3-2-11
24-0.5-3-28
24-2-1-4
12-0.7-36
12-0.5-2-23
6-1-1-35
12-0.8-42
12-0.5-2-61
6-1-1-43
12-0.9-75
NT

b

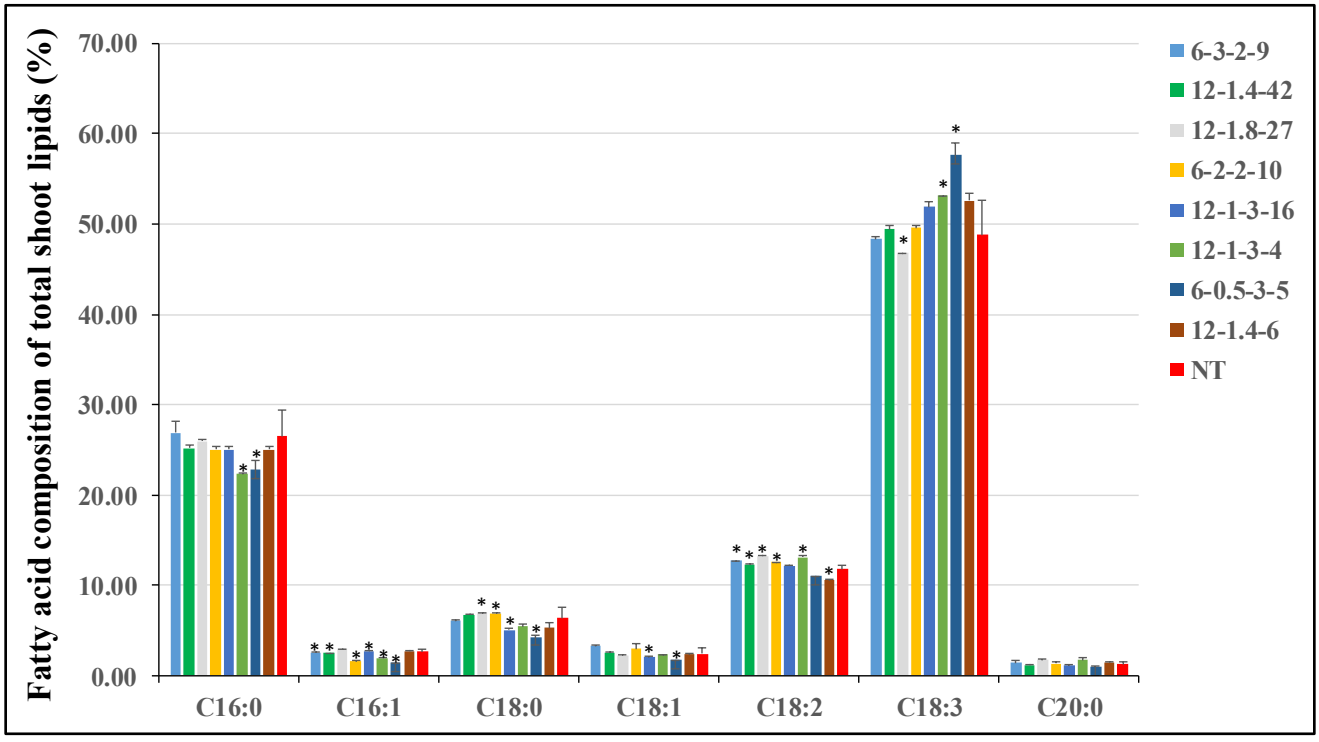
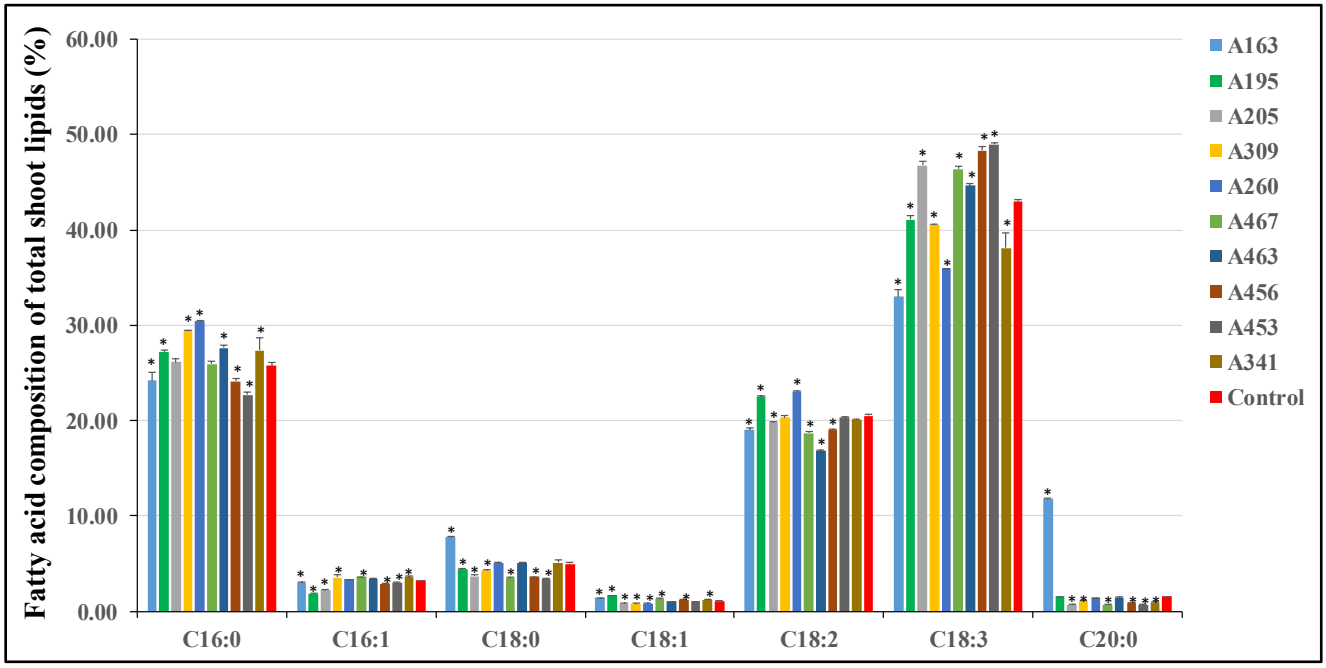

Fatty acid composition of total shoot lipids (%)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
C16:0
C16:1
C18:0
C18:1
C18:2
C18:3
C20:0
6-3-2-9
12-1.4-42
12-1.8-27
6-2-2-10
12-1-3-16
12-1-3-4
6-0.5-3-5
12-1.4-6
NT

Figure 4

a


Fatty acid composition of total shoot lipids (%)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
C16:0
C16:1
C18:0
C18:1
C18:2
C18:3
C20:0
A163
A195
A205
A309
A260
A467
A463
A456
A453
A341
Control

b

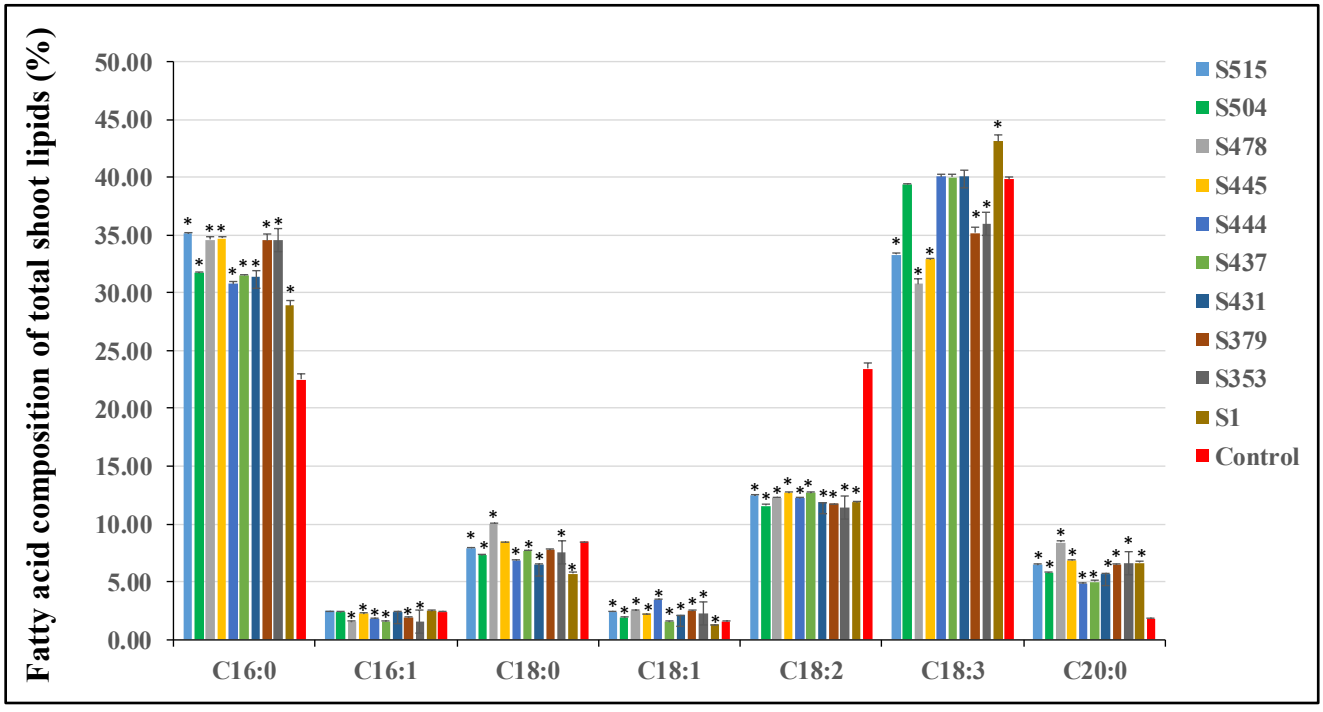

Fatty acid composition of total shoot lipids (%)
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
C16:0
C16:1
C18:0
C18:1
C18:2
C18:3
C20:0
S515
S504
S478
S445
S444
S437
S431
S379
S353
S1
Control

Figure 5

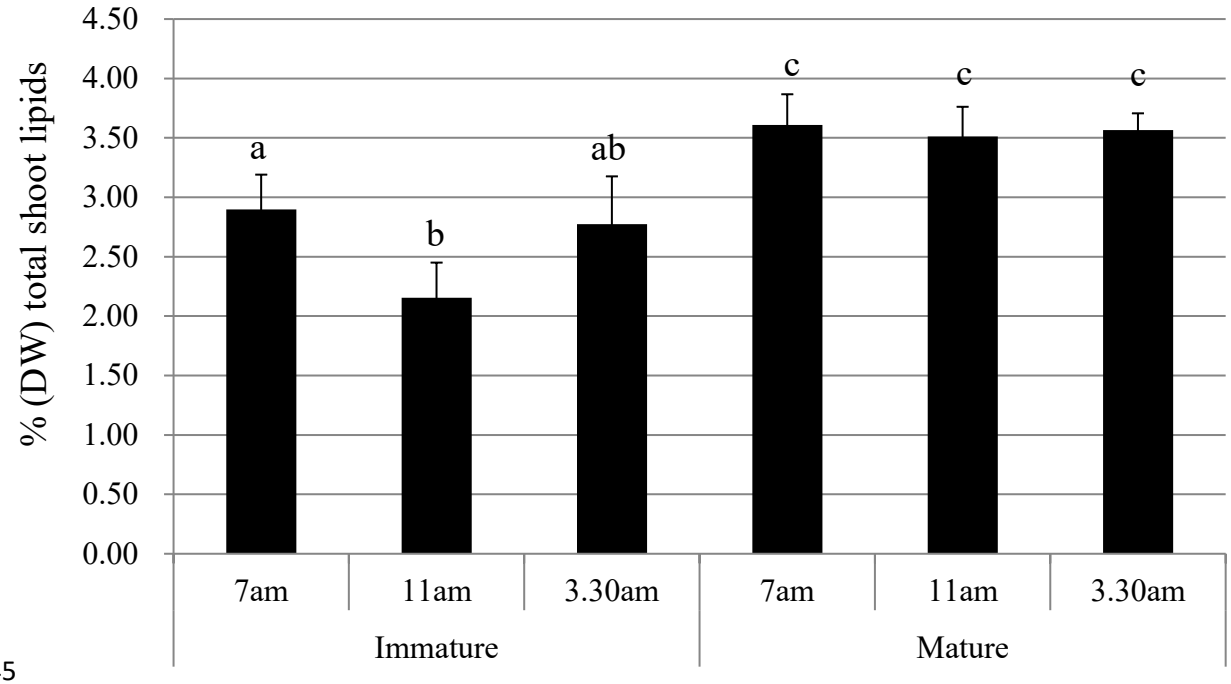
737 Supplementary table 1. Total shoot lipid content and protein content (% dry weight (DW) of
738 selected alfalfa and sainfoin plants of the parent population.

Forage crop	Plant ID	Lipid content (%DW)	Protein content (%DW)
Alfalfa	6-1-1-35	5.10±0.17 [b]	30.48±1.32
	12-0.7-24	4.92±0.08 [b]	28.41±2.13
	6-1-1-87	4.92±0.09 [b]	30.01±1.87
	12-0.5-2-76	4.825±0.22 [b]	26.26±2.61
	12-0.8-42	4.75±0.19 [b]	31.37±1.12
	9-3-2-11	4.75±0.12 [b]	28.35±2.04
	24-0.5-3-2	4.725±0.22 [b]	28.55±2.11
	6-1-1-43	4.575±0.22 [b]	26.63±3.02
	12-0.9-75	4.6±0.14 [b]	27.83±2.3
	12-0.5-2-23	4.575±0.22 [b]	30.81±1.32
	24-1-3-12	4.55±0.20 [b]	27.40±3.11
	12-0.8-35	4.625±0.09 [b]	28.41±2.01
	12-0.9-72	4.562±0.18 [b]	30.82±1.23
	12-0.5-1-27	4.475±0.15 [b]	28.35±1.33
	12-0.5-2-61	4.487±0.10 [b]	28.31±1.91
	24-2-1-4	3.8±0.25 [b]	29.29±2.1
	NT6h	3.5±0.28[a]	28.32±2.43
	NT12h	3.04±0.25 [a]	29.31±1.22
	NT24h	3.19±0.12 [a]	27.83±2.82
Sainfoin	12-1-3-4	3.16±0.10 [d]	26.81±2.11
	6-2-2-10	3.15±0.15 [d]	26.97±1.87
	12-1-8-27	3.1±0.07 [d]	26.68±1.57
	12-1-4-6	3.05±0.18 [d]	26.63±1.54
	6-3-2-9	2.9±0.12 [d]	28.3±2.65
	6-0.5-3-5	2.71±0.09 [d]	24.19±2.76
	12-0.7-36	2.7±0.08 [d]	28.14±2.87

	12-1.4-42	2.65±0.13 [d]	25.02±2.13
	12-1-3-16	2.52±0.11 [d]	26.52±1.78
	NT6h	1.79±0.21 [c]	26.89±1.68
	NT12h	1.91±0.15 [c]	27.24±2.14
	NT24h	1.96±0.14 [c]	28.66±1.23

739 Values represent three biological and technical replicates each. No treatment (NT)) denote the
740 mean values of approximately 100-200 control plants treated with 0% EMS for 6 h, 12 h and 24
741 h, respectively. Differences in total shoot lipid content and protein content in selected plants
742 were compared with plants without EMS treatment (NT). Means with different lower case letters
743 are significantly different according to LSD at $P < 0.05$.

744



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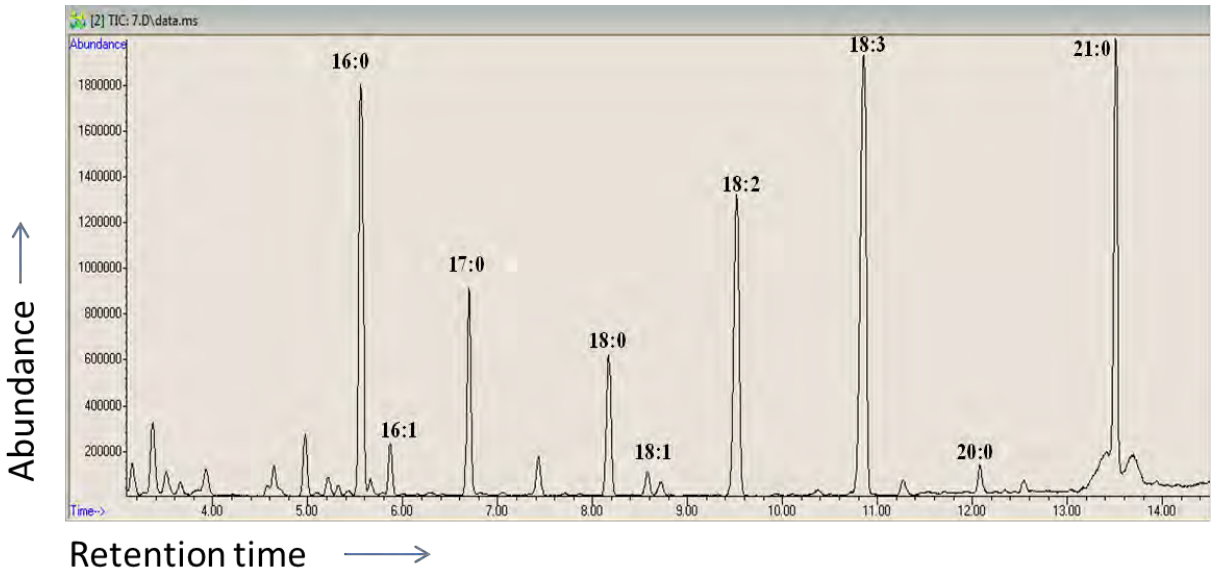
746 Supplementary figure 1



747

748 Supplementary figure 2