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RESEARCH ARTICLE

Development and characterization of ethyl methane sulfonate (EMS) induced mutant population of grass pea with yield, quality and forage traits

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ABSTRACT

Grass pea (*Lathyrus sativus* L.) is an important protein-rich annual legume crop that can survive harsh environment conditions. Genetic improvement of grass pea is highly important for expanding its production, and mutation breeding can be a valuable tool in creating additional genetic variability. In the present study, we treated totally 3000 seeds of widely cultivated grass pea cultivar, Gürbüz-2001, with two different concentrations (0.5% and 1%) of mutagen EMS for 4 hours. The germination rates were calculated as 71.6% (1074 plants) and 42.4% (636 plants) for 0.5% and 1% EMS concentrations in M_1 generation, respectively. We identified several mutant phenotypes such as seedling growth defects, abnormal branching, chlorophyll mutants, absence of flowering, and sterility in M_1 and M_2 populations. A total of 40 mutant plants which were superior than control variety in terms of vegetative components from M2 population were selected for agronomic, quality and forage traits analysis. Plant height of mutant lines ranged from 48 (GMP29) to 164 cm (GMP4), with an average value of 109.7 cm. One of the most interesting features of some mutant lines was the significant increase in 1000-seed weight over the control value, contributing to higher yield. The seed yield ranged from 7.19 to 87.18 g, and the lines GPM6 and GMP11 showed superior values compared to the control (13.6 g), indicating the positive effect of mutation in obtaining desired traits. There were promising mutants that showed higher levels of protein content among mutated lines. The β-ODAP (β-N-Oxalyl-l-α,β-diaminopropionic acid) content of grass pea seeds ranged from 0.139% to 7.39% in selected M_2 population, while the control cultivar was 0.523%. GPM6 and GPM11 were important for forage breeding with their high crude protein content, low ADF (acid detergent fiber) and NDF (neutral detergent fiber) ratios, as well as high seed and biological yield. The beneficial traits of these mutants obtained in this study should be followed to integrate as genetic resources for use in grass pea improvement programs.

Keywords: Legume; Mutagen; Mutation; Variation; β-ODAP

INTRODUCTION

Sustainability in agricultural production to ensure food security and to meet the ever-increasing population's food needs under global climate change is a big challenge in many countries (Smith and Gregory, 2013). Plants that can grow in droughty and poor soils are therefore highly valuable for productivity. From this perspective, grass pea (*Lathyrus sativus* L.) is an ideal and valuable legume, especially for poor farmers, that is known for its drought tolerance, and thrives with minimal external input (Yan et al., 2006; Vaz Patto and Rubiales, 2014). This resilient crop originated from southwest and central Asia, later spreading to Mediterranean regions (Kislev, 1989). It is commonly cultivated and is one of the cheapest protein sources, especially in developing countries (Enneking, 2011). Grass pea is also used to recover marginal lands and offers an alternative to wheat in the areas over exploited by cereal production in Europe (Polignano et al., 2009). This highly sought-after crop contains about 18–34% protein in its seeds (Rizvi et al., 2016), which is higher than other legumes, such as faba beans and lupine (Petterson et al., 1997). Grass pea is used worldwide as a pasture crop, green fodder, and animal feed (Kumar, 1997). It can also perfectly fix 108-125 kg ha^{-1} nitrogen into the soil annually (Peoples et al., 1995). Despite its agricultural and nutritional advantages, total production of grass pea is lower than other major legume and forage crops. Factors such as unsuitability for mechanized harvesting, limited adoption of new crop production techniques (Pandey et al., 1996), high β-ODAP content (Kumar et al., 2011), late maturity, and low seed yield are the main reasons for

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preventing this crop from large cultivation worldwide. In addition, grass pea has been poorly documented by the international scientific community, research areas, and the industry (Lambein et al., 2019).

The high presence of a neurotoxin (ODAP) in grass pea seeds is one of the major obstacles to its genetic improvement (Dixit et al., 2016). This non-protein free amino acid causes function disorder in motor neurons and leads to a disease known as lathyrism (Lambein and Kuo, 2009). Many researchers (Akalu et al., 1998; Zhao et al., 1999; Kuo et al., 2000; Vaz Patto et al., 2006) have reported the disadvantage of high β-ODAP content in grass pea. However, given the increasing need for resilient food crops, improvement of grass pea is important for researchers (Vaz Patto and Rubiales, 2014). Breeding studies on the development of improved varieties with low β-ODAP content have therefore been conducted to sustain grass pea production (Hanbury et al., 1999; Abd El Moneim et al., 2001; Kumar et al., 2011). Although the β-ODAP content of the Lathyrus species is mostly genetically controlled, it can also be greatly affected by environmental and climatic conditions (Tadesse, 2003) such as drought, salinity, and water (Jiao et al., 2011). Furthermore, maternal cytoplasmic effects on β-ODAP concentration were reported (Abd El Moneim et al., 2001).

Increasing the genetic potential of grass pea for qualitative and quantitative characteristics is one of the major aims in most grass pea breeding studies (Dixit et al., 2016). Broadening the genetic diversity of these characteristics is highly important for crop improvement because the use of only a few elite genotypes in improvement studies reduces the genetic variation, leading to a narrow gene pool. Discovering and developing crop characteristics is therefore highly significant for increasing genetic diversity for sustainable breeding programs (Yol et al., 2018). Grass pea has a narrow-range genetic diversity because of interspecific incompatibility and self-pollination (Nerkar 1976). Induced mutagenesis is an important breeding technique that can be used to create additional genetic variability, which is useful for developing new lines for specific aims (Rybinski 2003). This method has been utilized to create variation for qualitative and quantitative traits in grass pea (Ramezani et al., 2017). With the mutations, significant differences were recorded in the mean of chlorophyll (Singh and Sadhukhan, 2019) and β-ODAP (Talukdar and Biswas, 2006) content in mutant lines. Rybinski (2003) monitored that mutagen decreased the plant height, pod length and parameters of yield structure. The fertile mutant was obtained with less number of primary and secondary branches, reduced pod and seed size, lower yield and late maturity compared to control in the study conducted by Waghmare et al. (2001). Similarly, mutagen effect was particularly observed in reducing plant habit variability as dwarfism (Prasad and Das 1980). Glutathione (GSH)-deficient (gshl-1) and ascorbate-deficient semi-dwarf (asfL-1) mutants were also developed by Talukdar (2012a, b). Previous studies clearly showed that mutagens highly affected agronomic traits and it should be important alternative to increase genetic variation in grass pea. From this perspective, the objectives of this study were (i) improve desired traits related with yield, forage and quality, (ii) obtaining low β-ODAP content, and (iii) selection of elite lines and (iv) contributing the genetic diversity of grass pea by using the EMS chemical mutagen.

MATERIALS AND METHODS

Genetic material

The grass pea cultivar Gürbüz-2001 was used as a genetic material in this study. It was registered by the Field Crops Central Research Institute in Turkey. The agronomic traits of Gürbüz-2001 for the main stem length, 1000-seed weight, biological yield, and grain yield ranged from 35 to 50 cm, 100 to 120 g, 200 to 550 kg da-1, and 90 to 220 kg da-1, respectively. The plant has a wide adaptation ability and contains about 20% crude protein. Before the mutagen applications, the original seeds of Gürbüz-2001 were grown in two generations to ensure homogeneity.

EMS treatment

About 3000 seeds were previously imbibed in tap water for 12 hours at room temperature. The 1500 seeds were soaked in ethyl methane sulphonate (EMS) at two concentrations, 0.5% and 1% (v/v), for 4 h at 25^{o}C, while 250 seeds were used as a control (untreated). Then, seeds were thoroughly washed with deionized water three times and rinsed extensively in running water overnight to reduce the negative effects of the EMS mutagen.

Field trials

M_1 and M_2 plants were grown in the experimental field of Akdeniz University at Antalya, Turkey (30^{o}38'E, 36^{o}53'N) (Fig. 1). Major soil characteristics in the trial field were as follows: sandy loam soil texture with pH 7.2, 2.4% organic matter, 22.8% CaCO3, 5.2 mg kg^{-1} of P, and 275 mg kg^{-1} of K. Climate data, including monthly temperature, precipitation, and humidity, were recorded by the State Meteorology Station (Antalya, Turkey). As shown in Table 1, average temperature, precipitation, and relative humidity were 16.22 °C, 886.3 mm, and 71.08% in 2019 and 16.26°C, 781.5 mm, and 68.90% in 2019-2020, respectively.

After mutagen application, EMS-treated seeds (M_1) were immediately sown in the experimental field with an inter-row spacing of 100 cm and an intra-row spacing of 15 cm in March 2019. The seeds of Gürbüz-2001 were also sown in every 25 rows as a control. M1 plants were harvested individually in July 2019, and seeds and the vegetative parts of the plant were collected, packed, air dried, and stored. Seeds collected from individual M1 plants were sown as M2 families in the same field in two rows of 1 m length with a row-to-row distance of 100 cm and plant-to-plant distance within a row of 15 cm in November 2019. Standard agronomic practices were conducted for all plots in two experimental years. M_3 seeds from each M_2 plant were harvested in June 2020, then packed and stored for further study.

Data collection for traits

Grass pea descriptors (Campbell, 1997) were used for recording qualitative and quantitative traits. In M_2 generation, selected plants were characterized with the following characteristics: plant height, pod number per plant, pod length, seeds number, and weight per main stem and seed number and weight per plant. In addition, crude protein, acid detergent fiber (ADF), neutral detergent fiber (NDF), and β-ODAP were determined from the seeds. All obtained data from the M_2 generation were subjected to basic statistics using Microsoft Excel 2013.

β-ODAP analysis

In the first stage of analysis, seed samples were homogenized using a blender. Homogeneous samples were divided into 1-g portions in individual 50-ml sample tubes. Then, 25 ml extraction solution, 0.1% (v/v) formic acid in water: methanol (50:50) (v/v), was added to the sample tube. For recovery studies, a standard was added to the tube at this stage. The mixture was extracted using Ultra-Turrax for 2 minutes at 10,000 rpm. Extracted samples were centrifuged at 4 oC at 4000 rpm for 10 minutes. The supernatant was passed through a 0.2-µm PTFE membrane filter. Filtered samples were diluted with a mobile phase and injected at 10 µl volumes to LC-MS/MS. β-ODAP was purchased from ChemFaces, China, with high purity (>98%). After extraction, β-ODAP content was identified with the UHPLC-MS/MS method which has been detailed in the study by Arslan et al. (2017).

Fig 1. The photo from the experimental area.

Chemical analysis

Nitrogen content was determined by the Kjeldahl method, and the crude protein ratio was calculated using a conversion factor of 6.25. ADF and NDF concentrations were determined according to standard laboratory procedures of forage quality analysis outlined by Ankom Technology. ANKOM F57 filter bags were used for ADF and NDF analysis in this study. Total digestible nutrients (TDN), dry matter intake (DMI), digestible dry matter (DDM), and relative feed value (RFV) were estimated (Horrocks and Vallentine, 1999).

Flower and seed coat color

Mutants affecting flower color were detected by observing the plants from budding to flowering stages in M_2 generation. Seed coat color of grass pea was defined using the RHS mini color chart. Griesbach and Austin (2005) recommended the use of the Royal Horticultural Society Colour Charts (RHSCC) for describing the color of biological samples.

RESULTS

Development of mutant grass pea population

Two different EMS mutagenesis treatments, 1% and 0.5% for 4 h, were tested on 3000 grass pea seeds in this present study. The germination rate was calculated as 71.6% (1074 plants) for 0.5% EMS concentration in M1 generation. Among them, 240 plants died at early stages and 129 plants had produced no seeds, although they flowered. In other EMS treatment (1%), the germination rate was about 42.4% (636 plants) and a significant number of plants (346) died at a vegetative stage with that dose. The lethality of the grass pea population was therefore about 43.0% under EMS mutagen application. The altered phenotype was under 1% compared to original seeds. We also observed mutation effects in M_2 population. These changes include chlorophyll mutants, absence of flowering, increased/decreased height, wilting, and sterility (Fig. 2). In total we collected 905 M_2 seeds, among which 705 and 200 M_2 seeds were obtained with 0.5% and 1% EMS treatment, respectively.

Agronomic traits

In order to analyze the agronomic traits, 40 mutant plants that were superior than control variety in terms of vegetative components from the M_2 family were selected. The large variation was monitored among selected mutant lines for agronomic traits, which were presented in Table 2. Plant height of mutant lines ranged from 48 (GMP29) to 164 cm

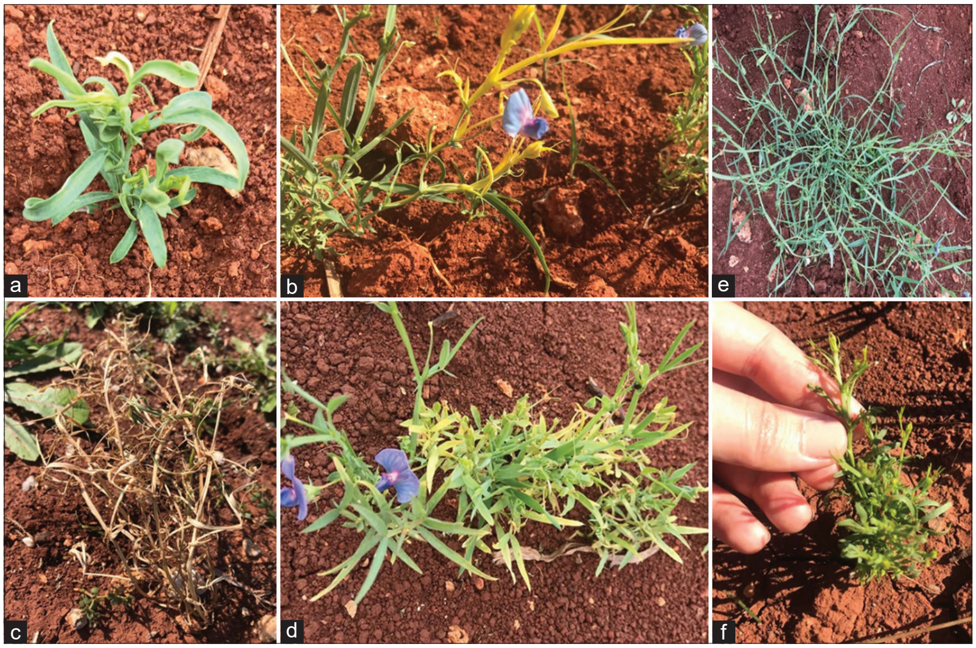

Fig 2. Mutation effects on grass pea. a) abnormal leaf (M1 plant) b) chlorosis (M1 plant) c) wilting (M1 plant) d) abnormal branching (M1 plant) e) spreading growth type (M2 plant) f) dwarf type (M2 plant).

(GMP4), with an average value of 109.7 cm (Table 2), while the average value was 75 cm for the control, Gürbüz-2001. Interestingly, some mutants showed about twice the plant height of the control (Table 2). Looking at the pod traits (pod number, pod length, pod width), we observed increased mean values compared to the control average (Table 2). The highest seed yields were found in GPM6 and GPM11 with values of 87.18 and 6.4 g in selected M2 lines, respectively, with a mean of 22.65 g/plant. There was also great variation among mutant lines with respect to biological yield, ranging between 13.12 (GPM38) and 197.42 g (GPM6), with an average value of 53.06 g. Gürbüz-2001, the control cultivar, had lower mean values for seed yield and biological yield, which were 13.6 and 19.27 g/plant, respectively. The individual plants GPM39 and GPM10 had the highest 1000-seed weight with values of >200 g. The results shown in Table 2 indicated that the mean of this trait in the mutant population was higher than the control plant.

Quality and forage traits

Although there was no β-ODAP-free mutant line, great variation was observed for this neurotoxic nonprotein amino acid among selected mutated grass pea lines in M_2 generation (Table 2). It ranged between 1.39 (0.139%) (GPM37) and 7.39 mg/g (0.739%) (GPM13), with about 77% of the selected mutant population showing higher mean values than the control, which had 5.23 mg g^{-1} β-ODAP content. Nine mutants had protein content of over 30%; the mean of the mutant population was 21.86%. Table 2 showed that the peak values of ADF and NDF are 10.70% and 19.91%, with averages of 8.76% and 15.92%, respectively. Unlike other traits, similar mean values were measured for ADF and NDF traits in the control cultivar compared to selected mutant population. The feed digestibility and nutritional values of grass pea mutant lines were 87.54-91.71, 6.03-9.30, 80.57-83.08, and 386.18-596.02 for total digestible nutrients, dry matter intake, digestible dry matter, and relative feed value, respectively.

Flower color and seed traits

The seed shape, coat color, coat creaking, and flower color of mutant grass pea lines were separately evaluated because of large variation (Table 3). Gürbüz-2001 (control) has a blue flower color and brown, non-cracking square seeds. However, 35% of the selected mutant population differs from this cultivar in terms of flower color (Table 3). In particular, six mutant lines showed extreme difference with pink and violet colors (Table 3). Remaining lines were characterized by blue (23 lines), white (5 lines), a mixture of blue and white (4 lines), and white stripes on the blue (2 lines) flower colors. Among the mutated grass pea lines, three main coat colors (dark brown, brown, and green) were observed, with sub-color groups of Brown-RHS N199A (13 lines), Brown-RHS 199A (14 lines), Brown-RHS N199C (6 lines), Brown-RHS 199C (5 lines), Dark Brown RHS-200B (1 line), and Green-RHS 194A (1 line). The figures for flower and seed coat colors of selected mutant lines are shown Fig. 3 and Fig. 4, respectively. The seed shapes were classified under four groups as spherical, rhomboid + obtriangular, square, and obtriangular (Table 3). In addition, seed coat cracking was detected in four lines (GMP8, GMP14, GMP17, and GMP40) in the mutant population.

Fig 3. Mutation effects on flower color in grass pea. a) control cultivar color (blue) b) white (M2 plant) c) violet (M2 plant) d) pink (M2 plant).

Fig 4. Mutation effects on grass pea seeds from selected M2 plants.

DISCUSSION

Grass pea is an underutilized legume crop which provides food and nutrition security to consumers in many developing countries (Lambein et al., 2019). Despite its importance, it has limited genetic variation (Dixit et al., 2016) because of self-pollination and interspecific incompatibility (Nerker et al., 1976). Mutation breeding is an alternative approach to creating additional genetic variability, especially for economic traits (Rybiński, 2003). However, the success of this method relies on the effectiveness and efficiency of the mutagen used (Arisha et al., 2015). In the present study, we applied two different EMS concentrations (0.5% and 1%) on grass pea seeds and the germination rate was drastically reduced to about 30% at higher concentrations, showing that the effect of a mutagen depends on the concentration applied (Siddique et al., 2020). Reduction in germination rate might be the result of altered enzyme activity (Khan and Goyal, 2009). In accordance with our result, the increase in dose/concentration of the mutagenic treatments negatively affected the germination rate of grass pea (Singh and Sadhukhan, 2019), rice (Talebi et al., 2012), and soybean (Espina et al., 2018). We identified differences among mutant phenotypes, including anormal stem growth, changes in plant height, alteration in colors for the vegetative part, and sterility in M_1 plants. A type of visible mutation was observed in one plant that had a range of yellow–green color characterizing chlorophyll deficiency, which is an important indicator of the effectiveness of a mutagen (Arisha et al., 2014; Espina et al., 2018; Siddique et al., 2020; Chen et al., 2020). Chlorotic mutants identified in this study should be used to identify chlorophyll-related gene function and regulation of its genes in grass pea.

We obtained a considerable variation for all studied traits after application of EMS treatments in M_2 generation. Plant height is an important morphological feature that directly affects biological and grain yield (Lambein et al., 2019). The characterization studies showed that plant height of grass pea ranged between 30.22-56.00 cm (Basaran et al., 2013), 46-153 cm (Turk et al., 2007), 44.13-50.73 cm (Kosev and Vasileva, 2019), and 61-96 cm (Mihailovic et al., 2013). In the present study, high divergence was obtained for plant height (48 to 164 cm), showing the effect of mutation on this trait. In particular, GPM4 has a plant height twice as high as the control, and this mutant might be useful in understanding the regulatory mechanisms of plant growth with the hybridization and mapping approach. The chemomutagens were also particularly efficient in inducing plant habit variability as low and high branching forms according to Pradas and Das (1980). This was confirmed by our result that the main stem number varied between 3.0 and 29.0 in the M2 population. Its range was also higher than some reports, which obtained main stem numbers of 5-11.8 (Mihailovic et al., 2013), and 3.73-6.00 (Kosev and Vasileva, 2019). The trait of 1000-seed weight is highly related to seed yield in grass pea (Aksu et al., 2021). In the present mutation study, a wide variation was observed for this trait; however, some lines had lower values than the control cultivar. This negative effect might be a source of difference in physiological and biological processes related to yield (Borovsky et al., 2013).

On the other hand, one of the most interesting features of some mutant lines was the significant increase in 1000-seed weight over the control value, contributing to a higher yield. Compared to our peak value (220 g), Tadesse and Bekele (2003) and Sayar and Han (2015) found that the largest weight was 91.0 and 136.5 g for this trait, respectively. A higher 1000-seed weight is also an important trait because larger seeds are preferred in some domestic markets. GPM39 and GPM10 had >200 g 1000-seed weight, and they therefore should be evaluated as promising lines in breeding studies for their economic interest. Although grass pea is used as a pasture crop, green fodder, and animal feed, it is mainly cultivated for seed yield (Grela et al., 2010). Therefore, one of the main targets in grass pea breeding is to increase seed yield (Campbell et al., 1994). In our M_2 population, the seed yield ranged from 7.19 to 87.18 g, and the lines GPM6 and GMP11 showed superior values compared to control (13.6 g), indicating the positive effect of mutation in obtaining desired traits. These lines also had the highest biological yield among the mutated M_2 lines, and they should be followed in future to integrate as genetic resources for potential higher biological and seed yield genotypes in breeding studies.

Grass pea is an important food source; therefore, the characterization of seed composition and biochemical compounds can be useful to identify novel features in mutant phenotypes. The changes in seed composition, such as increase in protein content, are highly important in grass pea as a legume crop. There were promising mutants that showed higher levels of protein content among mutated lines (Table 2). Mutant GPM37 exhibited >35% crude protein content, which is one of the highest values in grass pea compared to studies conducted by (Campbell et al., 1994). Unfortunately, its seed yield was low compared to the control cultivar, Gürbüz 2001. While GPM37 would not be optimal as a cultivar, it could be used as a parent in crossing programs to improve protein content, especially in the studied climatic area. An interesting result in our study is that GPM4 exhibited 9.08% protein content, which was about three times lower than the control plant. These two extreme phenotypes for protein content are thus highly important for identifying QTLs and functional analysis of corresponding genes. Besides yield and seed composition traits, breeding studies on grass pea have focused on estimating the varying levels of β-ODAP and eliminating or reducing the content of this toxin (Dixit et al., 2016). The β-ODAP content of grass pea seeds ranged from 0.139% to 7.39% in selected M2 population, while the control cultivar had 0.523%. This result indicates mutagen effects on seeds for this trait. Similarly, lower β-ODAP values than the control seeds were obtained after mutagen application (Talukdar, 2009). Assessment of grass pea seeds worldwide showed a wide variation in the β-ODAP content of the seeds, with ranges of 0.1-0.3% (Jeswani et al., 1970), 0.02-1.20% (Abd-El-Moneim et al., 2000), 0.02–0.54% (Fikre et al., 2008) and 0.02–2.59% (Kumar et al., 2011). The mutant lines that had low β-ODAP content, as identified in this study, might be evaluated under different conditions to obtain lower values for this trait. In particular, GPM37 had the lowest and highest values for β-ODAP and protein content, respectively. This genotype therefore should be stabilized and later integrated into breeding studies as a parent genotype for combining low ODAP content, high seed yield, and protein con-tent. The ADF concentration values include cellulose and lignin, while NDF value refers to the total cell wall and consists of the ADF fraction plus hemicellulose (Acikgoz et al., 2013). ADF and NDF values should be lower for monogastric and ruminant animals. The minimum values for ADF (7.47%) and NDF (12.96%) were lower than the control. We also obtained lower ADF and NDF than the study conducted by Basaran et al. (2011), which stated that the minimum values were 28.80 and 62.1, respectively. The other trait, relative feed value, is an index used to calculate the intake and energy rate of forage and is obtained from digestible dry matter and dry matter intake (Ayan et al., 2010). According to Hay Market Task Force of American Forage and Grassland Council standards, the genotype is classed as premium quality when it has protein content >19, ADF <31%, NDF <40%, and RFV >151. The 23 mutant lines in the M2 population meet these standards; there-fore, they should be classed as premium with respect to forage quality. Among them, GPM6 and GPM11 were considered the most promising lines for forage breeding with their high crude protein content, low ADF, and NDF ratios, as well as high seed and biological yield. They can also be used as a parent in hybridization studies to pyramiding different traits in grass pea.

Great variation was obtained for flower color after mutagen application in M2 generation. Similar effects were observed in the study conducted by Ramezani et al. (2017), which detected different flower color mutations such as pink, purple, white, and yellow detected against blue, which is a natural color in control progenies. Campbell (1997) stated that flower color in grass pea is generally highly correlated with the seed color: the blue, pink, or red flowers usually produce speckled, colored seeds, whereas the white flowers are associated with white-to-creamy yellow seeds. However, there was no strong correlation between flower and seed coat color in our study, which might be a sourced of mutation effect on these traits.

CONCLUSIONS

According to the findings obtained from the research, agronomic and forage traits are highly affected by EMS mutagen in grass pea. As a result, it was found that all traits showed wide variation after mutagen application and we obtained superior lines compared the control cultivar for almost all traits. These lines could be used directly and also integrated into hybridization programs indirectly to develop recombinant genotypes showing promise. They might also be evaluated in genomic and mapping studies to identify related genes/QTLs for studied traits.

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Authors' contributions

Engin YOL (EY) and Mehmet Arslan (MA) wrote the manuscript, planned the research and performed statistical analysis. Emine Doğan (ED) conducted field studies and collected data. All authors discussed the result and contributed to the final manuscript.

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