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Egyptian Clover Genotypic Divergence and Last Cutting Management Augment Nutritive Quality, Seed Yield and Milk Productivity

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Abstract: Under a changing climate, harvesting management and exploiting the genotypic divergence of Egyptian clover cultivars offers a biologically viable solution to sustainably boost the milk productivity of dairy animals. Two multi-season field trials were executed under semi-arid conditions whereby the first experiment aimed to assess the potential of Egyptian clover cultivars (Berseem Agaiti, Anmol and SB-11) for nutritional quality attributes and the digestibility of green forage and hay, forage palatability and milk productivity of buffaloes fed on hay. In the second field investigation, new promising line, SB-11, was tested for seed production potential under varying dates of the last harvesting regimes (10, 20 and 30 March along with 9 April and 19 April) owing to a sharp hike in temperature. In terms of the nutritive value of green forage and hay, SB-11 remained superior for recording the maximum crude protein (CP), ash, fat and nitrogen-free extract except dry matter (DM) content that was exhibited by the Anmol cultivar. Additionally, SB-11 remained unmatched by giving a minimum crude fiber (CF), while Berseem Agaiti yielded the lesser nutritive forage by producing 4% and 2% higher CF than SB-11 and Anmol, respectively. Moreover, SB-11 recorded the maximum digestibility of CP and DM. Furthermore, SB-11 exhibited a 6% and 9% higher palatability along with 8% and 11% higher milk production than Anmol and Berseem Agaiti, respectively. Additionally, 20 March surpassed the rest of the cutting dates by exhibiting 7%, 23%, 50% and 207% more seed yield than 10 March, 30 March, 9 April and 19 April, respectively, indicating quite a pronounced effect of the last cutting management on the seed production potential of Egyptian clover. The research findings suggest SB-11 as a promising genotype for bridging the nutritive gap of quality feed (forage and hay) for buffaloes along with addressing the seed production challenge of Egyptian clover.

Keywords: biomass yield; crude protein; correlation analysis; leguminous forages; palatability

1. Introduction

The changing climate, global warming, abiotic stresses, rapidly increasing human population, and decreasing land under forage crops owing to skyrocketing demand for staple crops have jeopardized dairy animal's nutritional status globally [1]. Sustainable supplies of milk and meat primarily depend on the provision of quality forage in sufficient quantities throughout the year. The cereal forages, including maize, millets, sorghum, oat, barley, triticale, etc., are deficient in protein and for attaining potential milk production, so dairy animals need to be fed expensive, protein-rich concentrates. This multiplies cost of production and diminishes the economic returns of dairy farmers [1,2]. Leguminous forages are best suited to cope with the nutritional challenge of dairy animals, provided their nutritional quality attributes are maintained through agronomic management. Among forage legumes, Egyptian clover (*Trifolium alexandrinum* L.) is a succulent annual crop that has been cultivated as forage and green manure crop primarily in the Nile valley's alkaline soils, USA, Turkey and Indo-Pak subcontinent [1,3]. It may be sown in sole cropping, intercropping with cereals and cover crop in rotation with staple crops. Being a leguminous crop, it tends to boost soil fertility, contributes to the reclamation of saline soils and thrives well in semi-arid conditions [1,4,5]. Under a changing climate scenario, it has emerged as a strategic forage resource as it fits well within a variety of low-input sustainable forage systems. In terms of nutritional quality (protein and mineral contents), Egyptian clover forage surpasses all cereals and many leguminous forage crops [6]. Additionally, it encompasses shallow taproot, hollow and branching stems that bear alternate leaves [6–8]. Furthermore, it entails an unmatched regenerative capacity and has the potential to supply several cuttings of nutritious and palatable forage for dairy animals.

Egyptian clover cultivars are varied significantly in terms of nutritional traits, especially crude protein, which directly determines the productivity of milch animals [1,4,7]. It was inferred that the screening of Egyptian clover genotypes for nutritive traits results in the isolation of genotypes having a higher dry matter production and protein content with a significantly lower crude fiber content (fiber tends to deteriorate forage quality by lowering digestibility) [1,7]. Salama et al. [1] reported that the Ghiza-6 genotype of Egyptian clover recorded better nutritive value, especially in protein content, than Sakha-4, Helaly and Gemmeza-1 cultivar and, thus, suggested that genotypic divergence must be exploited for attaining highly nutritive forage. In addition, it was opined that phenotypic divergence existed among clover genotypes, which influenced its palatability of forage and, thus, imparted a significant influence on milk productivity of dairy animals. Furthermore, Iqbal et al. [4] opined that Egyptian clover hay (air-dried feed) prepared from high-nutritive cultivars hold potential to sustainably bridge the lean period of forage during the summer months when green forages are not available, and thus, dairy animals productivity sharply decreases. However, to the best of our study, research findings are scant regarding the nutritional profiling of Egyptian clover genotypes grown under semi-arid conditions for forage and hay, and, resultantly, dairy animals suffer drastically during the summer months in Pakistan, leading to milk scarcity and exorbitant price hikes.

Despite numerous advantages offered by Egyptian clover, one of the downsides is its cross pollinated nature, as its flowers need pollination agents such as honeybees in order to produce seeds, which has led to seed production challenges [9,10]. Moreover, genetic variability has remained scant in *Trifolium* species owing to the narrow genetic base, and thus, these have been termed as shy-seeder or scant seed producers. Recently, climate change, global warming, the unabated use of toxic pesticides, and the disruption of bees' niche have led to a serious decline in indigenous bee populations, and, ultimately, new challenges have emerged in the form of sub-optimal fertilization, unprecedentedly higher pollen sterility and the post-fertilization abortion of newly developing seeds [9,11].

Recently, the changing climate scenario in Pakistan has resulted in a prolonged warm season that has considerably shortened the cool season, leading to the significant disruption of seed production. Under the semi-arid conditions of South Asia, biologically viable and economically feasible solutions have remained scant for boosting the seed production of Egyptian clover. Under changing pedo-climatic conditions, agronomic management strategies for Egyptian clover have remained unattended, and emerging climate change requisites their re-evaluation for bolstering seed production potential. Among agronomic practices, harvest management is one of the most crucial factors that greatly influence plant physiology and the productivity expression of clover genotypes. Previously, it has been inferred that dry matter yield, seed production capacity and assimilates partitioning of Egyptian clover remained dependent on the phenological stage at which the crop was harvested [10,12]. However, these studies have provided scant information pertaining to the dynamics of growth, regrowth and last cutting of Egyptian clover genotypes. The regrowth of clover species following the defoliation or initial 2–3 cuttings is a complex process governed by environmental factors' interactions with morphological and physiological factors, which determine the nutritional attributes and seed production potential.

Previous research findings including by Khaiti and Lemaire [13] concluded that assimilates partitioning between roots and shoots varied significantly by genetic make-up, climatic conditions and soil fertility status, which ultimately determined the productivity. Likewise, Blaikie and Mason [5] inferred that the root: shoot ratio significantly influences the growth and morphological development of white clover, while cuttings cause disturbances to the synchronous development of below- and above-ground biomass. However, the partitioning of biosynthesized assimilates towards reproductive parts determines the seed yield of leguminous crops [14]. The selection of cultivars having rapid regrowth through the utilization of organic reserves, assimilates and proteins that get stored in crowns and roots might contribute to producing highly nutritious forage along with successful seed production [13,15]. Additionally, Martiniello et al. [8] opined that shortening the interval of cutting coupled with a sharp increase in daytime temperature causes a serious decline in root reserves, which negatively affects seed development and yield. It was also inferred that seed-filling exhibited strong sink and primarily received photosynthates from leaves, and thus, the need to investigate the last harvest time of Egyptian clover was stressed. The last cutting management might manipulate and redistribute assimilates leading to achieving a high seed output. Previously, it was also emphasized that leaving crops for seed production after three harvestings yielded a 28% higher seed yield compared to four harvestings, as reported by Yadav et al. [16], Iannucci [17] and Sardana and Narwal [18]. So, it is of strategic pertinence to optimize the last cutting time for the Egyptian clover crop left for seed production to avoid the adverse effects imparted by high temperatures on pollination and fertilization. In the Punjab and Sindh provinces of Pakistan, there is sharp increase in temperature, especially in the middle of March and April, which has re-invited researchers' interest to optimize the last cutting of Egyptian clover to boost seed production.

Therefore, to ensure food security under a changing climate, it becomes pertinent to bridge the research gaps regarding the nutritional profiling of Egyptian clover genotypes and boost seed production potential through last cutting management, keeping in view rapidly hiking temperatures during the months of March and April. It was hypothesized that genotypic and phenotypic divergence might exist among Egyptian clover genotypes, and the optimization of harvesting date owing to the quick switching of weather conditions in the months of March and April might yield nutritious forage and hay along with a higher seed yield. Thus, the present multi-year field studies aimed to explore the genetic divergence among Egyptian clover genotypes for nutritional traits of green forage and hay under semi-arid conditions. Additionally, another objective was to evaluate the palatability of different genotypes and influence of hay feeding on the milk productivity of buffaloes. Lastly, it was also aimed to determine the most superior last cutting date for boosting the seed yield of Egyptian clover on a sustainable basis.

2. Materials and Methods

2.1. Meteorological Features and Physico-Chemical Description of Experimental Locality

The multi-year field trials were undertaken at Fodder Research Institute, Sargodha, Punjab province, Pakistan during 2018–2019 and 2019–2020. The geographical coordinates of the trial's locality are demonstrated in Figure 1. The meteorological characteristics pertaining to the precipitation and temperature of the study locality during the crop growth seasons have been presented in Table 1. The study locality entails harsh climatic conditions [19] characterized by extreme summers and cold winters. There were high temperatures during October, and thereafter temperature fell and again increased significantly during the last cutting months (March and April) of Egyptian clover during both seasons.

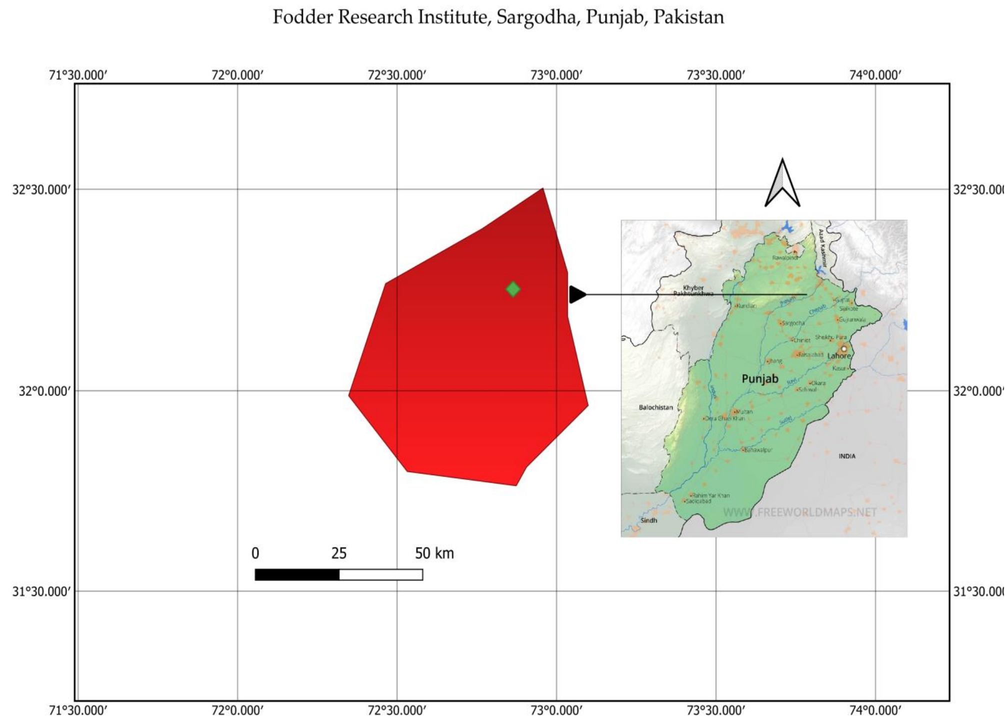

Figure 1. The trial's location (Sargodha, Province of Punjab, Pakistan) prepared using QGIST software whereby green dot presents the proximate location of experiment, while half arrow shows the north direction.

Table 1. The meteorological features (precipitation and average maximum and minimum temperature) of the study area during growth seasons of Egyptian clover.

Month	Average Maximum Temperature (°C)	Average Minimum Temperature (°C)	Rainfall (mm)
	2018–2019		
October	28.3	15.2	0
November	25.7	13.7	24
December	23.2	12.9	12
January	21.1	10.1	13
February	25.8	14.6	5
March	29.2	15.3	21
April	34.4	19.1	17
	2019–2020		
October			3
November	27.1	13.2	13
December	26.8	12.7	8
January	21.9	10.9	7
February	23.4	15.4	21
March	28.5	17.2	41
April	32.1	18.4	29

Before the sowing of the crop (Egyptian clover), different analyses were performed for determining the physico-chemical traits of the experimental block. The soil samples were collected from the depths of 0–15 cm and 15–30 cm, while collection was performed from the middle of the block as well as from four corners. Subsequently, all the soil samples collected from both soil depths were manually homogenized, shade dried, grounded and finally sieved with the help of a sieve of 2 mm pore size. For pH estimation, soil mixing with water was completed in the ratio of 1:2.5 in order to prepare the paste, while a glass electrode was used for the estimation of pH [20]. The conductivity meter was put into use to determine the electrical conductivity (EC) of the soil samples [21]. Additionally, organic carbon (OC) was also estimated by following the wet oxidation method, while the Walkley–Black protocol was used in order to assess the soil's organic matter (OM) [22]. Among macronutrients, total nitrogen (N) was determined using a Kjeldahl apparatus for performing the distillation process and H_2SO_4 (concentrated acid) titration [20,21]. Olsen's method was used for phosphorous (P) content determination, which involved a reaction of 0.5 N $NaHNO_3$ with soil: extractant paste in a ratio of 1:10 at 8.5 pH in a system containing H_2SO_4, and subsequently a spectrophotometer (882 nm) was used [23]. Finally, the ammonium acetate extraction method involving the shaking of soil samples with ammonium acetate solution of 0.5 M for 30 min resulted in the displacement of K+ ions that were detected by a flame photometer. In order to estimate the micronutrients concentrations, an extraction method involving the reaction of ammonium acetate solution (CH_3COONH_4) with the paste of soil samples (pH = 3.0) was executed for an estimation of the iron (Fe) content. In addition, the Fe content of soil samples was determined by following the methodology of colorimetry involving the use of a spectrophotometer (510 nm wave lengths). Moreover, boron (B), copper (Cu), zinc (Zn) and manganese (Mn) contents were also estimated using an extraction method with diethyle-netriaminepentaacetic acid [21–23]. The experimental soil's texture was clay loam having a pH and OM of 7.5 and 1.29%, respectively, indicating a higher requirement of fertilizers in order to achieve potential yield. The bulk density of the experimental soil was 1.31 cm^{-3}, while EC was 0.75 dS m^{-1}, indicating the soil was normal without salinity. As far as macronutrients of the soil samples were concerned, NPK concentrations were 79, 6.3 and 192 mg kg^{-1}, respectively. The micronutrients including B content (1.34 mg kg^{-1}), Mn (17.8 mg kg^{-1}), Fe (16.5 mg kg^{-1}), Cu (1.63 mg kg^{-1}) and Zn (1.34 mg kg^{-1}) were sufficient to support successful crop production.

2.2. Details of Treatments and Experiment's Execution

Two multi-year field investigations were executed in order to determine the nutritional profiling of indigenous varieties of Egyptian clover green forage and hay along with the seed production potential of a new candidate line under semi-arid conditions of Pakistan. The first trial was comprised of three cultivars including SB-11, Anmol and Berseem Agaiti, which were evaluated in terms of nutritional value of forage determined as crude protein, fiber, fats, ash and nitrogen-free extracts. The nutritional value traits were also determined for hay prepared from the same cultivars of Egyptian clover. Moreover, the palatability of green forage and impact of hay on the milk productivity of buffaloes were also investigated. In the second multi-season field trial, five different last cutting dates', including 10, 20, 30 March and 9 April and 19 April, impact on the seed production potential of candidate line SB-11 was tested, keeping in view the phenomenon of rapid weather switching in the month of March (mostly temperature hikes quickly with each passing day in March, which directly affects crop growing conditions). The experimental design for both experiments was a randomized complete block design (RCBD) with four replications. In both experiments, the net plot size of experimental units was 6 m $\times$ 5 m after the exclusion of the area of two-foot wider walking paths and field bunds of one and half feet width. Additionally, 5 feet fallow areas were kept between the experimental plots, while the fallow area of 5 m was maintained among replications. Regarding fertilization, urea, diammonium phosphate (DAP) and sulphate of potash (SOP) were applied at the rate of 110 kg ha^{-1}, 170 kg ha^{-1} and 125 kg ha^{-1}, respectively. The seeds of Egyptian clover cultivars were subjected to hydro-priming (pre-sowing seed soaking in sterilized water for 12 h) for ensuring vigorous seed germination, as suggested by Iqbal et al. [24]. Thereafter, hydro-primed seeds were dried under shade on muslin cloth and subsequently stored at 10 °C.

In order to prepare a finer seed-bed, three ploughings were performed using tractor-driven ploughs, while each plough was followed by planking using wooden planks. Egyptian clover cultivars were sown using 20 kg ha^{-1} as per the treatment. The sowing dates of the trials were 15 October and 20 October during first and second sowing seasons, respectively.

2.3. Response Variables Recordings

Pertaining to the data collection of response variables for both experiments, ten randomly selected plants from the inner rows of experimental plots were utilized for computing mean values to be used in further statistical analyses. For the first experiment, nutritional attributes of green forage and hay were determined by following standard protocols. The crude protein (CP) content of forage and hay were estimated with the help of a macro-KJeldahl apparatus, which assessed the nitrogen concentration that was then multiplied with a constant factor of 6.25 to calculate CP [25,26]. Likewise for evaluation of crude fiber content (CF), a digestion method involving H_2SO_4 and NaOH was followed. Furthermore, the ashing of forage and hay samples at 600 °C in a muffle furnace was performed for the total ash estimation [24,27]. Moreover, dry matter (DM) percentage was calculated through the determination of moisture content and DM by subjecting hay and forage samples to drying the sample above 100 °C, and thereafter moisture losses were measures using Equation (1) [20]. After the estimation of moisture percentage, dry matter percentage was computed using Equation (2). The nitrogen-free extract (NFE) depicts various non-structural carbohydrates including sugars and starches and was estimated for forage and hay samples using Equation (3). The ether extract (EE) shows fats and lipid contents of forage and hay samples that were estimated by subjecting the samples to ether solvent in order to remove lipid compounds from the samples. Similarly, the CP digestibility (CPD) and DM digestibility (DMD) of forage and hay were also determined using Equations (4) and (5), respectively [22]. Acid detergent fiber (ADF) shows lignin and cellulose fractions of the forage and hay samples, which deteriorate the nutritional value of the feed, and it was estimated by the sample's boiling in the acid detergent solution in order to remove the soluble portion and resultantly insoluble fraction as ADF was calculated. Likewise, CPD was estimated by calculating the difference between protein intake and protein lost in feces by following Equation (5) [20,22].

In order to prepare the hay of Egyptian clover cultivars, green forage was subjected to sun-drying to bring the moisture content to 13% as per the field curing methodology suggested by Iqbal et al. [4]. The green forage was spread in the dry field on an elevated cemented platform as a precaution to restrict moisture flow towards the drying place. The side shifting of drying fodder was performed after every three days gently while taking precaution to minimize leaf shattering. In order to determine the forage palatability of Egyptian clover cultivars, nine buffaloes having similar intakes of green forage of 65 kg on a per day basis were sorted out from the pool of over three dozen animal units after 15 days of consecutive feeding and appropriately tagged. The same animal units were monitored for milk production for 15 consecutive days, and thereafter those were provided hay of Egyptian clover cultivars in order to determine the influence of feed on milk production for 20 consecutive days.

For the second experiment, the plant height was estimated using a tailor's tape from the base of the plant to the uppermost leaf's tip. At each harvest, the number of capsules was recorded using m^2 quadrate in each experimental plot. Likewise, the number of seeds per capsule was estimated by randomly selecting ten heads from each treatment unit, and their average was then computed. In addition, 1000 seeds' weight was determined by manually counting the seeds at each harvest and weighing them using a digital balance. The seed yield was estimated after the harvesting of all plants in every unit, which were separately bundled and weighed using a spring balance in the field. Thereafter, the seed yield of all experimental plots was separately determined, and then seed yield was converted into a per hectare basis using Equation (6).

$$\text{Moisture percentage} = \frac{\text{PrDSW} - \text{PsDSW}}{\text{Sample weight}} \times 100 \tag{1}$$

where

PrDSW= Pre-drying sample weight
PsDSW = Post-drying sample weight

$$\text{DM percentage} = 100 - \text{moisture percentage} \tag{2}$$

$$\begin{aligned}\text{NFE percentage} = 100 - (&\text{water percentage} + \text{CP percentage} + \text{CF percentage} \\ &+ \text{Ash percentage} + \text{EE percentage})\end{aligned} \tag{3}$$

$$\text{DMD (\%)} = 88.9 - (0.779 \times \text{ADF percentage}) \tag{4}$$

$$\text{CPD (\%)} = \text{CP intake (\%)} - \text{CP lost in feces} \tag{5}$$

$$\text{Grain yield of Egyptian clover} = \frac{\text{Yield per plot} \times 10{,}000\ \text{m}^2}{\text{Plot area (m}^2\text{)}} \tag{6}$$

2.4. Statistical Analyses

The data were recorded for all response variables under study that were thoroughly arranged, and subsequently, statistical analyses were performed firstly by employing Bartlett's test, which demonstrated non-significant effects of the year. Therefore, year-wise data (2018-19 and 2019-20) were transformed into averaged values in order to execute further statistical analyses for estimating the significance among experimental treatments [28,29]. Thereafter, Fisher's analysis of variance (ANOVA) technique was employed for the estimation of generalized significance, while treatment means were compared by putting into use Tukey's Honest Significant Difference (HSD) test at the level of 5% probability using a computer-run SAS statistical package (9.2 Version, SAS Institute, Cary, NC, USA). The Pearson's correlation was also conducted using the same statistical package, while Microsoft's Excel program was utilized for preparing correlation figures as scattered points [30].

3. Results and Discussion

3.1. Experiment 1

3.1.1. Dry matter and Nutritional Attributes of Forage

The results pertaining to nutritional quality attributes revealed that Egyptian clover cultivars differed significantly, as Anmol cultivar remained unmatched for recording the maximum dry matter (DM) (Table 2). It was followed by the candidate line SB-11, which showed 1% lesser DM, while Berseem Agaiti showed 1.9% and 0.8% less DM compared to Anmol and SB-11, respectively. The crude protein (CP) content of forage is one of the most important nutritional quality traits and determines the feed value, and the line SB-11 surpassed rest of the cultivars by recording 1.15% and 2% higher CP than Anmol and Berseem Agaiti. The following cultivar, Anmol, gave a higher CP than the least-performing cultivar of Berseem Agaiti. Additionally, the crude fiber (CF) content decreases the nutritional value of the forage, and the Berseem Agaiti cultivar gave the maximum CF content, which was

2.3% and 4% higher than Anmol and BS-11. The minimum CF was recorded for SB-11 and it was significantly lesser than following the cultivar of Anmol. Pertaining to the fat content (Ft) of berseem clover cultivars, candidate line SB-11 outperformed the rest of the cultivars by exhibiting the maximum Ft, while it was followed by Berseem Agaiti. The minimum Ft was exhibited by the Anmol cultivar.

Table 2. Nutritional attributes of Egyptian clover cultivars sown under semi-arid conditions of Sargodha, Punjab, Pakistan (means of 2 years).

Variety	DM (%)	CP (%)	C. Fiber (%)	Fat (%)	Ash (%)	NFE (%)
SB-11	12.81 ± 0.13b	18.28 ± 0.26a	18.44 ± 0.18c	3.99 ± 0.09a	14.92 ± 0.51a	44.57 ± 0.82a
Anmol	13.90 ± 0.28a	17.13 ± 0.53b	20.03 ± 0.63b	3.19 ± 0.31c	14.04 ± 0.14c	42.71 ± 0.72b
Berseem Agaiti	12.05 ± 0.11c	16.25 ± 0.37c	22.35 ± 0.59a	3.53 ± 0.19b	14.19 ± 0.47b	41.88 ± 0.79c

Values having different letters within same column vary significantly at 5% probability level.

Furthermore, the total ash (Ta) presents mineral constituents of the forage, and SB-11 showed the greatest Ta; it was followed by Berseem Agaiti, while Anmol could not perform at par to other cultivars as far as Ta content was concerned. Regarding nitrogen-free extract (NFE), which represents various non-structural carbohydrates (starches, sugars, etc.), SB-11 exhibited the maximum NFE, and it was followed by Anmol, while Berseem Agaiti showed the minimum NFE content (Table 2). Besides nutritional quality traits, the digestibility of feed significantly influences the overall impact and value of the feed. It was observed that SB-11 recorded the highest crude protein digestibility (CPD), which was 2% and 6% higher than Anmol and Berseem Agaiti cultivars, respectively (Table 3). The following cultivar, Anmol, gave 4% more CPD than Berseem Agaiti, which could not perform at par to other cultivars as far as CPD was concerned. Pertaining to dry matter digestibility (DMD), SB-11 remained unmatched by recording a maximum DMD that was 7% and 4% higher than Anmol and Berseem Agaiti, respectively. Berseem Agaiti followed it by exhibiting 3% higher DMD than Anmol. In addition to nutritional traits, contents and digestibility, palatability, which presents the likelihood of feed by ruminants, constitutes one of the most strategic factors determining the nutritional value of the forages. The results revealed that SB-11 outperformed rest of cultivars by exhibiting 5% and 9% higher palatability than Anmol and Berseem Agaiti, respectively (Table 4). Moreover, Berseem Agaiti recorded a 3% higher palatability than Anmol, which remained inferior to rest of cultivars.

Table 3. Digestibility of crude protein (CPD) and dry matter (DMD) of berseem clover cultivars sown under semi-arid conditions of Sargodha, Punjab, Pakistan (means of 2 years' values).

Variety	CPD (%)	DMD (%)
SB-11	70.26 ± 0.27a	68.65 ± 0.15a
Anmol	68.48 ± 0.19b	61.86 ± 0.41c
Berseem Agaiti	64.09 ± 0.43c	64.92 ± 0.11b

Values having different letters within same column vary significantly at 5% probability level.

Table 4. Palatability of Egyptian clover cultivars sown under semi-arid conditions of Sargodha, Punjab, Pakistan for dairy buffaloes. (Total of 9 buffaloes; 3 buffaloes fed forage of each cultivar, and then 2 years' values averaged).

Variety	Offered Forage (kg)	Consumed Forage (kg)	Leftover Forage (kg)	Palatability (%)
SB-11	90	73	17	81.1
Berseem Agaiti	90	68	22	75.6
Anmol	90	65	25	72.2

The DM of forage is one of the most critical traits that determine the feed value of any feed stuffs [31]. Our findings remained in agreement with those of Martiniello et al. [8], who inferred that phenotypic divergence existed among tested genotypes, as clover genotypes belonging to Italian origin were found to be superior. owing to having higher DM yields compared to Egyptian genotypes. Based on these findings, it was suggested to explore the genetic divergence of clover for sorting out genotypes having a higher DM. In contrast to our findings, whereby Anmol surpassed other cultivars in terms of DM, Tufail et al. [32] inferred that Berseem Agaiti remained superior by yielding the highest DM of other cultivar under investigation. However, in agreement to our findings, Ranjbar [33] opined that over 21% higher DM might be achieved by sorting genetically superior cultivars of Egyptian clover. Besides genetic potential, it was suggested that agronomic management and favorable climatic conditions might yield over 41% more DM than low-potential cultivars sown under a suboptimal production technology package [34]. Likewise, Iannucci and Martiniello [35] reported that different genotypes responded differently to frequent harvesting, while few clover genotypes boosted the regeneration growth and DM production compared to low yielding cultivars.

In addition to DM, CP content improves the nutritive value of the forage and has been reported to be in antagonistic association with CF content, which decreases the digestibility and nutritional value of the forage [36]. Previous studies have provided evidence that, regarding the CP content of Egyptian clover, little genetic divergence was recorded; however, the number and the quality of leaf fraction served as the most strategic factor in determining the CP content, while a lesser leaf fraction coupled with a higher shoots ratio resulted in a greater CF content [24,27,36]. It was also argued that, being a defoliation-tolerant species, Egyptian clover recorded robust regrowth after harvesting with significantly higher leaf: stem values, leading to a higher CP and lower CF content [37,38]. Contrastingly, relatively smaller changes in the digestibility and CP concentration of the leaf during the growing season were recorded, and thus, minimal quality differences existed among the tested cultivars of clover subjected to varying harvest management systems. Likewise, it was inferred that as leaves were unmatched in terms of nutritive value compared to stems and thus genotypes of Egyptian clover, having a higher number of leaves and leaf area might be promoted to achieve higher CP and lower CF contents [37,39]. Amato et al. [40] inferred that the frequent cutting of Egyptian clover resulted in a higher regrowth of leaves, which led to a greater leaf: stem ratio, and ultimately the nutritive value of forage was increased. Previous findings also reported that the genetic potential for higher nutrient use efficiency caused a greater accumulation of amino acids (the building block of protein), and ultimately, superior CP, fats and ash contents were observed. Moreover, ash content presents mineral constituents of the forage, and considerable genetic divergence was recorded among Egyptian clover genotypes as per our research findings that was presumably owing to a better canopy structure and roots architecture, which boosted mineral uptake from the soil solution, and higher ash and NFE contents might be achieved. Besides nutritional quality traits such as protein, fiber, fat and ash contents, it is the digestibility that determines the nutritional value of the forage, as suggested by Iqbal et al. [40]. In this field trial, the candidate line SB-11 remained unmatched in terms of DM and CP along with CPD and DMD, indicating its genetic superiority over the rest of the cultivars. These findings corroborate with those of [41,42] who provided research-based evidence that annual Egyptian clover cultivars differed genetically in terms of CP biosynthesis and digestibility, owing to varying amino acids' accumulations having different compositions, which ultimately affects the digestibility of CP.

Similarly, the research findings of this field experiment are also in concurrence with those of [43,44], which opined that dry matter digestibility varied significantly among clover species and that it was presumably owing to lesser fiber contents, which increased the dry matter disability of forage. Moreover, palatability, which depicts the likelihood of ruminants for feed, serves as one of the chief traits of nutritional quality, as a low palatability of highly nutritious feed remains ineffective in giving the desired productivity of milk and meat [4]. Our research exhibited that the line SB-11 gave the highest palatability and a significantly lower leftover forage, presumably owing to an acceptable taste, aroma and absence of toxic chemicals, which increased the consumption of forage. These findings are in line with previous studies [45–47] whereby it was inferred that the presence of a bad aroma, stem hardiness, lesser leaves and higher stem ratio resulted in the lesser consumption and low palatability of forage.

3.1.2. Nutritional Quality of Hay and Buffalo Milk Productivity

The analytical assays pertaining to nutritional quality traits of hay prepared from Egyptian clover cultivars exhibited that significant genetic divergence was present among cultivars under evaluation. The highest quality hay was demonstrated by candidate line SB-11 in terms of crude protein, which was 1.03% and 2.56% higher compared to the CP exhibited by hay prepared from the Anmol and Berseem Agaiti cultivars, respectively. In addition, the same cultivar recorded a 1.26% higher CP than the most inferior cultivar Berseem Agaiti, as far as the CP content of the hay was concerned. The crude fiber (CF) content reduces the nutritional value of the hay, and the SB-11 line showed the highest CF, which was 1.18% higher than the following cultivar of Anmol. However, Berseem Agaiti surpassed other cultivars by recording the minimum CF content. As far as the fat content (Ft) of berseem clover was concerned, SB-11 remained unmatched by giving the maximum Ft, while the Berseem Agaiti cultivar followed it by recording 0.31% less Ft content. The least Ft content was exhibited by the Anmol cultivar and was 0.53% and 0.22% less than the SB-11 and Berseem Agaiti cultivars, respectively. Furthermore, the total ash (Ta) content, which actually represents mineral constituents of the feed, was found to be the highest in hay prepared from the Anmol cultivar that was 1.13% and 1.31% more than SB-11 and Berseem Agaiti cultivars, respectively. Moreover, the hay prepared from the SB-11 line remained superior to Berseem Agaiti cultivar hay by recording a 0.18% higher Ta content. Pertaining to the nitrogen-free extracts (NFE) of the hay, Berseem Agaiti exhibited the maximum NFE, and it was followed by the Anmol cultivar. The candidate line SB-11 showed 1.41% and 5% less NFE in comparison to the Anmol and Berseem Agaiti cultivars, respectively (Table 5). As far as the milk productivity of buffalo fed on the hay of Berseem cultivars was concerned, the candidate line SB-11 remained superior as it boosted milk production by 0.6 L and 0.8 L compared to Anmol and Berseem Agaiti hay, respectively (Table 6). The minimum increase in milk production was imparted by the hay prepared from the Anmol cultivar of Egyptian clover.

Table 5. Nutritional quality attributes of hay prepared from Egyptian clover cultivars sown under semi-arid conditions of Sargodha, Punjab, Pakistan (means of 2 years' values).

Variety	CP (%)	Crude Fiber (%)	Fat (%)	Ash (%)	NFE (%)
SB-11	17.89 ± 0.62a	32.84 ± 0.10c	2.98 ± 0.18a	11.26 ± 0.44b	37.23 ± 0.41a
Anmol	16.86 ± 0.49b	35.66 ± 0.36b	2.45 ± 0.11c	12.39 ± 0.21a	34.64 ± 0.34b
Berseem Agaiti	15.24 ± 0.51c	37.94 ± 0.29a	2.67 ± 0.37b	11.08 ± 0.15c	32.07 ± 0.55c

Values having different letters within same column vary significantly at 5% probability level.

Table 6. Milk production of buffalos fed on hay prepared from Egyptian clover cultivars sown under semi-arid conditions of Sargodha, Punjab, Pakistan (means of 2 years' values).

Variety	Milk Production before Trial (Liters) (Average of 15 Days)	After Trial (Liters) (Average of 20 Days)
SB-11	7.0	8.1
Anmol	7.0	7.5
Berseem Agaiti	7.0	7.3

Hay is the air-dried fodder and might serve as a nutritional source of feed for dairy animals when green and succulent forages vanish owing to a harsh climate (freezing temperatures or scorching heat of the sun). Among quality traits, crude protein has been regarded as one of the most vital traits [27], and hay prepared from Egyptian clover line SB-11 exhibited the maximum CP content, perhaps owing to a higher CP content recorded for green herbage of the same line, which might be attributed to a superior genetic potential; higher nutrient content, especially nitrogen uptake capacity by virtue of an extensive root network; wider canopy facilitating a higher photosynthetic rate; and the increased biosynthesis of amino acids, leading to a higher CP content [27,33]. Additionally, it has been inferred that hay preparation through air drying, as conducted in our trial under optimal conditions regarding temperature, prevents the degradation of protein, and resultantly, hay having an equivalent CP of green forage might be achieved. Furthermore, it was reported that over-drying results in leaf shattering, and resultantly CP content decreases significantly; thus, it was suggested that appropriate drying to bring the moisture level down to 13% may retain the CP content in hay [16,32]. Previous studies have reported a reverse relationship between CP and CF, as a serious decline in the nutritional value of the forage occurred with rising CF contents [1,6,15]. The hay of different cultivars exhibited varying CF contents, as SB-11 showed the minimum CF content, and it might be interpreted that other cultivars only produced a smaller number of leaves but also thicker stems, and ultimately a higher CF was witnessed in hay as well. A previous study by Ranjbar [33] reported that hay prepared from thicker stemmed crop plants recorded a significantly higher CF content owing to the presence of more cellulose, hemi-cellulose, etc., while hay of cultivars having a greater leaf number and area recorded higher a CP and lower CF content. Similarly, it was suggested that cultivars of Egyptian clover, owing to a genetic difference, recorded varying values of fats and total ash contents; however, it was suggested that the higher fats and total ash, which presents mineral constituents, improved the nutritional value of the hay [1,24,27,34]. However, it was also observed that suboptimal drying in the case of hay and inappropriate fermentation conditions for silage preparation reduced the fats and total ash contents by a considerable level. Likewise, the maximum NFE of hay was recorded for SB-11 forage, which had a higher NFE, and thus the appropriate drying of the forage, however, caused a reduction in NFE; however, this might be attributed to unavoidable loss as different types of carbohydrates and starches get restructured and de-structured during the drying process, and thus drying is bound to reduce the NFE content. Pertaining to the milk productivity of buffaloes fed on the hay of Egyptian clover cultivars, SB-11 remained outstanding by producing the maximum milk, presumably owing to improved nutritional quality of the hay. The protein content of hay has been reported to be directly involved in boosting milk and meat production, while fiber-rich feed results in a lowering of the milk productivity, as inferred by Muñoz et al. [48] and Colmenero and Broderick [49]. Similarly, dairy animals need fats and different types of minerals such as calcium, magnesium, iron, etc., to keep ongoing different functions of the body, which also support milk production and the overall health of dairy animals [1,48–50]. The candidate line SB-11 forage surpassed other cultivars in terms of fat and NFE contents, and ultimately these higher traits might be attributed to nutritious hay which, resulted in higher milk production. Previous studies have reported that the nutritional composition of the feed influences nutrient ingestion as well as rumen metabolism, and consequently, high-protein diets reduce the acetate: propionate ratio and enhance the protein synthesis that is a rich source of energy for the cow, thus imparting positive effects on milk yield [1,49,51].

3.2. Experiment 2

3.2.1. Yield Attributes and Seed Yield

The promising candidate line of SB-11 exhibited a stronger influence of last cutting dates on yield attributes and seed yield of Egyptian clover (Table 7). The results revealed that last harvesting on 19 April exhibited the tallest plants that remained statistically at par to harvesting performed on 9 April. The following treatment (last harvesting on 30 March) remained statistically equivalent to last cutting performed on 20 March, while the most inferior harvesting date (10 March) recorded the minimum plant height. The number of productive capsules is one of the most strategic yield attributes, and last cutting on 20 March remained unmatched in comparison to the rest of the harvesting dates. The last cutting on 10 March followed it by recording 2% less capsules. The minimum number of capsules was recorded for forage cutting on 19 April and was 126% less than the most-performing, last harvesting date of 20 March. The number of seeds per capsule serves as a reliable indicator to estimate the final seed yield of Egyptian clover, and the last cutting on 20 March remained unmatched in terms of seeds per capsule, which recorded a 73% higher value than the least performing cutting date of 19 April. The following harvesting date was 10 March, which performed statistically at par to the last cutting on 9 April. Pertaining to 1000 seeds' weight, the last cutting on 20 March surpassed the rest of the harvesting dates by exhibiting a 64% higher seed weight compared to the least productive harvesting date of 19 April. After 20 March, there was a persistent decline in the 1000 seeds' weight of Egyptian clover as 30 March and 9 April harvestings recorded 31% and 48%, respectively, less 1000 seeds' weight than the most-performing harvesting date of 20 March. As far as the seed yield of Berseem clover was concerned, the 20 March harvested crop recorded the maximum seed yield, which was 7% higher than the following last cutting date of 10 March. The same last harvesting date remained effective in boosting seed yield of Egyptian clover by 206% than the most inferior last cutting date of 19 April. Moreover, the last cutting date of 30 March yielded a 23% higher seed production compared to the last cutting conducted on 9 April. Overall, the 20 March harvested crops gave the maximum seed yield, while a delayed last cutting (19 April) caused a significant loss to the seed yield of Egyptian clover. These findings corroborate with those previously reported results of Asmaa et al. [52], Din et al. [53] and Singh et al. [54], whereby the plant height of Egyptian clover was inferred to be primarily a genetically controlled trait that was also influenced by agronomic management plans. However, it was concluded that time and crop stages of previous harvestings determine the plant height of Egyptian clover [52,55].

Table 7. Yield attributes and seed production of new promising candidate line SB-11 of Egyptian clover as influenced by varying last cutting dates under semi-arid conditions of Sargodha, Punjab, Pakistan (means of 2 years' values).

Last Cutting Dates	Plant Height (cm)	Number of Capsule (m^2)	Number of Seeds Capsule^{-1}	1000 Seeds' Weight (g)	Seed Yield (t ha^{-1})
10 March	71.17 ± 0.23c	451.83 ± 0.67b	41.00 ± 0.18b	2.71 ± 0.17b	0.548 ± 0.34b
20 March	82.50 ± 0.42b	460.50 ± 0.11a	45.00 ± 0.10a	3.75 ± 0.33a	0.586 ± 0.17a
30 March	84.34 ± 0.17b	408.66 ± 0.37c	43.50 ± 0.33c	2.86 ± 0.43c	0.477 ± 0.20c
9 April	90.83 ± 0.39a	379.67 ± 0.20d	41.50 ± 0.21b	2.52 ± 0.23cd	0.390 ± 0.41d
19 April	91.50 ± 0.50a	199.17 ± 0.31e	26.00 ± 0.47d	2.28 ± 0.63d	0.191 ± 0.11e

Values having different letters within same column vary significantly at 5% probability level.

Pertaining to the number of capsules and seeds per capsule, it was found that the source–sink relationship remained dynamic and varied by the crop stage and harvest management [53,56,57]. In addition, it was revealed that after the harvesting of berseem clover, shoots became the prime sinks, which were supplied assimilates by the roots and leftover crowns, which served as assimilate sources. Thus, a cycle of periodic storage of synthesized assimilates, their utilization and reutilization were repeated frequently during the crop growth season and greatly influenced the capsule development of the berseem clover [52,58–60]. Moreover, it has been revealed that intensive harvesting management involving two cuttings of Egyptian clover annually remained effective in increasing the florets number by over 14%. Conversely, a better optimization of the second-last harvesting time provided sufficient time to the crop, which led to a significant enhancing of the plant height along with leaf and capsule numbers per plant, as inferred by Puri et al. [61]

and Martiniello [62]. Similar findings have also been presented by Yadav et al. [16] and Din et al. [53], who opined that the last cutting time of Egyptian clover might result in a higher number of seed capsules and seeds per capsule by virtue of a comparatively longer reproductive phase availability, while a higher temperature in the case of delayed last harvests restricted the photosynthesis process and photosynthates transfer from sources of sinks, which led to a decline in the number of seeds per capsule, as inferred by Surinder [63], Kandil et al. [64] and Fulkerson et al. [65].

Besides the harvesting time of the last cutting, the cutting height of previous harvests imparted a significant influence on seed development and seed weight. It might be interpreted that environmental factors, especially suboptimal temperatures, might be attributed as the prime reason behind harvesting time impact on seed yield as it either limits or promotes plant's growth and, hence, the seed yield, as suggested by Mezoni, et al. [53]. As per our trial's findings, the last harvesting taken late resulted in a lower 1000-seed weight and yield, presumably owing to a shorter time available for seed setting under quickly hiking temperatures. Moreover, a rising air temperature coincides with low humidity, which results in a decline in pollination activity and fertilization rate [66–70]. Furthermore, lesser bee activity causes poor pollination, the infertility of pollen and seed development termination, leading to weak seed setting [63,65,71]. Along with the last cutting time management, the low seed production of Egyptian clover might also be attributed to poor-quality seed sowing and following inappropriate agronomic practices especially related to soil fertility and moisture management. Moreover, seed production potential by Egyptian clover was noticeably decreased at an occurrence of high temperature at a procreative phase as rising temepraturе also resulted in the decline in insect activity during the blooming span [72–77]. El-Zanaty [41] opined that 1000-seed weight and seed yield in Egyptian clover were affected by the sowing date, number of cuttings and, more importantly, by the last cutting date. The Egyptian clover growers keep on taking cuttings later after the onset of summers owing to a shortage of forage scarcity, which causes less foliage retention, poor blossoming and ultimately a smaller number of grains per capsule and seed productivity. Likewise, the 1000-seed weight reduction as observed in our trial with a delay of the last cutting could be presumably attributed to a variety factors such as temperature hikes and the disruption of the photoperiodism mechanism, which adversely affects the duration of vegetative and reproductive phases along with a decline in pollinators. The superiority of last cutting on 20 March suggested that this genotype was moderately tolerant to higher temperatures, and it might entail some genetic advantages, meriting its inclusion in future breeding programs. The seed production increment was at the expense of herbage yield, and therefore, last cutting time management has been suggested as the most vital factor for balancing green herbage yield and seed production. Thus, last cutting management might be utilized to manage the trade-off (seed in lieu of forage), depending on the requirement of forage or seed production, as suggested by Sardana and Narwal [18].

3.2.2. Correlation Analysis of Yield Attributes with Seed Yield

The correlation analysis indicates whether yield attributes are linearly associated with seed yield or have an indirect association. The results exhibited that plant height was inversely associated (R^2 = 0.48) with the seed yield of Egyptian clover, indicating that taller plants tend to reduce the seed yield of Egyptian clover (Figure 2A). Contrarily, number of capsules per plant had a significantly stronger linear relationship (R^2 = 0.96**) with the seed yield of Egyptian clover as the number of capsules varied within 199–460 and produced a seed yield in the range of 0.19–0.58 t ha^{-1} (Figure 2B). Interestingly, the maximum number of capsules per plant (560) exhibited a 205% higher seed yield compared to the corresponding value recorded by the minimum number of capsules per plant. Moreover, the 1000-seed weight was also found to be strongly directly associated (R^2 = 0.80**) with the seed yield of Egyptian clover, as a 64% increment in 1000 seeds' weight resulted in a 0.35 t ha^{-1} improvement in seed yield (Figure 2C). It might be inferred that taller plants tend to have more sinks in vegetative parts, which reduces assimilates' movement towards reproductive parts, and ultimately, seed yield is reduced [6]. These findings were in contradiction with those of Salama et al. [1], who reported that taller plants remained effective in boosting water and fertilizers' use efficiencies, which lead to the higher productivity of Egyptian clover cultivars. However, our findings corroborate with the conclusion of previous studies whereby the number of capsules per plant was reported as the vital yield attribute of clover species [24,38]. Likewise, it was inferred that seed weight directly contributes to total seed yield, and thus it might be used as a reliable indicator to estimate the seed yield of Egyptian clover cultivars [1,6,38]. It was opined that, besides superior genetic potential, the appropriate agronomic management and favorable climatic conditions contributed towards a higher 1000 seeds' weight, and ultimately, seed yield was multiplied. Moreover, Italian genotypes of clover remained instrumental in effectively utilizing farm-applied resources compared to Egyptian clover genotypes, and resultantly superior productivity was recorded for cultivars of Italian origin [1].

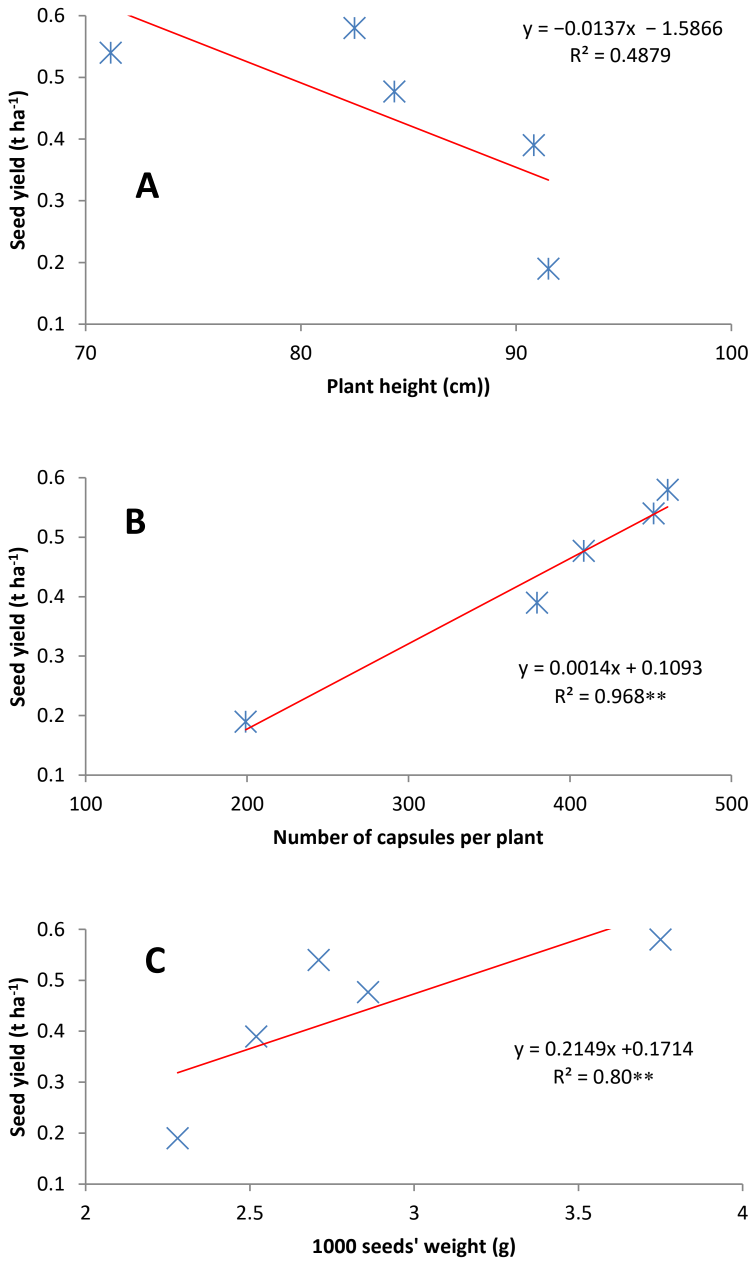

Figure 2. Correlation analysis indicating seed yield relationship with (**A**) plant height, (**B**) number of capsules per plant and (**C**) 1000 seeds' weight of Egyptian clover under semi-arid conditions of Pakistan. (** presents highly significant relationship at 5% probability level).

4. Conclusions

The research findings of this multi-year field trial remained in line with the postulated research hypothesis, as significant genetic divergence existed among Egyptian clover cultivars pertaining to dry matter percentage, nutritive quality of green forage and hay, digestibility of forage, and palatability for dairy buffaloes. The candidate line SB-11 outperformed the rest of the cultivars in terms of nutritional quality traits, especially higher crude protein, fats, nitrogen-free extract and lower crude fiber contents of green forage and hay. The same line was unmatched as far as the palatability of green forage along with protein and dry matter digestibility were concerned. Interestingly, buffaloes fed on SB-11 hay recorded a noticeable increase in milk production, indicating bright perspectives of boosting the milk productivity of large ruminants through the provision of quality feed. Based on these findings, it may be inferred that considerable genotypic divergence existed among Egyptian clover cultivars, and its exploration could constitute sustainable strategy for boosting milk productivity. In addition, the SB-11 line was further tested for seed production under varying last cutting management regimes, and results exhibited that a last cutting taken on 20 March remained instrumental in boosting the yield attributes of Egyptian clover, including the number of capsules and grains per capsule along with the 1000-seed weight, which led to the maximum seed yield of over half of a ton per hectare. It was also inferred that yield attributes except plant height were linearly associated with the seed yield of Egyptian clover line (SB-11) under investigation. Additionally, these findings exhibited that the optimization of last cutting date management keeping in view local agro-climatic conditions could serve as promising strategy for boosting the seed production of Egyptian clover under semi-arid conditions of Pakistan and other regions having similar agro-climatic conditions. Moreover, these findings might also serve as a baseline to utilize the SB-11 line in breeding programs for the development of high-yielding clover genotypes having the potential to thrive well under the changing climate scenario. However, there is a dire need to exploit a greater number of candidate lines for maximizing the nutritional quality on a sustainable basis, leading to enhanced milk and meat productivity for ensuring the nutritional security of a skyrocketing population.

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