

Effects of Fertilisation on Grass and Forb Gamie Reproduction in Semi-natural Grasslands

Michele Scotton (✉ michele.scotton@unipd.it)

University of Padua

Valentina Rossetti

University of Padua

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Abstract

Studying the effects of fertilisation on the seed production of grassland species can help understand the vegetation changes and biodiversity losses due to soil eutrophication. On a temperate hay-meadow, the seed production of fifteen grasses and seventeen forbs was studied under three fertilisation treatments: 0-0-0, 0-54-108 and 192-108-216 kg N, P₂O₅ and K₂O respectively, per year. Fertile shoots collected at the seed maturation stage were analysed for all main traits of the gametic reproduction. On average, forbs produced more ovules and viable seeds per shoot (199 and 65, respectively) than grasses (112 and 35, respectively). Fertilisation increased the number of inflorescences per shoot in both grasses and forbs and had a limited but variable effect on germinability and viability in the two functional groups: viability increased in grasses but often decreased in forbs. This pattern resulted in 55% and 11% increases in viable seed production in grasses and forbs, respectively. At the higher level of fertilisation, shoot density was positively related to the number of viable seeds per shoot in grasses and to the seed size in forbs. Higher grass seed dormancy likely allowed for later grass seed germination in autumn when vegetation cover was low, contributing to the dominance of a few light-seeded nutrient-demanding grasses and poor forb number in fertilised meadows.

1. Introduction

Extensively managed semi-natural grasslands have high plant and animal richness and play a central role in the conservation of biodiversity; the rapid decrease in these habitats in the last few decades is of great concern for European environmental policy (EEA 2004).

An issue critical for the conservation and restoration of semi-natural grasslands is the seed production of the species that compose them. In-depth study of the gametic reproduction of grassland species could reveal why grassland species react to different cultivation practices and clarify the degree to which grassland vegetation evolves following changes in management (Schütz 2000). The effects of anthropogenic increases in terrestrial soil nutrient concentrations (Galloway et al. 2004) must be investigated to clarify the possible effects of soil eutrophication on the biodiversity and functioning of grasslands. The persistence of forbs is at particular risk owing to soil eutrophication as forbs reproduce primarily by seed, whereas most grasses can reproduce through both agamic and gametic reproduction, propagate through tillering (Klotz et al. 2002), and greatly benefit from high soil nutrient levels (Gillet 1980). The patterns of grassland species seed production are also crucial knowledge for planning efficient seed harvests aimed at restoring species-rich grasslands (Scotton, 2009).

At the fertile shoot level, the output of gametic reproduction is the amount of viable seeds produced during a plant's life. This quantity is influenced by several reproductive traits: number of inflorescences per shoot, flowers per inflorescence, and ovules per flower, which determine the number of ovules per shoot (i.e., the seed yield potential); the proportions of ovules transformed to filled seed (seed set), and filled seeds which are viable (fulfilment of the yield potential) (Pearson and Ison 1997).

The above-mentioned traits are primarily genetically determined but can also be influenced by environmental factors (Hampton and Fairey 1997). The influence of soil fertility has received particular attention because of its importance for seed propagation and, more recently, its increase in terrestrial soils as a consequence of human activities (Galloway et al. 2004). Past studies have highlighted the degree to which soil fertility can affect the individual traits that determine seed yield. In grasses, for example, high nitrogen (N) supply can enlarge the inflorescence by increasing the number of flowers per spikelet or the number of spikelets per shoot (Hampton and Fairey 1997). High phosphorous (P) and potassium (K) availability was found to indirectly enhance flower formation through increasing the concentration of cytokinins in the plant (Engels et al., 2012). Stephenson (1981) reported that seed set was generally increased by higher availability of the macronutrients necessary for fertilised ovules to develop into seeds. However, Wiens (1984) found that seed set was more dependent on genetic factors. N and NP fertilization were also reported to indirectly influence seed viability in outcrossing species because it decreased the attractiveness of the nectar to pollinators (Ramos et al. 2018), therefore increasing self-pollination and consequently increasing the number of unviable seeds (Arista and Talavera 1996). Fenner (1986) reported that seed size and mineral concentration are not typically affected by soil fertility, so that even in infertile soils, seedling recruitment is not negatively affected.

The gametic reproduction of temperate grassland species has primarily been researched within the field of seed propagation, where fertilisation is the primary agronomic practice applied to influence seed yield (Rolston et al. 1997). Moderate fertilisation rates are typical to avoid an excess of vegetative growth that competes energetically with reproductive development (Hebblethwaite and Ivins 1977), and one, two, or all three primary macronutrients may be added, dependent upon the species being propagated and the base soil fertility. In semi-natural grasslands, fertilisation is also the management strategy with the strongest influence on grassland traits (Klapp 1971). Fertilisation levels have widely varying effects on gametic reproduction strategies and on seed yields, which can vary greatly by species, quantity of fertilizer, and time of distribution (Scotton et al. 2009). However, these effects can diverge significantly from those found in the propagation of forage seed, for the following reasons:

- 1.) grasslands are composed of many species with contrasting phenological strategies and different reactions to the same fertilisation;
- 2.) different aims of grassland cultivation (forage vs. seed yield) entail different management practices (e.g., earlier harvesting to obtain higher-quality forage);
- 3.) in grasslands, fertilisation is typically organic and all macronutrients are applied together, often at a very high rate.

Despite the importance of gametic reproduction in grasslands, no comprehensive studies have been conducted at the community level to address the effect of fertilisation on the primary plant species and species functional groups of these ecosystems in temperate climates, so that it is difficult to make any research hypotheses except for the general assumption that the soil nutrient-enrichment could more

favourably enhance the gamic production in grasses than in forbs.. This study therefore focused on the gamic reproduction of an important type of temperate grassland with the intent to:

- analyse the reproductive traits of the main grassland species and the two functional groups (grasses and forbs);
- identify groups of species with similar gamic reproduction behaviours and responses to fertilisation;
- and identify correlations among reproductive traits, defining different reproductive strategies and quantify their responses to fertilisation.

2. Materials And Methods

2.1 Site, meadow, and fertilisation

The grassland utilized in this study was located in Sedico (BL: 420 m a.s.l., eastern Italian Pre-Alps), where the annual mean temperature is 10.6°C and the annual rainfall is approximately 1366 mm (389, 326, 401, and 250 mm in spring, summer, autumn, and winter, respectively). The site was level and had an alluvial calcareous substratum. The soil was sandy-loam textured with 12.2% gravel content, 14.6% total carbonate content, and a pH of 7.5. Since 1977, a section of the meadow has been used for a fertilisation trial organised as three completely randomized blocks with 24 m² plots and twenty-seven treatments obtained by combining three levels of yearly N, P, and K applications per ha: 0, 96, and 192 kg N as ammonium nitrate; 0, 54, and 108 kg P₂O₅ as triple superphosphate; and 0, 108, and 216 kg K₂O as K sulphate. Since 2010, the grassland has been cut twice per year and surveyed for seed production in three treatments: no fertilisation (000), fertilization with no N and intermediate levels of P and K (011), and fertilisation with the highest nutrient rates (222).

The vegetation of the three treatments (Annex 1) corresponded to the following meadow types: type 000, vegetation intermediate between a poor-soil form of the *Arrhenatherum elatius* meadow (Ar0) and a *Bromus erectus* meadow (Br), with high species richness and low legume abundance; type 011, an Ar0 meadow with high species richness and legume abundance; and type 222, a grass-rich form of the *Arrhenatherum elatius* meadow with low species richness and legume abundance.

2.2. Plant sampling and laboratory analysis

During the two growth periods within each of the years from 2012–2017, fertile shoots were sampled from the three fertilization treatments. 15–30 shoots (5–10 in each plot) from each flowering species were collected at the optimal seed maturation stage (most fruits/inflorescences still intact, i.e., no seed shedding). At the sub-plot level, all fertile shoots were collected at the time of meadow mowing on one 1-m² sub-plot per plot. Collected shoots were put separately per species into porous paper bags, dried, and preserved in a refrigerator until laboratory analysis.

During the autumn and winter after collection, the 15–30 shoots of each species were analysed for the number of compound and/or simple inflorescences and the flowers per simple inflorescence or shoot. For

species with flowers or inflorescences too numerous to be rapidly counted, an inflorescence length or diameter which could be related to the number of flowers was also measured (e.g., the panicle length in grasses). In sample flowers, intact fruits, or simple inflorescences, the number of ovules per flower and the number of ovules transformed to seed were observed under a binocular microscope. Mature seeds were weighed and tested for germinability and viability according to ISTA (2003). Germination trials were performed with three seed samples per species, which were placed on filter paper in petri-dishes and moved to a germinator for 4 weeks (8 hr light / 25°C and 16 hr darkness / 15°C) with weekly observation and extraction of germinated seeds. At the end of the germination test, seeds that had not germinated were checked for viability with the tetrazolium test. Total viability was calculated as the sum of germinability and viability of non-germinated seeds.

All shoots collected on the sub-plots were counted and measured for the number of inflorescences and flowers. When inflorescences and flowers were too numerous to be counted rapidly (e.g., in all grasses), only the same length/diameter measured on the 15–30 shoot samples was recorded.

A more detailed description of the laboratory analyses is available in Scotton (2018).

2.3. Data analysis

The value of each reproductive trait was calculated for each year and growth period at the plot level for each species. The values of the traits describing the size of the reproductive system were obtained from the shoots collected on the sub-plots. However, for species with too many flowers per shoot, a relation was calculated between the flowers per shoot and the length/diameter of the inflorescences measured on the 15–30 shoot samples. This relationship was then used to calculate the flower number for each shoot. The number of ovules per flower, the portion of ovules transformed to seed (ovule site utilisation), the 1000-seed weight, germinability, and viability were calculated from the results of lab analyses of the 15–30 shoot samples.

Because all the species collected were not always present in the six study years, only the thirty-two species (fifteen grasses and seventeen forbs: Table 1) found in at least three of the study years were considered in this paper to obtain enough reliable results.

The statistical analyses were performed at the levels of individual species and the two grassland functional groups (grasses and forbs). Nine reproductive traits describing the whole process of gametic reproduction were considered: number of simple inflorescences per shoot, flowers per simple inflorescence, ovules per flower, ovules and viable seeds per shoot, OSU (ovule site utilization), percent viability, germinability, and seed weight. Percent dormancy (the difference between percent viability and germinability) and the shoot density recorded in the subplots were also considered in some analyses.

Only sixteen species were present in all of the fertilisation treatments, presenting a challenge in the tests that included all of the species together because a balanced among-treatments comparison was only possible by discarding the data from species not present in all of the treatments. To overcome this issue, we assumed that due to symbiotic N-fixation, the high presence of legumes in the PK treatment was

equivalent to an N fertilisation of about 3.5 kg per percent legume abundance (Scotton et al. 2002: Dietl and Lehmann 2004). Therefore, treatment 011 (30% more legumes present than in treatment 222: Annex 1) was regarded as an N addition of 105 kg per ha per year. The values of the reproductive traits were then calculated for two fertilisation levels, low (LowFert) and high (HighFert). For species present in only two fertilisation treatments (000–011 or 011–222), LowFert and HighFert were matched to the two treatments. For species present in three fertilisation treatments, LowFert was 000 and HighFert was the average between 011 and 222. Statistical analysis considering only the species present in all fertilisation treatments yielded a similar pattern of fertilisation effects to those found in analysis of the two separate fertilisation levels. The analysis of the two fertilisation levels was therefore utilized because it was representative of a larger number of species.

Table 1

Species studied for reproductive traits in a grassland fertilisation trial in the Italian eastern Alps. 8

GRASSES	Code	Fertilisation treatment (NPK level)			FORBS	Code	Fertilisation treatment (NPK level)		
		0 0 0	0 1 1	2 2 2			0 0 0	0 1 1	2 2 2
Anthoxanthum odoratum	AnOd	x	x	x	Achillea roseo-alba	AcRo	x	x	x
Cynosurus cristatus	CyCr	x	x	x	Clinopodium vulgare	ClVu	x	x	x
Dactylis glomerata	DaGl	x	x	x	Trifolium pratense	TrPr	x	x	x
Festuca pratensis	FePr	x	x	x	Centaurea nigrescens	CeNi	x	x	x
Holcus lanatus	HoLa	x	x	x	Rhinanthus freynii	RhFr	x	x	x
Trisetum flavescens	TrFl	x	x	x	Salvia pratensis	SaPr	x	x	x
Briza media	BrMe	x	.	.	Silene vulgaris	SiVu	x	x	x
Brachypodium rupestre	BrPi	x	x	.	Cerastium fontanum	CeFo	x	x	x
Avenula pubescens	AvPu	x	x	.	Medicago lupulina	MeLu	x	x	x
Festuca rupicola	FeRu	x	x	.	Ranunculus acris	RaAc	x	x	x
Arrhenatherum elatius	ArEl	.	x	x	Plantago media	PlMe	x	x	.
Bromus hordeaceus	BrHo	.	x	x	Primula veris	PrVe	x	x	.
Carex contigua	CaCo	.	x	x	Stachys officinalis	StOf	x	x	.
Lolium perenne	LoPe	.	x	x	Knautia drymeia	KnDr	x	x	.
Poa trivialis	PoTr	.	x	x	Leontodon hispidus	LeHi	x	x	.
-	-	-	-	-	Leucanthemum vulgare	LeVu	x	x	.

-	-	-	-	-	Rumex acetosa	RuAc	.	x	x
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Statistical analyses were conducted to: 1. study the fertilisation effect on the reproductive behaviour of individual species and the two species groups of grasses and forbs; 2. find and characterise grasses and forbs with similar behaviours and similar responses to fertilisation; and 3. identify multispecies correlations among reproductive traits and the possible effects of fertilisation on their patterns.

For the first aim, the fertilisation effect was tested for the reproductive traits of each individual species through application of a mixed linear model under a repeated measure approach. In the model, fertilisation treatment, year, and block were input as class factors, and a plot identifier was used as a random factor. In case of significant fertilisation effects, the among-treatment differences were tested using the Tukey multiple comparison adjustment. Prior to performing the mixed model, data were checked for homoscedasticity and normality and, if necessary, log-transformed.

From the individual species mixed models, a table was calculated containing the frequency of cases with fertilisation effects (three levels: no, positive, or negative) for each reproductive trait and species group. To check if grasses and forbs differed for the obtained frequencies, for each trait a chi-square test on the frequency table “fertilisation effect x species group” was performed.

In a following set of analyses, the effect of the grassland functional group (grasses or forbs) on the multi-year means of each reproductive trait was tested with general linear models (GLM). Prior to the analysis, the data were sometimes log-transformed to mitigate homoscedasticity and normality problems. In these analyses, species were considered as replicates within the species group (therefore not included as a class factor) and the fertilisation level was input as a class factor. The effect of the fertilisation level on each reproductive trait was tested separately for the two species groups. In this case, the GLM included both fertilisation level and species as class factors.

To characterise grasses and forbs with similar behaviours and similar responses to fertilisation, subgroups of grasses and forbs with similar reproductive behaviour were defined with cluster and principal component analysis (CA and PCA, respectively) performed on the table “species x reproductive traits averaged across fertilisation treatments”. For the CA analysis, the standardised reproductive traits and species were clustered using the similarity ratio and the minimum variance method (Wildi and Orłóci 1996). For PCA, the standardised reproductive traits were log-transformed to reduce the weight of the traits with high values, and the data were centred by species (Leps and Šmilauer 2003). CA and PCA were also carried out to find groups of species with similar responses to fertilisation. In this case, for each reproductive trait the data used were the percent value of HighFert compared to LowFert. The data were clustered and ordered with the same methods as above, but were not transformed prior.

Possible determinants of the response to fertilisation were investigated by relating the percent values of the ovules or the viable seed number per shoot found in HighFert compared to LowFert (variables Y) to the following explanatory (X) variables: average values of the reproductive traits, Ellenberg bioindicator

values (Pignatti 2005), and percent variation of shoot density. The relationships were fitted according to a linear regression approach for grasses and forbs together or separately and checked for the parametric assumptions of residual normality and homoscedasticity. For the percent differences HighFert-minus-LowFert of individual species OSU, seed germinability, viability and weight, one-way analyses of variance were performed where three traits of the species reproductive biology (type of reproduction, breeding system and pollen vector (Annex 1): Klotz et al. 2002) were used as categorical factors. A GLM approach was also used in this case.

Multispecies correlations were analysed by in-pairs relating the reproductive trait values of individual species averaged across fertilisation treatments and years. Fertile shoot density recorded in the subplots. was used as a supplementary characteristic. Nonlinear relationships were made linear with a log-transformation. Because the purpose of the analysis was not to predict one trait from the other but to efficiently summarise the relationships between traits, the standardised major axis (SMA) approach was used instead of the linear regression method (Warton et al. 2006). The analyses were performed for grasses and forbs both together and separately. In order to verify if fertilisation could affect the characteristics of the evaluated relationships, a second set of SMA analyses were performed by separating the two fertilisation levels and the lines obtained were tested for common slope and elevation according to Warton et al. (2006).

The year effect will be reported in a forthcoming paper and is therefore not discussed here, despite its inclusion in other statistical analyses.

The software used were SAS (1985) with procedures MIXED, GLM, REG, and UNIVARIATE, CANOCO (Ter Braak and Smilauer 2002), Mulva-5 (Wildi and Orloci 1996), and R 3.0.0 (Core Team R 2013) with package SMATR.

3. Results

3.1 Frequency and amount of the fertilisation effect in grass and forb functional groups

The frequency of significant fertilisation effects was highly variable depending on the reproductive trait (Fig. 1 calculated from Annex 2). Fertilization effects were more frequently significant for the size traits of the reproductive system than for biological traits (OSU and viability), and were generally positive. For biological traits, the effect was often both positive and negative.

Between grasses and forbs, individual size and biological traits determining viable seed production per shoot did not display significantly different frequencies of responses to fertilisation, but the resulting viable seed production per shoot increased more frequently in grasses due to more frequent increases of ovules per shoot and no decrease in viability, in contrast to frequent decreases in viability in forbs.

The seed quality traits (1000-seed weight and germinability) were frequently affected by fertilisation, both positively and negatively.

Grasses displayed a greater number of simple inflorescences per shoot than forbs (47 vs. 4: Fig. 2), but fewer flowers per inflorescence (3.4 vs. 43) and ovules per flower (1 vs. 10). Overall, the number of ovules per shoot was significantly higher in forbs (193 vs. 112). OSU and viability did not differ between the two functional groups. Forbs produced on average more viable seeds per shoot than grasses (65 vs. 35). Percent dormancy (difference between percent viability and germinability) was significantly higher (26 vs. 18%) for grasses than for forbs (data not shown).

In grasses, fertilisation significantly increased the size of the reproductive system (Fig. 2.1). The increase was exclusively due to a higher number of spikelets per shoot, which elicited a 37% increase in the number of ovules per shoot. Fertilisation also increased viability, but did not increase OSU. The resulting increase of the viable seed production per shoot was a positive gain of 54% (43 vs. 28 seeds). Fertilisation had a weak positive effect on the seed quality traits, increasing the seed size by 2% and the germinability by 10%. Dormancy was not affected by fertilisation (data not shown).

In forbs, fertilisation increased the number of ovules per shoot by 30% (Fig. 2.2). However, viability in fertilized plants decreased significantly (5%), resulting in an overall increase in viable seeds per shoot of only 11% (68 vs. 61 seeds), a weaker effect than in grasses. Seed quality traits were also increased by fertilisation, but more weakly than in grasses. Due to the simultaneous decrease of viability and increase of germinability, dormancy was significantly reduced by 4% (data not shown).

3.2. Grass and forb groups with similar reproductive behaviour and response to fertilisation

Amongst the grasses, CA (Annex 3) and PCA identified two main groups. The first group (positive PC1 values in Fig. 3A) included species with a high number of viable seeds per shoot but low seed weight, and the second group (negative PC1 values) was composed of species with opposite behaviours. In the first group, species with positive PC2 values had few spikelets per shoot but high OSU and viability, while species with negative PC2 values had many spikelets per shoot but low OSU and viability. In the second group, species with negative PC2 values produced the lowest number of viable seeds per shoot due to particularly low OSU values.

In terms of response to fertilisation, PCA and CA created three groups. The viable seed production per shoot displayed notable increases due to a much higher number of spikelets in species in the low-left part of the PCA graph (Fig. 3B), and increased due to higher seed viability in species at the right side the PCA graph. The third group (high-left part in Fig. 3B) displayed a less pronounced increase of the viable seed yield.

In forbs, PCA (Fig. 4A) and CA (Annex 4) identified the same pattern of variation found for grasses (i.e. high seed size and low viable seed number vs. the inverse result). PCA on PC2 identified a second

strategy differentiation of many ovules per ovary and few flowers per inflorescence vs. the inverse. The first species group displayed large seed sizes but low viable seed numbers (right side of graph). In the second group (upper portion of graph) the seed size was on average small and the viable seed number was high due to a high number of ovules per ovary. In a third, more numerous group (central-left low side of the graph), the seed size was also relatively low, but the ovule and viable seed numbers were high due to the presence of flower-rich inflorescences.

In terms of response to fertilisation, the most influential traits were the number of inflorescences per shoot, the number of flowers per inflorescence, and viability. The effect was strongly positive for the species group at the right side of the PCA graph, primarily due to higher viability (Fig. 4B). Species with intermediate PC1 values had a weaker production increase. In the third group (more negative PC1 values) the viable seed production decreased as a consequence of lower seed viability.

No relation was found between the percent value of the number of ovules or viable seeds per shoot in HighFert as compared to LowFert (y) and average values of reproductive traits or Ellenberg's bioindicators (x). In forbs, a positive relationship was found between the percent increase of the fertile shoot density (Y) and of the number of viable seeds per shoot (X) in HighFert compared to LowFert ($\ln(Y) = 1.74\ln(X) - 4.09$; $R^2=0.30$, $p = 0.026$).

Breeding system and species group significantly influenced the response of seed viability to fertilisation (Fig. 5). Viability in HighFert was 9% higher than in LowFert for facultative or obligate autogamous species but 4% lower for obligate or primarily outcrossing species ($p = 0.0032$); viability was also 4.2% higher for grasses and 3.1% lower for forbs ($p = 0.05$).

3.3. Multispecies correlations among reproductive traits

The following negative relationships were noted linking reproductive traits to each other:

- the number of simple inflorescences was related to the number of flowers per simple inflorescence in both grasses and forbs (Fig. 6A);
- the number of ovules per flower was related to the number of flowers per shoot in forbs (Fig. 6B);
- the number of flowers per spikelet was related to OSU in grasses (Fig. 6C);
- and the number of viable seeds per shoot was related to seed size in both grasses and forbs (Fig. 6D).

Lines of best fit were power functions with negative exponents in 6A, 6B, and 6D, and linear in 6C. Relationships did never differ between the two fertilisation levels, therefore, only one common line was calculated. When fitting was performed separately, fertilisation moved the fitted line in the direction of the primary variation in the involved traits. This effect was particularly evident for the seed size to seed number relationship (Fig. 6D).

In grasses, shoot density was positively related to the number of viable seeds per shoot (Fig. 6E) and, due to the inverse relationship between viable seed number and seed size (Fig. 6D), negatively related to seed size (not shown). In forbs, the shoot density was positively related to the ratio between seed size and number of viable seeds per shoot (Fig. 6F).

4. Discussion

4.1 Average values of grass and forb reproductive traits

The results of this study are not directly comparable with most studies on the seed production of temperate herbaceous species, most of which do not take all primary reproductive traits and grassland species into account. A comparison can be made to a study performed with similar methods at a grassland in the same region, with similar altitude, temperature, and rainfall but an earlier time of seed maturation (May instead of June) (Scotton 2018). This comparison revealed a high multispecies correlation (Pearson $r > 0.9$) for most size traits of the reproductive system. Only the number of inflorescences per shoot in forbs was less strictly correlated ($r = 0.61$), likely due to the indeterminate growth of the flowering shoot in forbs. This trait is strongly affected by site-specific weather and climate conditions, as found in most temperate legumes by Puri and Laidlow (1984).

Among the seed quality traits, the seed size and OSU from the two studies were rather well correlated in both functional groups (on average $r = 0.88$ for seed size and $r = 0.64$ for OSU). Germinability and viability were well correlated in forbs ($r = 0.91$ and 0.82 , respectively) but uncorrelated in grasses despite similar across-species averages. The latter finding may indicate a significant effect of the primary factor differentiating the two study sites, i.e. the photoperiod length during seed maturation (June vs. May), which can elicit both positive and negative effects depending on the species (Baskin and Baskin 2014). This finding also confirms the particularly strong dependence of germinability and viability on the climatic conditions during seed maturation reported for grasses by Aamlid (1992).

4.2 Response of grass and forb reproductive traits to fertilisation

The overall size of the reproductive system (viable seed number per shoot) was significantly affected by fertilisation more frequently in grasses than in forbs (38% and 10% of species, respectively). However, in both functional groups, the traits more strongly and frequently affected were the number of inflorescences per shoot and the seed viability.

This study highlighted a much greater fertilisation effect on the spikelet number per shoot than on the flower number per spikelet. Past studies in the field of seed propagation showed contrasting results, with cases of stronger responses for the former and the latter trait (e.g., in *Festuca pratensis* and *Lolium perenne*: Hill and Watkin 1975). The relative fertilisation effect on the two traits is likely species-specific, but the greater effect on spikelet number per shoot found here may be dependent upon the intrinsic characteristics of permanent grasslands. Temperate perennial grasses are dual induction species, and

the contribution to the seed production of the following year is highest for shoots that attained the maximum development (i.e., maximum number of buds at the shoot apex) during the late summer and autumn of the previous year (Aamlid et al. 1997). The greater spikelet number per shoot observed here in the fertilized treatments was therefore likely to be due to the faster shoot development (i.e., more buds) induced by fertilisation during several months of the second part of the growing season, a period for grass shoot growth that is longer in permanent grasslands than under seed propagation conditions.

In forbs, a positive fertilisation effect was found in the inflorescence number per shoot (as in grasses) and on the flower number per inflorescence. The positive fertilisation effect on the number of inflorescences could be due to enhanced vegetative growth (i.e., high number of axillary buds) and increased floral differentiation (higher proportion of branches within an inflorescence) and was found in both legume and non-leguminous forbs (Durner 2017; Zhang et al. 2014). Higher number of flowers per inflorescence were found in more fertile soils at both taxonomic and ecological levels. For example, the head size of different *Helianthus* species was shown to be directly related to the fertility of the typical species habitat (Mason et al. 2017) and the flower number per inflorescence in plants from the same population was higher in *Ipomopsis aggregata* (Burkle and Irwin 2009), *Leucanthemum vulgare* and *Trifolium pratense* (Korevaar 2013) growing on more fertile soils.

Seed quality traits were affected by fertilisation less frequently and to a lesser extent than inflorescence size traits. OSU was, in most cases, independent of fertilisation. This result is not uncommon in seed propagation studies of grassland species (see results for *Lolium perenne* in Hampton and Fairey (1997) and Marshall and Ludlam (1989)), but is not consistent with the Stephenson's (1981) general view of seed set being normally limited by resource availability. Instead, it is in agreement with the results of the Wiens' study (1984), which found that resource limitations are critical for flower production, but seed set is genetically determined. For the species studied here, the flower number increased but OSU remained the same under the HighFert treatment.

Seed viability was more clearly affected by fertilisation than OSU. Discussing this result is difficult as to our knowledge, no studies have reported the effects of fertilisation on viability because many researchers consider this trait to be equivalent to or a proxy for germination, and viability is typically investigated through germinability tests (Copeland and McDonald 1995). However, viability and germinability were found here to be strictly correlated ($r^2 = 0.83$). Studies have reported positive, no, or negative fertilisation effects on germinability (see the review by Gray and Thomas 1982), as obtained here. In the current study, the variation in fertilisation effects was found to be largely related to the breeding system, as in most cases viability increased in obligate or facultative autogamous species, particularly in the wind-pollinated grasses, whereas it decreased in allogamous species, particularly in insect-pollinated species. Past studies showed that fertilisation can improve seed germination by increasing the nutrient concentration in seeds (e.g., Cheplick and Sung 1998). This finding could explain the behaviour of most grasses, for which viability increased but does not explain the reduced viability of most forbs under HighFert. Two compatible hypotheses are possible to explain the result for forbs. The first refers directly to the allogamous character of the involved species. Studies have found that fertilisation can change pollen

and nectar quality, reducing their attractiveness to pollinators (Nye et al. 1973; Ramos et al. 2018), and that fewer pollinator visits can result in increased self-fertilization with a consequent decrease in seed viability in allogamous species (Arista and Talavera 1996). The second hypothesis posits that lower forb seed viability is due to competition from other species (Allison 2002). In our study, this competition could have come from grasses, which increased greatly in the fertilised treatments.

4.3 Multispecies correlations among reproductive traits

All of the negative relationships found between the reproductive traits can be interpreted as trade-off behaviours due to allocation of resources to one function over another (Fenner and Thompson 2005). PCA showed that for both grasses and forbs, the primary differentiation in strategy was between species yielding many light seeds with low probability to produce seedlings able to win the competition of the established plants and specie producing a few heavy seeds, whose seedlings had a higher probability of survival (Fig. 6D) (Jakobsson and Eriksson 2000). Figures 6A and 6B illustrate additional trade-off behaviours found at the shoot level. The relationship illustrated in Fig. 6C, found only for grasses, represents a trade-off example within the same reproductive structure. Species with many flowers per spikelet showed lower average OSU values due to the frequent sterility of the highest flower in the spikelet and the failure of fertile flowers to develop to filled seed (data not shown), likely due to an intra-spikelet competition for resources. Significant relationships similar to those described above were found in the work of Scotton (2018), which confirms their strength and consistency.

An effect of fertilisation on the described trade-offs is visible from the point distribution in Fig. 6 but was never robust enough to reach the level of statistical significance. However, Fig. 6D details a result of particular interest: among the traits defining the primary trade-off strategy, i.e. seed number and seed size, the seed number underwent the highest positive changes due to fertilisation. This could imply a competitive advantage for grasses, which showed a great increase in seed number per shoot following fertilisation than forbs.

No relationship was found between any reproductive trait and the shoot density in any species group at LowFert. This result confirms that species adapted to poor soils rely more on vegetative than sexual reproduction (Fujita et al. 2014). Instead, two significant, positive relationships were found only in HighFert between reproductive traits measured at different levels of fertilisation. In forbs, the shoot density (population level) was positively related to the seed size (see also Scotton, 2018) and to the number of viable seeds per shoot in grasses (shoot level). As seed production per shoot and seed size were strongly negatively correlated, the two relationships indicated that in HighFert the shoot density of the two functional groups was regulated in inverse ways by the seed number/seed size trade-off.

Forbs that thrived in HighFert had a high seed size, even if their seed production per shoot was lower. The main species behaving in this way were *Centaurea nigrescens*, *Knautia drymeia*, and *Rhinanthus freynii*. As previously indicated, larger seed size can increase species recruitment by increasing the probability of seedling survival in closed vegetation (Jakobsson and Eriksson 2000). The result obtained here indicates that this is the case particularly in a fertilised meadow and for forbs, likely because forb seedlings from

large seeds can better overcome the recruitment obstacle represented by the strong competitive ability of grass species in the high-N environments approximated by the HighFert treatment (Shaver & Chapin 1986). This finding does not negate the importance of seed production per shoot, because it increases the baseline number of seeds proceeding to the establishment stage. This would explain why for forbs the shoot density was found to be significantly related to the fertilisation-related increase of the number of viable seeds per shoot.

Contrary to forbs, grasses with the highest shoot density were characterised in HighFert by large numbers of viable seed per shoot. The main species displaying this behaviour were the light-seeded *Holcus lanatus*, *Poa trivialis*, and *Trisetum flavescens*. One possible explanation, supported by the results of both this study and that of Scotton (2018), is that a higher percentage of dormant seeds allows grasses to germinate and establish in autumn, the predominant time in which grass seeds germinate (Stampfli and Zeiter 2008). Strong subsequent tillering occurring under the mild autumn climate (Gillet 1980) would allow many grass tillers to vernalise during winter and, therefore, enter the reproductive stage in the subsequent spring (Aamlid et al. 1997). In autumn, when the growth of established vegetation is low, many grass seeds could successfully establish to the seedling stage, and the competitive advantage of large seeds would be less important. This effect could be reinforced by the soil surface covered by vegetation in the autumn (= space free for seedling establishment), being particularly low in fertilised meadows (e.g., D'Ottavio et al. 2002).

The relationships between reproductive traits and shoot density discussed above were not found for the low fertilisation level. This result indicates that on infertile soils with low above-ground biomass, competition from established plants is less of a problem and stress-tolerance against soil nutrient shortages becomes more important.

5. Conclusions

Although considerable among-species variability was observed, inflorescence size traits were strongly modified by fertilisation, while less significant effects were observed on the stage of seed maturation.

The size of inflorescences generally increased due to fertilisation, particularly in terms of the number of inflorescences per shoot in both species groups and the number of flowers per inflorescence in forbs.

The efficiency of the transformation of ovules to viable seeds generally increased in grasses but more often decreased in forbs. Seed viability differed most between the two functional groups, as it was positively affected by fertilisation or remained the same in grasses but frequently decreased in allogamous insect-pollinated forbs. Seed set was generally not influenced by fertilisation.

On average, fertilisation caused a significant increase of the viable seed production per shoot in grasses (+ 55%), but not in forbs.

Fertilisation did not change the trade-off relationships between individual reproduction traits. However, our results showed that reproductive traits had important effects on the species composition of the grassland and that these effects varied depending on the level of the soil fertility. In the treatments with no or little fertiliser, none of the considered traits was found to affect the shoot density in either functional group, confirming the relatively low importance of reproduction by seed in low soil fertility grasslands. At higher levels of soil fertility, significant but inverse relationships were found for the two species groups. The species with higher shoot densities were those with the largest number of viable seeds produced per shoot in the case of grasses and those with higher seed size in the case of forbs. The higher dormancy of grass seeds and the lower soil vegetation cover of the fertilised meadows during the last part of the growing season likely explain the first result. The ability of forb seedlings to overcome the competition of grass was likely the mechanism behind the second result.

In light of the increasing nutrient enrichment of world soils due to atmospheric deposition of nitrogen and higher anthropogenic fertilisation inputs, the results of this study highlight that reproductive traits and their responses to changing soil nutrient composition will play an important role in shaping the species composition of the grasslands of the future, and provide an explanation for the low diversity of insect-pollinated allogamous forbs in grasslands with nutrient-rich soils.

Declarations

Authors' contributions

MS conceptualization, investigation, formal analysis, writing, reviewing and editing. VR investigation, and reviewing. All authors read and approved the manuscript.

Competing interests

The authors declare no financial or non-financial competing interests.

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Data availability statement

The raw data of the study can be found online within the supporting information of this paper (Annex 2).

Additional statements

The experimental research and the collection of plant material was done according to relevant institutional, national, and international guidelines and legislation. No grassland species considered in the research is included in the list of endangered species according to the IUCN, European Union, Italian national and regional classifications. The collection of plant specimen was done with permission of the grassland owner during the hay-making operations for forage production which do not need a special permission from the concerned local authorities. The plant species were identified by the first author, Michele Scotton. A voucher specimen of each plant species considered in the research was stored in the laboratory of the authors' Department (DAFNAE) and the authors have provided an ID number for each voucher specimen.

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Figures

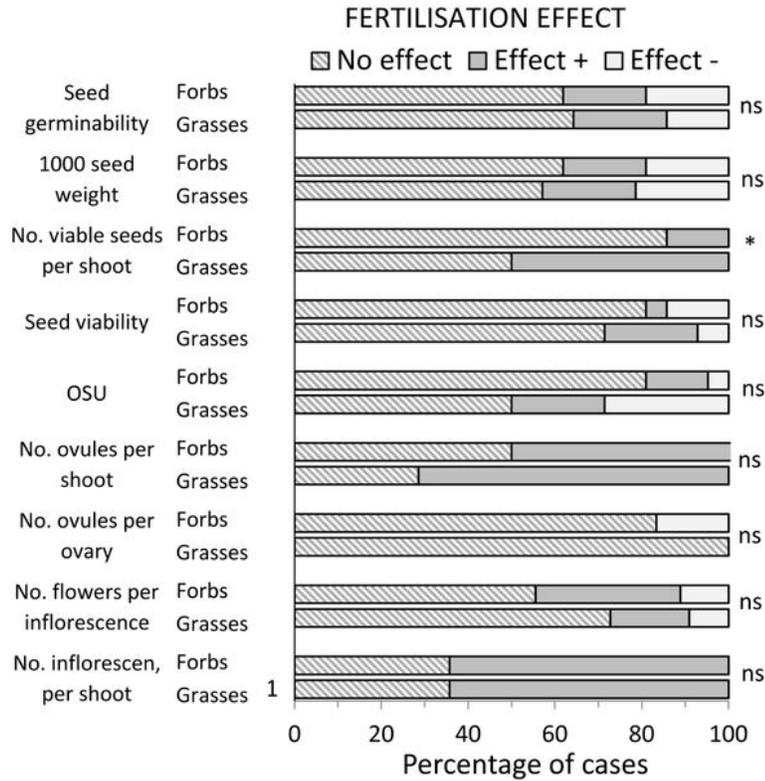


Figure 1

Frequency of different effects of fertilisation on the reproductive traits of fifteen grasses and seventeen forbs in a grassland fertilisation trial in the Italian eastern Alps. No effect, Effect +, and effect - denote statistical insignificance, and positive or negative effects, respectively. ns and * indicate no significance or significance at $p \leq 0.05$ of the chi-square test for the association between species group (grasses and forbs) and type of fertilisation effect.

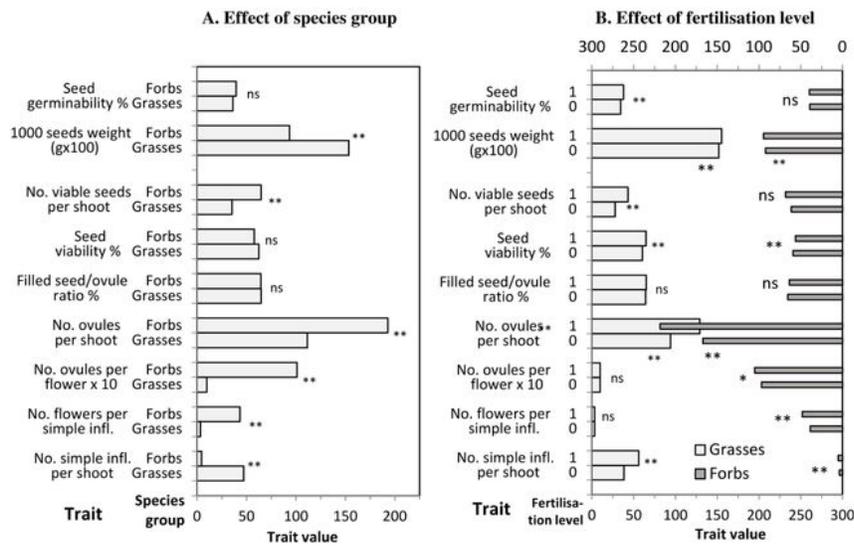


Figure 2

Mean values and among-years variability of reproductive traits of fifteen grasses (left graph) and seventeen forbs (right graph) in a managed grassland. In the left side of each graph the letters represent the results of the Tukey's test for the comparison with the corresponding values of the other species group (different capital letters mean significant difference at $p \leq 0.01$). In the right part of each graph, the two bars represent the fertilisation level means and ns, *, and ** indicate the Anova results being not

significant, significant at $p \leq 0.05$, and significant at $p \leq 0.01$, respectively. Individual species values are shown in Annex 2.

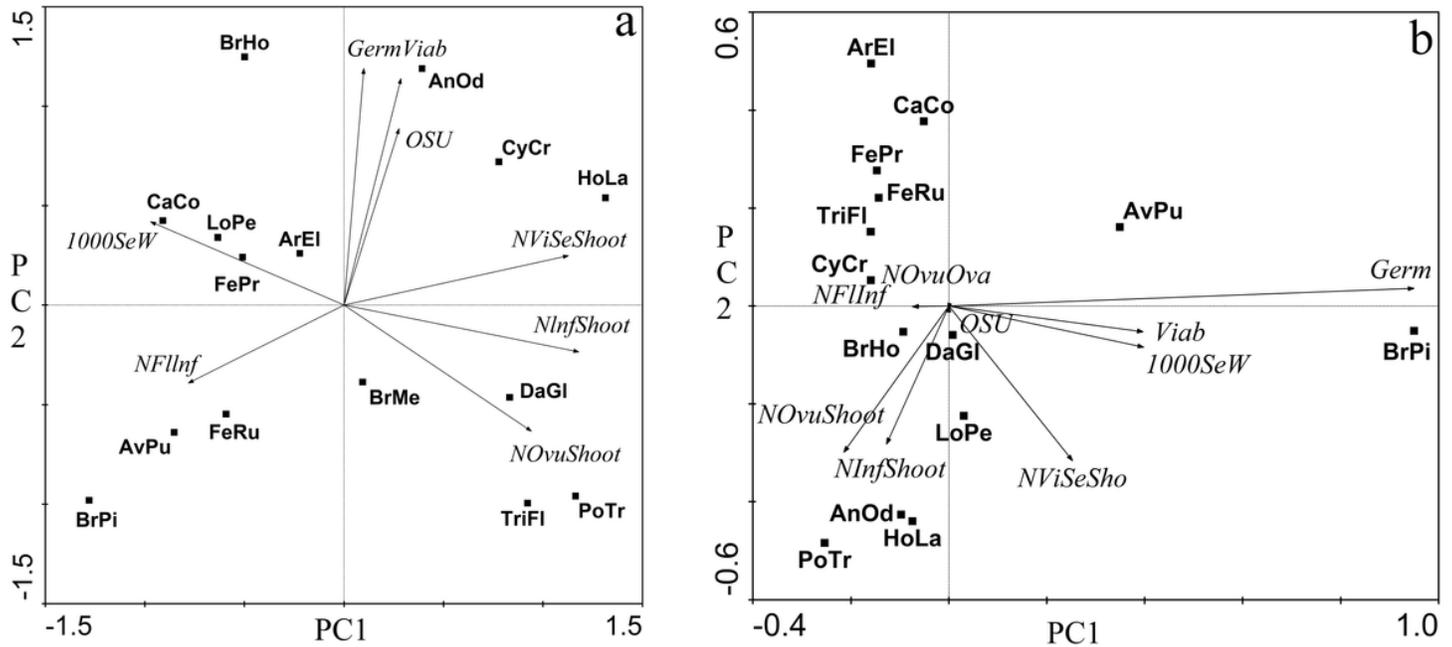


Figure 3

Principal component analysis (PCA) of the reproductive traits of fifteen grasses in a fertilisation trial in a semi-managed grassland in the eastern Italian Alps. PCA was performed on log-transformed trait values averaged across fertilisation treatments in graph A and on percent values of the reproductive traits found in the high fertilisation level compared to the low fertilisation level (i.e. response to fertilisation) in graph B. The variability explained by PC1 and PC2 was 43.6% and 29.5% in graph A and 73.1% and 15.2% in graph B, respectively. Species codes in Table 1. Trait codes: 1000SeW, 1000 seeds weight; NFlInf, number of flowers per inflorescence; Germ, %germinability; Viab, %viability; OSU, ovule site utilisation; NViSeShoot, NInfShoot, NOvuShoot, number of viable seeds, inflorescences, and ovules per shoot, respectively.

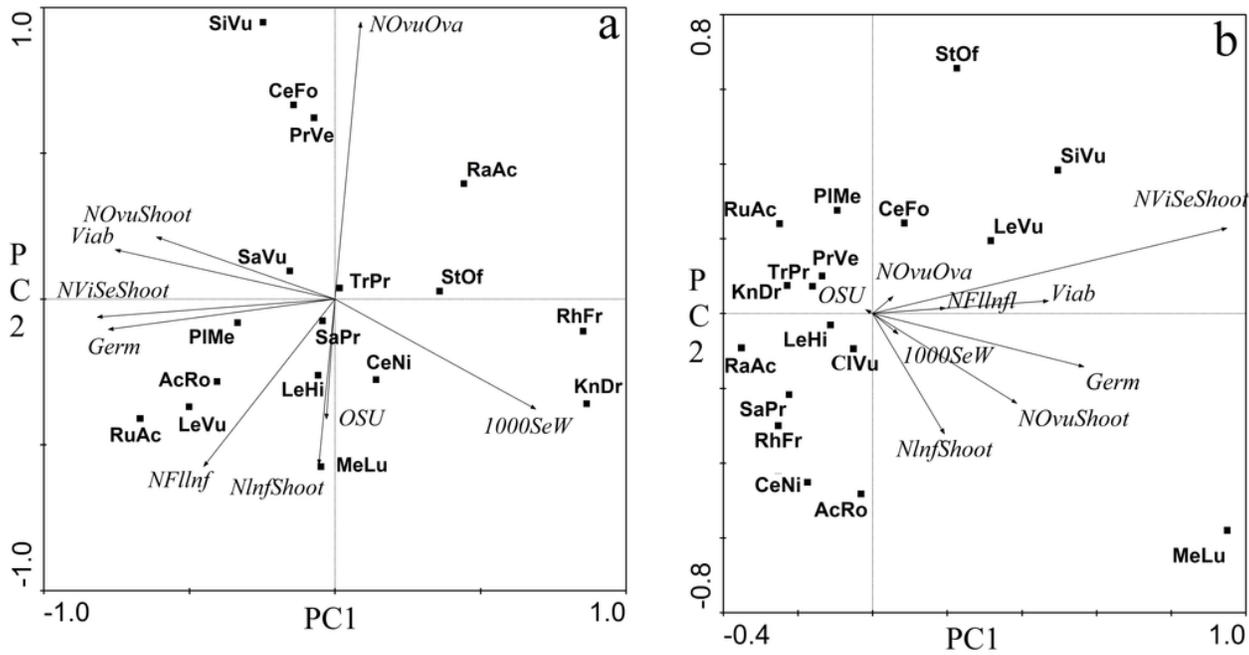


Figure 4

Principal component analysis of the reproductive traits of seventeen grassland forbs. Data from a fertilisation trial in the Italian eastern Alps. In graphs A and B, PCA was performed as explained in the title of Fig. 3. The variability explained by PC1 and PC2 was respectively 32.9 % and 22.1 % in graph A and 72.6 % and 10.4 % in graph B, respectively.

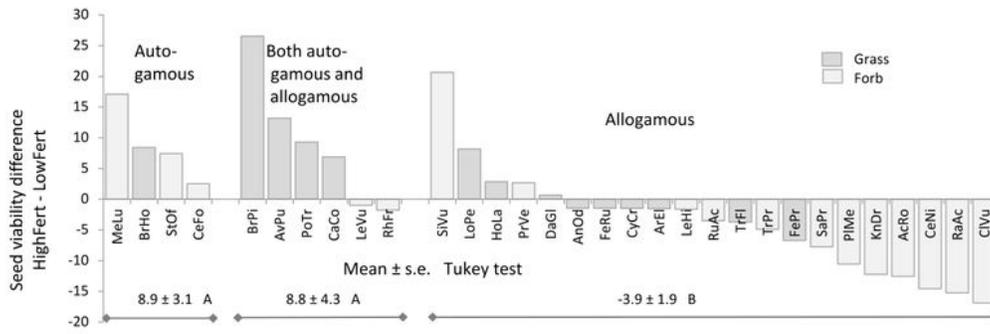


Figure 5

Relationships between variation of viability at higher compared to lower fertilisation level and breeding system of thirty-one grassland species. Data from a fertilisation trial in the Italian eastern Alps. Species codes in Table 1.

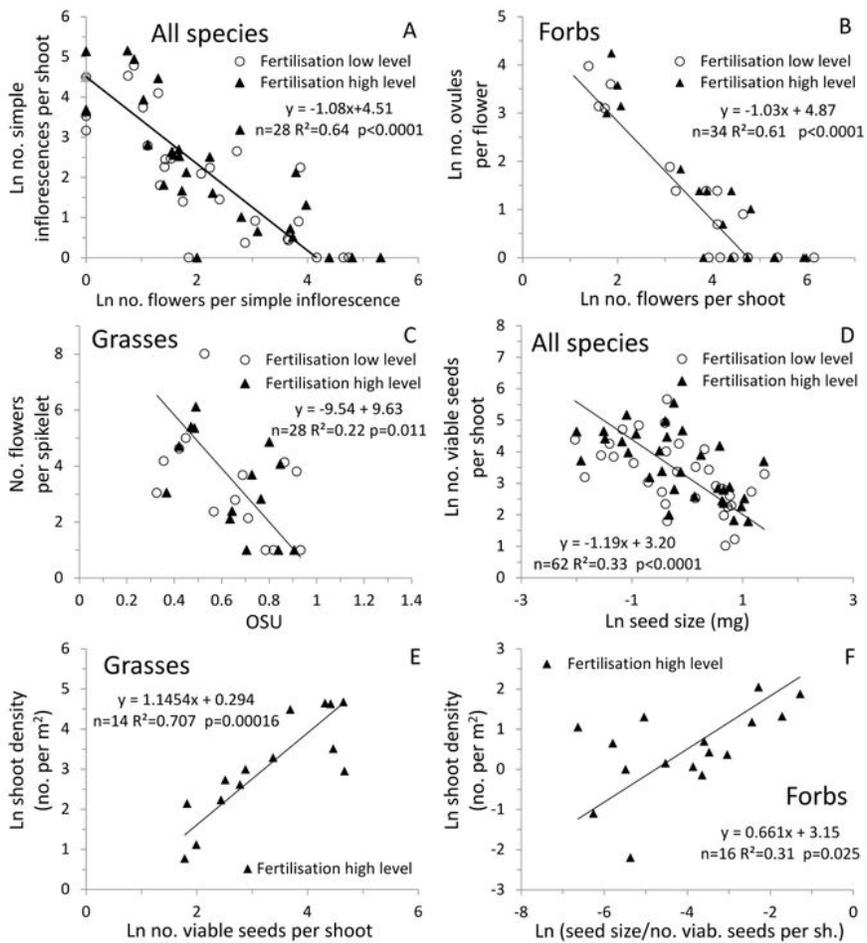


Figure 6

Inter-species relationships between reproductive traits of thirty-two grassland species based on data from a fertilisation trial in the Italian eastern Alps. Lines obtained with the standardized major axis method. In F, the outlier value of *Cerastium fontanum* was excluded from the calculation of the line.

Supplementary Files

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