

Effects of defoliation frequency and nitrogen fertilization on the production and potential for persistence of *Dactylis glomerata* sown in multispecies swards

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Abstract

Management decisions should facilitate the dominance of C₃ perennial grasses over annuals. This study examined the effects of defoliation frequencies and nitrogen fertilization on the productivity and potential for persistence of *Dactylis glomerata* L. (DG cocksfoot, perennial) in multispecies swards. Treatments were randomly applied to 24 mini-swards of DG + *Bromus willdenowii* Kunth (BW prairie grass, annual/biennial) in a factorial design of four defoliation frequencies, based on number of leaves per tiller, by two nitrogen winter fertilization levels (N⁻ or N⁺). Regardless of fertilization, very frequent and repeated defoliations were related to decreases of about 43% of aboveground biomass and frequent defoliations with decreases of about 44% of vegetative tillers associated with horizontal space occupation and potential for persistence. Nevertheless, differences in DG aerial productivity or reserves were not detected between frequent and optimal defoliation frequencies. Combined effects of N⁺ and optimal frequency were related to root biomass increment of about 200%, compared with frequent defoliation, associated with competitiveness and survival of DG. Optimal defoliation frequency would have ecological but not production advantages, compared with frequent defoliations. The results are

discussed in terms of more objective decision-making in the management of multispecies swards.

Keywords: optimal defoliation frequency, leaf stage, *Bromus willdenowii* Kunth, tillering, root biomass

Introduction

Grassland that consists mainly of perennial grasses and legumes has several advantages compared to that dominated by annual species (Kemp *et al.*, 2000; Kemp and King, 2001; Laidlaw and Teuber, 2001). These may include more efficient use of resources, better ground cover and less seasonal variability in primary production (Kemp *et al.*, 2000; Garden and Bolger, 2001; Harris, 2001; Lazenby and Tow, 2001; Sanderson *et al.*, 2002). Management decisions, including fertilization and defoliation strategies, may result in changes in the floristic composition of grassland with possible changes in intra- and interannual variability of productivity.

In humid temperate pastoral systems of Argentina, swards of C₃ grasses [*Dactylis glomerata* (cocksfoot, DG) and *Bromus willdenowii* (prairie grass, BW)] are sown in association with legumes at the beginning of autumn. The annual prairie grass establishes rapidly (Harris, 2001; Sanderson *et al.*, 2002) and may exclude the perennial cocksfoot by competition. We have previously discussed the morphogenetic and structural mechanisms involved in the early competitive interactions between these species. Prairie grass showed higher leaf elongation rate, green lamina length and pseudostem height and therefore restricted the vertical space occupation of DG and also its root colonization. In addition, the neighbouring annual plants caused a

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delay in leaf appearance rate which limited the tiller appearance rate and horizontal space occupation of both species during sward establishment (Gatti *et al.*, 2013, 2015). In consequence, the authors pointed out the advantage of using a low seeding rate of the annual grass to avoid negative effects on the establishment success of the perennial grass. Moreover, early nitrogen (N) fertilization of this sward provides green forage towards the end of the winter (Gatti *et al.*, 2009) which attenuates the intra-annual productive variability (Kemp and King, 2001) and ameliorates the forage deficit that occurs in the local pastoral systems during that period. If the forage increment in response to N addition succeeds simultaneously with the displacement of perennial grasses, in this case of DG (Suding *et al.*, 2005), the question arises of how to manage defoliation in these temperate multispecies swards, during establishment and in the critical first months of sward development (Skinner, 2005).

Defoliation management during the critical first year after sowing affects sward success in the long term. In multispecies swards, the optimal defoliation frequencies of one component species may not be the best for the other species in the mixture. The defoliation frequency most suited to one species might have a negative effect on the competitive ability and potential for persistence of the other species (Madakadze *et al.*, 1999; Fulkerson and Donaghy, 2001). This situation could adversely affect perennials sown in mixtures with highly competitive annual species. On the other hand, the recovery of species after defoliation not only depends on the intrinsic capacity of each species and the characteristics of defoliation, but also on the biotic and abiotic environment – light, water and nutrients – in which the plant grows (Richards, 1993). The negative impact of defoliation on the performance of the plant is usually greater in conditions of environmental stress than in more favourable conditions (Sugiyama and Nakashima, 1995). Nitrogen fertilization not only affects the competition of species but also their responses to defoliation (Remison and Snaydon, 1980). Therefore, it might be expected that the impact of high defoliation frequencies on DG plants, sown in mixtures with the annual BW, could be different in situations with N fertilization compared with unfertilized conditions.

Optimal frequency of defoliation in a grass is based on morphogenetic parameters, i.e. number of living leaves and leaf lifespan. It is recommended to allow the full expansion of the maximal number of live leaves per tiller between two defoliations (Lemaire and Agnusdei, 2000; Fulkerson and Donaghy, 2001). This means, in the case of DG, keeping around four to five living leaves per tiller (Fulkerson and Donaghy, 2001; Rawnsley *et al.*, 2002; Turner *et al.*, 2006a,c), whereas for BW, four to six living leaves per tiller

(e.g. Hume, 1991a; Duru and Ducrocq, 2000a; Gatti *et al.*, 2015). The optimal frequency allows maximum growth rates, recovering of reserves, resumption of root growth, increased tillering, forage quality to be maintained and improved the persistence of the species (e.g. Fulkerson and Slack, 1994; Fulkerson and Donaghy, 2001; Turner *et al.*, 2006a; Lemaire *et al.*, 2009). This ontogenic stage of the tiller, or regrowth age, coincides with the start of the senescence of the first leaf produced after the last defoliation (i.e. the oldest leaf of the tiller). This period is expressed by means of the leaf lifespan (Fulkerson and Slack, 1994; Berone *et al.*, 2008). Perennial species have leaves with a higher mean leaf lifespan than annuals, 478–700 vs. 338–583 growing degree-days for DG and BW respectively (e.g. Duru and Ducrocq, 2002; Berone and Dreher, 2007; Gatti *et al.*, 2013). In this context, species differential leaf lifespan shows the dilemma about which species will be taken as a reference and which is the number of leaves per tiller in this species.

Numerous studies have assessed the effect of the frequency of defoliation on physiology, growth and feed quality in species monocultures using the leaf stage approach (e.g. Fulkerson and Slack, 1994; Fulkerson and Donaghy, 2001; Rawnsley *et al.*, 2002; Turner *et al.*, 2006a; Donaghy *et al.*, 2008). In contrast, this approach has been poorly evaluated in multispecies swards (except, e.g. Turner *et al.*, 2006c). The aim of this study was to analyse the effects of winter nitrogen fertilization and the frequency of defoliation on the productivity and potential for persistence of DG in swards of C₃ annual and perennial grasses. It focuses on the population level organization and discusses the morphogenetic, structural and functional mechanisms during the critical period of sward development on the months after establishment (medium-term). Our main hypotheses were as follows: (i) more frequent defoliation frequencies than the optimum required by the perennial grass *D. glomerata*, when applied during winter and early spring, will negatively affect its growth, relative ground cover and potential for persistence in the swards, (ii) these effects will be independent of the defoliation frequency applied during the subsequent spring–summer period is appropriate for *D. glomerata*, and (iii) the negative effect of high frequencies of defoliation on *D. glomerata*, as a perennial grass in competition with an annual, will be greater in non-fertilized swards than in N-fertilized swards.

Materials and methods

Environmental conditions

The experiment was carried out in the experimental field of the School of Agronomy, University of Buenos Aires

(FAUBA, 34°35S, 58°29W). The chemical analysis of the upper soil horizon (0–20 cm) indicated a pH (H₂O 1:2.5 in water) of 5.85, electrical conductivity of 0.20 dS m⁻¹, carbon content (Walkley and Black method) of 12.9 mg g⁻¹ and nitrates (SNEED) of 31.6 mg kg⁻¹. The content of extractable P (Bray and Kurtz, 1945) in the upper layer showed a spatial distribution in patches, between 5.3 and 10.9 mg kg⁻¹. The soil content was increased to 20 ppm so that the plants received between 59 and 95 kg of P ha⁻¹ at sowing, respectively, according to Rubio *et al.* (2012). The experimental period from sowing (8 April 2010) until the last record (21 January 2011) was 288 d (Table 1). Air temperatures were recorded with a data logger, which values ranged from 14.6 to 13.2 and 24.3°C during initial, training and recovery subperiods (see details later in experimental period). The mean temperature was 15.9 ± 6.1°C (mean ± s.d.). Total precipitation was of 796 mm during the experimental period, 279.1, 327.8 and 189.1 mm during the mentioned subperiods. Photosynthetically active radiation (PARi = μmol m⁻² s⁻¹) was recorded over the sward and within it at the level of the ground level at 12:00 h (Sims *et al.*, 2005), using a radiometer with a 50-cm-long aluminium vane (photon flux = μmol m⁻² s⁻¹; see details later in experimental period).

Plant material and experimental design

Twenty-four experimental units (mini-swards) of five alternate lines of 0.24 and 0.175 m apart (about 0.21 m²) were sown on 8 April 2010. All the experimental units had the same initial floristic composition: three lines of the grasses *B. willdenowii* cv. Fortín Pergamino (BW = prairie grass, annual/biennial species) and *D. glomerata* cv. Porto (DG = cocksfoot, perennial species) and the other two lines of the legume *Trifolium pratense* cv. Redgold (TP = red clover). The sowing design implied a density of 214 plants m⁻² (approximately 35% of DG + 15% of BW). Seedlings were manually sown into a sequence that involved all the possible combinations of intra- and interspecific competition that can be established between the two grasses. The two lines of TP had seven plants (67 plants m⁻²). The sowing arrangement of the mini-swards can be seen in Figure S1. Weeds were manually removed; mini-swards were irrigated to avoid water deficits.

After emergence, the density was manually reduced to obtain approximately 200 seedlings of grass per m², with 1.5 cm between plants in the line (Gatti *et al.*, 2011). The treatments were randomly applied to the mini-swards, in a factorial arrangement with two levels of fertilization (N⁻ = non-fertilized; N⁺ = fertilized with 170 kg N ha⁻¹ applied in winter) and four defoliation frequencies, resulting from the combination of the grass species (BW or DG) and the number

of regrowth leaves per tiller, whenever regrowth of the species reached either two or four leaves per tiller, BW 2L, DG 2L, BW 4L and DG 4L [the last was the optimal defoliation frequency; control treatment] (See Introduction and later, determination of the regrowth status required). The design was a randomized complete block with three replicates. The blocks controlled two sources of variation: the topographic position and the initial content of phosphorus (P) of the soil (See before in Environmental conditions).

Experimental period

Following Berone *et al.* (2008), the experimental period was subdivided into three subperiods: (i) initial, since emergence to first cut, (ii) training and (iii) recovery. The observations spanned the last two subperiods (see Table 1). Initial subperiod began on July 8 (89 d after sowing, 67 d after seedling emergence and approximately 1000 growing degree-days (GDD) when it was considered that both species were established). Observations started after the mowing of the mini-swards. At the time of this initial cut, DG had an average number of 3.75 leaves tiller⁻¹ and 7 tillers plant⁻¹, whereas BW had 4.44 leaves tiller⁻¹ and 13 tillers plant⁻¹; these data indicate an average of 1625 tillers m⁻² in each mini-sward, 816 and 809 tillers m⁻² of DG and BW, respectively, and a radiation interception of 69.39 ± 6.09%.

During the training subperiod, defoliation and fertilization treatments were applied according to the randomization. Nitrogen fertilization was applied on 16 July (97 d after sowing, 75 d after emergence and 8 d after the initial cut) in midwinter. At that time, the soil NO₃⁻ content was 31.6 mg kg⁻¹. The treatments of high defoliation frequency were applied prior to the frequencies considered optimal for the grass species. They were defined as half of the optimal number of leaves per tiller (i.e. 2L). Thus, during this subperiod, the mini-swards were cut twice at the stage of two new leaves per tiller (BW 2L and DG 2L, treatments with high defoliation frequency and repeated defoliation) and once at the stage of four leaves (BW 4L and DG 4L). During the recovery period, plants of BW or DG were allowed to reach four new regrowth leaves per tiller, and after that, DG plants were harvested. Final harvest of DG plants was carried out according to each treatment (see Table 1).

Determination of the regrowth status required

The above-mentioned levels of frequency of defoliation (two or four leaves) were determined based on the assumption that the optimal interval between

Table 1 (a) Dates of defoliation, defoliation intervals (ID) or rest periods, duration of the training subperiod and number of leaves per tiller in *Dactylis glomerata* (DG). The records obtained in the high defoliation frequencies correspond to the first and second cut. (b) The recovery period ends at the date of final harvest.

Fertilization	N ⁻			N ⁺				
	2L	4L	4L	2L	BW	DG	BW	DG
Leaves.tiller⁻¹ + Reference species	BW	DG	BW	DG	BW	DG	BW	DG
Training (a)								
Cutting date/s	1 September and 12 October	10 September and 25 November	20 September	4 October	26 August and 1 October	6 September and 10 November	15 September	4 October
ID (d)	55 + 33 = 88	64 + 70 = 134	74	88	49 + 36 = 85	60 + 56 = 116	69	88
ID (GDD)	483 + 515 = 998	599 + 1188 = 1787	728	924	413 + 466 = 879	536 + 893 = 1429	660	924
Leaves in DG	<2	2	≈3	4	<2	2	≈3	4
Recovery (b)								
ID (d)	49	57	58	64	53	72	63	59
ID (GDD)	946	1384	954	1213	957	1692	1022	1096
Date of final harvest	1 December	21 January	17 November	7 December	24 November	21 January	17 November	2 December

Dates of final harvest, intervals between defoliations and duration of the recovery subperiod. Values of defoliations intervals are expressed in growing degree-days (GDD) from the initial cut (8 July) during the training subperiod and from the date of the last cut during the recovery subperiod. Effect of defoliation frequencies (combination between species: DG or *Bromus willdenowii* (BW) and number of regrowth leaves per tiller: 2L or 4L) and the effect of winter nitrogen fertilization (two levels: N⁻ = non-fertilized; N⁺ = fertilized with 170 kg N ha⁻¹). BW 2L and DG 2L: high frequencies twice defoliated during training subperiod; BW 4L: high frequency once defoliated and DG 4L: optimum defoliation frequency for DG [control treatment].

defoliations (or optimal rest period) is the reciprocal of the frequency of defoliation (Lemaire *et al.*, 2009) for each species. It was considered to be the time required for the full expansion of the maximum number of live leaves per tiller after defoliation. In this study, it was assumed that both species were in their optimal leaf stage, at four leaves per tiller. The treatments of high frequency were defined as half of the optimal number of leaves per tiller (i.e. 2L). Since BW has a shorter phyllochron than DG, the emergence and elongation of four new leaves per tiller required lesser time between defoliations; so BW 4L was considered a treatment with high frequency but not repeated defoliation compared with DG 4L (see Table 1).

Data collection

The number of fully expanded leaves was recorded on six tillers of the grass species, identified with rings of different colours in each mini-sward. Tillers of different ages were randomly chosen in plants of the central line of each mini-sward. Next, all the plants of the mini-swards were cut when at least 50% of the marked tillers reached the number of leaves per tiller (Berone *et al.*, 2008). The plants were hand-cut with scissors, at the height of the ligule of each leaf to remove all the leaf tissue (Davidson and Milthorpe, 1966), which represented intense defoliation (Hirata and Pakiding, 2002; Cullen *et al.*, 2006; Lestienne *et al.*, 2006). The DG leaves harvested on each date were oven-dried at 65°C until constant weight and then weighed. The minimum height of the remaining sheaths was 39.2 mm for DG, being 55% higher in N⁺ than in N⁻.

During both the training and recovery subperiods, the lamina appearance (visible leaf tip) per tiller was recorded weekly (Duru and Ducrocq, 2000a,b) in three of the marked tillers in each mini-sward. At the end of the experimental period, whole plants of DG were harvested 3 h after sunrise to avoid the confounding effect of daily fluctuation in the concentration of water-soluble carbohydrates (WSC; Fulkerson and Slack, 1994; Turner *et al.*, 2006b; Berone *et al.*, 2008). The root of the plants was manually excavated up to 20 cm deep, the surrounding ground was carefully removed, and the species root was rinsed with water (Fang *et al.*, 2012). The material harvested was separated into root and aerial parts, and the latter in turn subdivided into leaves and pseudostems (leaf sheaths plus elongating leaves at the base; Lee *et al.*, 2010). The material was oven-dried (65°C) to constant weight and then weighed to estimate dry weight of leaves, pseudostems and roots. The content of WSC – fructans – (Fulkerson and Slack, 1994) in the pseudostems was determined in the laboratory of the

Center of Research and Services in Animal Nutrition of the School of Agronomy of the University of Buenos Aires (Buenos Aires, Argentina).

Response variables and statistical analysis

The response variables were estimated for the training and recovery subperiods and for the total observational period. The phyllochron and the regrowth of leaves were measured in both subperiods. In the treatments with high frequency and twice defoliated, the value of the variable was the average weight (according to the thermal time) of both cuts.

The phyllochron (GDD leaf⁻¹ at tiller level) represents the thermal time until the leaf appears through the previous leaf sheath and it is usually used to describe and understand the development of grasses (Wilhelm and McMaster, 1995). The phyllochron for each subperiod was estimated as the inverse of the slope of the linear regression between the number of leaves and the thermal time, with a base temperature of 0°C (Bartholomew and Williams, 2005; Fustec *et al.*, 2005). The regrowth of leaves (mg GDD⁻¹ m⁻²) was the difference in the gross biomass (green + dead) of leaves between defoliation events; initial regrowth leaf biomass was always 0 mg m⁻² due to the methodology of defoliation (intense defoliation). Reproductive biomass (inflorescences) was not recorded.

Results were analysed by analysis of variance (ANOVA) with repeated measurements according to a multivariate approach, with a significance level of 5%, following a randomized complete block design according to a 2 × 4 factorial arrangement with two fixed factors: fertilization (two levels: N⁻ = non-fertilized and N⁺ = fertilized with 170 kg N ha⁻¹ applied in winter) and defoliation frequencies [four levels: combination of two grass species (BW or DG) and two possible number of regrowth leaves per tiller (2L or 4L)] with a total of 24 mini-swards (*n* = 3) and the response variable associated with the period as repeated measurement (two levels: training and recovery).

For the whole-observational period, all the rates were expressed by units of thermal time (GDD) to eliminate the differential effect of temperature on the different dates of harvest (Table 1). The aboveground biomass, root biomass, tiller density and WSC were measured at the time of the final harvest. The rate of aboveground biomass accumulation (or productivity; mg GDD⁻¹ m⁻²) was the sum of leaf regrowth and the biomass of pseudostems (living and dead) obtained in each mini-sward in each defoliation event throughout the experimental period (between July 8 and the defoliation and final harvest) (Poff *et al.*, 2011), expressed per unit of thermal time and surface (mg

GDD⁻¹ m⁻²). The rate of root biomass accumulation was calculated as the root dry weight relative to the thermal time at the final harvest (mg GDD⁻¹ m⁻²). We differentiated between the root biomass up to 15 cm (0–15 cm) and that recorded at a depth >15 cm (15–20 cm).

Before the initial cut and at the final harvest, tiller density was measured by counting the total number of vegetative and reproductive tillers of DG in each mini-sward (Scheneiter *et al.*, 2008). The rate of net accumulation of vegetative tillers was the difference between the number of new tillers that appeared during the experimental period, minus that at the time of the initial cut, and the variable was expressed per unit of thermal time and unit of surface (tillers GDD⁻¹ m⁻²).

Water-soluble carbohydrates were determined in the pseudostem dry matter of the perennial grass from each mini-sward because their concentration was greater than in the roots (Fulkerson and Slack, 1994; Turner *et al.*, 2006a,c; Berone *et al.*, 2008). The method of determination was based on separating WSC from fructans by washing with ethanol 80% (Faichney and White, 1983). In these conditions, low-molecular-weight sugars were solubilized while reserve sugars were insoluble. Then, fructans were gelatinized by treatment at 100°C in water and subsequent hydrolysis in acid medium (Hall, 2003). The separated and solubilized reserve carbohydrates were determined through colorimetry by the anthrone method (Yemm and Willis, 1954). WSC contents were expressed per individual or tiller, given that it is more reliable than their expression as concentration of energy (% of dry-matter DM; Turner *et al.*, 2006c), to assess the levels of reserve energy. As a result, the rate of accumulation of WSC was expressed as mg tiller⁻¹ GDD⁻¹ m⁻².

The ANOVA for the whole period considered only two factors: fertilization (two levels) and defoliation frequencies (four levels). The normality of the data was assessed using the Shapiro–Wilks test and the homogeneity of variances using the Levene's test. Means were compared using the Tukey's test with $\alpha = 0.05$ when significant interactions were found. The InfoStat version 1.1 professional package was used to perform the statistical analyses (Di Renzo *et al.*, 2008).

Results

Radiation intercepted conditions

During the training subperiod, the radiation intercepted by the sward prior to the first cut ranged from 80 to 91% when plants were frequently defoliated

(2L) in N⁻ and from 88 to 97% in N⁺ (Figure S2b and c). The intercepted radiation decreased substantially after the first cut, especially in N⁺ (N⁻: 40% vs. N⁺: 26%). The BW 4L swards intercepted between 95 and 97% of the incident radiation at the time of defoliation, in N⁻ and N⁺ respectively. The DG 4L swards intercepted 98% of the incident radiation both in N⁻ and in N⁺ (October 4; Figure S2a).

Training and recovery subperiods

Morphogenetic mechanism – phyllochron of the grass species

The phyllochron of the grass species was differentially affected by the defoliation frequency ($F = 52.37$; $P < 0.0001$) and by the interaction between fertilization and the period ($F = 9.84$; $P = 0.0073$; Figure 1). Highly frequent defoliation (from 4L to 2L) increased the phyllochron of the species in a similar manner in both subperiods ($P = 0.99$): 24% in BW (BW 2L: 210.4 vs. BW 4L: 169.6 GDD leaf⁻¹) and 54% in DG (DG 2L: 388.2 vs. DG 4L: 251.6 GDD leaf⁻¹; Figure 1a). In contrast, fertilization had different effects in both subperiods: during the training period, we did not establish differences in the phyllochron of the grass species between N⁻ and N⁺ (ca. 214 GDD leaf⁻¹), whereas during the recovery period, N⁺ increased it by 28% (N⁻: 259.2 vs. N⁺: 332.7 GDD leaf⁻¹; Figure 1b).

Functional mechanism – leaf regrowth

Leaf regrowth of DG was affected by defoliation ($F = 16.28$; $P = 0.0001$) and nitrogen fertilization ($F = 5.17$; $P = 0.04$), but it was similar between both subperiods and did not present interaction of the other factors with period ($P > 0.05$; Figure 2). Highly frequent twice defoliated (2L) decreased 69 and 39% leaf regrowth of DG when BW had 2L and when DG had 2L respectively (39.8 BW 2L and 79.2 DG 2L compared with BW or DG 4L: 129.55 mg GDD⁻¹ m⁻²; Figure 2a). Nitrogen fertilization increased leaf regrowth of DG by 30% (N⁻: 82.1 vs. N⁺: 106.9 mg GDD⁻¹ m⁻²) (Figure 2b).

Whole-observation period

Functional mechanisms

Rate of aboveground biomass accumulation. Aboveground (blades + sheaths) productivity of DG decreased between 37 and 49% at the high frequencies twice defoliated as compared with the defoliation of the perennial species at 4L

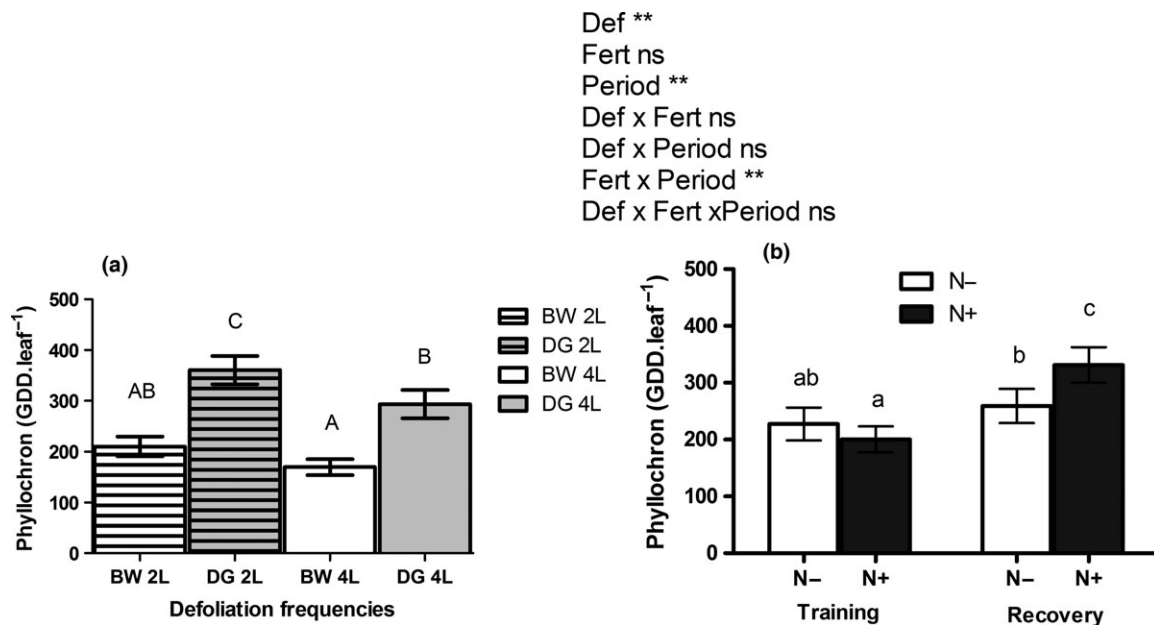


Figure 1 Phyllochron (growing degree-days per leaf = GDD leaf⁻¹) of *Bromus willdenowii* (BW) and *Dactylis glomerata* (DG) for the training and recovery subperiods. (a) Shows the effect of defoliation; open bars correspond to defoliation at the stage of four regrowth leaves per tiller (4L), whereas hatched bars correspond to defoliation at the stage of two regrowth leaves per tiller (2L); (b) shows significant fertilization and subperiod interaction ($P < 0.05$); white bars represent the N⁻ treatment, whereas black bars represent the N⁺ treatment. Def, defoliation; Fert, fertilization. Defoliation terms: the first abbreviation is the name of each species (BW or DG); second abbreviation is number of regrowth leaves per tiller at defoliation (4L or 2L); e.g. DG 4L. Data are means \pm s.e.; P : ** $P < 0.01$; * $P < 0.05$; ns, non-significant effect. Base temperature = 0°C.

(DG 2L: 184 and BW 2L: 150 vs. DG 4L: 293 mg GDD⁻¹ m⁻²; $F = 8.90$; $P = 0.0015$). However, differences in productivity between BW 4L vs. DG 4L were not detected (high frequency but not repeated defoliation, once defoliated) (BW 4L: 263 vs. DG 4L: 293 mg GDD⁻¹ m⁻²). Also, we did not detect statistically significant fertilization effects on the rate of aboveground biomass accumulation ($P > 0.05$) (Figure 3a).

Rate of belowground biomass accumulation. The rate of belowground biomass accumulation was differentially affected by defoliation under the two levels of fertilization evaluated (defoliation frequency \times fertilization interaction; $F = 12.31$; $P = 0.0003$; Figure 3b). The rate of belowground biomass accumulation was unaffected by defoliation frequencies in N⁻. In contrast, in N⁺ and high defoliation frequencies, twice (BW 2L and DG 2L) and once defoliated (BW 4L) during training subperiod, decreased belowground biomass by 70% compared with DG 4L (DG 4L: 40 vs. the other treatments: 12.18 mg GDD⁻¹ m⁻²). Belowground biomass up to 15 cm followed a similar pattern to the one described for total belowground biomass. However,

belowground biomass 15–20 cm was much higher (650% of increment) in the BW 4L treatment, regardless of fertilization, compared with the rest of the defoliation treatments (BW 4L: 1.20 vs. rest: 0.16 mg GDD⁻¹ m⁻²; $F = 14.17$; $P = 0.0002$).

Structural mechanisms

Rate of net accumulation of vegetative tillers. The rate of accumulation of vegetative tillers of DG decreased by 44% when defoliation frequencies were higher than the optimum for DG (DG 4L: 0.19 tillers GDD⁻¹ m⁻²) (Figure 3c). Under optimal defoliation (DG 4L), the density of reproductive tillers of DG also increased 84% with respect to the other treatments (data not shown). Nitrogen fertilization did not affect the final density of tillers ($P > 0.05$).

Rate of accumulation of reserve carbohydrates (WSC). The level of reserve WSC accumulation of DG increased by 962% in BW 4L as compared with highly frequent and twice defoliated (BW 4L: 0.035 vs. BW

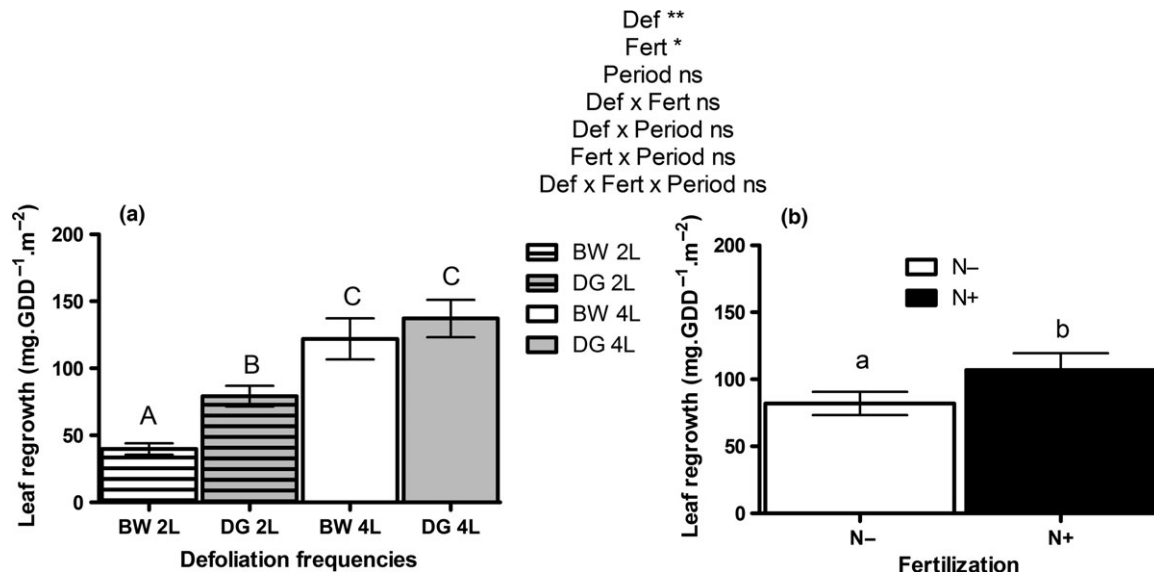


Figure 2 Leaf regrowth ($\text{mg GDD}^{-1} \text{m}^{-2}$) of *Dactylis glomerata* for the training and recovery subperiods. The effect of period was non-significant ($P < 0.05$). (a) Shows the effect of defoliation frequency; open bars correspond to defoliation at the stage of 4 regrowth leaves per tiller (4L), whereas hatched bars correspond to defoliation at two regrowth leaves per tiller (2L). (b) Shows the effect of fertilization; white bars represent the N⁻ treatment, whereas black bars represent the N⁺ treatment. Def, defoliation; Fert, fertilization. GDD, growing degree-days. Defoliation terms: the first abbreviation is the name of each species (BW or DG); second abbreviation is number of regrowth leaves per tiller at defoliation (4L or 2L); e.g.: DG 4L. Data are means \pm s.e. and P : ** $P < 0.01$; * $P < 0.05$; ns, non-significant effect.

2L/DG 2L: $0.0032 \text{ mg tiller}^{-1} \text{ GDD}^{-1}$; $F = 6.28$; $P = 0.0063$). DG 4L showed an intermediate situation: $0.0220 \text{ mg tiller}^{-1} \text{ GDD}^{-1}$). We did not detect a significant nitrogen fertilization influence on the level of WSC ($P > 0.05$; Figure 3d).

Discussion

Partially in contrast to our first hypothesis, it was only the highest defoliation frequencies (plants twice defoliated in the period), such as BW 2L and DG 2L that negatively affected the perennial species *D. glomerata* compared to DG 4L, considered its optimal frequency. Leaf regrowth (Figure 2a), aboveground biomass (Figure 3a) and WSC reserves (Figure 3d) were negatively affected. However, high frequency (plants once defoliated in the period) – BW 4L – did not have a similar negative effect on DG growth when compared with the highest frequencies. Despite the fact that BW 4L led to reduce accumulation of vegetative tillers (Figure 3c) and of root biomass only when nitrogen was applied (Figure 3b), DG showed similar leaf regrowth (Figure 2a), aboveground biomass (Figure 3a), WSC reserves (Figure 3d) and better root depth development >15 cm compared with the optimal DG 4L.

In accordance with the second hypothesis, the negative effects of higher defoliation frequencies applied during the training subperiod remained, even when the optimal defoliation frequency was applied during the following period of recovery. In contrast to the third hypothesis, the negative effects of the high frequencies of defoliation on the perennial grass remained similar in both the non-fertilized and fertilized swards. The addition of nitrogen had significant effects independently of the defoliation frequencies: it increased the phyllochron during the recovery subperiod and the leaf regrowth during the training and recovery subperiods. Nitrogen fertilization improved root biomass accumulation when defoliation was carried out at DG 4L (Figure 2b). However, the nutrient supply did not affect the accumulation of vegetative tillers (Figure 3c).

Effect of highly frequent and repeated defoliation (plants twice defoliated)

The highest frequencies (high and repeated defoliation, BW 2L and DG 2L) were applied 39 d and 511 GDD and 28 d and 388 GDD, respectively, earlier than the optimal one (Table 1). They had negative effects on the perennial *D. glomerata* with respect to DG 4L.

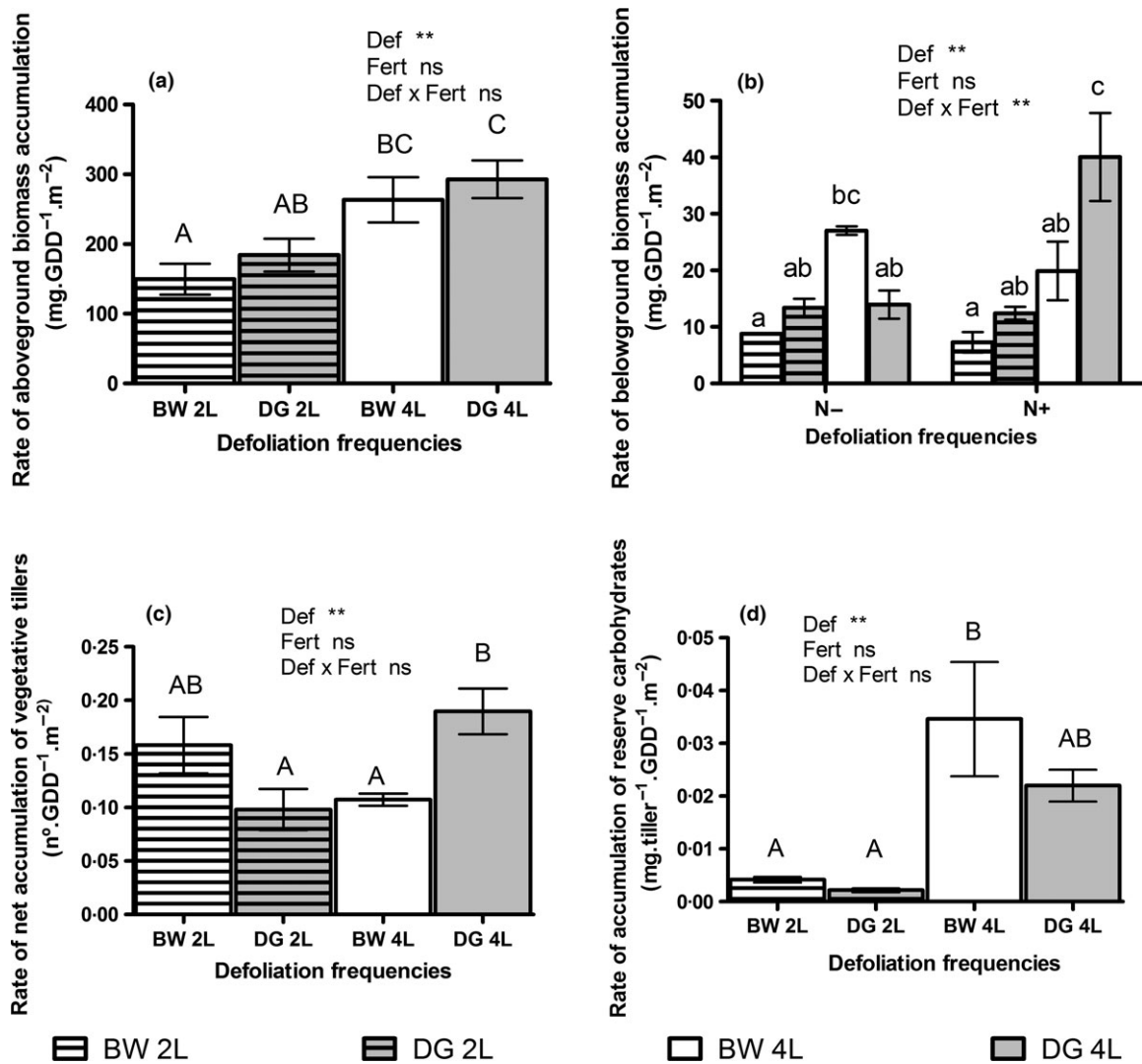


Figure 3 Productivity and ecological-associated variables during the whole-observation period in *Dactylis glomerata*. Accumulation rates of (a) aboveground biomass (mg GDD⁻¹ m⁻²), include living and dead leaves and pseudostems (vegetative biomass), (b) root biomass (mg GDD⁻¹ m⁻²), (c) vegetative tillers (tillers per degree-days m⁻²) and (d) reserve carbohydrates (WSC, mg tiller⁻¹ GDD⁻¹ m⁻²). (a, c and d) show no significant fertilization effects ($P > 0.05$), and (b) shows significant defoliation \times fertilization interaction ($P < 0.05$). Def, defoliation; Fert, fertilization. GDD, growing degree-days. Defoliation terms: the first abbreviation is name of each species (BW or DG); second abbreviation is number of regrowth leaves per tiller at when defoliation (4L or 2L); e.g.: DG 4L. Data are means \pm s.e. and P : ** $P < 0.01$; * $P < 0.05$; ns, non-significant effect.

The negative effects they had on *D. glomerata* remained even when the optimal defoliation frequency was applied during the following period of recovery. These highest frequencies were probably responsible for the lengthening of the species phyllochron, especially in the case of DG 2L (DG 2L: 388.2 vs. DG 4L: 251.6 GDD leaf⁻¹; Figure 1a). These results may provide the explanation for the lower number of

potential sites for tillering, according to the shown rate of net accumulation of vegetative tillers (Figure 3c). Phyllochron data are superior to reported values of 160–204°C d (e.g. Hume, 1991a; Rawnsley *et al.*, 2002; Turner *et al.*, 2006a; Berone and Dreher, 2007). The phyllochron increment showed by the application of high defoliations frequencies was an opposite trend to that reported for *Lolium perenne* (Gautier *et al.*,

1999). Frequent defoliations diminished the phyllochron in long-leaved populations of perennial ryegrass, and it was not modified in short-leaved ones.

Besides the phyllochron increment, the low level of WSC (Figure 3d) should have decreased the leaf elongation rate once leaves were outside the sheaths (Hazard and Ghesquière, 1995; Berone *et al.*, 2008) and it might negatively affect leaf regrowth (Figure 2a). With fewer leaf production and shorter leaves, it may be expected that there would be reduced carbon acquisition and, hence, a low rate of aboveground biomass accumulation of the perennial grass at 2L (Figure 3a).

Effect of winter nitrogen fertilization

The consequences of high defoliation frequencies were similar with and without nitrogen fertilization. Addition of N improved root biomass accumulation at optimal defoliation based on the leaf stage of four leaves, DG 4L (see discussion later; Figure 3b). These results differ from those of Sprague and Sullivan (1950) who reported a major root biomass in unfertilized compared with fertilized *D. glomerata* intermittently defoliated. Nitrogen fertilization caused phyllochron increments of both grass species only during the recovery subperiod (Figure 1b) and leaf regrowth rates in both subperiods (Figure 2b). These fertilization effects on the phyllochron of DG would be associated with the gradual increase in the length of the pseudostem (Duru and Ducrocq, 2000b; Duru *et al.*, 2000). It has been reported previously that, in addition to the effect of N supply, during the recovery period, the higher temperature recorded after the end of November can also increase the phyllochron (Wilhelm and McMaster, 1995; Table 1).

The positive effect caused by nitrogen fertilization on leaf regrowth may be explained by the higher leaf elongation rate and the higher total leaf length of the plant, both N responses reported many times by other authors (e.g. Gastal *et al.*, 1992; Cruz and Boval, 2000; Lemaire and Agnusdei, 2000; Gatti *et al.*, 2013, 2015). These effects are probably associated with the sharp depletion of the intercepted radiation of the sward [(up to about 26% in N⁺ vs. ca. 40% in N⁻); Figure S2], because leaf blades were exclusively harvested (see Materials and methods). On the other hand, the positive effect on N on the leaf blade length justifies fertilization with 170 kg of nitrogen ha⁻¹ in winter in these temperate-humid swards. A key limitation of these soils with high organic matter content is the NO₃⁻ restriction due to low temperatures winter (8–10°C average mean air temperatures) which diminished the mineralization rates. Despite the fact that nitrogen fertilization promoted leaf regrowth, it did

not affect WSC content (Figure 3d), probably because nitrogen addition would be significantly effective on this variable in the long term (White, 1973; Fulkerson and Donaghy, 2001).

Defoliation and fertilization practices in the first year of the sward. Productivity and ecological relevance of the results

Undoubtedly, the highest defoliation frequencies had negative effects on *D. glomerata*. Meanwhile, leaf regrowth (Figure 2a) and thus, aerial productivity (Figure 3a), was indifferent to the defoliation management within the range of frequencies evaluated (BW 4L ≈ DG 4L), with or without nitrogen fertilization. Therefore, rest period shortening of about 30% of its optimum value: 924 GDD (Table 1) do not have negative impact on DG aerial productivity.

Contrary to expectations, WSC contents of DG defoliated at BW 4L were slightly better than in DG 4L (Figure 3d); this was because DG begins to accumulate reserves once the tiller has two leaves and then they were used towards new tillers differentiation (Turner *et al.*, 2006c; Figure 3c). Despite WSC reserves having been related to persistence (Nurjaya and Tow, 2001), a better tiller density, meaning a more horizontal space occupation and coverage (Hume, 1991a,b), might also be associated with potential persistence. Therefore, with and without N fertilization, there would be a certain amount of compensation between BW 4L and DG 4L: BW 4L promoting WSC content and fewer tillers and DG 4L promoting less WSC and more tillers. Both responses have clear ecological relevance associated with the potential of species to persist in the community.

However, longer rest periods – DG 4L – improve the rate of root biomass accumulation (Figure 3b) when nitrogen was applied. This is positive, as any factor that improves the development of the root has a positive effect on the total growth, due to water and nutrients absorption and competitiveness improvements (Fulkerson and Donaghy, 2001). Furthermore, higher root development may favour potential persistence in stressed conditions like the first year of summer water deficit (Fulkerson and Donaghy, 2001; Nurjaya and Tow, 2001).

Therefore, the frequency of defoliation DG 4L would maximize belowground productivity only in N⁺ (Figure 3b) and optimize the ecological parameters associated with the improvement of the relative coverage and potential to persist of the population of the perennial grass in the community. But DG 4L did not maximize neither aerial productivity (Figure 3a) nor WSC reserves (Figure 3d). Nevertheless, the extrapolation of these responses can present some limitations

associated principally with the different time periods analysed in the diverse treatments, so the tests should be repeated both in different seasons and across different sites.

Conclusions

The highest defoliation frequencies – BW 2L and DG 2L – applied during the first winter and early spring period of the sward, negatively affected the population of the perennial grass *D. glomerata*, regardless of whether defoliation frequency was defined following the number of leaves attained by the annual or the perennial grass species. However, this effect was minor when the defoliation frequency was slightly higher than the optimal for DG i.e. BW 4L frequency. The disadvantage of the highest defoliation frequencies was represented by increments of phyllochron and decrements of leaf regrowth rates, aerial biomass accumulation rates and WSC contents, regardless nitrogen addition.

Nitrogen fertilization increased the phyllochron during recovery subperiod and the leaf regrowth during training and recovery subperiods. However, N fertilization did not affect the accumulation of vegetative tillers.

The defoliation frequency of DG 4L did not maximize either the aerial productivity or the WSC reserves. It may maximize belowground productivity when nitrogen fertilization is applied and, hence, it may favour a major potential persistence and competitiveness during the first summer drought stress. Also, it seems to promote ecological parameters associated with the potential improvement of the relative coverage and persistence of the perennial grass population in the community. These results, referred to defoliation and fertilization strategies during the critical first year, can contribute to an efficient design and management of multispecies temperate swards. The responses shown cannot be generalized to subsequent stages of the sward.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Diagram of the sowing arrangement of the mini-swards, identical in their initial floristic composition, in which the optimal frequency and high frequency of defoliation for each grass species were considered: *Dactylis glomerata* (DG) and *Bromus willdenowii* (BW). ♦ DG and • BW; × *Trifolium pratense*; TP; G line of grasses and L line of legumes.

Figure S2. Photosynthetic active radiation intercepted by the canopy (%) during the training subperiod (a) defoliations performed in four regrowth leaves per tiller (4L), (b) defoliation in two leaves (2L) in the non-fertilized treatment (N⁻) and (c) defoliation in 2L in fertilized treatments (N⁺). The arrows indicate the dates of defoliation. C1 = first cut and C2 = second cut in the treatments with repeated defoliation, twice defoliated in 2L.