

Sink strength manipulation in branches of a Mediterranean woody plant suggests sink-driven allocation of biomass in fruits but not of nutrients in seeds

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Abstract The relative autonomy of branches within an individual plant may favor different resource allocation responses after reproductive losses. The assessment of these reproductive strategies at the branch level before their integration at the plant level would provide more insight into how plants deal with reproductive losses. Here, we present a field experiment to assess changes in the allocation strategies at the branch level after sink strength manipulation in a Mediterranean woody plant (*Cistus ladanifer*). We applied three levels of removal of developing fruits (75, 25 and 0 %) on branches of the same plants, and measured their effects on resource allocation (biomass, nitrogen and phosphorus) and seed production after controlling for the effects of branch diameter and leaf weight per branch. Our results suggest that after experimental fruit thinning, *C. ladanifer* branches presented a sink-driven allocation of biomass to fruits but this was not the case for the allocation of nutrients to seeds, which

could be driven by competition with leaf biomass. Reductions in biomass per fruit resulted in a reduction in seed output since the average weight per seed remained constant. From these results, it could be suggested that an heterogeneous distribution of fruit losses among the branches within a crown would produce a higher impact on reproductive output than a more equitable distribution.

Keywords Allocation · Reproductive investment · Phosphorus · Nitrogen · Trade-off · Branch autonomy · *Cistus ladanifer*

Introduction

Plants present different mechanisms to deal with unpredictable losses of reproductive tissues and diminish the impact on their fitness. Some species can compensate for these losses by the production of new reproductive structures in the same season (Pilson and Decker 2002; Lowenberg 1994; Wise et al. 2008). In other cases, however, surplus resources—mainly photosynthates, but also nutrients—are stored for subsequent reproductive events (Sadras 1996; Wise et al. 2008) or reallocated among the remaining reproductive structures (Fang et al. 2006; Trueman and Wallace 1999).

The study of these reproductive allocation strategies should contribute to our understanding of diverse aspects of life-history evolution in plants (Obeso 2004). However, the balance of costs and benefits of any of these strategies is usually measured as the overall seed production per plant (Devlin and Stephenson 1987), which overlooks the modular structure of plants (Silvertown 1989). In the concept of modularity, a plant's crown is made up by a hierarchical series of semi-autonomous subunits made up of elements

Tragically, our dear friend Luis Balaguer passed away on March 19, 2014. We dedicate this contribution to his memory.

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called modules. The branch is the largest subunit in this hierarchy (Valladares 1999). The allocation of resources for reproduction usually takes place within these subunits (Acosta et al. 1997). In addition, losses of reproductive tissues are not usually homogeneously distributed across the crown and; therefore, branches of the same plant could be differentially affected. Under this perspective, the analyses of reproductive losses and subsequent reproductive reallocation of resources at the plant level represents the integration of investment decisions exerted at different levels of the hierarchy: number and size of fruits per branch, allocation to different reproductive fractions within fruits (fruit wall, seeds) and different number and size of seeds per fruit. Therefore, a more detailed assessment of reproductive allocation strategies in plants after reproductive losses can be achieved by considering branches rather than individual plants as the basic units.

In perennial woody plants, branches have been considered as autonomous units in terms of carbon supply (Watson 1986). In addition, the independence of branches within a plant has been reported even in highly demanding phenological phases such as bud break (Landhäusser 2011), flowering (Oitate et al. 2011) and fruiting (Marsal et al. 2003; Volpe et al. 2008). Even fruits have been reported to contribute to their own carbon supply through the photosynthesis of their green parts (Hoch and Keel 2006). Nevertheless, developing fruits can also obtain carbon and nutrients either from current photoassimilates produced in neighboring branches or from reserves stored in other parts of the plant (Obeso 1998; Hoch 2005).

In this paper, we present a field experiment to assess the impact of reproductive tissue losses on reproductive allocation patterns in branches of *Cistus ladanifer* L., a Mediterranean woody plant. We removed developing fruits to increase current resource supply at the branch level and measured their effects on resource allocation and seed production at the fruit level. We hypothesize that if developing fruits receive resources based on the supply of current resources, treated branches should produce heavier fruits, higher seed weight per fruit or higher average weight per seed, since the same amount of resources should be partitioned among a lower number of fruits. On the other hand, if fruits receive resources based on their demand, these reproductive variables may be reduced, since the sink strength would be decreased. We have specifically examined whether removal of young fruits affected (1) fruit weight; (2) fruit wall weight; (3) seed weight per fruit; (4) seed-to-fruit wall ratio; (5) average weight per seed; and (6) seed nitrogen (N) and phosphorus (P) content. We used fruit thinning rather than flower thinning to avoid interferences with the pollination process and to obtain a stronger effect on resource supply. Fruits present a higher cost than flowers (El-Keblawy and Lovett-Doust 1996;

Lord and Westoby 2006) and have been identified as major sinks, not only for photosynthates (Génard et al. 2008) but also for water (Marsal et al. 2002, 2008) and nutrients (Obeso 2012).

Materials and methods

Study area and species

Cistus ladanifer is an obligate seeder, depending on the soil seed bank for post-fire seedling establishment (Calvo et al. 2005). Cross-pollination, usually by insects, is required for fruit production, which are lignified globular capsules with 7–10 valvae, containing hundreds of seeds (Talavera et al. 1993). In our study area, flowering takes place in May over a few days and the opening of fruits begins in late summer.

The experimental plot was located in Monte de El Pardo, Madrid, Spain (40°30'N; 3°43'W; altitude: 694 m). The climate is Mediterranean, with a moderate continental component; mean annual rainfall is 550 mm and average temperature is 13.58 °C. Soils in the study area are acidic and nutrient-poor, characterized as luvisols (FAO-UNESCO soil classification system) presenting deep ABC profiles, a poorly developed humus layer and a high degree of erosion resulting from human use (Nombela et al. 1994).

Experimental design

We carried out all fieldwork in a natural patch of *C. ladanifer*. We selected 24 individuals at random with the following restrictions: (1) they must have a main stem (i.e., not bifurcated at the base); and (2) at least four branches with fruits growing from the main stem (basal branches). Within each individual, we randomly selected three basal branches and assigned them to one of our three treatments. These treatments were: (1) high level of fruit thinning, 75 % of fruits removed; (2) low level of fruit thinning, 25 % of fruits removed; and (3) control, no fruits removed. We applied treatments on a single day in May 2009, when pollination had finished and fruits had just begun to develop.

We collected samples in September 2009, when fruits were completely lignified and dry but before seed dispersal. We measured branch diameter for all 72 branches (three per plant) as the maximum diameter at the insertion with the stem. As soon as these measurements were taken, each branch was first introduced into a plastic bag to avoid loss of material, then cut and carried to the laboratory.

We removed all the leaves on each branch and weighed them after oven-drying at 60 °C. After removing all fruits, we randomly selected a single non-predated fruit from each branch. For each fruit, we measured total fruit dry weight.

We then opened the fruit and obtained seed weight per fruit and fruit wall weight. In addition, we calculated the seed-to-fruit wall weight ratio for each fruit. We also obtained the average weight per seed as the average of the weight of 4 groups of 25 seeds each. Finally, we separated a subsample of 35 mg of seeds per fruit (roughly 200 seeds) for chemical analyses. We first performed seed digestion following the Kjeldahl method (Radojevic and Bashkin 1999) and later, we analyzed the supernatant with an automatic chemistry analyzer to obtain total N and P content on each seed sample (mg/g of dry seed) (Skalar 4000 SAN System, Segmented Flow Analyzer; Skalar, Breda, The Netherlands; Equipment located in Nutri-Lab <http://www.nutri-lab-urjc.es>).

Statistical analysis

To avoid the confounding effect of branch size, leaf biomass and treatment on fruit and seed features, we analyzed data by means of General Linear Models (GLM) with the software package Statistica 10 (StatSoft, Inc 1984–2002). We used fruit removal treatment as a fixed factor, plant as a random factor and two covariates, branch diameter and total leaf dry weight. *F* values were calculated using the appropriate error terms following the Satterthwaite's method of denominator synthesis. All non-significant interactions ($p < 0.05$) were hierarchically removed from the model. Significant main effects were further analyzed using Fisher's LSD test when appropriate. Variables were transformed logarithmically when necessary to satisfy the criteria of normal distribution and homogeneity of variances.

Results

Fruit thinning produced a significant negative effect on fruit weight, seed weight per fruit and seed-to-fruit wall ratio (Table 1). Branches from the high fruit-thinning

treatment produced lighter fruits than controls and lower seed weight per fruit and seed-to-fruit wall ratio than both controls and low fruit-thinning treatments (Fig. 1). However, there were no treatment effects on seed features and branches from different treatments presented the same average weight per seed, with the same N and P content (Table 2, Fig. 2).

There was no significant effect of branch diameter on fruit features (Table 1). Nevertheless, there were significant treatment \times branch diameter interactions (Table 1). All slopes were positive but there were differences in slope values among all treatments for all fruit features ($p < 0.05$). Accordingly, branch diameter did not affect average weight per seed either, but positively affected both seed N and P content.

Finally, total leaf weight per branch was not related to any fruit feature or to average weight per seed. Nevertheless, leaf weight was negatively correlated with both seed N and P content (Table 1).

Discussion

The experimental reduction in the number of developing fruits of *C. ladanifer* performed in our study strongly influenced the allocation of biomass to fruits, but not the allocation of nutrients (N and P) to seeds. We found that the decrease in fruit weight should be mainly due to a reduction in seed weight per fruit since fruit wall weight remains almost constant among treatments. This reduction in seed weight per fruit along with the constancy of average weight per seed must imply a reduction in seed number per fruit (i.e., reproductive output) when branches are heavily thinned. The constancy of average weight per seed among different fruit-thinning treatments for all ranges of branch size and leaf weight per branch suggests that this feature may be subject to strong stabilizing selection. In addition, it has been reported that variations in seed size of *C.*

Table 1 General linear model results for the test of whether fruit features varied with removal treatments (75, 25 and 0 %)

Effect	Fruit weight			Fruit wall weight			Seed weight per fruit			Seed-to-fruit wall ratio		
	<i>F</i>	<i>df</i>	<i>p</i>	<i>F</i>	<i>df</i>	<i>p</i>	<i>F</i>	<i>df</i>	<i>p</i>	<i>F</i>	<i>df</i>	<i>p</i>
Plant	5.079	23, 19	<0.001	3.661	23, 19	0.003	6.123	23, 19	<0.001	3.781	23, 42	<0.001
Treatment	7.824	2, 19	0.003	3.653	2, 19	0.045	10.348	2, 19	0.001	4.739	2, 42	0.014
Branch diameter	2.983	1, 19	0.094	2.322	1, 19	0.139	3.001	1, 19	0.093	0.585	1, 42	0.449
Total leaf weight (Log)	0.245	1, 19	0.627	0.006	1, 19	0.941	0.971	1, 19	0.337	2.151	1, 42	0.15
Treatment \times branch diameter	8.37	2, 19	0.002	3.525	2, 19	0.049	12.169	2, 19	<0.001	7.126	2, 42	0.002
Plant \times branch diameter	5.02	23, 19	<0.001	3.785	23, 19	0.002	5.744	23, 19	<0.001	–	–	–

The plant \times branch diameter interaction were removed from the seed-to-fruit wall ratio model because of a lack of significance
 Bold font indicates statistical significance

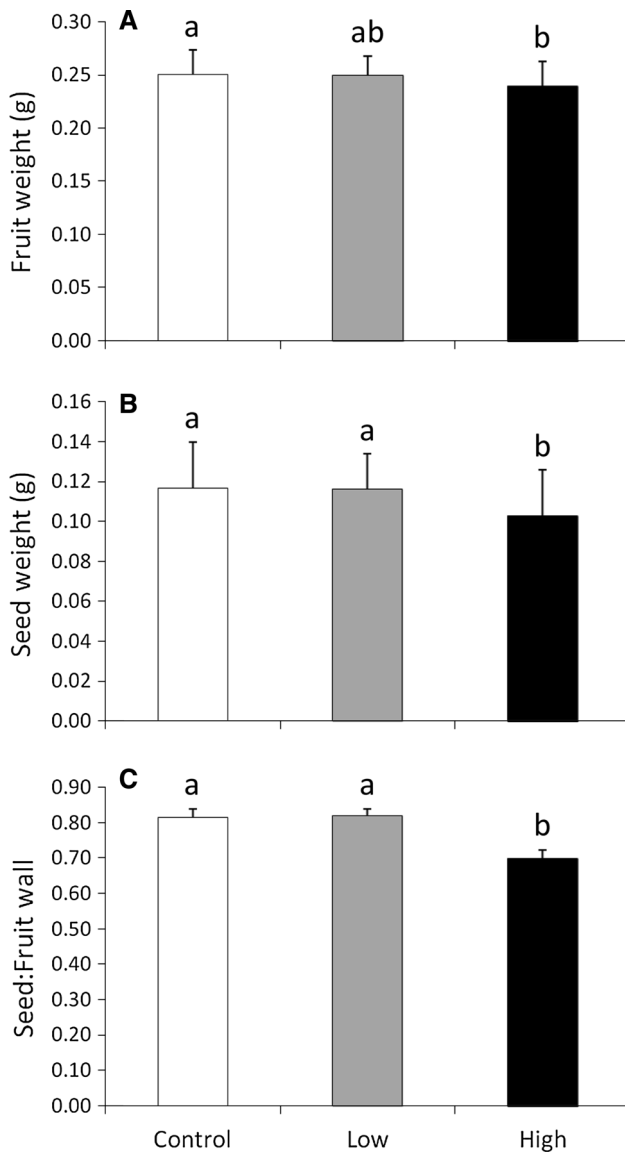


Fig. 1 Adjusted means (\pm standard error) of fruit weight (a), seed weight per fruit (b) and seed-to-fruit wall ratio (c), as a function of fruit removal treatment. Different letters above the error bars correspond to significant pair-wise contrasts using the Fisher's LSD test ($p < 0.05$)

Table 2 General linear model results for the test of whether seed features varied with fruit removal treatments (75, 25 and 0 %)

Effect	Average weight per seed			Seed N content			Seed P content		
	<i>F</i>	<i>df</i>	<i>p</i>	<i>F</i>	<i>df</i>	<i>p</i>	<i>F</i>	<i>df</i>	<i>p</i>
Plant	2.564	23, 44	0.004	1.768	23, 44	0.052	1.691	23, 44	0.066
Treatment	1.132	2, 44	0.332	0.509	2, 44	0.605	0.485	2, 44	0.619
Branch diameter	0.138	1, 44	0.712	5.811	1, 44	0.02	7.344	1, 44	0.01
Total leaf weight (Log)	0.551	1, 44	0.462	7.634	1, 44	0.008	8.863	1, 44	0.005

Bold font indicates statistical significance

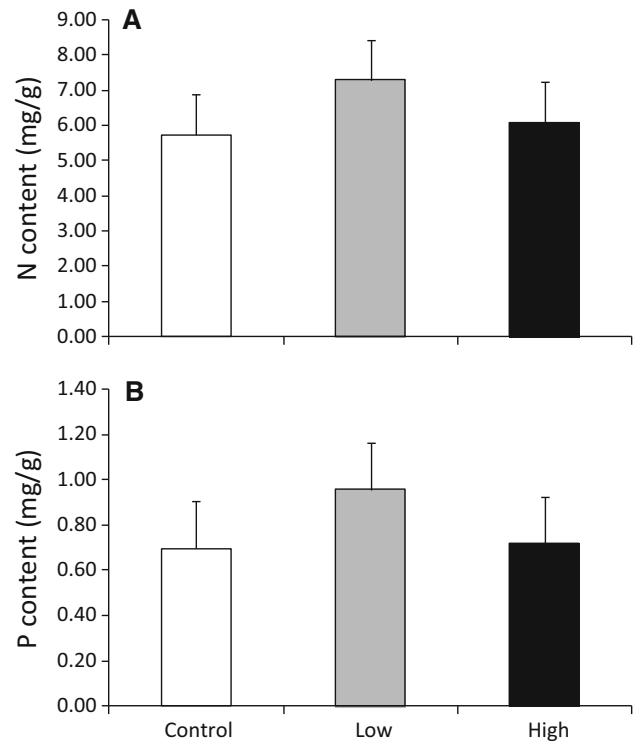


Fig. 2 Adjusted means (\pm standard error) of seed N content (a) and seed P content (b) as a function of fruit removal treatment

ladanifer are linked to ontogenetic development and may have relevant consequences for recruitment in this species since different-sized seeds present different levels of success under different germination scenarios. Heavy seeds disperse closer to parent plant and perform better after fires; and light ones disperse further and have the advantage between fire recruitment events (Delgado et al. 2001, 2008).

Our results could suggest that the allocation of biomass and, indirectly, the reproductive output per fruit in *C. ladanifer* are not limited by the supply of available resources. Instead, resource use for seed production would seem to depend on the demand imposed by developing

fruits on each branch. A 25 % removal of developing fruits did not produce any significant effect, but the 75 % removal significantly reduced reproductive output per fruit. From these results, it could be suggested that branches respond as independent subunits. Nevertheless, our design do not allow to determine if carbon in fruit biomass comes from the same branch or a neighboring one: Therefore, additional experiments with girdling or isotopic labelling are needed in order to confirm branch autonomy in this species (i.e. Lacoïnte et al. 2004; Hoch and Keel 2006). Finally, it could be also suggested that low levels of reproductive losses did not affect reproductive output of the remaining fruits, but large fruit losses induced reproductive depression in that branch. The physiological mechanisms implied might be related to a reduction in sink strength and hormonal signals as it has been reported for other species (Heuvelink 1997).

Overall, our results suggest that understanding the effects of the predation of developing fruits on reproductive output at the plant level is not trivial. The same level of fruit predation may produce different impacts depending on the distribution of predated fruits on the different branches of the plant. If fruit predation is concentrated on a few branches the impact will be higher than if it is evenly distributed among all the branches, since massive fruit losses on a branch may lead to reduced seed production through reproductive depression. Similar scaling problems could arise when integrating the effects of leaf herbivory on reproductive losses since it has been reported that within a tree, fruit abortion is positively related to folivory intensity (Hochwender et al. 2003).

Despite the effects observed on biomass allocation, treatments did not affect N and P concentration in seeds, either directly or through interaction with branch diameter. These results suggest that nutrient acquisition by branches for reproductive investment does not depend on reproductive sink strength and is consistent with the idea that N allocation is normally source driven (Millard and Grelet 2010). Nevertheless, the content of these nutrients in seeds was strongly correlated with branch diameter. This result can be explained through the well known relationships between branch diameter and xylem area increasing both water and nutrient transport.

Since branch diameter is related to nutrient content but not to average weight per seed. Larger branches did not produce larger seeds but nutrient-richer seeds. Although biomass is usually used as a surrogate of investment in nutrients, our results suggest that this cannot be generalized, at least in the assessment of reproductive performance (see also Obeso 2012). Furthermore, it has been reported that seed concentration of nutrients such as N and P is positively related to seedling fitness (Austin 1966; Bolland and Paynter 1990). The advantage of nutrient-rich seeds in

a fire-prone species such as *C. ladanifer* may be related to resistance to high pre-germination temperatures. It has been suggested that the release of heat shock proteins is related to heat shock resistance (Hanley et al. 2001).

In addition, our results show that, after controlling for branch diameter, the higher the total leaf weight the lower the seed N and P content. Leaves need high quantities of N and P to perform photosynthesis and nitrogen fertilized *C. ladanifer* plants present higher photosynthetic capacity (Supplementary Data Figure S1 based on own unpublished data). Therefore, it could be suggested that there should be a strong competition among developing fruits and leaves in terms of N and P allocation (i.e. a trade-off between the photosynthetic and reproductive functions).

In conclusion, *C. ladanifer* branches present a sink-driven allocation of biomass to fruits but this is not the case for nutrients allocated to seeds, which seem to be more affected by competition with leaf biomass. Fruit thinning at the branch level did not affect average weight per seed, which implies that reductions in biomass per fruit produced a direct reduction in reproductive output. From these results, it could be highlighted that the way fruit predation rate per individual plant is partitioned among the different branches is relevant to the total seed output at the plant level. The more aggregated the fruit losses in a few branches, the higher the impact on reproductive output.

Author contribution statement JAD and MDJ were responsible for conception and design of the study and along with PC performed fieldwork. PC carried out measurements and chemical analyses. All of the authors contributed to analysis and interpretation of the data. PC and JAD prepared the draft of the article. MDJ and LB provided a critical revision of the article for important intellectual content. All of the authors approved the final version of the article to be submitted.

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