

Persistent cotyledons and multiple-stem resprouting as strategies of oak seedlings for tolerating herbivory damage

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Background and aims – Large cotyledonary reserves are plant adaptations to tolerate elevated herbivory during the recruitment phase. Oak seedlings have large cotyledons that promote epicotyl resprouting after it is browsed by herbivores. Several studies indicated that browsing can induce multiple stem resprouting, but these data are usually anecdotic. Thus, whether multiple stem resprouting is linked to cotyledonary reserves, or whether it improves seedling establishment, are questions that remain largely unexplored. This study tests the hypothesis that, after the primary stem has been clipped, those seedlings that retain their cotyledons resprout faster and display multiple-stem resprouting responses, also improving their survival and growth, as compared with seedlings without cotyledons.

Methods – We performed a greenhouse experiment in which seedlings of an endemic Mexican oak, *Quercus xalapensis* Humb. & Bonpl. (Fagaceae), were subjected to epicotyl clipping. A total of 200 clipped seedlings were used in this experiment and cotyledons were excised on half of the seedlings. Resprouting, survival and growth rates were monitored on both groups of plants, also counting the number of new stems they developed.

Results – Seedlings with and without cotyledons displayed different resprouting responses. Seedlings that retained cotyledons resprouted from the base of the plant, while seedlings with excised cotyledons resprouted from the fraction of the epicotyl that remained attached to the plant after clipping. Resprouting, survival and growth rates were higher in seedlings that retained the cotyledons. Only those seedlings that retained their cotyledons developed more than one new stem. Survival in these latter seedlings increased with the number of stems, while their growth rates showed the opposite pattern.

Conclusions – Our results indicate that large cotyledonary reserves and multiple stem resprouting may confer advantages to oak seedlings recruiting in habitats with elevated herbivory pressure.

Key words – Aerial shoot, browsing, herbivory tolerance, *Quercus*, seed reserves, seedling growth, seedling recruitment, survival.

INTRODUCTION

Recruitment of tree seedlings is crucial for maintaining the structural integrity of forest ecosystems, but this stage of the plant life cycle faces several environmental hazards before contributing with new individuals to populations. Beneath forest canopies, tree seedlings are exposed to low levels of light and soil nutrients, as well as intense intra- and inter-specific competition for these resources (Leishman & Westoby 1994, Bonfil & Soberón 1999, Yi & Wang 2016). It was proposed, however, that seeds with large cotyledonary reserves constitute an adaptation of seedlings to deal with these adverse conditions (Foster 1986). This hypothesis relies on the assumption that large cotyledons compensate the pho-

tosynthetic deficits and the low supply of mineral nutrients of seedlings, extending their chances of surviving until favourable conditions to growth occur beneath forest canopies (Leishman & Westoby 1994, Kitajima 2003, Bruun & Ten Brink 2008).

Besides these environmental constraints, tree seedlings can also face strong predation pressure by herbivores (Bonfil 1998, Dalling & Harms 1999, Gómez et al. 2003, Rizkalla & Swihart 2007, Badano et al. 2015), which is another important selective pressure operating on them (Foster 1986). It is well documented, however, that seedlings with large hypogeal cotyledons can resprout after their aerial shoots are clipped by herbivores (Andersson & Frost 1996, Harms & Dalling

1997, Hoshizaki et al. 1997, Bonfil & Soberón 1999, Dalling & Harms 1999, Kabeya & Sakai 2003, Barchuk et al. 2006) and this has led to the hypothesis that large cotyledonary reserves may also constitute an adaptation to tolerate herbivory (Green & Juniper 2004, Yi & Liu 2014). Nevertheless, several forest-dwelling granivores remove and consume the cotyledons of seedlings because they still have an elevated nutritional value (Sonesson 1994, Gómez et al. 2003, Drexhage & Colin 2003), and this may preclude the resprouting of aerial shoots (Zhang et al. 2017).

Oak trees (*Quercus* spp., Fagaceae) are conspicuous large-seeded species in most temperate forests of the northern hemisphere (Nixon 1993, Pulido 2002). All oak species have hypogeal cotyledons and several authors have analysed the role of cotyledonary reserves on the performance of oak seedlings (e.g. Sonesson 1994, Andersson & Frost 1996, Bonfil 1998, García-Cebrián et al. 2003, Gómez et al. 2003, Yi & Liu 2014). Most of these studies concur in the suggestion that these large persistent cotyledons promote resprouting of aerial shoots in oak seedlings that have been browsed by herbivores. Additionally, some studies have documented that aboveground herbivory damage can stimulate multiple resprouting in oak seedlings, leading to multi-stemmed plant morphologies (Collet et al. 1997, Drexhage & Colin 2003). However, whether persistent cotyledonary reserves are responsible of this multiple resprouting response, or whether it contributes to seedling establishment after herbivory damage, are questions that remain largely unexplored.

This study focused on these issues in order to provide a better understanding of the role that hypogeal cotyledonary reserves of oak seedlings play after herbivory damage. We hypothesize that, after the primary stem of seedlings is browsed by herbivores, those individuals that retain their cotyledons resprout faster and display multiple stem resprouting, as compared to seedlings without cotyledons. Further, we propose that retention of cotyledons improves the survival of these seedlings and enhances the growth of the resprouted stems. To test these hypotheses, we performed a greenhouse experiment in which seedlings of an endemic Mexican oak species, *Quercus xalapensis* Humb. & Bonpl., were subjected to simulated aboveground herbivory damage (clipping of the aerial shoot) and cotyledon removal.

MATERIALS AND METHODS

Acorn collection

The acorns of *Quercus xalapensis* that we used to develop the seedlings required for the experiment described below were collected in a well preserved sub-humid oak forest located in the Huasteca Region of the state of San Luis Potosí, México (21°52'N 99°21'03"W, 840 m a.s.l.). Mature acorns were collected beneath the canopies of twenty randomly selected individuals in October 2014, after they were released from parental trees. We collected 100 mature acorns beneath each tree (2000 acorns in total) and carried them to the laboratory. Since acorns may lose their viability in the field because of seed desiccation (oak seeds are extremely recalcitrant) or because they are parasitized by insects or fungi (Ramos-Palacios et al. 2014), we used the floatability test to

separate viable and unviable acorns (Gribko & Jones 1995). For this, acorns were placed in 20 L containers filled with water during 2 h. After that, sunken acorns were assumed as potentially viable, while floating acorns were assumed as unviable because of seed desiccation or parasitism (Gribko & Jones 1995). This resulted in a batch of 768 potentially viable acorns (only 38 % of collected acorns were viable).

Experimental design

We performed a greenhouse experiment to assess whether cotyledonary reserves promote shoot resprouting after the epicotyl of oak seedlings is clipped, as well as to assess whether cotyledon retention induces multiple-stem resprouting responses. On 21 Oct. 2014, all potentially viable acorns retained after the floatability test were sowed in individual seedbed bags (250 mL) filled to $\frac{3}{4}$ of their capacity with a mixture of soil of the study site (90 %) and perlite (10 %). Acorns were buried at 2 cm depth to emulate the behaviour of scatter-hoarding animals that disperse them, such as mice and squirrels (Smallwood et al. 2001). Seedbed bags were watered every two days applying 100 mL of water to each of them. Bottoms of bags were perforated to allow drainage of excess water. The emergence of the epicotyl was observed between 14 and 20 days after sowing in c. 50 % of these acorns. This resulted in a cohort of 340 similarly aged seedlings.

These seedlings were grown in the greenhouse during 90 days, until most of them fully developed their first set of leaves. This allowed us to ensure that emerged seedlings started the establishment process, as several of them can die before developing leaves (Zavala-Chávez 2001). On 19 Jan. 2015, a group of 200 seedlings were selected for the herbivory simulation experiment. In this experiment, we only included seedlings with fully developed leaves, while the remaining seedlings were donated to local organizations for reforestation programs. The epicotyl of selected seedlings was clipped 2 cm above the soil using surgical scissors to emulate the effects that herbivores have on seedlings establishing in the field (E.I. Badano, pers. obs.). This eliminated the apical buds and all leaves of seedlings. To assess the relevance of cotyledonary reserves on shoot resprouting, these structures were removed from half of the seedlings. For this, we carefully dug around the seedling stem until finding the cotyledons and excised them using scalpels. This resulted in two groups of 100 seedlings subjected to two different damage treatments: (1) removal of the aerial shoot with retention of cotyledons; and (2) removal of the aerial shoot with excision of cotyledons.

Seedbed bags with seedlings subjected to these two damage treatments were randomly distributed on an experimental table located at the centre of a greenhouse following a rectangular arrangement (10 × 20 experimental units, spaced 10 cm each other). The watering regime of seedlings was the same as that applied during acorn germination (100 mL of water every two days). Resprouting of aerial shoots was assessed on all seedlings every two days, from 19 Jan. to 29 Apr. 2015 (100 days of monitoring). Effective resprouting was assumed when one or more stems larger than 1 cm were observed emerging from seedlings. Seedlings that did not resprout

during this period were assumed to have died. In both damage treatments, we also monitored the survival of resprouted seedlings until the end of the experiment. We assumed that resprouted seedlings had died when the apical buds of their new stems were completely withered. At the end of the experiment, seedlings were harvested to measure the length of each resprouted stem with digital callipers. This allowed us to estimate the net aboveground growth rate of each resprouted stem by computing the ratio between its length and the number of days elapsed since its emergence.

Statistical analyses

To assess whether stem resprout rates differed between seedlings with and without cotyledons we used a failure time analysis. For this, all seedlings had a value of 0 (zero) at the beginning of the experiment, but these values became 1 (one) on the date in which the new stems emerged. Those seedlings that did not resprout during the experiment retained their zero-value. With these data, the Kaplan-Meier method (Kaplan & Meier 1958) was used to calculate the resprout rates of seedlings across time for each damage treatment. These values were later compared between treatments (with and without cotyledons) with the Cox-Mantel two-sample test (Kleinbaum & Klein 2012). The same statistical procedures were latter used to compare the survival rates of resprouted seedlings between damage treatments. In this case, however, the seedlings received a value of 1 (one) on the date in which

they resprouted (i.e. the initial date) and this value became into 0 (zero) on the date of their death (i.e. when their apical buds were withered).

A failure time analysis was also performed to assess whether the number of newly developed stems influences the survival of resprouted seedlings. Because this multiple resprouting response was only observed in seedlings that retained the cotyledons, the individuals from this treatment were grouped according to the number of new stems they produced. This resulted in three groups of seedlings: single-stemmed, two-stemmed and three-stemmed seedlings. For this reason, the generalized Gehan-Wilcoxon test for multiple samples (Kleinbaum & Klein 2012) was used to compare survival among the different groups of seedlings. After that, the Cox-Mantel two-sample test was used to perform pairwise comparisons between seedling groups (Kleinbaum & Klein 2012).

To compare net aboveground growth rates, seedlings with excised cotyledons were included as a single treatment because all of them just developed one new shoot. However, as several seedlings that retained the cotyledons developed more than one new stem, they were included as different treatments depending on the number of new stems they produced. We did this because we were suspicious that growth rates could differ among seedlings with different numbers of stems. These data were compared among treatments with a one-way ANOVA, in which the net growth rates of the different aerial shoots produced by multi-stemmed seedlings were averaged

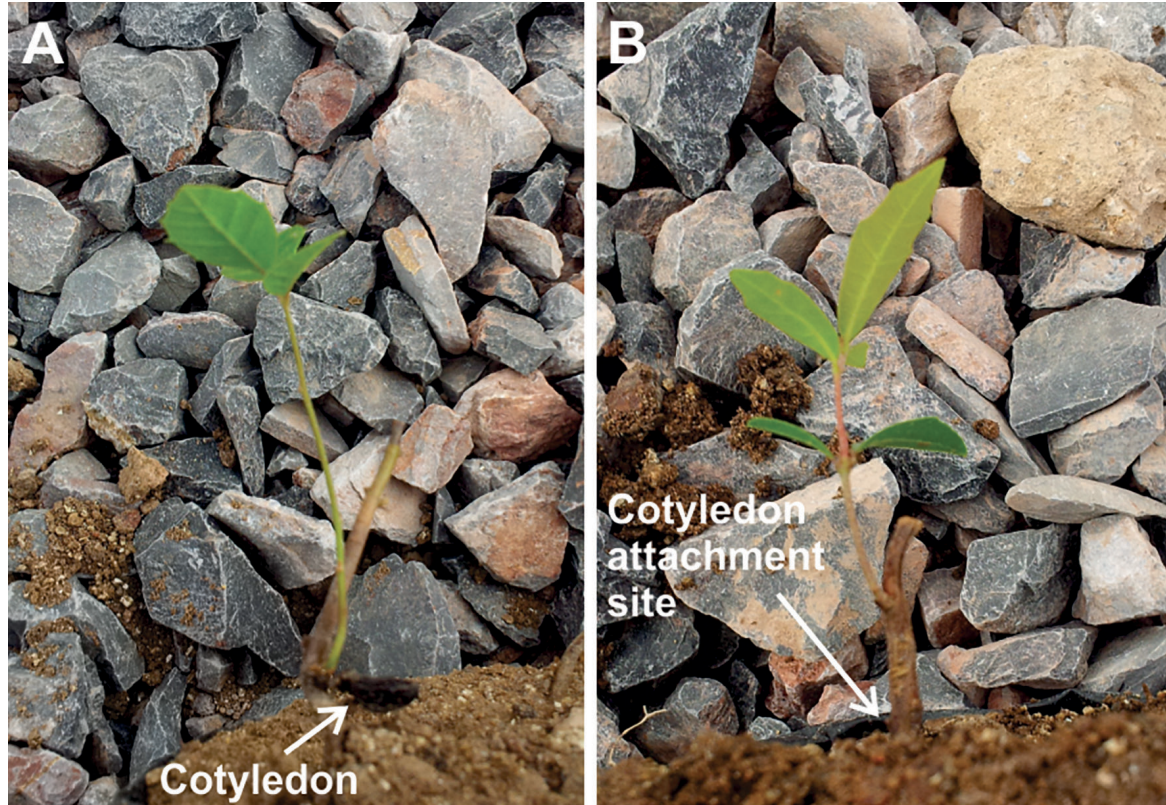


Figure 1 – Resprout responses of *Quercus xalapensis* seedlings subjected to simulated browsing damage (epicotyl clipping). Stem resprouting always occurred from the base of the plants in seedlings that retained the cotyledons (A). Conversely, resprouting in seedlings with excised cotyledons always occurred from the fraction of epicotyl that remained attached to the plant (B).

to avoid pseudo-replication. The *post-hoc* Tukey test was later used to assess differences between seedling groups. To verify the consistency of these results, an additional one-way ANOVA was conducted but, in this case, we only included the fastest-growing stem of each multi-stemmed seedling. All statistical analyses described in this section were conducted in R 3.3 (R Foundation 2016).

RESULTS

Two differential and distinctive resprouting responses were observed on oak seedlings subjected to the different damage treatments. Resprouting in seedlings that retained the cotyledons always arose from the base of the plant, from the section where the epicotyl attaches to the cotyledons (fig. 1A). Conversely, the new stems in seedlings with excised cotyledons always emerged above this site, more specifically from the fraction of the epicotyl that remained attached to the plant after the damage treatment was applied (fig. 1B).

Stem resprouting increased with time and stabilized around 40 days in both damage treatments (fig. 2). However, resprout rates in seedlings that retained the cotyledons were significantly higher than in seedlings with excised cotyledons (Cox-Mantel statistic = 2.574, d.f. = 1, $p = 0.010$). At the end of the experiment, resprouting was observed in 53 % of the seedlings that retained the cotyledons, while the remaining seedlings of this treatment were assumed to have died. Among these seedlings, 49 % developed a single new aerial shoot, 32 % developed two new shoots, and 19 % developed three new shoots. On the other hand, only 29 % of the seedlings with excised cotyledons resprouted during the experiment and, in all cases, these plants developed a single new stem.

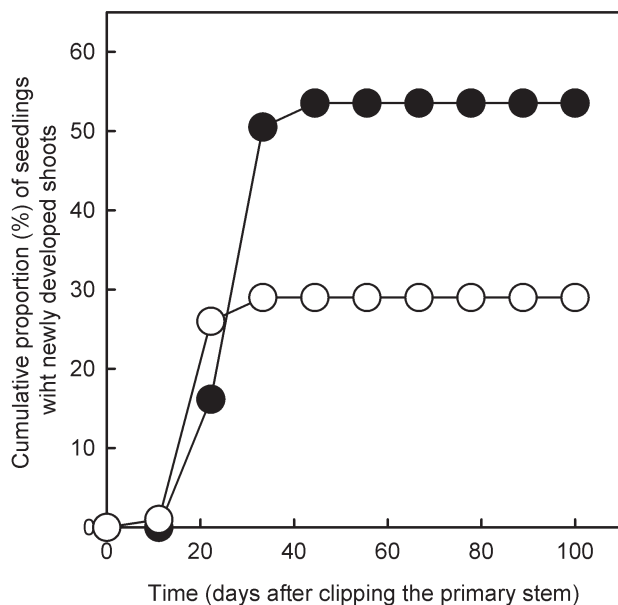


Figure 2 – Resprouting rates of seedlings of *Quercus xalapensis* that retained the cotyledons (black symbols) and seedlings with excised cotyledons (white symbols).

Resprouted seedlings that retained the cotyledons had higher survival rates than those with excised cotyledons (Cox-Mantel statistic = 4.605, d.f. = 1, $p < 0.001$). Survival of oak seedlings that retained the cotyledons gradually decreased during the first 45 days after their resprouting but, after this period, survival stabilized around 66 % (fig. 3). Conversely, survival of seedlings with excised cotyledons abruptly decreased during the first 28 days, in which 65 % of resprouted seedlings died (fig. 3). Survival rates of seedlings with excised cotyledons decreased slower after these initial 28 days and stabilized around 24 % after 62 days (fig. 3).

In seedlings that retained the cotyledons, survival rates differed among individuals that developed different numbers of aerial shoots (Gehan-Wilcoxon statistic = 6.269, d.f. = 2, $p = 0.043$). Pairwise comparisons indicated that seedlings with three new stems had significantly higher survival rates than seedlings with two or a single new stem (fig. 4). No differences were found between seedlings that developed two or a single new stem (fig. 4).

Net aboveground growth rates differed among groups of resprouted seedlings. These findings concurred between the analysis including the average growth rates of multi-stemmed seedlings ($F_{(3,38)} = 94.898$, $p < 0.001$) and the analysis including the fastest-growing stem of each multi-stemmed seedling ($F_{(3,38)} = 92.0310$, $p < 0.001$). In both cases, those seedlings that retained the cotyledons and developed a single new stem had significantly higher aboveground growth rates than seedlings of all other groups (fig. 5). In the other extreme were the seedlings with excised cotyledons, which always developed a single new stem and had significantly lower growth rates than seedlings of all other groups (fig. 5). Seedlings that retained the cotyledons and developed two or three new shoots did not differ in their growth rates (fig. 5).

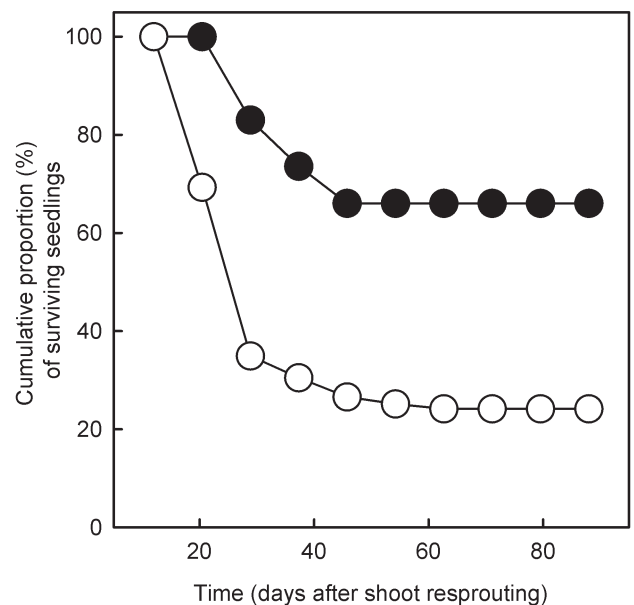


Figure 3 – Survival rates of resprouted seedlings of *Quercus xalapensis* that retained the cotyledons (black symbols) and seedlings with excised cotyledons (white symbols).

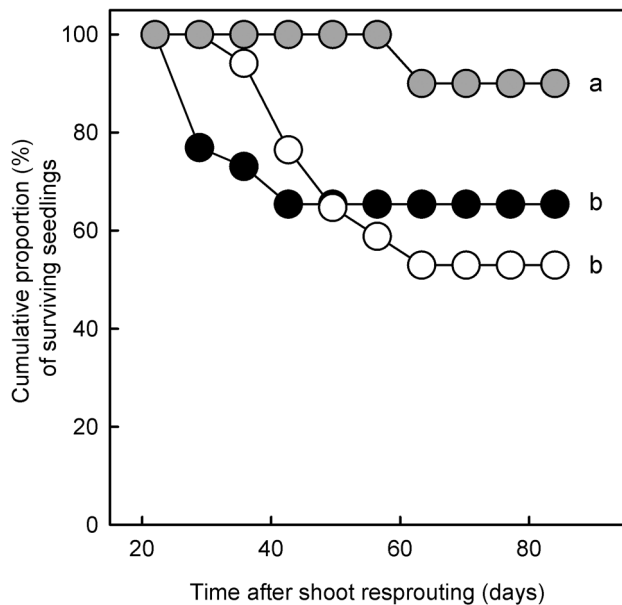


Figure 4 – Survival rates of resprouted seedlings of *Quercus xalapensis* that retained the cotyledons and developed a single new stem (black symbols), two new stems (white symbols) or three new stems (grey symbols). Different letters on the side of survival curves indicate significant differences between groups of seedlings (Cox-Mantel test $\alpha = 0.05$).

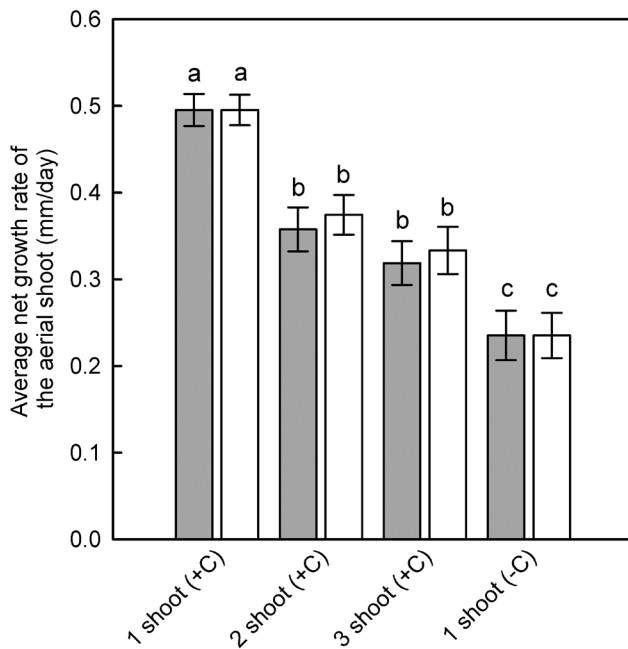


Figure 5 – Net aboveground growth rates (averages \pm 95 % C.I.) of resprouted seedlings of *Quercus xalapensis* that retained the cotyledons (+C) and developed different numbers of stems (1, 2, or 3 new shoots), and seedlings with excised cotyledons (-C). The figure shows the results of the ANOVA conducted with the average growth rates of multi-stemmed seedlings (grey bars) and the ANOVA conducted with the fastest-growing stem of multi-stemmed seedlings (white bars). Different letters above bars indicate significant differences between groups of seedlings (Tukey test $\alpha = 0.05$).

DISCUSSION

The results of this study indicate that seedlings of *Q. xalapensis* can resprout after their epicotyl has been clipped, and also show that this process occurs either when cotyledons are retained or when they are excised. Nevertheless, resprouting occurred faster, survival was higher and growth rates of resprouted stems was enhanced in seedlings that retained the cotyledons. These findings concur with the proposal that large hypogeal cotyledonary reserves confer advantages to seedlings that recruit in habitats with elevated herbivory pressure (Bonfil 1998, Green & Juniper 2004, Yi & Liu 2014).

Although these results highlight the relevance of cotyledonary reserves for prompting the recovery of oak seedlings after putative herbivory damage, it is also important to note that up to 29 % of seedlings with excised cotyledons resprouted after the epicotyl was clipped. This suggests that other nutrient reserves, besides the cotyledons, can be involved in the resprouting process. On this issue, some authors reported that seedlings of large-seeded species from tropical and temperate forests can quickly replace their aerial stems if they are browsed by animals, even when cotyledons are also predated (Sonesson 1994, Dalling & Harms 1999). This independence between shoot resprouting and cotyledonary reserves was attributed to the quick allocation of nutrients from cotyledons to the root development and, thus, seedling roots act as reservoirs of nutrients for the development of aerial structures (Sonesson 1994, Dalling & Harms 1999). In our case, the root was the only plant organ that seedlings with excised cotyledons retained after epicotyl clipping. Therefore, the resprouting of these seedlings suggests that root reserves may partially contribute to shoot recovery. Further, resprouting in seedlings with excised cotyledons always occurred from the fraction of the epicotyl that remained attached to these plants (see fig. 1), suggesting that dormant lateral buds of the epicotyl take the apical dominance after the apical bud is removed. This resprouting response in seedlings with excised cotyledons could be a strategy for optimizing the use of resources after herbivory damage (Kabeya & Sakai 2003). Thus, it can be proposed that, in absence of cotyledonary reserves, resources accumulated in other plant organs (e.g. the taproot) can be allocated to the development of structures in which the seedlings have already invested (e.g. undifferentiated lateral buds of the epicotyl), and this could minimize the energetic cost of developing new structures. Nevertheless, the low survival rates and the smaller growth rates of resprouted seedlings with excised cotyledons indicate that nutrient reserves accumulated in other plant organs would not be enough to ensure their establishment after their aerial structures are damaged.

Conversely to that observed in seedlings with excised cotyledons, shoot resprouts in seedlings that retained their cotyledonary reserves always occurred from the base of the plant (see fig. 1). This suggests that these seedlings do not invest resources in the damaged epicotyl. In turn, they seem to prioritize the development of completely new stems. Indeed, because several of these seedlings developed more than a new stem (up to three shoots), it can be proposed that their cotyledonary reserves overcompensate the loss of the

primary stem. This multiple resprouting response has been reported in other species with hypogeal cotyledons (e.g. Collet et al. 1997, Dalling & Harms 1999, Drexhage & Colin 2003, Green & Juniper 2004) and some authors suggested that this occurs because the loss of the primary stem stimulates the meristematic activity in the root collar, which may simultaneously initiate the growth of several dormant buds (Collet et al. 1997, Drexhage & Colin 2003). In our case, the seedlings with excised cotyledons did not develop this multiple resprouting response (all of them developed a single new stem from the epicotyl), which suggests that this response depends on the availability of cotyledonary reserves. As far as we are aware, this is the first study documenting this interdependence between multiple stem resprouting and cotyledonary reserves after oak seedlings are damaged by herbivores, but more research is required to elucidate the physiological mechanisms that trigger this process.

Besides improving resprouting, the retention of cotyledonary reserves also increased the survival of oak seedlings and enhanced the growth of the newly developed stems. Similar responses were reported for *Quercus laurina* in Mexico (Bonfil 1998) and *Quercus mongolica* in China (Yi & Liu 2014), where cotyledon removal dramatically reduced the survival and growth of resprouted seedlings. However, to the best of our knowledge, no previous studies have reported differential survival rates in resprouted seedlings that developed different numbers of aerial shoots. In our case, oak seedlings that retained the cotyledons and developed up to three new stems had higher survival rates than seedlings that developed two or a single new stem. This resprouting response could be conceived as a “multiple bet-hedging” strategy of oak seedlings for tolerating elevated herbivory pressure, where the development of several stems after epicotyl clipping could increase their chance of retaining at least one stem if seedlings are damaged again. Nevertheless, more experiments are required to determine whether this resprouting response actually confers advantages for the establishment of oak seedlings.

As mentioned above, those seedlings that retained their cotyledons had higher aboveground growth rates than seedlings with excised cotyledons. This also highlights the relevance of persistent cotyledonary reserves for the restitution of seedling structures that are lost because of external factors (Bonfil 1998, Green & Juniper 2004, Yi & Liu 2014). However, in seedlings that retained the cotyledons, the aboveground growth rates were higher in plants that developed a single new shoot, as compared to plants that developed two or three new shoots. These results could be mirroring a trade-off between the number of resprouted shoots and their growth, where cotyledonary reserves are allocated to the development of several shoots of small size or the development of a single large-sized new shoot, as these two commitments could not be achieved simultaneously.

This study overall suggests that persistent cotyledonary reserves and multiple stem resprouting may constitute strategies of oaks to deal with elevated herbivory pressure during the seedling stage. However, because the evidence supporting this hypothesis was obtained from a greenhouse experiment using a single species, it remains to be elucidated whether seedlings in the field exhibit similar responses, or

whether these responses are consistent across different tree species. Developing this knowledge is critical for a better understanding of the processes that regulate the maintenance of forest ecosystems, especially in face of the climatic changes expected during this century.

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