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Retention of cotyledons is crucial for resprouting of clipped oak seedlings

SUBJECT AREAS:
FOREST ECOLOGY
STABLE ISOTOPESXianfeng Yi^{1,2} & Changqu Liu²¹College of Life Sciences, Jiangxi Normal University, Nanchang 330022, China, ²College of Agriculture, Henan University of Science and Technology, Luoyang, 471003, China.Received
21 February 2014Accepted
29 April 2014Published
3 June 2014Correspondence and
requests for materials
should be addressed to
X.F.Y. (yxf@jxnu.edu.
cn)

Although resprouting plays an important role in facilitating persistence of damaged seedlings, the functional attributes of cotyledons and taproots during resprouting of 1-year oak seedlings are not well explored. In this study, cotyledons were removed from *Quercus mongolica* seedlings to explore resprouting in response to simulated disturbance as a function of shoot clipping, and to examine the resprouting ability in relation to timing of clipping and cotyledon removal. Isotope labeling experiments were also performed to evaluate contribution of the cotyledons and taproots to resprouting. Regardless of timing of shoot clipping, seedlings successfully resprouted provided their cotyledons were not detached. Clipped seedlings were less likely to resprout when cotyledons were removed. Seedlings clipped at earlier development stage exhibited higher resprouting capacity than those clipped at later stage. Cotyledon removal, more than timing of clipping, decreased the dry masses of newly-resprouted shoots. However, no significant influences of cotyledon removal and timing of clipping were found on the dry masses of roots, suggesting the importance of cotyledons for resprouting. Roots became functional and accumulated more soil nitrogen after shoot clipping and cotyledon removal, representing a double security-based strategy for the clipped seedlings to resprout despite the importance of cotyledons.

Seedlings, especially those at early stage of development (e.g., 1-year seedlings), are regarded as one of the most vulnerable for the recruitment and colonization of various plant species^{1–3}. Whether seedlings can survive and successfully establish usually relies on various biotic and abiotic factors that can threaten seedling survival^{4,5}. Seedling predation has been widely documented in a number of plant species^{6–8}. Moreover, shoot clipping and cotyledon damage of seedlings by rodents and other herbivores shows negative impacts on seedling survival and development^{6,9–13}.

Feeding on the early stages of seedlings (clipping or removing cotyledons) has been reported in a number of plant species^{2,14–16}. Seed reserves play an important role in improving resprouting and development of seedlings^{17–19}. Different from other species, a large amount of seed biomass remains in the cotyledons of *Quercus* species with large acorns^{20,21}. Although most oak species have hypogeal cotyledons and do not protrude their acorns above the ground after germination, vertebrate animals of different taxa exert high level of predation pressure on their seedlings⁶, because they are able to locate the cotyledons using the seedlings as a cue^{14,22–24}.

From the evolutionary view, seedlings are expected to extract energy reserves from the attached seeds as quickly as possible to reduce their attractiveness to potential predators²⁵. However, dry masses of oak acorns do not change significantly even after germination^{20,21}. This may suggest that the amount of metabolizable biomass in cotyledons acts as backup reserves for growth and survival of seedlings in the presence of various environmental disturbances^{11,26–29}. Among these disturbances, seedling browsing and clipping by livestock and rodents have apparently contributed to seedling damage in the fields^{6,30,31}. However, resprouting is the main regeneration mechanism of most *Quercus* species after severe disturbances³². Oak seedlings are not necessarily killed because they resprout readily after loss of their shoots^{23,33}. Resprouting of damaged seedlings can be largely attributed to two storage organs of young seedlings, cotyledons and taproots. In the field, oak seedlings may be shoot clipped, or cotyledon removed, or even both by a number of vertebrate animals^{6,20}. Previous studies mainly concerned the resprouting capability of clipped seedlings or the effects of cotyledon removal on seedling survival^{19,34–38}, there have been few studies concerning the relative importance of the taproots and cotyledons as storage organs during germination to establishment stages^{17,39}. Moreover, there has been little research about the specific effects of timing of clipping and cotyledon removal upon oak seedling establishment, growth, and survival⁴⁰.

In this study, we carried out an outside experiment with simulated shoot clipping to examine the importance of the reserves in cotyledons during resprouting of *Quercus mongolica* seedlings artificially clipped at different stages of seedling development, i.e., shoot emergence, full development of the first leaf, and the completion of the first flush.



Our study aimed to test four hypotheses: (1) The retention of cotyledons is crucial for resprouting of clipped seedlings; (2) The role of cotyledons in supporting resprouting varies depending on the development stages of seedlings; (3) The role of taproots is less important than the cotyledons in supporting resprouting; and (4) The reserves in cotyledons can be backup energy for resprouting before the storage function of roots ultimately replaces that of cotyledons.

Methods

Experimental protocol. The experiment was carried out in the Field Experimental Base of the Institute of Zoology, CAS (mean elevation of 750 m, 45°58'N, 129°08'E) in the Dailing District, Yichun City, Heilongjiang Province, northeastern China. Mature acorns of *Q. mongolica* were collected in Dongfanghong Forest Park in a seed masting year 2012. After storage at 4°C for 10 months, 560 germinating acorns with ~0.5 cm radicle were randomly selected in June 2013. The germination substrate was brought to moisture capacity before sowing. Ten acorns were evenly sown 1–2 cm deep in each plastic container (15 cm in radius and 20 cm in height) filled with forest soil excavated from where the acorns were collected. The 56 containers were randomly assigned to three cohorts (1, 2, and 3). Cohort 1 with 24 containers was randomly allocated into 3 groups (I, II, and III), with each consisting of 8 containers. Similarly, the 24 containers of cohort 2 were divided into another 3 groups (IV, V, and VI), whereas cohort 3 covered the other 8 containers. All containers were maintained under natural conditions in an enclosure with a diurnal temperature range of c. 18–26°C, and watered regularly as necessary. To determine the effect of cotyledon loss on soil nitrogen allocation in seedlings, each container was evenly applied with 100 ml fertilizer solution containing 13.17 mmol/L KNO₃ and NH₄Cl (enriched to 10 atom %¹⁵N, Shanghai Laiang Biotech Co., Ltd., China). After germination, a shoot-clipping and cotyledon removal treatment was performed to examine the relative contribution of the cotyledons and taproots to resprouting. To examine resprouting capability with respect to seedling development stages, we randomly assigned the three groups in cohort 1 (I, II, and III) to three clipping treatments, respectively (Table 1). Clipping involved the removal of all materials 0.5 cm above substrate level using surgical scissors at different development stages of seedlings. In cohort 2, seedlings in the three groups (IV, V, and VI) were shoot-clipped in the same way; however, cotyledons of all seedlings were also removed when clipping. The removed cotyledons were collected for dry mass measurement. Cohort 3 had only one group (VII) and was treated as control without shoot clipping and cotyledon removal (Table 1).

Growth parameters. Seedling resprouting percentage of each container in cohort 1 and 2 was measured 2 months after clipping at the end growing season. Then, all of the groups were harvested at the same time and individual plants of the seven groups were extracted from the containers and separated into cotyledons, shoots and roots. All parts of seedlings were cleaned under running water and oven-dried (70°C for 48 h) for measurement of the dry masses of roots, shoots and remnant cotyledons. The cotyledons, root and shoot (i.e., epicotyl and leaves) of each seedling were weighed separately to the nearest ±0.01 g. All seedlings in each container were mixed together and averaged for data analysis between the seven groups.

Stable N isotope analyses. Dry roots of seedlings in each container were separately ground and dispatched to isotope ratio spectrometer for isotopic analysis using elemental analyzer/continuous flow isotope ratio mass spectrometry as described by Bidartondo et al.⁴¹. Samples were analyzed for N stable isotope abundances at the Laboratory of Stable Isotope Spectrometer, Chinese Academy of Forestry Sciences. Interface between element-analysis meter and spectrometer was Flash EA1112 HT (Thermo Finnigan, USA). Operation condition: oxidizing furnace temperature was 900°C, reducing furnace was 680°C, pillar temperature was 40°C. The resulting N₂

were purified in a vacuum line and injected in a Finnigan MAT Delta V advantage spectrometer (Thermo Fisher Scientific, Inc., USA) fitted with double inlet and collector systems. The results are expressed in δ¹⁵N relative to the standards in the conventional δ per mil notation as follows:

$$\delta^{15}\text{N} (\text{‰}) = \left[\left(\frac{{}^{15}\text{N}}{{}^{14}\text{N}} \right)_{\text{sample}} / \left(\frac{{}^{15}\text{N}}{{}^{14}\text{N}} \right)_{\text{standard}} - 1 \right] \times 1000$$

where ¹⁵N/¹⁴N are the isotopic ratios of sample and standard (atmospheric nitrogen). The overall analytical precision was ±0.2‰ including both sample preparation and analysis.

N content calculations. A mixing isotope model was used to determine the contribution of isotope-labelled N in soil to the total seedling N⁴². ¹⁵N applied was assumed to be the main source of soil N, while unlabeled N in seedlings came exclusively from the N reserved in acorns⁴³. The N allocation patterns were evaluated based on seedling development stages, shoot clipping, and cotyledon removal:

$$N_{\text{soil}} \% = \left(\frac{{}^{15}\text{N}_p - {}^{15}\text{N}_A}{({}^{15}\text{N}_F - {}^{15}\text{N}_A)} \right) \times 100$$

where N_{soil} %, ¹⁵N_p, ¹⁵N_A and ¹⁵N_F were the proportion of soil N allocated into seedlings, the ¹⁵N isotope abundance of the roots, cotyledons and the fertilizer (10 atom %), respectively. The natural ¹⁵N isotope abundance of the cotyledons was estimated at 0.36722 ± 0.00075 atom % in this study).

Statistical analyses. We used Statistical Package for the Social Sciences (SPSS 16.0) for data analyses. Each container was considered a replicate for statistics. Two-way ANOVA was used to see if there was difference in the resprouting percentages between group I, II, III, IV, V and VI, following arc-sine transformation. General linear model was used to determine if there were differences in the proportion of soil N allocated into seedlings between group I, II, III, VI and VII. Groups IV and V were not included in the comparison because clipped seedlings in these two groups failed to resprout and all biomass rotted. The same procedure was applied to test the differences in dry masses of shoots, roots, and remnant cotyledons, respectively.

Results

Two-way ANOVA analyses showed that cotyledon removal significantly decreased the resprouting capability of clipped seedlings of *Q. mongolica* ($F = 621.529$, $df = 1$, $P < 0.001$) (Fig. 1). Clipped seedlings were less likely to resprout without the support of cotyledon reserves, suggesting that the amount of stored reserves in cotyledons is crucial to support resprouting during early seedling development. Despite the severity of the clipping at the completion of the first flush, a few seedlings in group VI successfully resprouted. We also found significant effect of timing of clipping on seedling resprouting ($F = 4.178$, $df = 1$, $P = 0.022$). No resprouting occurred in the clipped seedlings in group IV and V where the cotyledons were also removed. In group I, II, and III that cotyledons were not removed, clipped seedlings demonstrated high resprouting capacity throughout the development stages. However, a different picture was found in group IV, V, and VI where the cotyledons were removed from the clipped seedlings (Fig. 1).

The dry masses of cotyledons collected from seedlings of the 7 groups differed significantly ($F = 33.985$, $df = 6$, $P < 0.001$) (Fig. 2). The cotyledon dry masses of group I, II, and III were much lower than those of group IV, V, VI, and VII. However, no difference in cotyledon dry mass was detected between group IV, V, VI, and VII.

Table 1 | Experimental protocol of seedling clipping and cotyledon removal in this study

Cohorts	Groups	Treatment protocol	Sample size	Date of clipping or cotyledon removal	Date of harvesting	% survival
1	I	Seedlings with shoots clipped when the shoots emerged (SE+C-S)	8	11–13, Jul. 2013	10, Sept. 2013	98.2
	II	Seedlings with shoots clipped when the first leaf fully developed (FLD+C-S)	8	17–19, Jul. 2013	10, Sept. 2013	95.7
	III	Seedlings with shoots clipped when the first flush completed (FFC+C-S)	8	22–25, Jul. 2013	10, Sept. 2013	81.0
2	IV	Seedlings with shoots and cotyledons removed when the shoots emerged (SE-C-S)	8	11–13, Jul. 2013	10, Sept. 2013	0
	V	Seedlings with shoots and cotyledons removed when the first leaf fully developed (FLD-C-S)	8	17–19, Jul. 2013	10, Sept. 2013	0
	VI	Seedlings with shoots and cotyledons removed when the first flush completed (FFC-C-S)	8	22–25, Jul. 2013	10, Sept. 2013	16.3
3	VII	Seedlings without any clipping and acorn removal (+C+S)	8	-	10, Sept. 2013	100

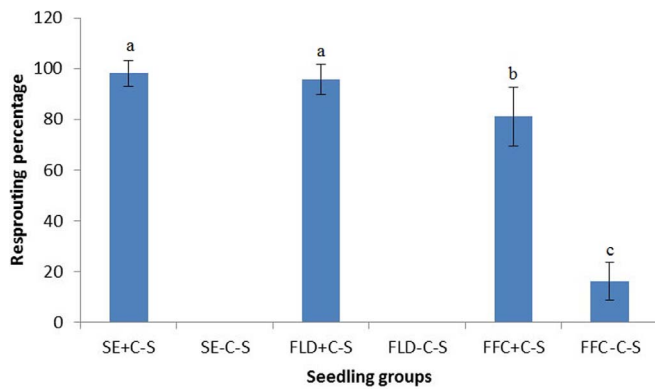


Figure 1 | Resprouting capability of clipped seedlings in response to cotyledon removal. SE: clipped when the shoots emerged; FLD: clipped when the first leaf fully developed; FFC: clipped when the first flush completed; +C: with cotyledons; -C: without cotyledons; +S: no shoot clipping; -S: shoot clipping. Data are expressed as mean \pm SE. Different letters on the histograms indicate significance at $P < 0.05$ level.

There were significant differences in the dry masses of shoots between the group I, II, III, VI, and VII ($F = 18.508$, $df = 4$, $P < 0.001$) (Fig. 3). The dry masses of newly resprouted shoots of group 6 were much lower than those of group I, II, III, and VII, respectively. The dry masses of newly resprouted shoots of group I and II were much higher than those of group III and VII, respectively. However, the dry masses of roots showed another pattern ($F = 3.071$, $df = 4$, $P = 0.029$) (Fig. 3). No difference was found between group I, II, III, and VI; however, the dry masses of roots of group II, III, and VI were much lower than those of group VII, respectively.

Based on the isotope mass balance model, we found that seedlings with different treatments showed considerable differences in the proportion of soil nitrogen ($F = 29.748$, $df = 4$, $P < 0.001$) (Fig. 4). More soil nitrogen was allocated into seedlings in group III than those in group I, and II, showing the effect of development stages on nitrogen allocation strategies. No difference was found in soil nitrogen allocation between group I, II, and VII. However, much more soil nitrogen was allocated into seedlings of group VI, compared with group I, II, III, and VII, respectively (Fig. 4).

Discussion

Leaf or stem browsing and cotyledon removal are two possible dangers that influence growth and survival of oak seedlings^{40,44}. Our

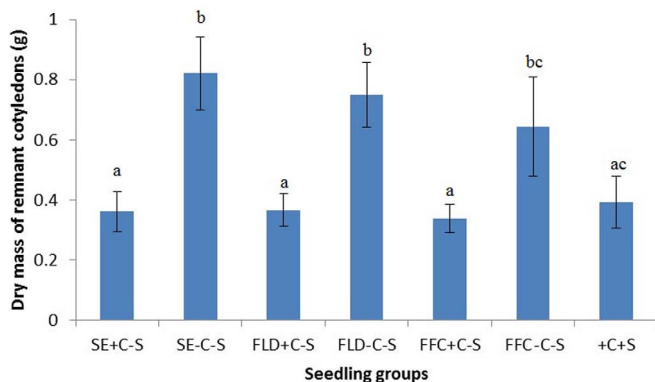


Figure 2 | Changes in dry mass of cotyledons of clipped and unclipped seedlings. SE: clipped when the shoots emerged; FLD: clipped when the first leaf fully developed; FFC: clipped when the first flush completed; +C: with cotyledons; -C: without cotyledons; +S: no shoot clipping; -S: shoot clipping. Data are expressed as mean \pm SE. Different letters on the histograms indicate significance at $P < 0.05$ level.

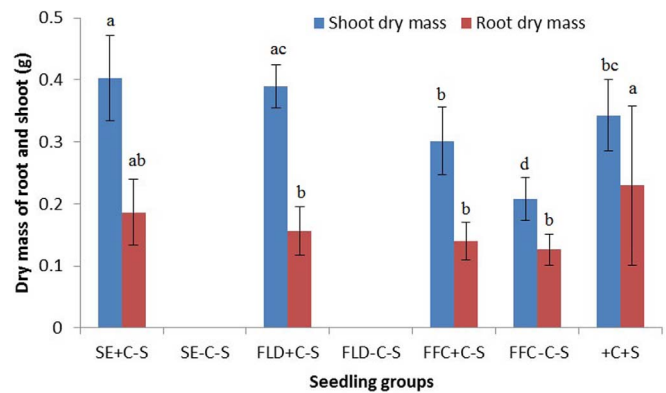


Figure 3 | Dry mass of shoots and roots of clipped and unclipped seedlings in response to cotyledon removal. SE: clipped when the shoots emerged; FLD: clipped when the first leaf fully developed; FFC: clipped when the first flush completed; +C: with cotyledons; -C: without cotyledons; +S: no shoot clipping; -S: shoot clipping. Data are expressed as mean \pm SE. Different letters on the histograms indicate significance at $P < 0.05$ level.

results show that the cotyledons play an important role in influencing the resprouting capability of clipped seedlings of *Q. mongolica*, further supporting the observation of Kabeya and Sakai¹⁷. However, our observations do not support the previous study that found that the cotyledons were not of vital importance for regrowth of the seedlings of *Quercus robur*⁴⁴. This inconsistency may be attributed to different clipping stages in these studies.

Regardless of timing of shoot clipping, clipped seedlings were able to resprout provided their cotyledons were attached. With the reserve support of attached cotyledons, clipped seedlings in group I, II, and III resprouted successfully. The timing of shoot clipping showed no significant effects on resprouting capability of seedlings with cotyledons in group I, II, and III, indicating that the cotyledons contain enough energy reserves to support resprouting although substantial biomass in the shoots has already been clipped. This hypothesis was supported by the finding that dry mass of cotyledons of clipped seedlings in group I, II, and III decreased significantly during resprouting compared with those in group IV, V, and VI. However, many biotic and abiotic factors affect seedling growth and survival, the resprouting and growth of clipped seedlings in natural conditions may be challenged by other environmental factors, i.e., water stress and light availability⁴⁵⁻⁴⁷.

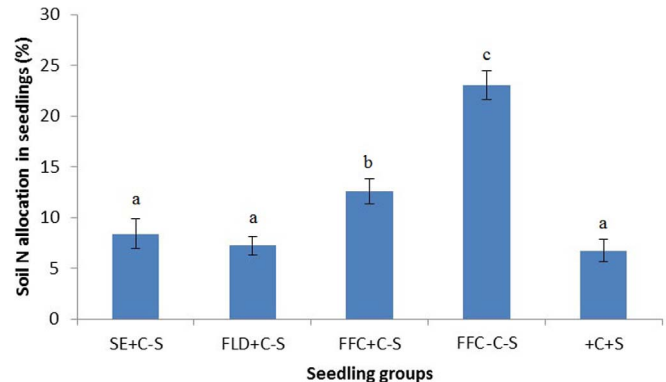


Figure 4 | Soil nitrogen allocations into clipped and unclipped seedlings in response to cotyledon removal. SE: clipped when the shoots emerged; FLD: clipped when the first leaf fully developed; FFC: clipped when the first flush completed; +C: with cotyledons; -C: without cotyledons; +S: no shoot clipping; -S: shoot clipping. Data are expressed as mean \pm SE. Different letters on the histograms indicate significance at $P < 0.05$ level.



As anticipated, clipped seedlings without cotyledons have been found to resprout only in group VI that the cotyledons were removed at the completion of first flush. The failure of clipped seedlings to survive at early development stages suggests that the roots of clipped seedlings are unable to generate sufficient amounts of reserves to support resprouting when their cotyledons are removed. However, if the seedlings are not clipped, the attached cotyledons appear to contribute little to oak seedling growth and survival⁴⁸. These indicate that retention of cotyledons is more important for ensuring seedling resprouting than for supporting seedling growth and survival. Although the dry mass of the cotyledons decreased as the seedlings developed as seen in group IV, V, VI, and VII, there is no enough evidence for significant difference between group I, II, III, and VII, implying that a proportion of dry masses are not accessible to seedlings although resprouting is expected under generalized environmental hazards^{29,49–52}. These results echo the previous observation that the transfer of resources from cotyledons to seedlings appeared to be completed at a very early stage⁴⁰. Therefore, the redundant biomass has been regarded as structural carbohydrate or food rewards to attract animals for seed dispersal²⁰.

We found no evidence that shoot clipping exhibited negative effects on seedling performance in terms of dry mass of the newly-sprouted shoots in group I, II, and III, compared with the control group VII. However, in group VI that the cotyledons were also removed, the dry mass of newly-sprouted shoots was significantly lower than those of group I, II, III, and VII. Moreover, the dry mass of roots showed no significant differences between group I, II, III, and VI. These findings provide strong support for the crucial role of cotyledons in supporting resprouting of clipped seedlings^{27,53}. However, compared with group VII, group II, III, and VI were characterized by lower dry mass of roots, indicating that resprouting of clipped seedlings are also dependent on root reserves, but maybe not as important as the cotyledons.

Our results also showed that early clipping of shoots (e.g., shoot emergence, and full development of the first leaf) failed to enhance nitrogen absorption of seedlings (group I and II) from soil, compared with the seedlings in group III and VI. One potential explanation is that the roots are not functional to absorb substantial nutrition from soil at the early stages of seedling development. However, a low proportion (16.25%) of clipped seedlings without cotyledons in group VI resprouted and developed into seedlings. One potential explanation for this finding is that the roots have accumulated enough reserves and/or become functional to absorb nutrition from soil. Fully developed root systems of clipped seedlings in group III and VI may be highly capable to extract nitrogen from soil to support resprouting. This prediction can be supported by the findings that roots of clipped seedlings in group III and VI absorbed 12.6% and 23% nitrogen from soil, much higher than those in group I, II, and VII. There was evidence for the decrease in dry mass of roots in group III, indicating that the need to form new shoots surely decreased root growth²³. However, even at the end of growth season, significantly low level of soil nitrogen was found in the unclipped seedlings in group VII, implying that roots of 1-year seedlings only become functional to absorb nutrition from soil in the presence of negative disturbances, e.g., shoot clipping and cotyledon removal. These findings provide evidence that oak seedlings appear to depend upon cotyledon reserves of carbohydrate, P, K and Mg during most of the first year of growth^{39,54,55}. Oak seedlings are expected to become independent of the cotyledons at the end of the first growing season.

Conclusion

Although hypogeal cotyledons and taproots have been regarded as storage organs of oak seedlings, our results show that the role of cotyledons and taproots in supporting resprouting changes as the seedling develops. Without the support of cotyledons, clipped seedlings are unable to resprout. Moreover, the dry mass of cotyledons

decreased significantly before and after resprouting in the clipped seedlings, suggesting that cotyledons play a crucial role during shoot development stages. No significant difference found in the dry mass of roots and low level of soil nitrogen allocated into seedlings during resprouting suggest an alternative function of root storage in supporting resprouting. Roots are not functional in absorbing nutrition from soil in natural 1-year seedlings but become functional in supporting resprouting of clipped seedlings when the cotyledons are removed or at the completion of the first flush. Despite these, resprouting capability of seedlings acquired by cotyledons and taproots at early stage suggesting a double security-based strategy for the clipped seedlings to resprout in response to environmental disturbances.

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Acknowledgments

Funding for this study was supported by the National Natural Science Foundation of China (No. 31172101 and 41203018), the State Key Laboratory of Integrated Management of Pest Insects and Rodents (ChineseIPM1404), and the Program for New Century Excellent Talents in University (NCET-12-0693). We would like to thank Guoqiang Liu and Mingming Zhang for their help with the experiments. We declare no conflict of interest.

Author contributions

X.F.Y. conceived and designed the experiments. X.F.Y. and C.Q.L. performed the experiments. X.F.Y. and C.Q.L. analyzed the data and prepared all figures. X.F.Y. wrote the main manuscript. All authors reviewed the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Yi, X.F. & Liu, C.Q. Retention of cotyledons is crucial for resprouting of clipped oak seedlings. *Sci. Rep.* **4**, 5145; DOI:10.1038/srep05145 (2014).



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