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Original article

Acorn germination and seedling survival of *Q. variabilis*: effects of cotyledon excision

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Abstract

- We studied the effects of acorn cotyledon loss by insect damage and artificial excision on germination and seedling survival of *Quercus variabilis*, to determine whether insect infestation decreases acorn germination and seedling establishment; and to what extent cotyledon damage can affect acorn germination and seedling establishment.
- Artificial excision partially imitated the effect of insect damage on acorn germination and seedling survival, and indicated that insect damage did not significantly decrease germination rate and seedling survival of *Q. variabilis* acorns. The viability of infested but still viable acorns strongly depends on which sector weevil larvae feed on.
- The cotyledon reserves at the apex of acorns were more important than those at the base in supporting acorn viability and seedling establishment. The excision of apical cotyledon has the most detrimental effects on acorn germination activity. The relatively low effect of larval infestation on acorn viability was likely related to the high frequency of feeding and escaping at the base, which was closely correlated with differentiation in pericarp thickness in *Q. variabilis* acorns. These could explain the low decrease in acorn germination and seedling survival experienced by infested acorns.

1. INTRODUCTION

Oaks are large seeded tree species prevalent in the northern hemisphere (Harper et al., 1970), however, acorn germination, seedling performance and natural regeneration are usually to be affected due to their recalcitrant acorns (Doody and O'Reilly, 2008; McCreary, 1996; Tilki and Alptekin, 2006). Researchers have observed a failure in the natural regeneration of the dominant oak species over the last two decades (McCarthy et al., 1987; McDonald et al., 2003). Three major insect acorn pests are widely known in oak acorns, i.e., the acorn moth (Lepidoptera: Tortricidae), the gall wasp (Hymenoptera: Cynipidae), and the acorn weevil (Coleoptera: Curculionidae). Weevils of the genus *Curculio* are the major culprit (Collins, 1961; Downs and McQuilken, 1944; Kearby et al., 1986) and the most studied group of pests affecting oak regeneration (Andersson, 1992; Crawley, 1992; Crow, 1988; Riccardi et al., 2004; Siscart et al., 1999).

Several studies have shown that the acorns infested by insects experience lower germination than the uninfested acorns (Branco et al., 2002; Siscart et al., 1999; Soria et al., 1996), indicating that pre-dispersal acorn predation by insects is one of the constraints for the sexual regeneration of oaks (Crawley and Long, 1995; Oak, 1993; Oliver and Chapin, 1984; Yi and Zhang, 2008). However, large cotyledon reserves in acorns would act as a potential nutrition pool and help the seedling overcome the effect of insect infestation (Leishman and Westoby, 1994; Siscart et al., 1999; Tripathi and Khan, 1990). Most insect-infested acorns can still germinate to form a seedling despite one or both cotyledons are damaged by *Curculio* spp. and other larva of insects (Hirka and Csóka, 2004; Yi and Zhang, 2008). Although cotyledon reserve has been identified as main resource to improve the early development of the growing plant (Francisco et al., 2003), it remains a big question to what extent cotyledon loss can affect acorn germination and seedling establishment. Cork oak (*Q. variabilis*) is one of the most important oak species in a wide area of eastern Asia (e.g. China, Japan, and Korea). Acorns of cork oak and of many other oak species are frequently infested by weevils, moths and other insect larvae (Fukumoto and Kajimura, 2000). Field observations indicate that *Q. variabilis* produces large-sized acorns (2–6 g) and is usually attacked by single weevil larva (*Curculio* spp.) in each acorns. *Q. variabilis* acorns are supposed to easily overcome the negative effect of insect infestation on germination and seedling survival. However, only a few studies describe their damage to seedling establishment and ecological adaptability of acorns of *Q. variabilis* (Fukumoto and Kajimura, 2000).

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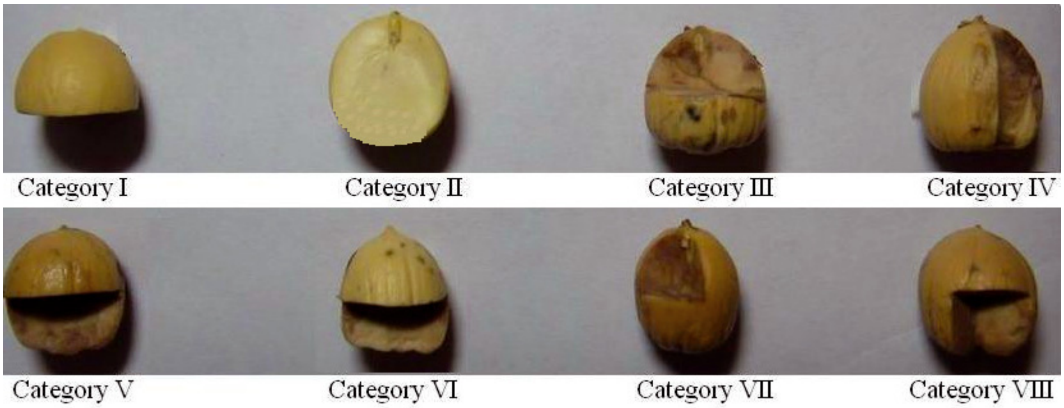

Figure 1. Eight categories of *Quercus variabilis* acorns after artificial cotyledon excision. Category I: excision of the base of both cotyledon (50%); Category II: excision of one cotyledon (50%); Category III: excision of apex of one cotyledon (25%); Category IV: excision of longitudinal 50% of one cotyledon (25%); Category V: excision of base of one cotyledon (25%); Category VI: excision of 50% of base of both cotyledon (25%); Category VII: excision of 50% of apex of one cotyledon (12.5%), and Category VIII: excision of 50% of base of one cotyledon (12.5%). (A color version of this figure is available at www.afs-journal.org.)

Since internal damage cannot be reliably predicted from external symptoms, damage may have been under- or overestimated in previous studies where only external damage symptoms were recorded. The aim of this study is to determin to what extent acorn predation by insects is limiting the sexual regeneration of *Q. variabilis*. To do that we studied the effects of acorn cotyledon loss by weevil larval damage and artificial excision on germination and seedling establishment. We address the following specific questions in this study: (1) Does insect infestation significantly decrease acorn germination and seedling establishment? (2) To what extent does cotyledon loss significantly affect acorn germination and seedling establishment? (3) Which sector of the cotyledon is the most important for germination and seedling growth.

2. MATERIALS AND METHODS

2.1. Acorn collection and infestation evaluation

To quantify the percentage of insect attack, 24 seed traps were deployed in the understory to measure seed fall under *Q. variabilis* crowns in late August 2008. Seed traps were made of a 1 m × 1.2 m polyester net (2 mm mesh) on a 0.5 m^2 metal frame. The frame was set on a thin wooden rod approximately 1.2 m above the ground to prevent predation by terrestrial vertebrates. Traps were established to catch acorns and other debris while letting rainfall pass through. One seed trap was placed 1–2 m from the trunk under 24 randomly selected *Q. variabilis* trees (diameter at breast height: 22–25 cm) prior to release of acorns. Traps were left in place until all ripe acorns had fallen. Length, width and fresh mass of each acorn were measured prior to dissection. Immediately after collection, all acorns were carefully evaluated in the laboratory, by removing the pericarp and dissecting and examining for the presence of insects. The number of larvae per acorn, pre-dispersal infestation rate, pericarp thickness, and feeding site occurrence were recorded. Insect infested acorns randomly collected in field were stored in sunlight free conditions at room temperature for 35 d, which allowed all weevil larvae to escape from infested acorns. The number of exit holes per acorn and exit hole occurrence frequency at different parts of infested acorns were recorded. The difference of pericarp thickness at the apex, middle, and base of acorns was determined to see whether there was a relationship between weevil feeding sites and pericarp thickness. The difference of acorn length, width, and fresh mass between infested and uninfested acorns was determined to see whether there was oviposition preference for large acorns. Uninfested acorns were collected for tannin analysis using Folin-Denis method according to AOAC (1990).

2.2. Germination tests

Uninfested and infested acorns were collected under the canopy of twelve *Q. variabilis* trees for germination and seedling survival experiments. Germination tests were performed on uninfested and infested acorns. Infested acorns with one exit hole were used to evaluate the effect of insect infestation on germination because more than 94% infested acorns had a single exit hole. In November 2008, 10 acorns from each category were buried in plastic pots (45 cm height × 50 cm diameter) at a depth of 2 cm. Pots were embedded in the soil in the field and water was supplied at regular interval. There were 5 replications of each category.

We selected uninfested acorns, which were the same size and fresh mass as those used in the above germination and seedling survival experiment, to imitate insect damage through artificial cotyledon excision. The pericarp of each acorn was opened along the longitudinal axis without injuring the cotyledons. The cotyledons were carefully taken out for artificial cotyledon excision. There were eight levels of artificial cotyledon excision which imitated damage in the cotyledons by *Curculio* spp. The excised percentage was determined by measuring the individual acorn size: Category I: excision of the base of both cotyledon (50%); Category II: excision of one cotyledon (50%); Category III: excision of apex of one cotyledon (25%); Category IV: excision of longitudinal 50% of one cotyledon (25%); Category V: excision of base of one cotyledon (25%); Category VI: excision of 50% of base of both cotyledon (25%); Category VII: excision of 50% of apex of one cotyledon (12.5%), and Category VIII: excision of 50% of base of one cotyledon (12.5%) (see Fig. 1). We ensured that the remaining

Table I. Characteristics of infested and uninfested acorns of *Q. variabilis*.

Acorn features	Uninfested		Infested
Length (cm)	2.05 ± 0.19		2.07 ± 0.18
Width (cm)	1.78 ± 0.16		1.82 ± 0.23
Fresh weight (g)	4.47 ± 1.04		4.26 ± 0.98
Seed crops (acorn/m^2)		8.08 ± 6.46	
Number of larvae per acorn		1.12 ± 0.05	
Number of exit holes per acorn		1.06 ± 0.03	
Pre-dispersal infestation (%)		44.86 ± 30.08	
	Non-damage	Light-damage	Severe damage
Relative frequency (%)	66.30 ± 8.37	17.90 ± 2.13	15.80 ± 2.86
	Apex part	Middle	Base
Tannin content (mg/g)	78.29 ± 6.95	–	72.42 ± 8.07
Pericarp thickness (mm)	0.70 ± 0.08	0.50 ± 0.06	0.46 ± 0.08
Feeding site occurrence (%)	5.00 ± 0.80	48.75 ± 2.60	54.55 ± 4.50
Exit hole occurrence (%)	10.87 ± 0.60	43.48 ± 2.10	45.65 ± 3.20

parts of the cotyledon were connected to the embryo after artificial excision for each acorn. The successfully excised cotyledons were sterilized by washing in 10% sodium hypochlorite solution for sterilization and then put back into the corresponding pericarps for later planting (50 acorns per category, total of 400 acorns). We also planted 50 acorns with unaltered cotyledons covered with opened pericarps as control in November 2008. In late March 2009, germination rate were initially recorded. Acorns were considered germinated when the hypocotyl and radicle protruded. At the end of June 2009, seedling survival rate, stem growth, and the number of leaves were measured.

2.3. Statistical analysis

Acorn germination dynamics was evaluated by using a general linear model (GLM) repeated measures test. To compare the differences in final germination rate, stem growth, and number of leaves between uninfested and infested acorns, or among the eight categories of cotyledon excision, the GLM univariate was used (fixed effects). GLM univariate (fixed effects) was also applied to test the difference of pericarp thickness at the apex, middle, and base of acorns; the difference of acorn length, width, and fresh mass between infested and uninfested acorns, and the difference of tannin concentration between the apex and base of acorns. Spearman correlation analyses were used to test the relationship between the epicarp thickness and the appearance percentage of oviposition site, feeding sites and emergence sites, respectively.

3. RESULTS

3.1. Insect infestation in acorns

In 2008, *Q. variabilis* produced 8.08 ± 6.46 acorns/m^2 (mean ± one standard deviation) in our study area (Tab. I). Acorn dissection indicated an average pre-dispersal infestation rate of 44.86 ± 30.08%. Additionally, 15.80 ± 2.86% of the acorns were severely damaged and 17.90 ± 2.13% of acorns were slightly damaged. There was no significant difference in acorn fresh mass ($F = 1.360$, df = 1, $P = 0.246$), length ($F = 0.245$, df = 1, $P = 0.621$), and width ($F = 1.581$, df = 1, $P = 0.211$) between infested and uninfested acorns (Tab. I). There was a significant difference in pericarp thickness at base, middle and apex ($F = 60.643$, df = 2, $P < 0.0001$). Pericarp thickness at the base and medial parts was significantly lower than that at the apex (all $P < 0.0001$); however, no difference was seen between the middle and base ($P = 0.061$). Our results showed that 5.00 ± 0.80%, 48.75 ± 2.60%, and 54.55 ± 4.50% of eggs or larvae were found feeding at the apex, middle, and base of acorns, respectively. Consequently, 10.87 ± 0.60%, 43.48 ± 2.10%, and 45.65 ± 3.20% of exit holes were found drilled at the apex, middle, and base, respectively (Tab. I). Weevil infestations were not often found feeding at (5.00 ± 0.80%) and emerging from (10.87 ± 0.60%) the base. The number of larvae and exit holes per acorn was 1.12 ± 0.05 and 1.06 ± 0.03, respectively (Tab. I). Spearman correlation analyses showed that epicarp thickness of the three parts of acorns was significantly and negatively correlated with the appearance percentage of oviposition site, feeding sites and emergence sites, respectively (all $P < 0.01$). No significant difference was found in the tannin concentration between the apex and base of acorns ($F = 0.910$, df = 1, $P > 0.394$).

3.2. Seed germination

Our results clearly indicated a significant difference in germination dynamics among the ten acorn categories ($F = 20.113$, df = 9, $P < 0.0001$). Uninfested acorns germinated much more quickly than those of acorns of category II, III, VII and infested acorns ($P = 0.05$, $P < 0.0001$, $P < 0.0001$, $P = 0.008$), but more slowly than those of I, IV, V, VIII ($P = 0.001$, $P = 0.005$, $P = 0.011$, $P = 0.032$) (Fig. 2). Infested acorns showed a much longer germination period than those of I, IV, V, VI, VIII without excision of apical cotyledon ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P = 0.032$, $P < 0.0001$). Despite total loss of basal cotyledon, germination time of category I were much higher than those of II, III, VI, VII with excision of apical cotyledons (all $P < 0.0001$). Germination time of category II lagged behind those of IV, V, VIII (all $P < 0.0001$). Category III acorns were slower than

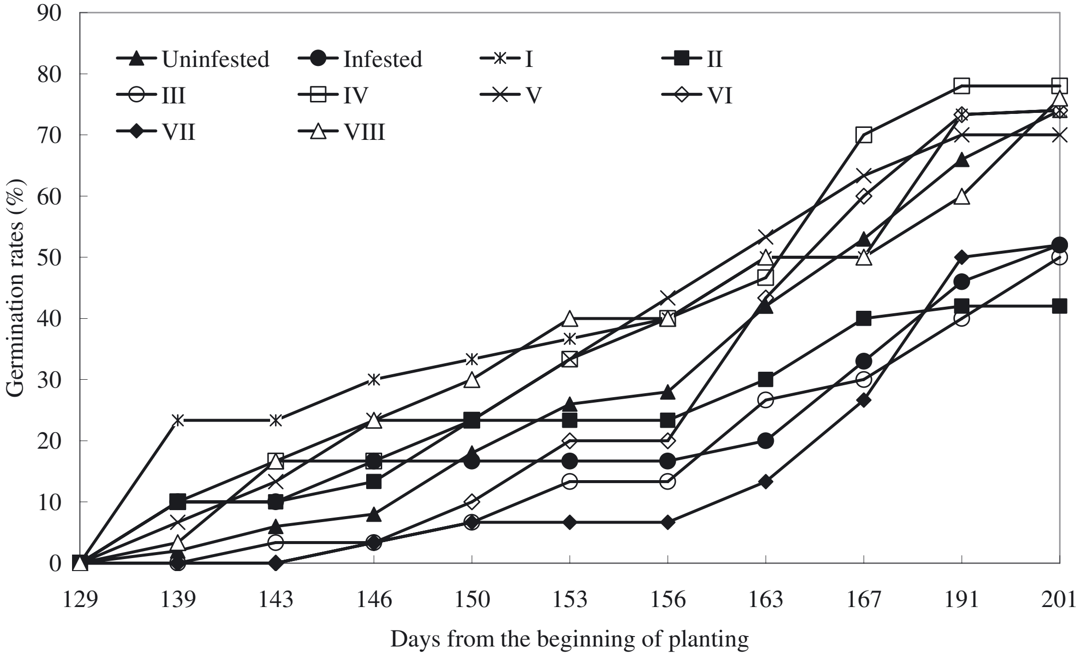

Figure 2. Germination rates (%) of *Quercus variabilis* acorns as a function of date with different degree of cotyledon excision or damage.

category II, IV, V, VI and VIII in germination time ($P = 0.028$, $P < 0.0001$, $P < 0.0001$, $P = 0.001$, $P < 0.0001$). No difference was found in the germination time of category IV, V, VI and VIII; but there was a significant lag period of germination schedule of category VII (all $P < 0.0001$).

However, insect damage caused a significant reduction in the final germination rate when compared to uninfested acorns ($F = 24.200$, df = 1, $P = 0.001$). The average germination rate of infested and uninfested acorns were 74 ± 4.8% and 52 ± 6.4% respectively (Fig. 2). Imitating cotyledon excision experiments showed that significant influence of cotyledon excision on germination rates of *Q. variabilis* acorns ($F = 3.814$, df = 8, $P = 0.002$). Germination rate of category II was significantly lower than those of category I, IV, V, VI, VIII and uninfested acorns respectively ($P = 0.002$; $P = 0.001$; $P = 0.007$; $P = 0.002$; $P = 0.001$; $P = 0.002$). Germination rate of category III was significantly lower than those of category I, IV, V, VI, VIII and uninfested acorns respectively ($P = 0.020$; $P = 0.007$; $P = 0.050$; $P = 0.020$; $P = 0.012$; $P = 0.020$). Germination rate of category VII was also significantly lower than those of category I, IV, VI, VIII and uninfested acorns respectively ($P = 0.032$; $P = 0.012$; $P = 0.032$; $P = 0.020$; $P = 0.032$) (Fig. 2). No significant difference in germination rate was detected among category I, IV, V, VI, VIII and uninfested acorns (all $P > 0.05$). Germination rates of category II, III and VII were not significantly different (all $P > 0.05$) (Fig. 3). These suggest that two groups of germination rates can be distinguished: high (uninfested, I, IV, V, VI, and VIII) and low (infested, II, III, VII), indicating that insect damage at the apex of acorns is more harmful than at the base in spite of the proportion of cotyledon loss.

3.3. Seedling survival

Our results revealed that seedling survival rates were 100% for all acorns except in category I and VI, with 4.54% and 4.35% seedlings failing to survive, respectively (Fig. 3). Stem growth was not significantly different between seedlings from uninfested and insect damaged acorns ($F = 2.543$, df = 1, $P = 0.120$). However, the number of leaves at the end of the 229 d period in seedlings which developed from uninfested acorns was significant lower than those from insect damaged acorns ($F = 3.814$, df = 8, $P = 0.002$) (Fig. 3), possibly reflecting the induction effect of insect damage on ramet tillering of infested acorns. With respect to imitating cotyledon excision, significant differences were found for stem growth and number of leaves in seedlings which developed from acorns with different degree of cotyledon excision ($F = 2.972$, df = 8, $P = 0.004$; $F = 2.759$, df = 8, $P = 0.007$) (Fig. 3). The stem height in seedlings from uninfested acorns was much higher than those from acorns of category I, II, III, VI and VII ($P < 0.0001$; $P = 0.042$; $P = 0.032$; $P = 0.005$; $P = 0.024$) respectively. The stem height in seedlings from acorns of category I was much lower than those from acorns of category IV, V, VI and VIII ($P = 0.000$; $P = 0.047$; $P = 0.006$; $P = 0.004$; $P = 0.004$) respectively. The number of leaves in seedlings from category VII acorns was much lower than those from acorns of category IV, VI and uninfested acorns ($P = 0.033$; $P = 0.037$; $P = 0.001$) respectively (Fig. 3). Therefore, it was evident that the feeding activity of larvae on acorns did not necessarily result in a negative effect on seedling growth. But the apical cotyledon loss by artificial excision would cause some negative effects.

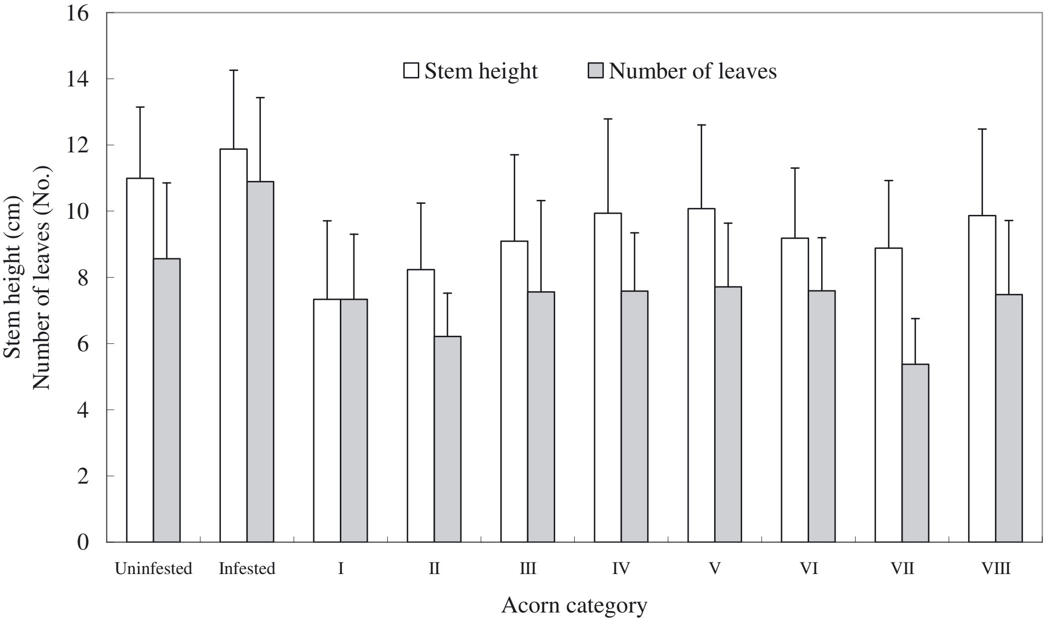

Figure 3. Number of leaves and stem height (cm) of *Quercus variabilis* seedlings from unifested, infested, and acorns with different degree of cotyledon excision or damage. Error bars represent one standard deviation.

4. DISCCUSION

Unlike the study of Branco et al. (2002), the present study confirmed the importance of weevil larvae on causing damage to acorns of *Q. variabilis* because insect infestation significantly decreased germination rates, which was similar to other studies with other *Curculio* or *Quercus* species (Cortés-Gimeno, 2003; Oliver and Chapin, 1984; Pulido and Díaz, 2003; Weckerly et al., 1989). The results were also consistent with the effect of cotyledon excision carried out by Frost and Rydin (1997), Milberg and Lamont (1997) and Bonfil (1998), but not with Andersson and Frost (1996) who have reported that cotyledon extirpation had no effect on growth in oaks. Our results indicated that cotyledon damage or excision was not likely to reduce survival rate of seedlings, well in agreement with the results of Branco et al. (2002) and Leiva and Fernández-Alés (2005), but inconsistent with the work of Fukumoto and Kajimura (2000) on acorns of *Q. variabilis* that hypocotyls and radicle survival is severly affected by high level of cotyledon loss. However, no significant difference in stem height and number of leaves was found between uninfested and infested acorns, failing to strengthen other similar studies (Branco et al., 2002; Fukumoto and Kajimura, 2000), which showed that early growth of young seedlings was greatly reduced with increasing acorn damage by insects. This is possibly due to the induction effect of insect damage on ramet tillering of infested *Q. variabilis* acorns.

Previous studies deal mainly with the effect of insect damage on seed germination and a few with hypocotyl and radicle survival based on the presence of exit holes or other external symptoms (Branco et al., 2002; Fukumoto and Kajimura, 2000). However, the internal damage cannot be reliably predicted from external symptoms (e.g., exit holes). In this study, we analyzed the effects of artificial excision of cotyledon on acorn germination and seedling vigour, as measured by stem growth and number of leaves. Our results showed that there was significant difference in germination rates and seedling establishment in different categories of artificial cotyledon excision, reflecting the effect of infestation site and damage intensity on acorn viability. Stem height of seedlings from acorns of category I, II, III, VI and VII was significantly lower than those from uninfested acorns, indicating that the excision or loss of apical cotyledon would cause significant reduction in seedling vigour, growth rate and number of leaves compared with basal excision at the same level of cotyledon loss, suggesting the more importance of apical cotyledon reserve in supporting acorn germination and seedling establishment. These observations implied that apical cotyledon tissues might have not been damaged by weevil larvae in most infested acorns in our experiments, because germination rate of infested acorns was decreased by only 30%, and stem height and number of leaves developed from infested acorns were similar to those from uninfested acorns. In addition, larvae of *Curculio* spp. were often found feeding at the base of the acorns (Tab. I), consistent with similar findings for other insect on other host species (Steele et al., 1993). However, we failed to see the correlation between unbalanced distribution of tannin and feeding sites in the apex and base, the differentiation of pericarp thickness seems to be responsible for the larval feeding activity in acorns and seedling establishment of *Q. variabilis* (Tab. I). Our results indicated that epicarp thickness of the three parts of acorns was significantly and negatively correlated with the appearance percentage of oviposition site, feeding sites and emergence sites, respectively. Thus, the larval feeding activity was concentrated on basal cotyledon and seldom results in injury to the apical embryo, consequently showing little effect on the capability of seeds to germinate. Alternatively, the weight losses caused by insect attack found in this study were negligible, which was similar to the results from Montoya and Iranzo (1997) and Soria et al. (1997), respectively. The relatively low effect of larval infestation on acorn viability we found in this study was likely related to the large size of *Q. variabilis* acorns (Siscart et al., 1999). Hence, insect infested acorns may have a greater chance of establishment than uninfested ones with high rate of predation in natural conditions, because rodents removed significantly more healthy than infested acorns (Xiao et al., 2003), and have a high degree of accuracy in rejecting infested seeds (Johnson and Adkisson, 1985).

In summary, our artificial cotyledon excision can partially imitate the effect of insect damage on acorn germination and seedling survival. Our results indicate that cotyledon reserves at the apex of acorns are more important than those at the base in supporting acorn viability and vigour. The relatively low effect of larval infestation on acorn viability that we found in this study was likely related to the high frequency of feeding and emerging at the base of acorns. The feeding activity was closely correlated with differentiation in pericarp thickness in *Q. variabilis* acorns. That could explain the low decrease in acorn germination and seedling emergence experienced by infested acorns relative to the uninfested ones.

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