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Cool Temperatures and Post-Germination Adaptations Enhance Seedling Recruitment in the Subalpine Oak *Quercus longispica* (Fagaceae)

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Abstract: *Quercus longispica* is a dominant shrub in the Himalayan subalpine region, demonstrating high levels of persistence despite high seed predation and extreme climatic conditions. However, its seed germination ecology and adaptations for seedling recruitment remain poorly understood. This study investigated the effects of temperature, water potential, and insect damage on seed germination and seedling establishment. Pre-germination seed traits and seed-to-seedling ontogeny were systematically analyzed. Our results demonstrated that seed germination percentages decreased with increasing insect damage across all temperature and water potential treatments. Cool temperatures (5–10 °C) yielded the highest germination percentages, potentially due to the suppression of parasitoid activity and mildew growth. While drought conditions also suppressed parasitoid activity, they significantly increased seed mortality. Despite a decline in seedling performance with increasing seed damage, overall seedling establishment remained robust. Several adaptive traits enable *Q. longispica* to persist in its harsh environment. Multi-seeded, non-apical embryos combined with rapid germination help embryos evade or escape damage from parasitism and predation. The rapid elongation of cotyledonary petioles pushes the embryo axis into the soil, with rapid nutrient and water transfer from the cotyledon to the taproot, thereby avoiding the threats of predation, drought, cold, and wildfire. Additionally, temperature-regulated epicotyl dormancy at the post-germination stage prevents the emergence of cold-intolerant seedlings in winter. This study provides the first comprehensive description of seed-to-seedling ontogeny in this Himalayan subalpine oak, offering crucial insights into the adaptive mechanisms that facilitate successful seedling recruitment in the challenging subalpine habitats.

Keywords: insect parasitism; germination; epicotyl dormancy; seedling establishment; ecological adaptation

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1. Introduction

Seed germination and seedling establishment are critical phases in the plant life cycle, as these processes are essential to the recruitment of new generations and the sustainability of plant communities [1]. However, these stages are particularly vulnerable to environmental stresses, often exhibiting extremely high mortality rates. Such high mortality exerts the strongest natural selection pressure throughout the entire life cycle [2,3]. Consequently, mechanisms that increase seed germination and seedling survival are subject to strong natural selection [3]. Through adaptive evolution, plant seeds have developed diverse strategies to mitigate unfavorable conditions, enabling successful seedling establishment in specific habitats [4]. Thus, seed germination and seedling establishment characteristics profoundly influence species distribution and ecological niche preferences [3].

The genus *Quercus* L., comprising approximately 470 species, is a keystone lineage in the Northern Hemisphere, exhibiting remarkable ecological divergence across temperate and subtropical forests [5,6]. Seeds of *Quercus* spp. display considerable variation in maturation timing, and responses to abiotic and biotic stresses at the post-dispersal stage, such as dehydration, temperature fluctuations, predation, and fire [7–9]. For instance, seeds from Mediterranean and semi-arid regions exhibit higher desiccation tolerance than those from humid forests [8,10,11]. Meanwhile, seeds from subtropical habitats are more sensitive to lower germination temperatures and exhibit higher physical defenses than those from temperate regions [9–11]. Furthermore, seeds of *Q. rubra* L. (section *Lobatae*) exhibit physiological dormancy and have greater fire resistance than those of *Q. alba* L. (section *Quercus*) without dormancy [12]. Importantly, seed germination traits also show variability in response to local environmental factors, even with intraspecific populations in different regions demonstrating great variation in their seed germination rhythms [13]. The strong association between habitats and seed germination traits in the genus *Quercus* suggests that local adaptation is a key driver of seed germination trait evolution in this lineage [14–16].

The *Quercus* group "Heterobalanus", also known as the Himalayan subalpine sclerophyllous oaks, is a unique lineage within *Quercus* section *Ilex*, comprising 5–7 species [5,17]. These species dominate mid- to high-elevation (1900–4600 m) forests in the Himalayas and southwest China [18–21]. They are well-adapted to harsh high-elevation conditions, such as low temperatures, drought, impoverished soils, and strong ultraviolet (UV) radiation [20,22,23]. Fossils of these oaks were found at 6000 m on the Qinghai-Tibet Plateau, where these species can no longer survive, providing direct evidence of the rapid uplift of the Himalayans during the Neogene [24]. Consequently, these oaks have drawn considerable interest from researchers across diverse fields, including plant ecology, physiology, geography, and evolutionary biology.

Despite their ecological significance, the nutrient-rich seeds of subalpine oaks are highly vulnerable to predation and environmental stress, likely due to their thin pericarp and small seed size [9]. Their high desiccation sensitivity and limited water retention capacity make them susceptible to drought immediately after post-dispersal. However, these seeds exhibit adaptation for rapid germination at low temperatures, with a base germination temperature range of −0.8 °C to 2.5 °C [9] and the ability to germinate even at −5 °C [25]. Such rapid germination is considered an essential "escape" strategy, protecting embryos from predation [26–28], desiccation, and freezing damage [29,30]. Furthermore, the cold, dry subalpine climate, lasting over five months annually (October–April), imposes extreme challenges to seedling establishment. However, the mechanisms underlying the seedling establishment and survival of subalpine oaks in their unique harsh subalpine habitat remain poorly understood.

Quercus longispica (Hand.-Mazz.) A. Camus, a dominant subalpine oak species, is widespread from the central Yunnan-Guizhou Plateau to the East Himalayas at elevations of 2000–3800 m, often forming shrubby landscapes on semi-arid, rocky limestone slopes [18]. Seeds of this species mature from late September to early October and are dispersed in October to early November, coinciding with cold weather, increased predation, and drought stress, and typically undergo rapid germination, with seedling emergence observed between March and May of the following year. Given the theory of phylogenetic niche conservatism [31], it is likely that *Q. longispica* shares adaptive seed traits with other subalpine oaks. However, detailed studies on the seed ecology of this species are lacking.

Our preliminary field observations revealed high levels of acorn parasitism in *Q. longispica*. A few previous studies have shown that lower environmental temperature and seed moisture content (MC) significantly reduce the survival of parasitic insect eggs and larvae [32,33]. We hypothesized that post-dispersal drought and low temperatures in *Q. longispica* may mitigate seed/seedling mortality caused by high parasitism rates.

This study comprehensively investigated the effects of temperature and water potential on seed germination and development in *Q. longispica* under varying levels of acorn damage. Additionally, we document the ontogenetic changes from germination to seedling establishment to understand how post-dispersal conditions influence recruitment success. Specifically, we aim to (1) describe the seed germination and post-germination characteristics of *Q. longispica*; (2) explore the interplay between temperature, water potential, parasitic insects, and seed germination; and (3) reveal the ecological importance of seed traits in seedling establishment within the subalpine environment. This study provides novel insights into the seed ecology of *Q. longispica*, enhancing our understanding of adaptive strategies that facilitate seedling recruitment within challenging subalpine habitats.

2. Materials and Methods

The general experimental design is illustrated in a workflow scheme of the experiments and treatments in the Supplementary Materials (Figure S1).

2.1. Location and Climate of the Study Area

The study area is located in Ye-Mao Mountain, Xiaoshao village, Kunming, Yunnan Province, China (25°13′ N, 102°44′ E, altitude 2384 m). This region experiences a subtropical plateau monsoon climate, influenced by the Indian summer monsoon [34–36]. From October to February, following seed dispersal (October–February), the average field temperature is 10 °C and increase to 15.7 °C during the seedling emergence period in spring (March–May). Annual precipitation is concentrated in the wet season (May–September), with 79.5% of the total precipitation occurring in this period. Monthly average precipitation is 160.0 mm in the wet season and only 29.6 mm in the dry season (October–April) [37]. Consequently, the region is highly fire-prone during the dry season [38]. The *Q. longispica*-dominated shrubland ecosystem in subalpine limestone areas is subject to a natural regime of mild to moderate wildfire disturbance (Figure S2). We located only eight mature, fire-unaffected trees (8–15 m tall, 15–25 cm diameter at breast height (DBH)) in this study area.

2.2. Acorn Collection, Characteristics, and Insect Parasitism/Damage Assessment

About 20 kg of fresh acorns were randomly collected from the eight fire-unaffected mature trees, which produced substantial acorns, on 5 October 2021. Acorns were sealed in zip bags and transported to the lab on the same day and were stored in a 4 °C refrigerator. Two days later, all the collected acorns were washed and immersed in tap water for one hour to screen the debris and discard the floating acorns (mostly aborted). The remaining acorns were categorized into three insect damage levels: (1) intact; (2) parasitized (with parasitic entry holes, no escape holes, and an intact embryo axis and cotyledons, containing insect eggs or young larvae); (3) damaged (with escape holes, embryo axis and cotyledons partially damaged, and may still contain mature larvae) [7]. Parasitic and escape holes were identified following the descriptions in Matsumura, et al. [39] and Canelo, et al. [40].

Thirty intact acorns were randomly selected, dissected into three components (cotyledon, pericarp, and embryo axis), then oven-dried at 103 °C for 17 h. The dissected cotyledon, pericarp, and embryo axis from each of ten acorns were weighed together to determine the dry mass (DM) and MC (fresh weight basis). The seed-to-defense allocation ratio (pericarp ratio, PR) was calculated as the ratio of pericarp DM to acorn DM [41]. The axis mass ratio (AMR) was calculated as the ratio of the embryo axis DM to the seed DM, and values were presented as percentages [9]. The length and diameter of the acorns were measured on 150 intact acorns. The number of seeds per acorn was counted after pericarp removal. Embryo axis position was observed via a longitudinal section of all seeds.

2.3. Effects of Temperature and Water Potential on Seed Germination

2.3.1. Effects of Temperature on Germination

To compare the effects of temperature on germination across different damage levels, intact, parasitized, and damaged seeds were germinated at six temperatures (5–30 °C, in 5 °C increments). For each temperature treatment, 100 seeds were divided into five replicates of 20 seeds each. Seeds were germinated on 1% agar in deionized water within lidded boxes in a growth chamber under a 12 h photoperiod and 80% relative humidity. Germination was defined as radicle protrusion of ≥2 mm and was assessed every two days until no new germination was observed for 30 consecutive days. Mildew growth on the substrate at each temperature was observed and photographed using a digital camera (SONY ILCE-7RM4A, Sony Corporation, Tokyo, Japan).

For the parasitized acorns, in addition to germination percentages, the incidence of larvae hatching (identified through escape holes and dissection) and parasitism-induced mortality during germination at each temperature were also recorded. Seed mortality was attributed to insect parasitism if (1) pre-emergence larvae exit, resulting in subsequent seed death; (2) larvae were found in ungerminated seeds at the end of the experiment.

2.3.2. Effects of Water Potential on Germination

The influence of water potential on seed germination was investigated for intact, parasitized, and damaged seeds. Different osmotic potentials (0 [control, deionized water], −0.2, −0.4, −0.6, −0.8, and −1.0 MPa) were generated with polyethylene glycol 6000 (PEG-6000) solutions [42]. Seventy-five seeds (five replicates of 15) were used for each osmotic potential. Germination was conducted in a growth chamber at 20 °C with a 12 h photoperiod. Seeds were germinated on filter paper saturated with the appropriate polyethylene glycol solution in lidded plastic boxes. Germination was monitored every two days, and the filter paper was replaced every 4 days to maintain stable PEG-6000 concentrations. Larvae hatching and parasitism-induced seed mortality were assessed following the same procedure as in the temperature experiment.

2.4. Impacts of Insect Damage on Seedling Establishment

Germinated intact, parasitized, and damaged seeds with similar growth status were planted into nonwoven fabric pots (30 cm depth and 30 cm diameter) filled with a substrate of 20% vermiculite, 20% perlite, and 60% peat moss. For intact and parasitized seeds, 300 seeds were divided into 10 replicates of 30 seeds each for seedling cultivation. For damaged seeds, 150 seeds were divided into five replicates of 30 seeds each for seedling cultivation. Potted germinated seeds were grown under a shaded greenhouse with a maximum photosynthetic photon flux density (PPFD) of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, temperature ranging from 15 °C to 38 °C, and relative humidity ranging from 75% to 90%.

The cotyledon petiole length, taproot length and diameter, stem height, DM of cotyledon, taproot, and leaves were measured immediately after seedling establishment (defined as first leaf pair appearance; Figure S3). Seedling establishment was monitored until no new seedlings emerged in any pots for 30 consecutive days.

2.5. The Seed-to-Seedling Transition

Intact seeds were germinated at room temperature and potted in the greenhouse to observe seedling establishment and post-germination traits. Establishment time was defined as when 50% of sown seeds established seedlings.

Twenty seedlings were sampled weekly for 56 days to track developmental growth sequences. After 56 days, due to slow seedling growth, sampling was suspended, and some seedlings were transferred to a transparent glass incubator for direct observation of underground growth until seedling establishment. Seedling development and key morphological changes were comprehensively observed and photographed using the same digital camera (SONY ILCE-7RM4A). The cotyledonary petiole, epicotyl length, stem height, and taproot length and diameter were measured using ImageJ (version 2.14.0) from digital images [43].

Due to the difficulty in directly measuring epicotyl length of ungerminated seeds and seedlings before establishment (first 14 days after germination), paraffin embedding and sectioning techniques were employed. Samples containing the embryo axis were fixed in FAA solution (50% ethanol–formaldehyde–glacial acetic acid = 90:5:5, volume ratio) at 4 °C for one week, then transferred to 70% alcohol, and stored at 4 °C until processing. Fixed samples were dehydrated in a graded ethanol series (30, 50, 70, 85, and 95%), followed by two 1 h incubations in 100% ethanol. The dehydrated samples were then infiltrated gradually with a xylene/ethanol series (25, 50, and 70% xylene in ethanol) for 1 h each, followed by two 30 min incubations in xylene. The samples were then embedded in Plus® Paraplast (Leica Microsystems GmbH, Wetzlar, Germany; melting point of 53 °C) and sectioned at 6 μm using a rotary microtome (Leica RM2155, Leica Microsystems GmbH, Wetzlar, Germany). After de-waxing, sections were stained with safranin-fast green and examined using a stereo microscope (Leica S9i) and an upright light microscope (Lecia DM3000LED, Leica Microsystems GmbH, Wetzlar, Germany).

To assess nutrient transfer from the cotyledon to taproot, 10 of the 20 samples from Experiment 1 were dissected into cotyledon and taproot parts. Dry mass content (DMC, dry weight/fresh weight × 100%) was determined for each tissue to assess nutrient transfer during development.

2.6. Response of Epicotyl Growth to Temperature

Germinated intact seeds with similar growth status were incubated at temperatures of 5–30 °C (on 5 °C increments), as well as 0 °C and −3 °C to simulate subalpine winter soil conditions under snow cover, which can fall below freezing. Thirty seeds per temperature were divided into three groups and were grown under the same conditions as the germination test. Seeds were placed on moist filter paper in plastic boxes for five months, approximating the time required for seedling emergence in the field, to observe the effect of temperature on epicotyl growth. Field observations indicate that *Q. longispica* seedlings typically emerge between March and May of the following year after germination, suggesting that epicotyls may undergo dormancy at low temperatures. Therefore, ten germinated seeds were processed for paraffin sectioning to measure epicotyl length and determine if significant changes occurred after the five months exposure.

Epicotyl emergence (shoot protruding from cotyledonary petiole, Figure S3) and the time to 50% emergence (T_{e50}) were recorded. Epicotyl emergence rates ($1/T_{e50}$) were regressed against temperature to estimate epicotyl dormant temperature (T_d). As the epicotyl of seeds incubated at −3 °C and 0 °C did not emerge during the 5-month observation period, paraffin sectioning was used to assess epicotyl lengths before and after inculcation

2.7. Statistical Analyses

The time to 50% germination (T_{g50}) at different temperatures was determined, and its reciprocal ($1/T_{g50}$) was calculated to express the germination rate [9]. Linear regression in R (version 4.3.1) was used to analyze the relationship between the epicotyl emergence rate ($1/T_{e50}$) and temperatures. The x-intercepts of the resulting regression lines were defined as the dormant temperature of the epicotyl emergence (T_d).

The Shapiro–Wilk normality test was performed on all datasets, and the results indicated that the distribution of $1/T_{e50}$ did not meet the assumption of normality ($p < 0.05$). A natural logarithm transformation (base e) was applied to $1/T_{e50}$, and subsequent statistical analyses were conducted. One-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) post hoc test was used to compare differences among means of applicable variables ($p < 0.05$) using R (version 4.3.1).

3. Results

3.1. Acorn Characteristics and Insect Parasitism/Damage Assessment

Fresh and dry weights of *Q. longispica* acorns were 1.32 ± 0.18 g and 0.55 ± 0.13 g (mean ± SE), respectively. The initial acorn MC was 58.33 ± 5.89%. Acorn length ranged from 0.93 to 1.87 cm (mean = 1.29 ± 0.42 cm), with a diameter ranging from 0.79 to 1.65 cm (mean = 1.12 ± 0.38 cm). PR and AMR were 0.24 ± 0.01 and 0.13 ± 0.02%, respectively. Detailed data of weight, MC, and DM for each acorn component were summarized in Supplementary Table S1.

In most acorns, the embryo axis was positioned below the style base at the apex (Figure 1a), but in 15.6 ± 2.33% of cases, it was located at the non-apical position, near the seed scare or lateral end (Figure 1b). Seed anatomy revealed that the embryo axis comprised the embryo positioned at the apex of the prominently developed cotyledonary petiole (Figure 1c). Multi-seeded instances occurred in 11.2 ± 2.5% of acorns, with two or three embryos observed per acorn (Figure 1d,e). Approximately two-thirds of acorns showed signs of insect activity, with 27.63 ± 2.97% parasitized and 39.24 ± 3.72% physically damaged. Evidence of insect activity included visible parasitic and exit holes on the pericarp surface (Figure 1f,g).

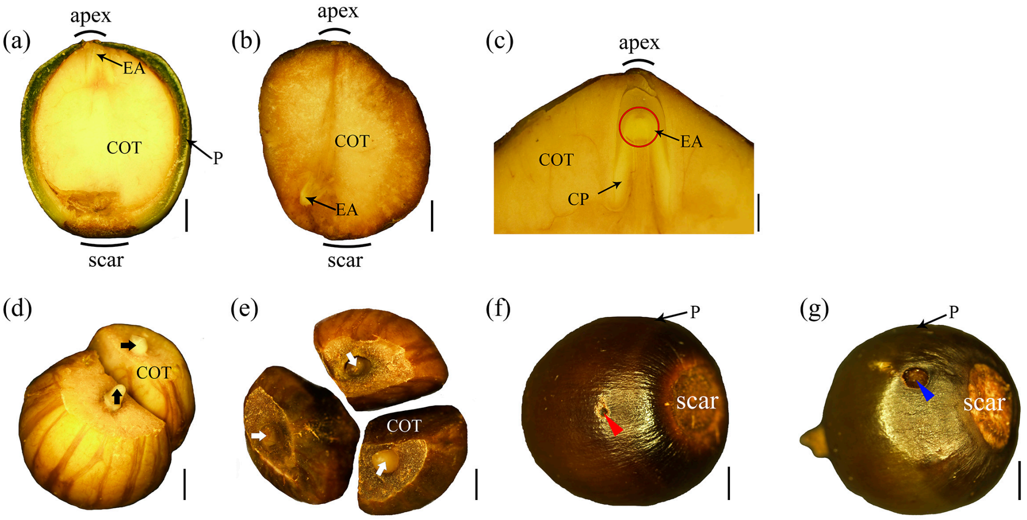

Figure 1. Acorn and seed traits of *Q. longispica*. (**a**) Embryo axis at seed apex (style base); (**b**) a basal embryo axis (close to seed scar); (**c**) embryo axis (marked with red circle) at the top of cotyledon

petiole; (**d**,**e**) two and three seeds in one acorn (black/white arrows indicate embryo axis); (**f**) parasite hole (red triangle); (**g**) escape hole (blue triangle). EA, embryo axis; COT, cotyledon; P, pericarp; CP, cotyledon petiole. Scale bar = 2mm in (**a**,**b**,**d**–**g**); scale bar = 1 mm in (**c**).

3.2. Effects of Temperature and Water Potential on Seed Germination

3.2.1. Effects of Temperature on Germination

Germination percentage declined with increasing insect damage across all temperature gradients (Figure 2a). Damaged seeds exhibited significantly lower germination compared to intact and parasitized seeds at all temperatures ($p < 0.001$). At lower temperatures (5 °C and 10 °C), germination percentages of intact and parasitized seeds were not significantly different ($p > 0.05$), but at higher temperatures (15–30 °C), intact seeds showed a significantly higher germination percentage ($p < 0.001$). Intact seeds achieved the highest germination percentage (85.0 ± 3.2%) at 10 °C, with no significant difference from 5 °C to 15 °C ($p > 0.05$). Germination of intact seeds decreased to 43.0 ± 3.4% at 30 °C. Both parasitized and damaged seeds exhibited the highest germination percentage at 5 °C (78.0 ± 2.4% and 34.79 ± 2.7%, respectively), with no significant difference between 5 °C and 10 °C ($p > 0.05$, Figure S4). Germination decreased sharply at higher temperatures, reaching approximately 50% at 20 °C and 32 ± 3.4% at 30 °C for parasitized seeds and only 2% for damaged seeds at temperatures above 25 °C (Figure S4). Mildew growth was abundant on intact and parasitized seeds at higher temperatures (15–30 °C), while it was significantly reduced at lower temperatures (5–10 °C). In damaged seeds, mildew growth was only significantly reduced at 5 °C (Figure S5).

Seed damage level did not significantly affect germination rates at any tested temperatures ($p > 0.05$). At 25 °C, all seeds exhibited the highest germination rates, with $1/T_{g50}$ values of 0.17 ± 0.02 d^{-1}, 0.16 ± 0.02 d^{-1}, and 0.19 ± 0.03 d^{-1}, for intact, parasitized, and damaged seeds, respectively (Figure 2b). Germination rates decreased at lower temperatures, with the $1/T_{g50}$ values dropping to 0.02–0.03 d^{-1} at 5 °C. However, seeds incubated at 30 °C also showed lower germination rates than those incubated at 25 °C.

In parasitized seeds, the percentage of seeds with hatched larvae and the parasitism-induced mortality increased with temperature from 5 °C to 25 °C (Figure 2c), peaking at 25 °C (44.0 ± 1.7% and 29.0 ± 0.9%, respectively) but with no significant difference to that at 20 °C ($p > 0.05$). At 30 °C, both hatched larvae and parasitism-induced death were lower than at 25 °C ($p < 0.01$). The lowest percentages of hatched larvae (9.0 ± 1.7%) and parasitism-induced death (3.0 ± 1.1%) were recorded at 5 °C (Figure 2c).

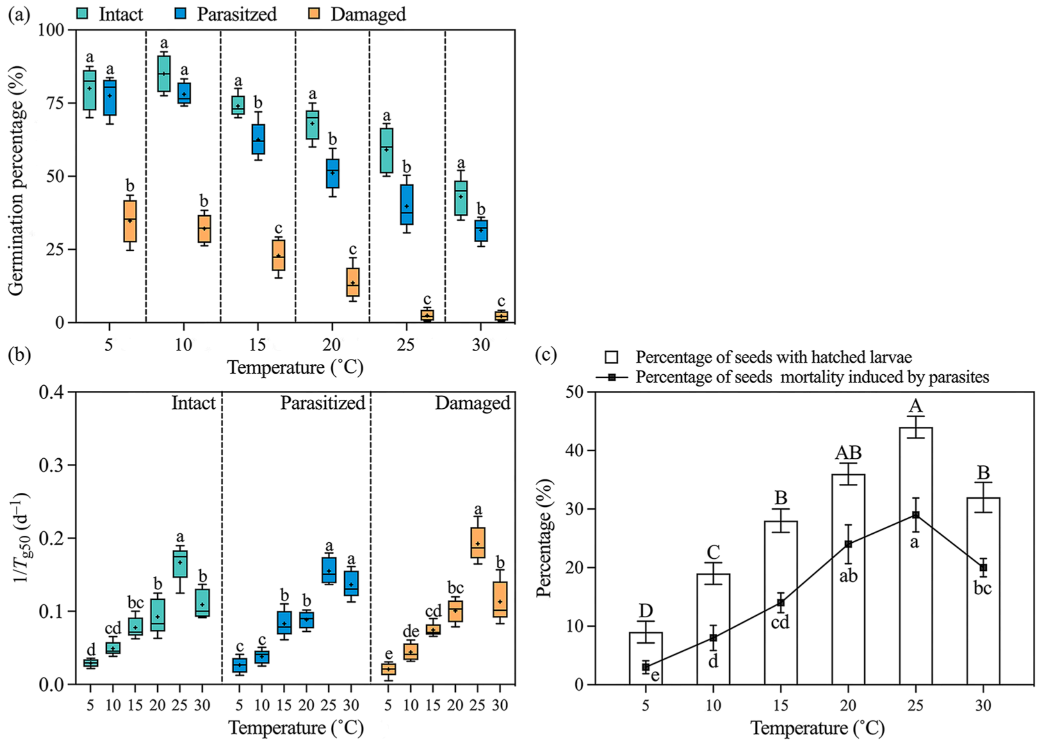

Figure 2. Effects of temperature on germination percentage and parasitism in *Q. longispica*. (**a**,**b**) Germination percentage and rate of seeds with different insect damage levels; (**c**) percentage of seeds with hatched larvae and parasitism-induced seed mortality. In figure (**a**,**b**), within each subplot, bars sharing the same letter are not significantly different (Tukey's HSD, $p < 0.05$). Box plots represent the interquartile range (25th–75th percentiles), with the median and mean shown by a solid line and a "+", respectively. Whiskers represent the 5th and 95th percentiles. In figure (**c**), data are presented as means ± standard error. Capitals denote significant differences in percentage of seeds with hatched larvae; lowercases denote significant differences in percentage of parasitism-induced seed mortality (Tukey's HSD, $p < 0.05$).

3.2.2. Effects of Water Potential on Germination

Germination percentage declined with decreasing osmotic potentials in seeds of all damage levels (Figure 3a). Maximum germination occurred at 0 MPa: 68.3 ± 3.6% for intact seeds, 47.1 ± 2.3% for parasitized seeds, and 17.0 ± 2.7% for damaged seeds. No significant differences were observed at −0.2 MPa ($p > 0.05$, Figure S4). At −0.4 MPa, the germination percentage decreased sharply to 27.0 ± 1.3% (intact), 15.5 ± 2.1% (parasitized), and 7.7 ± 1.1% (damaged). No germination was observed at −1.0 MPa in any seed type (Figure 3a).

In parasitized seeds, the percentage of seeds with hatched larvae and parasitism-induced mortality declined with decreasing osmotic potential (Figure 3b). The maximum percentage of seeds with hatched larvae (35.6 ± 1.8%) and the highest parasitism-induced seed death (27.0 ± 3.9%) occurred at 0 MPa (control), with no significant difference at −0.2 MPa ($p > 0.05$). When osmotic potential was decreased to −0.4 MPa, parasitism-induced seed mortality decreased significantly to 17.8 ± 1.8%, while no larvae emerged from seeds at −1.0 MPa (Figure 3b).

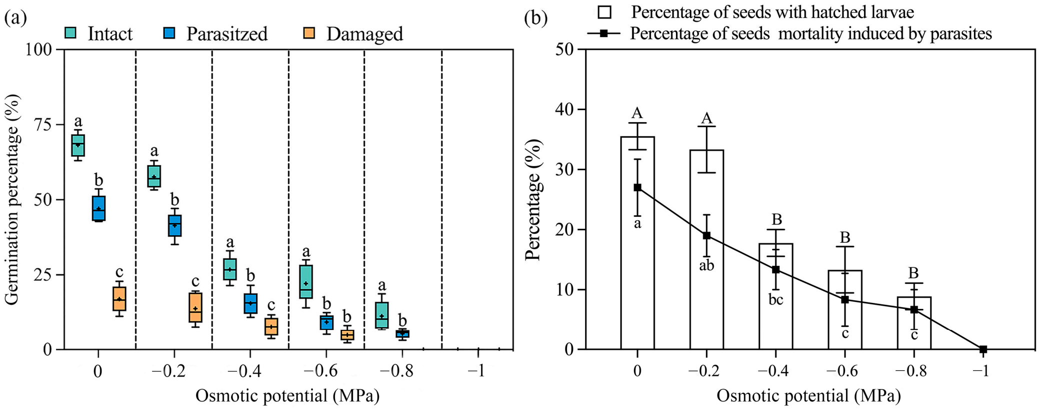

Figure 3. Effects of osmotic potential on seed germination percentage of *Q. longispica* and parasitism development in the acorns. (**a**) Germination percentage at different levels of insect damage. Within each subplot, bars sharing the same letter are not significantly different (Tukey's HSD, $p < 0.05$). Box plots represent the interquartile range (25th–75th percentiles), with the median shown as a solid horizontal line and the mean indicated by a "+"; whiskers extend to the 5th and 95th percentiles. (**b**) Percentage of seeds with hatched larvae and the parasitism-induced mortality of parasitized seeds; data are presented as means ± standard error. Capital letters indicate significant differences in the percentage of seeds with hatched larvae; lowercases indicate significant differences in parasitism-induced seed mortality (Tukey's HSD, $p < 0.05$).

3.3. Impacts of Insect Damage on Seedling Establishment

Seed damage level did not significantly influence seedling establishment ($p > 0.05$, Figure 4a), as long as seeds germinated. However, seedling growth was significantly affected by initial seed damage (Figure 4b–h).

Intact seeds exhibited significantly superior growth metrics, including for the cotyledonary petiole length, taproot length and diameter, stem height, and DMC of the taproot and leaf, compared to damaged seeds ($p < 0.001$, Figure 4). Parasitized seeds generally performed intermediately, with no significant differences from intact seeds in all matrices ($p > 0.05$, Figure 4). The seedling performance of parasitized and damaged seeds differs significantly only in the development of taproots, including the length, diameter, and DMC of the taproot ($p < 0.001$, Figure 4)

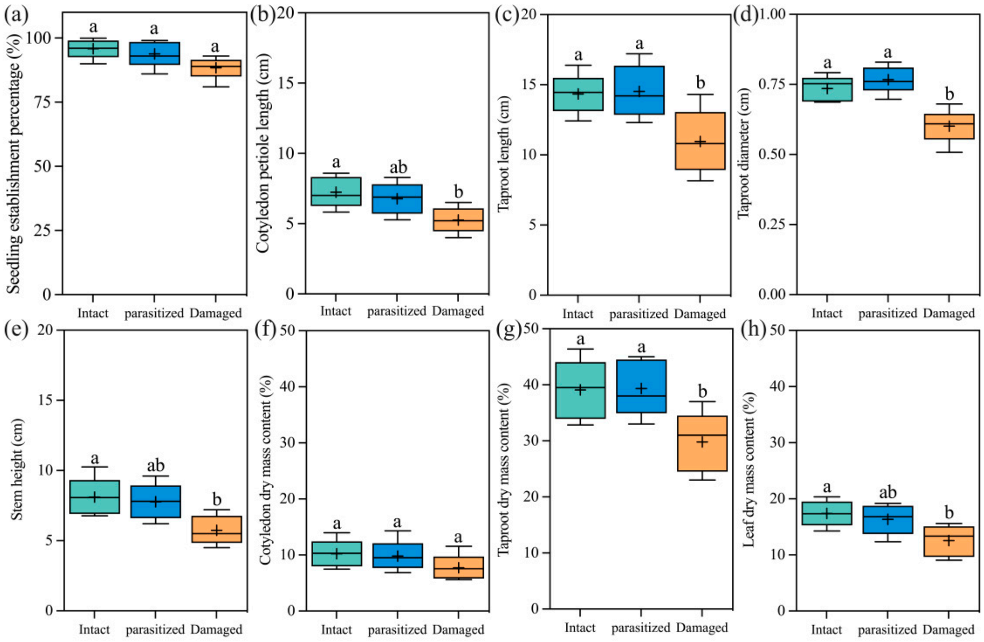

Figure 4. Effects of insect damage level on seedling establishment percentage (**a**) and seedling growth (**b**–**h**) in *Q. longispica*. Within each plant response variable of each subplot, bars sharing the same letter are not significantly different (Tukey's HSD, $p < 0.05$). Box plots represent the interquartile range (25th–75th), with the median and mean represented by a solid line and "+", respectively. Whiskers represent the 5th and 95th percentiles.

3.4. The Seed-to-Seedling Transition

Seed germination to seedling establishment required 110 days under greenhouse conditions (Figure S3). Cotyledonary petioles elongated rapidly after germination, reaching 6.52 ± 0.31 cm within 14 days (90% of final length). Subsequent growth over the following 21 days was slower, with an additional elongation of 0.59 ± 0.56 cm. No further elongation was observed thereafter (Figure 5a). Within the first 14 days after germination, the taproot also grew rapidly, reaching a length of 8.92 ± 0.97 cm (62.19% of final length). By day 35, the taproot length had further increased to 13.43 ± 0.66 cm. Subsequently, taproot elongation slowed significantly, with an additional increase of only 0.91 ± 0.62 cm at the end of seedling establishment (Figure 5a). Concurrently, taproot diameter increased notably in girth beginning on day 7 (Figure S3), reaching 0.51 ± 0.02 cm between day 7 and day 14 (69% of total increase). The growth rates then decelerated from day 14 to day 35, with a diameter increase of only 0.2 ± 0.03 cm (27% of total increase). From day 35 to seedling establishment, only a minimal increase of 0.03 ± 0.03 cm was observed (Figure 5a).

In mature ungerminated seeds, the cotyledon petioles were fused into a cylinder and hollow (separated) above the epicotyl (Figure 6a). The embryo axis length was 745 ± 35 μm, comprising an epicotyl of 55 ± 4 μm and a hypocotyl of 690 ± 26 μm. Seven days after germination in the greenhouse, the epicotyl reached 0.7 ± 0.1 mm in length (Figure 6b). At this stage, the leaf primordia formed (Figure 6b,c) and cotyledon buds were visible (Figure 6c). In the first two weeks after planting, the epicotyl grew rapidly to a length of 6.4 ± 0.5 mm (Figure 5a) but remained enclosed within the hollow cotyledon petiole (Figure 6d). By day 21, the epicotyl had elongated to 20.4 ±3.4 mm (Figure 5a) and emerged from the cotyledon petiole (Figure S3). At day 49, the epicotyl had reached 55.2 ± 5.3 mm (52% of the final length, Figure 5a). The epicotyl continued to elongate until the young shoot emerged above the soil surface and seedling establishment was complete, reaching 81.0 ± 4.8 mm (Figure 5a).

Cotyledon DMC continuously decreased from 41.8 ± 4.4% to 10.2 ± 1.2% during seedling establishment, a reduction of 75.6%. In contrast, taproot DMC continuously increased to 39.1 ± 0.9% during the establishment of seedlings (Figure 5b). The biomass transfer from cotyledons to the taproot was notably faster during the first 28 days after planting. Thereafter, no significant changes in DMC were detected in either the cotyledons or taproot (Figure 5b).

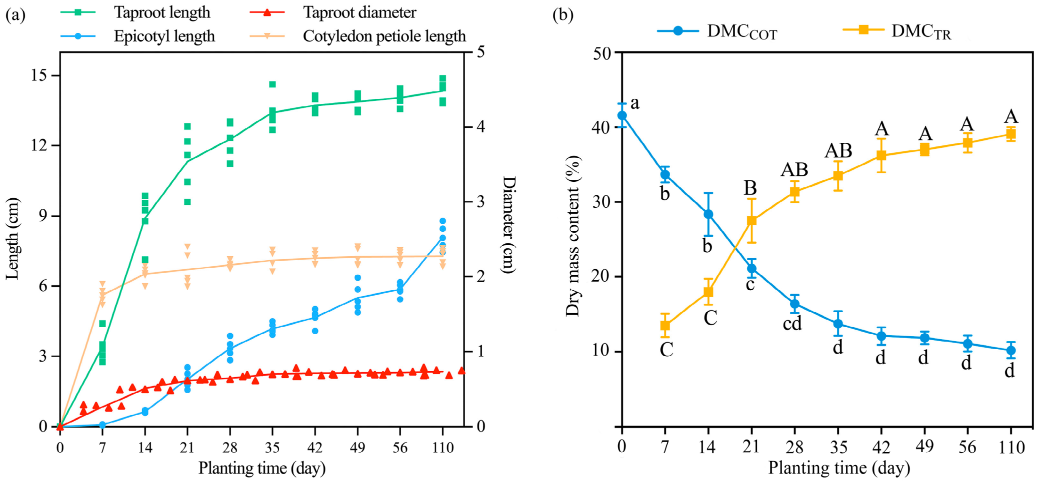

Figure 5. The cotyledon petiole length, epicotyl length, and taproot length and diameter (**a**) and DMC of cotyledon and taproot (**b**) in *Q. longispica* seedings at different post-germination stages in the greenhouse. Data are presented as means ± standard error (n = 10). Different capital letters indicate significant differences in the DMC of taproot; Different lowercases indicate significant differences in DMC of cotyledon (Tukey's HSD, $p < 0.05$). DMC$_{COT}$, DMC of cotyledon. DMC$_{TR}$, DMC of taproot.

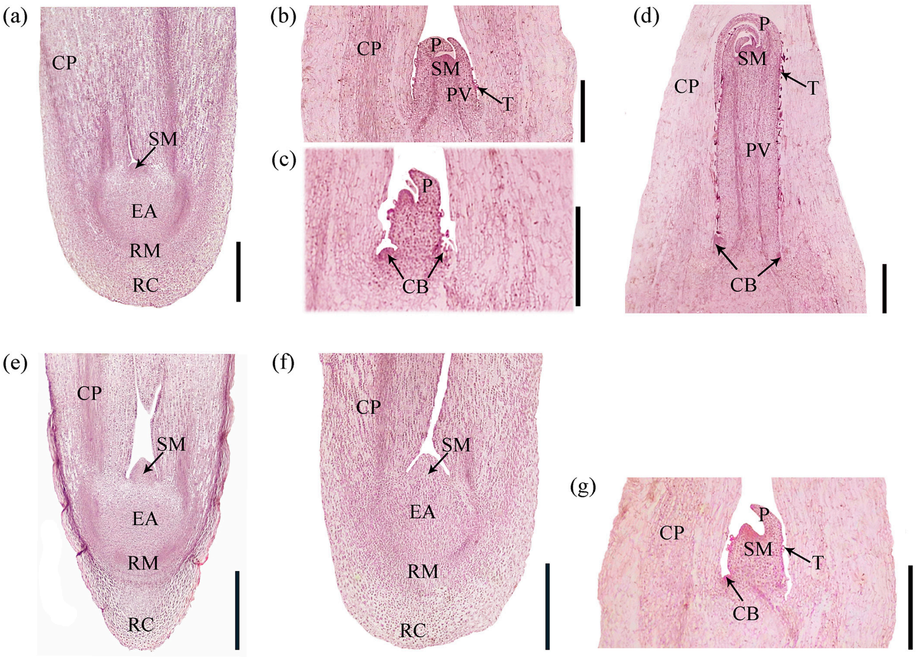

Figure 6. Morphology of embryo axis in paraffin sections of *Q. longispica* seeds: (**a**) mature embryo; (**b**,**c**) embryo development 7 days after germination; (**d**) embryo development 14 days after germination; (**e**) epicotyl at the onset of epicotyl dormancy; (**f**,**g**) epicotyl growth after 5-month culture at −3 °C and 0 °C, respectively. CP, cotyledonary petiole; P, leaf primordium; EA, embryo axis; PV,

provascular tissue; RM, root meristem; RC, root crown; SM, shoot meristem; T, trichome; CB, cotyledonary bud. Scale bars = 500 µm in (**a**–**c**) and (**e**–**g**) and 1 mm in (**c**).

3.5. Response of Epicotyl Growth to Temperature

Epicotyl elongation was temperature dependent. The initial seed epicotyl length was about 161.60 ± 15.13 µm (Figure 6e). At temperatures 0 °C and −3 °C, the epicotyl did not penetrate the cotyledon petiole even after five months (Figure 6f,g). At −3 °C, the epicotyl almost did not elongate, remaining at 169.60 ± 16.79 µm (Figure 6f). Although the epicotyl did not emerge from the cotyledon petiole at 0 °C, its length significantly increased to 366.8 ± 24.6 µm after 5 months of culture, a 127% increase (Figure 6g). Therefore, the threshold temperature for breaking epicotyl dormancy in *Q. longispica* was between −3 °C and 0 °C.

The epicotyl emergence rate ($1/T_{e50}$) increased linearly with temperature from 5 °C to 30 °C (Figure 7). Extrapolation of the linear regression to the x-intercept yielded a dormancy temperature (T_d) of −1.6 °C (Figure 7), consistent with the epicotyl development observed at −3 °C and 0 °C.

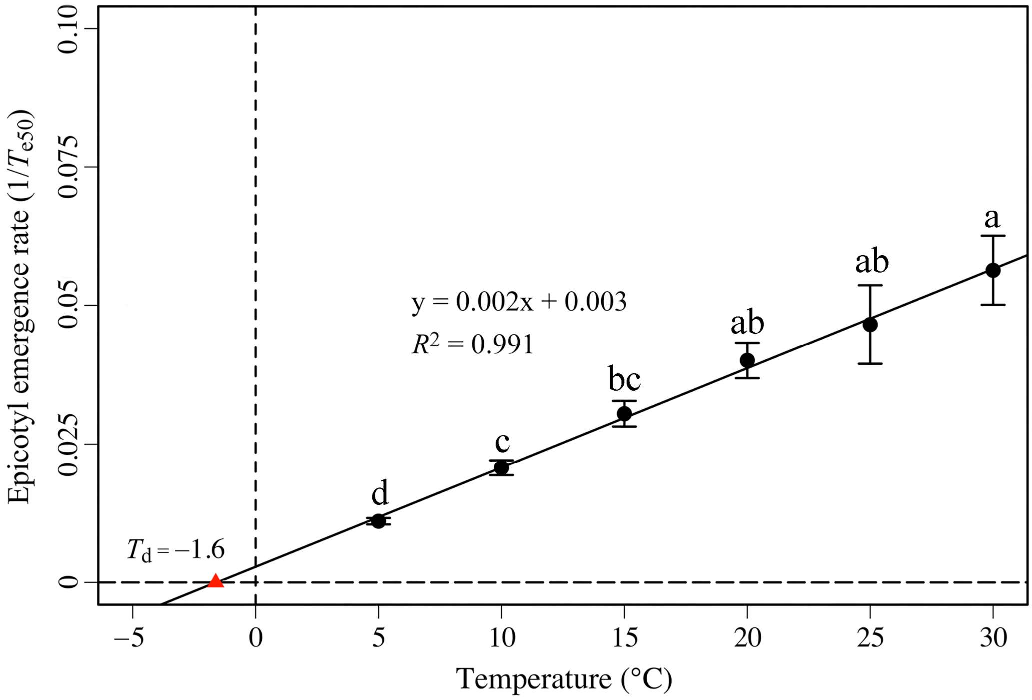

Figure 7. Effect of temperature on the epicotyl emergence rate ($1/T_{e50}$) of *Q. longispica*. The epicotyl dormancy temperature (T_d) was determined as the x-intercept (red triangle) of the linear regression equation ($y = 0.002x + 0.003$, $R^2 = 0.991$). Data were presented as means ± standard error. Means sharing the same letter are not significantly different (Tukey's HSD, $p < 0.05$).

4. Discussion

This study provided compelling evidence that the adaptation of *Q. longispica* seeds to germinate at low temperatures offers an ecological advantage by reducing seed mortality caused by insect parasitism and microbial infection. Despite their high desiccation sensitivity and vulnerability to predation, *Q. longispica* acorns employ a suite of adaptive strategies to ensure seed survival and seedling establishment in dry, high-predation subalpine habitats. The presence of multi-seeded and non-apically positioned embryos within each acorn ensures that some embryos remain viable for germination even after predation. Rapid germination and cotyledonary petiole elongation enables the seedling to quickly penetrate the litter and soil layers, thereby reducing their exposure to biotic and abiotic stresses. Rapid biomass transfer from the cotyledons to the taproot supports seedling survival under conditions of low temperatures, dehydration, and predation. Furthermore, temperature-dependent epicotyl growth, with inhibition at low temperatures and stimulation at higher temperatures, protects emerging seedlings from chilling and freezing injury. These integrated strategies represent a specialized adaptation to the selective pressure of subalpine habitats.

4.1. Adaptation Strategies Before Germination

Q. longispica seeds exhibit a wide germination temperature range (5–30 °C, Figure 2a) compared to oak species from warmer subtropical regions and temperate mesic forests [9]. Notably, its germination percentages are highest at lower temperatures (5–10 °C, Figure 2), despite slower germination rates (Figure 2b). This is consistent with the average field temperature (10.38 °C) during the germination period of *Q. longispica* seeds after dispersal (October–December) [37]. Low temperatures delay or inhibit egg hatching of common acorn parasites [32,44–49], such as weevils and moths [50,51], providing a crucial window for seedling establishment. Additionally, mildew growth is suppressed at lower temperatures [52], reducing microbial-induced seed mortality (Figure S5). Although, *Q. longispica* seeds demonstrate the highest germination rate at 25 °C (Figure 2b), suggesting this temperature is optimal for their germination metabolism. However, the 25 °C temperature range also favors parasitic insects and mildews, resulting in significantly higher seed mortality (Figures 2c and S5). This higher mortality at 25 °C reduces the number of seeds available for later germination, potentially contributing to the lower overall germination rates. In addition, this study confirms that pericarp damage from insect parasitism increases acorn susceptibility to microbial infections, especially at higher temperatures (Figure S5), consistent with findings on *Q. robur* L. [53]. Therefore, rapid germination at low temperatures is crucial for *Q. longispica* seeds to mitigate the impacts of high insect parasitism and damage in subalpine habitats.

This study showed that the germination percentages of *Q. longispica* decreased significantly with an osmotic potential below −0.2 MPa (Figure 3a), consistent with the findings in other high-mountain oaks of the “Heterobalanus” group [9]. According to phylogenetic niche conservatism theory, dehydration sensitivity is likely a shared characteristic among subalpine oaks, posing a high risk of post-dispersal seed mortality. However, rapid germination often allows high-mountain oak seeds to germinate before dehydration becomes critical [9]. In *Q. longispica*, mild dehydration may even suppress parasitic insect development, further reducing seed mortality (Figure 3b).

Pericarp thickness and seed size are crucial factors influencing plant resistance and tolerance to seed predators [54,55]. Acorns of the “Heterobalanus” group, which are generally smaller and have thinner pericarps than those of oaks from subtropical and temperate regions [9], may, therefore, be more vulnerable to predation [7,55–57]. Acorns of *Q. longispica* exhibit multi-seeded (Figure 1d,e) and atypical embryo positioning, with embryos located laterally or basally. Being multi-seeded and a non-apical embryo position likely enhance seed survival under predation [58,59], as viable embryos may remain undamaged, which has also been reported in other oak lineages, including section *Quercus* (white oaks), section *Lobatae* (red oaks) [59], and section *Cyclobalanopsis* [60,61]. This phenomenon be common across oaks [62], suggesting a convergent pattern of trait evolution. However, further case studies are needed to validate the relationship between being multi-seeded, a non-apical embryo position, and predation intensity.

4.2. Rapid Germination and Adaptation of Seeds After Germination

High water content and a large embryo axis typically promote rapid germination in subalpine oak seeds [9,63]. Despite having a relatively smaller embryo axis (AMR ≈ 0.13), *Q. longispica* seeds exhibit rapid germination rates, and they can even germinate by attaching to the branchlet on the tree based on our field observation. Notably, the embryo axis reported in other subalpine oaks is much longer (AMR = 0.43) [9]. This discrepancy may be due to differences in measurement methodologies as these studies include the cotyledonary petiole when measuring the embryo axis. Therefore, further research using homologous measurements of the embryo axis is needed to accurately assess its correlation with seed germination speed. Intact seeds of *Q. longispica* germinated faster than those of temperate and subtropical oaks, as they exhibit a mean $1/T_{g50}$ of 0.09 ± 0.02 d^{-1}, germinating 1.5-fold and 3-fold faster than other oak species from temperate and subtropical regions [9]. Following germination, cotyledonary petioles can elongate quickly to 6 cm in the first week (Figure 5) and push the embryo axis (plumule) and radicle deep into the soil, where the relatively stable temperature and humidity mitigate exposure to cold and drought conditions above ground. Similarly, in some white oak species (section *Quercus*), the cotyledon petiole elongation of 1–2 cm after germination can sufficiently prevent seedling injury during post-germination seed removal by seed predators [64,65]. The hollow cotyledonary petioles of *Q. longispica* can be easily detached from the taproot (Figure S3), thus protecting the developing seedling when rodents excavate the seed. Moreover, rapid accumulation of nutrients and water in the inflated taproot supports seedling establishment, even if the remaining cotyledonary parts are detached [66]. Our study found that partial cotyledon loss did not significantly affect seed germination and seedling establishment (Figure 4a). This may be because the nutrients deposited in cotyledon often significantly exceed the requirements for seedling establishment [67], and *Q. longispica* seeds rapidly transfer the remaining reserves to the taproot during the early stages of germination (Figure 5b). The rapidly developing taproot facilitates early nutrient uptake from the soil [30], thereby reducing the dependency of the seedling establishment process on cotyledon reserves.

A field survey revealed that *Q. longispica* communities frequently dominate post-wildfire landscapes (Figure S2). The thick bark and strong regenerative capabilities likely contribute to this resilience [68–70]. In southwest China, fire seasons coincide with acorn dispersal. Studies have shown that even mild fires can significantly reduce the survival rates of exposed seeds on the ground surface [12,71]. Burial depth is a key factor in mitigating fire damage [12,72], as the soil temperature 1 cm below the surface increases by only 9.6 °C during prescribed fires [73]. Deep burial of the plumule and taproot, facilitated by the long cotyledon petiole, protects the propagule from fire damage and other environmental stresses, such as drought, freezing temperature, and intense predation. Therefore, rapid cotyledon petiole elongation, which ensures the deep burial axis growing points (shoot and root), likely represents a crucial adaptive trait for survival and regeneration of *Quercus* in subalpine habitats.

Unfavorable cold and freezing temperatures persist in subalpine regions for over five months, and above-ground exposure of seedlings during this period can cause severe chilling and freezing damage [74]. Epicotyl growth in *Q. longispica* is temperature dependent. Growth ceases below −1.6 °C and is inhibited at temperatures below 10 °C, while temperatures above 15 °C stimulate rapid elongation (Figure 7). The average field temperature in this region from autumn to winter (October–February) is approximately 10 °C, while the spring (March–May) temperatures average 15.7 °C [37]. Under these conditions, *Q. longispica* seeds can exhibit the highest germination percentage after dispersal (Figure 2), and the epicotyl may grow slowly if not remaining dormant during winter (Figure 7). This temperature-regulated germination and epicotyl dormancy ensures that seedlings emerge during favorable seasons, reducing the risk of chilling and freezing injuries and maximizing the length of the growing period.

Similar germination patterns were observed in other (sub)tropical species, such as *Pittosporopsis kerrii* Craib (Icacinaceae) [75], *Quercus chungii* F. P. Metcalf [60], and *Attalea vitrivir* Zona (Arecaceae) [76]. This germination strategy, known as "remote-tubular germination", is considered one of the most primitive germination types in the palms (Arecaceae) [77,78], providing significant advantages in coping with predation, dehydration, and fires in dry tropical environments [77–80]. The convergent evolution of this germination strategy across diverse plant lineages highlights their substantial ecological advantages, particularly in enabling seeds and seedlings to withstand a wide range of environmental stresses.

5. Conclusions

The ability to germinate at low temperatures increases seed survival for *Q. longispica* by inhibiting insect egg hatching, suppressing larval development, and reducing mildew growth. Post-germination adaptations, including rapid cotyledon petiole elongation that pushes the embryo and radicle deep into the soil, rapid nutrient translocation from the cotyledon to taproots, and the temperature-regulated epicotyl growth, collectively enhance seedling survival under harsh subalpine conditions.

This study represents the first comprehensive investigation of germination and post-germination strategies in Himalayan high-mountain oaks (the "Heterobalanus" group), providing valuable insights into the adaptive mechanisms that support survival in high-elevation ecosystems. The convergence of similar germination strategies across diverse angiosperm lineages and habitats highlights their ecological significance in overcoming environmental stresses. Further comparative research on seed and seedling ecology will be vital for advancing our understanding of these adaptations and their evolutionary significance.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f16020261/s1: Figure S1: Workflow of the experiments and treatments of the germination test on *Q. longispica* in this study; Figure S2: Location of the sampling site (the left) and characteristics of the habitats (the right). *Q. longispica* is a dominant shrub in a wildfire-disturbed community of the karst areas. Red arrows show fire-damaged branches of *Q. longispica*, and the purple arrows show the new twigs; Figure S3: Phenotypic changes during the seed-to-seedling development of *Q. longispica*; Figure S4: Effects of temperature and osmotic potential on the germination percentage of *Q. longispica* seeds with varying levels of insect damage; Figure S5: Mildew growth under different temperatures and levels of insect damage at the end of the germination experiment; Table S1: Weight and moisture content of *Q. longispica* acorn components.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

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Conflicts of Interest: The authors declare there are no competing interests.

Abbreviations

The following abbreviations are used in this manuscript:

DM	Dry mass
DMC	Dry mass content
MC	Moisture content
PR	Pericarp ratio
AMR	The axis mass ratio
T_{g50}	Time to reach 50% of the final germination percentage
T_{e50}	The time when 50% of the seed epicotyl emergence
T_d	Epicotyl dormant temperature

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