Symbiotic germination and development of the myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed *Sebacina* spp.

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Summary

- Symbiotic germination and development of the fully myco-heterotrophic orchid *Neottia nidus-avis* were studied in 'packets' of seed placed adjacent to, or at varying distances from, adult plants in a *Fagus sylvatica* woodland.
- The distribution and identity of the fungal partner(s) of *N. nidus-avis* were investigated by internal transcribed spacer (ITS)-restriction fragment length polymorphism (RFLP) and sequence analysis of part of the 28S gene in fungal DNA extracted from adult plants from the UK and Germany, and from seedlings germinated *in situ*.
- Germination commenced in the spring, but only in the presence of a specific fungus, and occurred most frequently in plots containing adults of *N. nidus-avis*. Seedlings grew best in packets in which a large number of seeds germinated. Adults and seedlings of UK origin contained the same fungal partner whose 28S sequence most closely matched *Sebacina dimitica*. Plants of German origin contained a closely related, but distinct, fungus.
- The results provide the first definitive chronology of the development of *N. nidus-avis* and establish its critical dependence upon, and specificity for, the locally distributed *Sebacina*-like fungus that is required for germination and growth.

Key words: orchid, seedling development, fungal specificity, myco-heterotrophy, *in situ* germination, *Sebacina*.

Introduction

A prerequisite for early development in all myco-heterotrophic plants (MHPs) is colonization by an appropriate fungal symbiont (Leake, 1994). Achlorophyllous MHPs retain this absolute requirement for symbiotic association throughout their lives. It can be hypothesized that, as a consequence of this dependency, the distribution of MHPs will be strongly influenced by the availability of the fungus or fungi that initiate and maintain the development process. Unfortunately, because of the small size of their seeds (e.g. *Neottia nidus-avis*- 0.0022 mm^3) and seedlings it has proved difficult, until recently, to obtain reliable chronological characterization of the early events in the initiation of symbiotic development in MHPs. Most of what we know about them is therefore based upon observations of seedlings recovered from randomly taken soil samples.

The development of techniques enabling burial and sequential recovery of these minute seeds from defined habitats (Rasmussen & Whigham, 1993; Masuhara & Katsuya, 1994) has greatly increased the potential for systematic analysis of the early critical events in myco-heterotroph development. In an earlier paper we described the use of these techniques to evaluate the factors influencing germination of the achlorophyllous myco-heterotroph *Corallorhiza trifida* (McKendrick *et al.*, 2000a). Here, we present the results of an analysis of the symbiotic germination and development of another orchidaceous MHP, *Neottia nidus-avis* (L) Rich. This species, which normally grows in deep shade in beech, hazel, oak-hazel or occasionally yew or coniferous woodland, typically on calcareous soils (Fuchs & Ziegenspeck, 1925; Summerhayes, 1968), has fascinated botanists for many years. The first record of fungal infection in a myco-heterotrophic orchid was made on sections of *N. nidus-avis* roots by Reissek (1847). There

followed early, but unsystematic, accounts of the plant by Irmisch (1853), Prillieux (1856), Groom (1895), Bernard (1899, 1902, 1909), Fuchs & Ziegenspeck (1924, 1925, 1926) and Burgeff (1909, 1932, 1936), and these provided limited information on its germination and development.

The absence of photosynthesis in the orchid was reported by Drude (1873) and repeatedly confirmed both through evidence of the lack of critical light-harvesting pigments (Wilschke, 1914; Seybold & Egle, 1937; Montfort & Küsters, 1940; Reznik, 1958; Reznik *et al.*, 1969; Pfeifhofer, 1989), lack of carbon fixation (Montfort & Küsters, 1940; Reznik, 1958; Hudak *et al.*, 1997), and absence of O_2 evolution in light (Menke & Schmid, 1976). The absolute dependence of *N. nidus-avis* on myco-heterotrophic carbon assimilation from its fungal partners is therefore not in doubt, but neither the processes of symbiotic germination nor the chronology of seedling development have been reliably established and the identity of the fungal partner(s) remains unclear.

In the present experiments we analysed and characterized the development of *Neottia* in sequential harvests over a 33-month period following burial of seed packets at selected locations in and around a natural population of the plant in southern England. The DNA from fungi infecting both seedlings and adult plants at a site in England and in adults from Germany was extracted and analysed using internal transcribed spacer (ITS)-restriction fragment length polymorphism (RFLP) followed by sequencing of the fungal ITS and 28S regions.

Materials and Methods

Two experiments were carried out at Galley Down, Hampshire UK (National Grid Ref: SU 576 188), in a mature *Fagus sylvatica* L. wood with a large population of *N. nidus-avis* growing in soil over chalk. In the first experiment (Expt 1a) the chronology of germination, growth and development of *Neottia* were examined and the impact of the proximity of mature *Neottia* plants upon these processes was studied. In a supplement to this experiment (Expt 1b) a further set of packets was planted in the autumn and harvested 4 months later in order to establish whether germination first occurred in autumn/winter. In Expt 2 the effect of distance from established plants upon seed germination was further investigated in smaller plots.

Experiment 1a: the chronology of germination, growth and development of *Neottia nidus-avis* at distances of less than 1 m and greater than 5 m from flowering adults

Seeds of *N. nidus-avis* were collected from ripe capsules of the orchid growing under mature *F. sylvatica* on Galley Down, Hampshire, UK, on 7 August 1995. They were sown into seed packets constructed from photographic slide mounts and plankton netting of 53 µm pore size (Rasmussen & Whigham, 1993). There were *c.* 50–100 seeds per packet. On 8–10 August 1995 the seed packets were inserted vertically to a depth of *c.* 6 cm below the leaf litter in six 1 m^2 plots. In each plot, 100 packets were planted in a grid pattern using a 1-m^2 quadrat divided by string into 10×10 cm areas as a template (see McKendrick *et al.*, 2000a). Three of the plots (1, 2 and 3) contained a scattering of flowering spikes of *Neottia*, both within and immediately around the quadrat while three (4, 5 and 6) were situated at least 5 m from the nearest known *Neottia* plant. The six plots were dispersed throughout the wood.

A preliminary sample of eight packets was taken from each of plots 1 and 2 on 23 May 1996 after 9 months' incubation, to check whether germination had occurred. There were four subsequent harvests in which samples were taken from all plots. These took place 11 months (23 July 1996, *c.* 20 packets per plot), 18 months (12 February 1997 10 packets per plot), 23 months (23 July 1997 10 packets per plot) and 30 months (26 February 1998 *c.* 20 packets per plot) after sowing. A small sample of remaining packets was also taken from plots 2 and 3 in May 1998: these have been included when plotting the position of packets containing seedlings, but were not included in any statistical analyses.

Immediately following each harvest packets were returned to the laboratory where they were stored moist at 5°C. They were opened, within 3–4 d and examined microscopically to detect the extent of germination, and of seedling development. The contents of each packet were then mounted on a glass slide in 50% glycerol, sealed with clear nail varnish, and stored at 5°C for up to 3 wks, until the development of the seedlings could be characterized. Seedlings too large to be mounted in this way were measured fresh.

In order to determine the more detailed aspects of relationships between fungal penetration and germination it was necessary to remove the testa of the individual seedlings. While it was not feasible to attempt this procedure for all of the thousands of seeds counted in the study, testa removal was achieved in a subsample of 590 seeds from packets recovered in the first two harvests of Expt 1a. Dissecting needles were used to remove the embryos from the testa. The embryos were then examined under the light microscope at magnifications of ×250 and ×400. The length and breadth of each seedling was measured and presence or absence of fungal penetration was recorded. These data were used to establish, and to define, on the basis of these simple measurements, the key developmental stages of the seedlings following initiation of germination. At all of the later harvests, measurements were only made of seeds that had reached at least the first stage of development (see Table 1 for definition of stages).

The percentage germination was calculated for each packet, and the volume of each germinated seedling determined using the equation of Hadley & Williamson (1971) to provide a quantitative measure of development. Estimates of the volume of branched seedlings were made by applying the same

Table 1 Developmental stages of *Neottia nidus-avis*

Germination stage	Description of stage
0	*Ungerminated seed*
1	*Changes before rupture of testa*
1a	Embryo becomes more translucent and may or may not expand. No fungal penetration observed. May or may not lead to further development.
1b	Translucent embryo with fungal penetration of cells at the base of the embryo. Normally leading to further development.
1c	Expansion of embryo to a length ≥ 300 μm and breadth ≥ 200 μm together with formation of pelotons
2	*Changes after rupture of testa but before branching of protocorm* Protocorm initially torpedo-shaped; larger protocorms more pointed because of their prominent apical meristem (Fig. 1). No rhizoids produced, even at this stage.
3	*Changes after branching of protocorm* Rootlets develop from the protocorm and a differentiated shoot-bud develops from the apical meristem.

equation to the main body of the seedling and by adding the separately calculated volume of each individual branch. Analysis of variance, followed by Tukey multiple-comparison test was used to determine if there were significant differences in the mean (natural-log transformed) volume of seedlings in each of the early developmental stages. Linear regression was carried out to determine whether the size of the largest seedlings in a packet was related to the numbers of seeds that had germinated in the packet. This was to test the hypothesis that, where there were large numbers of seedlings in the same packet, rates of growth of germinated seedlings may be adversely affected by competition between individuals for the supply of carbon from the fungal partner.

The total volume of germinated seeds in each packet was calculated and plotted on a grid representing the quadrats in the field, so that any spatial patterns of germination could be observed.

Experiment 1b: determination of the time of germination in *Neottia nidus-avis*

This supplement to Expt 1a was carried out 1 yr later to define more precisely when germination took place.

Seeds were gathered in July 1996, and prepared as described in Expt 1a. Seed packets were constructed and planted on 26 October in three 100 × 50 cm plots situated around established *Neottia* plants. A sample of 10 packets per plot was removed on 12 February 1997 and a further sample, on this occasion of *c.* 25 packets per plot, was harvested on 23 July 1997.

Experiment 2: germination and development of *Neottia nidus-avis* seed in relation to distance from single adult flowering spikes

This experiment was designed to provide a more precise indication of the effect of the presence or absence of adult spikes, and the distance to adults on the distribution of fungal symbiont of the orchid, assessed by bioassay of seedling germination. Seeds were gathered on 23 July 1996, dried over calcium chloride at room temperature for 4 wks then stored in air-tight glass vials at 4°C until needed. Seed packets were constructed as in Expt 1 and sown either into three 50 × 50 cm plots each centred on a single flowering spike of *N. nidus-avis* and with no other spikes within 0.5 m of the plot, or into three plots of the same dimensions lacking adults. The latter were located at least 5 m from the nearest adult plant. Packets were inserted between 17 and 19 September 1996 at 10-cm intervals within these plots, there being 25 packets per plot. They were harvested on 24 July 1997. The presence or absence of germination was recorded and the distribution of the most advanced stage of development was mapped. The effect of distance from the nearest flowering spike (within 15 cm, between 15 and 30 cm and more than 5 m) on the frequency of seed packets in which germination took place was tested by χ^2 analysis.

Identification of fungi

Microscopic observations were made on the fungi associated with germinating seeds in sampled packets and of the fungi found on and infecting these seeds. DNA analyses were carried out on seedlings from eight packets harvested from Galley Down and on 13 adult plants of which 11 were from Galley Down and two from a beech forest, near Tübingen, Germany. Seedling DNA was extracted from large individuals (> 1 mm long) or from bulked material if individual seedlings were < 1 mm long.

Fresh or freeze-dried *N. nidus-avis* tissue was ground in sodium dodecyl sulphate (SDS) buffer with a plastic pestle and mortar and extracted with an equal volume of chloroform. The extract was purified using GeneClean (BIO 101, Inc., Carlsbad, CA, USA) glass milk as described in Taylor & Bruns (1997). The fungal ITS region of the nuclear ribosomal repeat was amplified with the general fungal primer ITS1F (Gardes & Bruns, 1993) combined with the universal primer

ITS4 (White *et al.*, 1990). The fungal ITS amplicons were digested, separately, with the restriction enzymes *Hin*fI, *Alu*I and *Mbo*I and the fragments were separated on 1.5% high resolution agarose gels (Sigma, St Louis, MO, USA) in 0.5× Tris-borate EDTA buffer. Samples yielding identical ITS-RFLP patterns for the three enzymes were considered to have been colonized by the same fungal species. Additional details of the fungal ITS-RFLP approach are provided in Gardes & Bruns (1996a), Karen *et al.* (1997) and Taylor & Bruns (1999a).

The fungal ITS regions were sequenced, using the same two primers, from samples representing each distinct ITS-RFLP pattern. Briefly, ITS amplicons were purified with the QIAquick PCR kit (Qiagen GmbH, Hilden, Germany), an Applied Biosystems Big Dye (Applied Biosystems Inc., Foster City, CA, USA) terminator kit was employed for cycle sequencing, and sequence separation was performed on an ABI 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Following McKendrick *et al.* (2000a) the resulting fungal ITS sequences were compared with sequences in the GenBank database in order to estimate the taxonomic affinities of each associate. Sequences of approximately 550 bp, spanning ITS1, the 5.8S gene, and ITS2, were submitted to the nucleotide–nucleotide search option of BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) using default settings (Altschul *et al.*, 1997).

Because no sequences with high similarity to the *Neottia* fungal ITS spacer regions were present in GenBank, a more conserved gene region was also used. The 5′ end of the 28S gene was chosen because fungal-specific primers are available and diverse unknown ectomycorrhizal fungi have been successfully identified to family or genus using this region (Taylor & Bruns, 1999b). Fragments spanning the ITS regions and part of 28S were amplified using the primer pair ITS1F and cNL2F (5′-GTTTCCCTTTTAACAATTTCAC-3′, kindly provided by Timothy M. Szaro). These were sequenced using Ctb6 (complementary to ITS4, 5′-GCATATCAATAAGCG-GAGG-3′) and cNL2F. The resulting 28S sequences were again compared with GenBank sequences using the NCBI BLAST website.

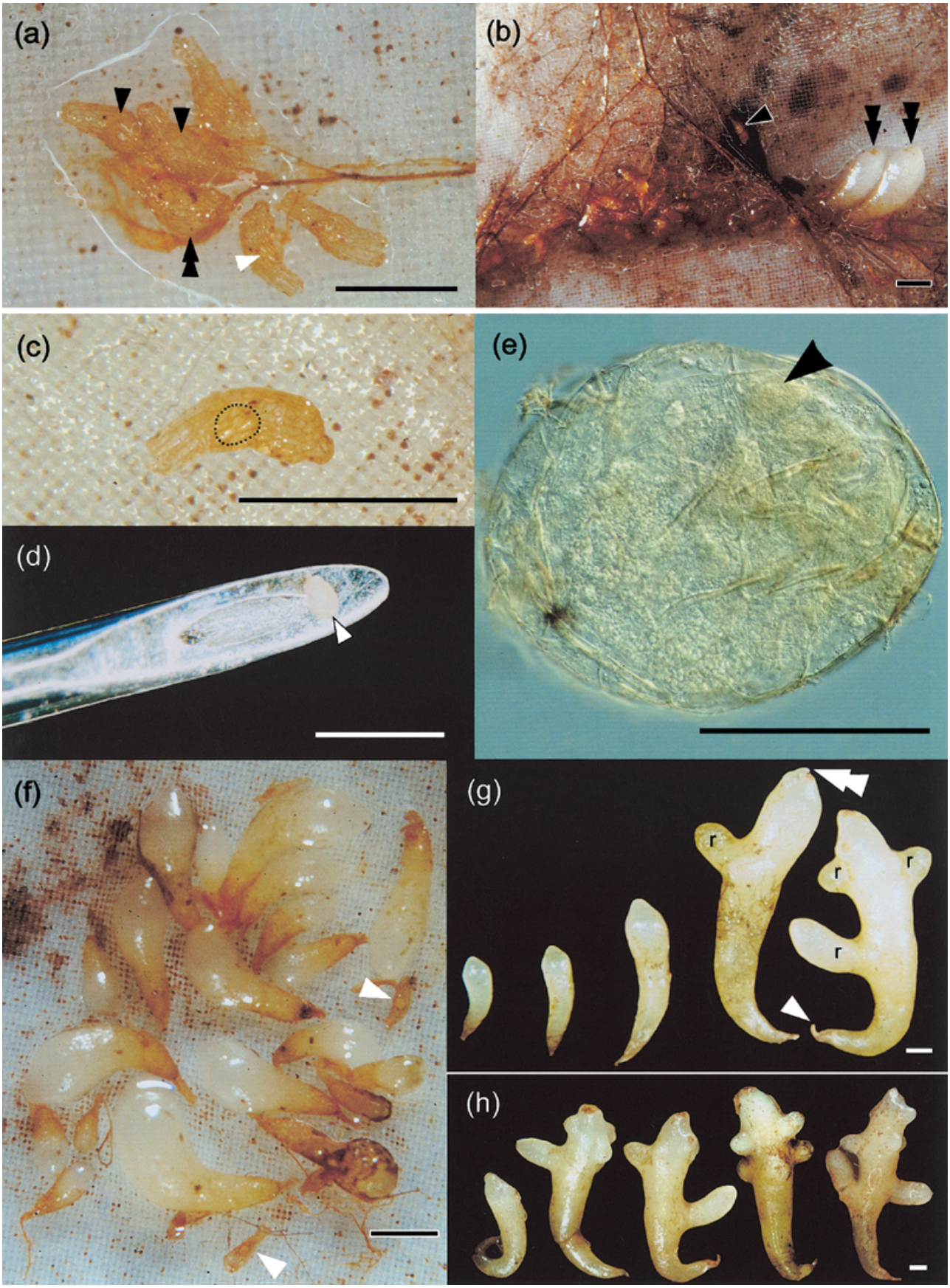
Results

Stages of germination in *Neottia nidus-avis*

The results of the detailed microscopic analysis of seeds and seedlings in packets recovered from Expt 1 enable definition of a series of developmental stages (Table 1 and Fig. 1). Of the 590 seeds that were examined after removal of their testa, 72% were ungerminated (Stage 0) and had a mean volume of 0.0022 mm^3 (Figs 2 and 3). The first visual evidence that germination has been initiated takes the form of a marked increase in translucence of the embryo within the testa. This is designated Stage 1a. Microscopic examination of whole or squashed embryos from which the testa was removed revealed no evidence of fungal penetration in a sample of 65 seedlings that reached this developmental stage. However, it is likely that the proximity of an appropriate fungus is a prerequisite for the onset of germination (see Identification of fungal symbionts). Stage 1a appears to be equivalent to that designated 'the imbibition stage' by some workers (Rasmussen, 1995). The mean size of seeds increased slightly to 0.0031 mm^3 at this stage. Although this was significantly larger than the mean size of ungerminated seeds (Tukey test, $P < 0.05$), individual seeds could not be assigned to this developmental stage simply on the basis of their length and breadth measurements since their volume distributions overlapped with those of ungerminated seeds (data not shown).

The next level of development (Stage 1b) is characterized by fungal penetration of cells at the base (micropylar end) of the embryo – there is no obvious suspensor in *N. nidus-avis* (Arditti, 1992). This stage was reached without any further increase of seedling size, the mean volume of seedlings ($n = 14$) being 0.0030 mm^3, which was significantly larger than the ungerminated seeds (Tukey test, $P < 0.05$) but not significantly different from the seedlings at stage 1a, in which there was no apparent fungal penetration (Tukey test $P > 0.05$). The small number of seedlings found in this developmental stage is almost certainly indicative of rapid progression to the next stage following infection of the seedlings by their fungal partner(s).

Fig. 1 Stages in development of *Neottia nidus-avis*. (a) Seeds in various stages of germination accompanied by orange-brown rhizomorph-forming fungus: Stage 0, ungerminated seed (white arrow); Stage 1, swollen embryo colonized by fungus (single black arrow); Stage 2, protocorm with ruptured testa (double black arrow). Bar, 1 mm. (b) A mesh packet with some seedlings at a more advanced stage of development and accompanied by a brown rhizomorph-forming fungus: Stage 1, swollen embryo colonized by fungus (single black arrow); Stage 2, protocorms that have fully ruptured their testa and have developed a well-defined apical meristem (double black arrows). Bar, 1 mm. (c) Detail of ungerminated seed showing the unexpanded embryo (circled by dotted black line) fully enclosed in the testa. Bar, 1 mm. (d) Germinated seed (Stage 1a, expanded seedling before fungal infection) on a dissecting needle following removal of the testa. Bar, 1 mm. (e) Detail of a germinated seedling (Stage 1c) with testa removed. Fungal pelotons have formed, one of which is indicated (black arrow). Bar, 0.25 mm. (f) Seedlings in various stages of development in a single packet. Development ranges from ungerminated seed (Stage 0, white arrows) to large Stage 2 seedlings. Note accompanying brown rhizomorph-forming fungus. Bar, 1 mm. (g) Progressive development of seedlings in a single packet ranging from Stage 2 (left side, unbranched seedlings) to Stage 3 seedlings with many rootlets (r) forming. The translucence of the apical meristem of the stage 2 seedlings results from the cells lacking both pelotons and starch accumulation in this region. Note the development of a shoot bud with a rudimentary scale-leaf on the largest seedling (double arrow). The testa of the original seeds are visible (single arrow shows one example). Bar, 1 mm. (h) Representative examples of seedlings at Stage 3, showing early development of the characteristic short rootlets that grow at right angles to the main axis and form the 'bird's nest' appearance from which the plant derives its name. Bar, 1 mm.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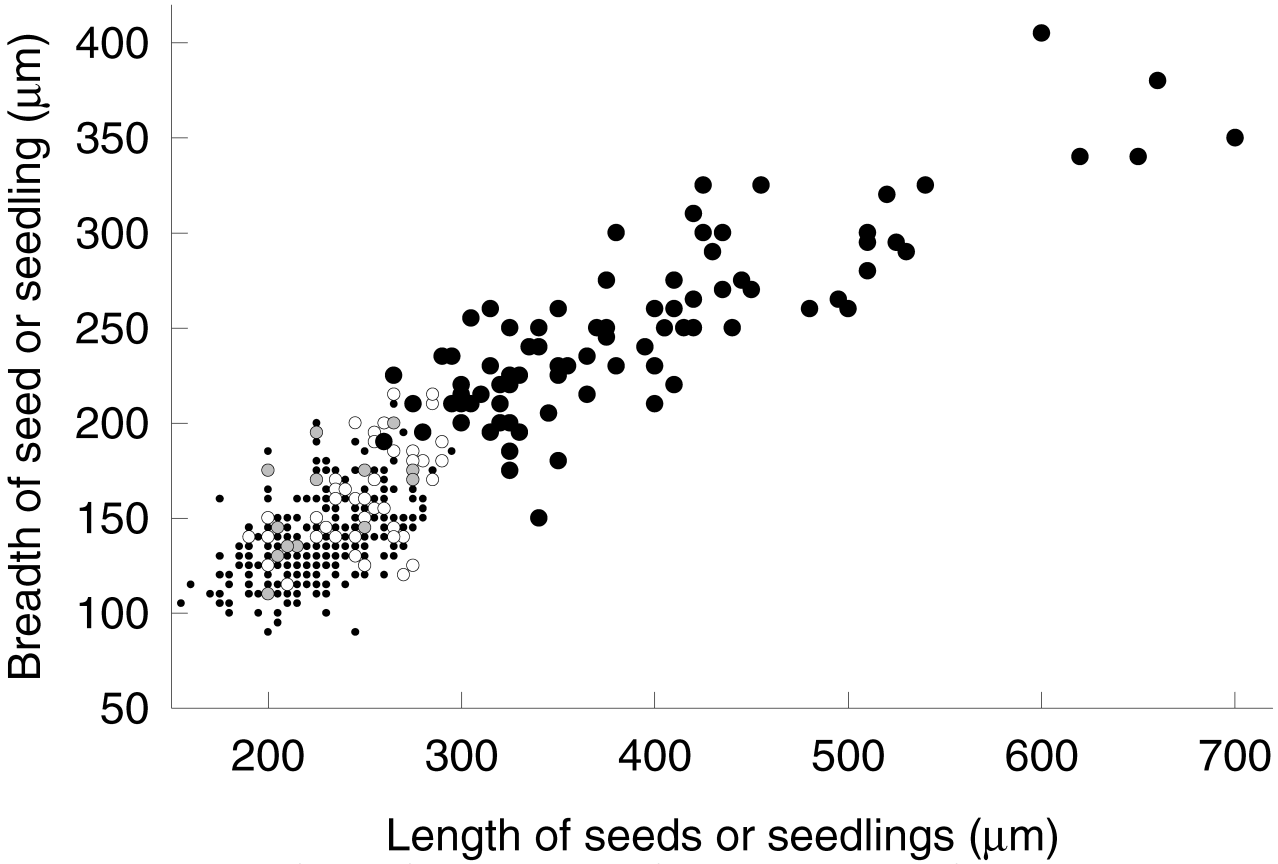

Fig. 2 The length and breadth of a sample of 590 seeds and seedlings of *Neottia nidus-avis* in transition from ungerminated seed (Stage 0, small filled circles) to seedlings containing fungal pelotons. The embryo becomes visibly less dense (Stage 1a, open circles), and is then infected by a fungus through the base of the embryo (Stage 1b, tinted circles). The infection then spreads to adjacent cells and fungal pelotons are formed, accompanied by expansion of the seedling (Stage 1c, large filled circles).

Following mycorrhizal infection, the length and width of the seedlings markedly increased in parallel with the development of fungal pelotons, initially in the cells adjacent to the attachment cells, and then more widely throughout the small protocorm (Stage 1c). It was possible to define this stage on the basis of measurements of length and breadth of the seedlings since, from the sample of 590 seeds/seedlings dissected from their testa, over 95% of the individuals with length greater than 300 µm and a breadth in excess of 200 µm contained pelotons. This definition excluded only three from a total of 88 seedlings that contained pelotons but were smaller than these dimensions (see Figs 2 and 3). The mean volume of the seedlings with pelotons at the first two harvests was 0.0119 mm^3, a value that was significantly larger than those found at any of the previous developmental stages (Tukey test, $P < 0.05$).

Since the numbers of seeds that needed to be checked for germination in the experiments was too large for the removal of the seed testa to be feasible as a routine procedure, and since stages 1a and 1b could only be reliably detected by this procedure, in the remaining studies only seeds that were greater

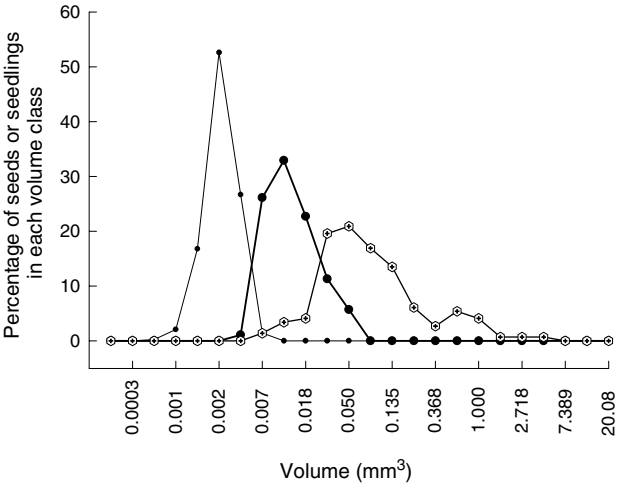

Fig. 3 The percentage of *Neottia nidus-avis* seeds (Stage 0, ungerminated: small filled circles) and seedlings at Stage 1c (infected: large filled circles) and Stages 2–3 (seedling expansion ruptures testa: circles containing crosses) in different volume size classes. Note the volume classes are on the natural log scale. Data for Stages 1a and 1b are not presented because they overlap the distribution of Stage 0. There were insufficient data for Stage 3 plants (branched protocorms) to present these separately but all seedlings with a volume greater than 1.64 mm^3 were branched.

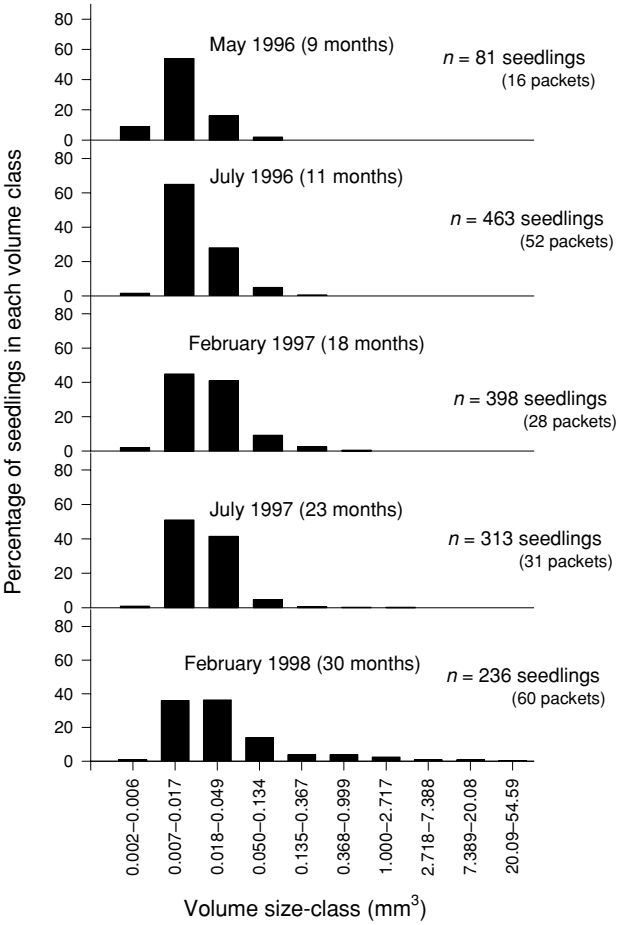

Fig. 4 The percentage of germinated seedlings in each volume size-class at each harvest. Note the volume size classes are on the natural log scale. The number of seed packets sampled at each harvest is indicated in parentheses.

than 300 µm long and 200 µm wide (i.e. Stage 1c and later developmental stages) were recorded as having germinated.

Embryos which have reached Stage 1c normally continue to expand until they burst through the testa as small, rhizoid free, unbranched protocorms which eventually become somewhat torpedo shaped (Fig. 1b,f,g). This is designated Stage 2. This stage is developmentally a continuation of Stage 1c and there is considerable overlap in the size-distribution of seedlings in these two stages (Fig. 3). Nonetheless, the definition of Stage 2 is a major convenience for the recording of germination since seedlings at this stage are easily recognizable under a binocular microscope and do not have to be measured to determine whether they are to be classed as germinated or not. Subsequent developments involve the production of side branches at right angles to the main axis of the protocorm and the formation of a cone-shaped apical bud, sometimes with leaf primordia (Stage 3; see Fig. 1g,h). Growth beyond this stage was not observed probably either because further development was constrained by the walls of the packet or because a longer period for growth was required.

Chronology of development

Experiment 1a The first set of 16 packets taken in May, 9 months after sowing, had between 7.9% and 24.4% of seeds germinating, the largest of which was more than 0.05 mm^3 (Fig. 4) and had reached Stage 2. The decrease in proportion of seedlings in the smallest volume size-class at the July harvest, and the increase in proportion in the 0.007–0.017, 0.018–0.049 and 0.05–0.134 mm^3 volume classes (Fig. 4) is evidence for progressive development of seedlings through the early summer months. By the following February, 18 months after sowing, the number of seedlings in the larger volume classes had increased further (Fig. 4), probably as a result of ongoing development of seedlings germinated in the previous year. The continued presence in the following July (23 months after packet insertion) of a large number of seedlings in the small volume (0.007–0.017 mm^3) cohort may be a result either of arrest in the development of embryos produced the previous year or of a second flush of germination in spring of 1997.

Throughout the period of sampling there is an increase in representation of seedlings in progressively larger volume classes (Fig. 4). The seedlings in volume class 0.05–0.134 mm^3 first appeared after 9 months, in the 0.135–0.367 mm^3 class after 11 months, in the 0.368–0.999 mm^3 class after 18 months, in the 1.000–2.717 mm^3 class after 23 months, and in the three classes from 2.718 to 54.59 mm^3, 30 months after sowing.

Experiment 1b No germination was observed in 30 packets harvested in February, 4 months after sowing, whereas in the 75 packets harvested in July, 12 months after sowing, 69% of the packets contained germinating seeds.

Germination, growth and development of *Neottia nidus-avis* at distances of less than 1 m and greater than 5 m from previously occurring flowering spikes (Expt 1a)

There were striking differences in seedling germination and development between the plots containing adult *N. nidus-avis* plants and those situated at least 5 m from the nearest adult (Table 2, Fig. 5). Whereas a high percentage of packets in all three plots situated in the vicinity of adults yielded seedlings, germination was seen in only one packet in a single plot (Plot 5) in the absence of adults and in that packet only 5% of seeds showed evidence of germination (Table 2). It should be stressed that germination was only detected in this case because the packet was included in the samples from which the testa was dissected from the seeds. Since the total volume of the seedlings in this packet was less than 0.007 mm^3 (Fig. 5) their development represents only Stage 1a in which there is no evidence of fungal penetration of the seed.

Table 2 Experiment 1a. Effect of the presence of adult plants on the germination of *Neottia nidus-avis* sown in *Fagus sylvatica* woodland on 8–10 August 1995

	With adults			Without adults		
	Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6
Percentage of packets in which germination occurred	96 (n = 54)	61 (n = 62)	55 (n = 58)	0 (n = 60)	2 (n = 60)	0 (n = 60)
Percentage germination per packet (excluding seed packets in which no germination occurred)	29 ± 2 (n = 51)	17 ± 4 (n = 37)	17 ± 3 (n = 36)	0 (n = 0)	5 (n = 1)	0 (n = 0)
Percentage germination of seeds in all sampled packets	28 ± 2	11 ± 2	11 ± 2	0 ± 0	0 ± 0	0 ± 0

Seed packets were buried in 1 m^2 plots, three of which contained adult plants of *Neottia* and three of which were greater than 5 m from the nearest adult *Neottia*. Samples were harvested 11, 18, 23 and 30 months after sowing. Germination is expressed as the mean percentage of packets in which germination occurred, the percentage germination in packets in which germination had occurred and the total percentage germination over all packets (± SE); n = number of packets.

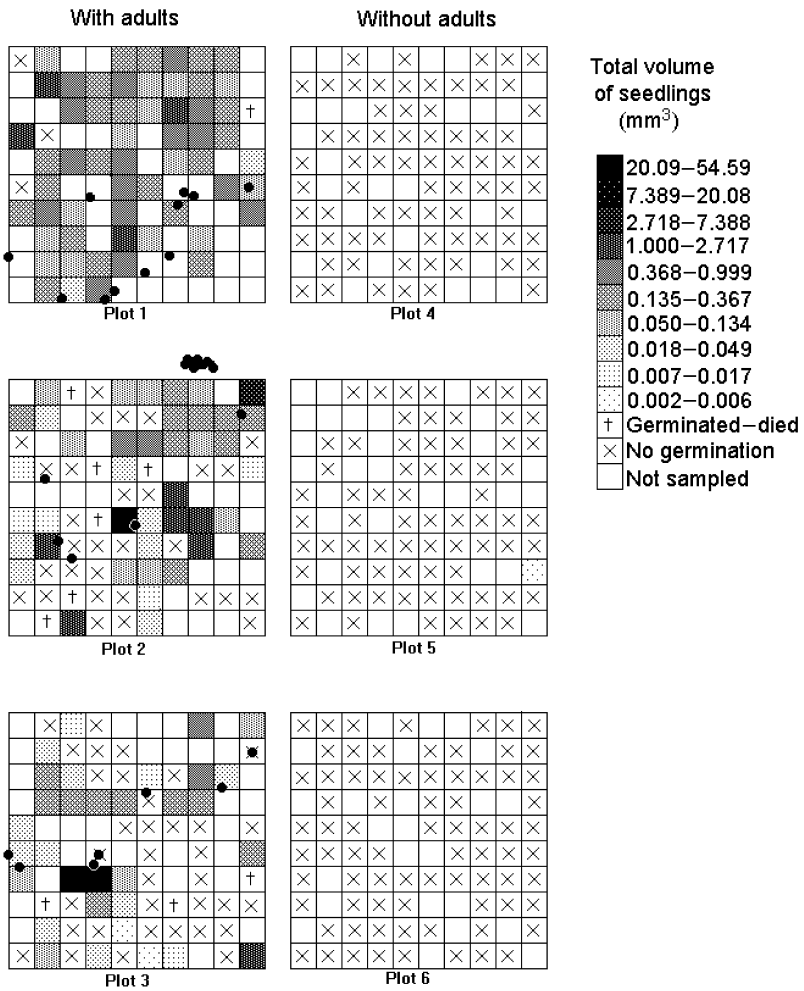

Fig. 5 The spatial distribution of sampled seed packets and the total volume of living seedlings in each packet planted within the 1 m^2 plots supporting *Neottia nidus-avis* (Plots 1, 2, 3) and in equivalent plots lacking adults (Plots 4, 5, 6) in Experiment 1a. The seedling volume increments are on the natural log scale. The data are from all harvests, and include samples taken in May 1998 (33 months). Filled circles indicate flowering spike of *N. nidus-avis* in July 1995.

In Plots 1, 2 and 3 between 11% and 28% of the seeds germinated, and these values rose to 17–29% if the seed packets in which there was no germination were excluded (Table 2). Plots 2 and 3 both yielded some packets with total seedling volumes in the highest category (20.1–55.0 mm^3) (Fig. 5). These plots were also characterized by high spatial variability of germination success, between 39% and 45% of packets contained no germinating plants, and the packets containing the largest plants were found alongside packets in which little or no germination occurred (Fig. 5). In Plot 1 there was a

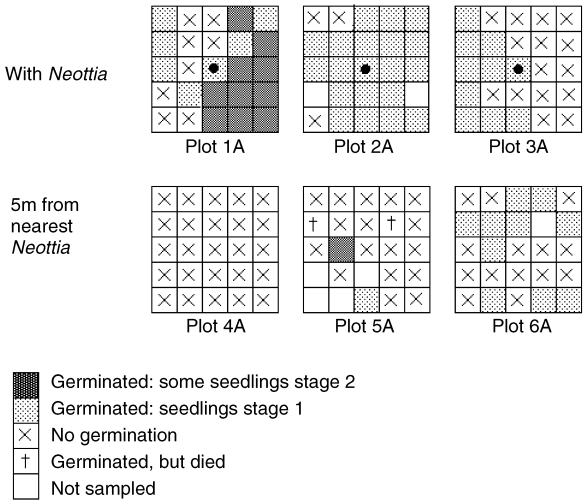

Fig. 6 Experiment 2. Position of packets planted within six 50 × 50 cm plots, three of which are positioned around a single flowering spike of *Neottia* (1A–3A), and three of which are 5 m from the nearest *Neottia* plant (4A–6A). The presence or absence of germination and the distribution of the most advanced stage in development are mapped. Packets were harvested in July 1997, 10 months after sowing in September 1996. Filled circles indicate the position of the flowering spike of *N. nidus-avis* in July 1996.

larger number, and more even distribution of packets containing cumulative volumes in the intermediate categories (0.38–1.00, 1.01–2.71 and 2.72–7.39 mm^3) and in this case only 4% of packets did not contain germinating seeds, these being located at the edge of the plot (Fig. 5).

While the results of Expt 1a. clearly indicated the importance of the proximity of adult plants for germination and development of *N. nidus-avis* at Galley Down, a more detailed analysis of the spatial patterns of germination in relation to distance from adults was not possible from this experiment. This was because the seed packets were not all harvested at the same time. In addition, these plots contained many adults so that it was difficult to relate germination to the effect of any particular adult. The second experiment was designed specifically to avoid these complications.

Germination and development of *Neottia nidus-avis* seed around and at distance from single adult flowering spikes (Expt 2)

Germination was observed in all plots established around single adult spikes (Fig. 6) and ranged from 40% of packets in plot 3A to 80% of packets in plot 2A. In plot 1A, development had proceeded to Stage 2 in 40% of the packets whereas in 2A and 3A no growth beyond Stage 1c was seen. Both germination and subsequent development were lower in plots more than 5 m from adult spikes (Fig. 6). There was no germination in Plot 4A. In Plot 5A, 4% of packets contained Stage 1 and Stage 2 seedlings and although in Plot 6A 40% of packets contained germinated seedlings these were all restricted to Stage 1. Over the areas of entire quadrats the frequency of packets containing germinating seeds was significantly higher in the plots containing adult plants (Fig. 6) than in those located more than 5 m from a visible adult (χ^2 = 34.2, df = 1, $P < 0.001$). In the plots containing adults, there was no significant difference between the frequency of packets with germinating seeds within 15 cm of the adults and those located 15–25 cm from the adult.

Growth of seedlings in relation to the numbers of seeds germinated in each packet

The volume of the largest seedling in each seed packet from Expt 1a. was plotted against the numbers of seeds germinated in each packet at the first and second harvests (Fig. 7a), the third harvest (Fig. 7b) and the fourth and fifth harvest (Fig. 7c). The data for the first two harvests showed a strong, positive, linear relationship (R^2 = 78.7% $P < 0.001$), between the growth of the largest seedlings and the numbers of seeds that germinated in the same packet. By the third harvest, the strength of the linear relationship had decreased (R^2 = 27.0%, $P < 0.01$) because of a wider scatter of the data, but the positive relationship was maintained (Fig. 7b). By the fourth and fifth harvests the largest plants had increased their volume by more than an order of magnitude and because of the wide scatter of the results there was no significant relationship (R^2 = 1.6% $P > 0.05$) between growth and seed number. It was notable, however, that at the first three harvests the largest seedlings were found in packets that contained more than 40 germinating seeds, yet by the final harvest the largest seedlings were found in packets that contained less than 20 living germinants.

Identification of fungal symbionts

In seed packets in which germination took place there was frequently observed a rhizomorph-forming fungus that varied from black-brown to bright orange-brown and was closely associated with the germinating seeds (Fig. 1a,b,f). The involvement of this fungus in seed germination was unproven. Microscopic examination of seedlings showed that the fungus routinely infecting seeds and often found on the testa of uninfected seeds in which germination had reached the imbibition stage (1a) was fine (typically 1.25 μm wide), hyaline, with infrequent septa and no clamp connections. The hyphal walls of the fine fungus varied from being hyaline to having a brick-red pigmentation. The fungus formed simple multistranded rhizomorphs in which the hyphae occasionally carried clear stellate crystals (possibly calcium oxalate) on their surfaces. A fungus with somewhat coarser hyaline hyphae (typically 2.5 μm wide) was also occasionally observed infecting the plants and in close association with the seed and seed coats.

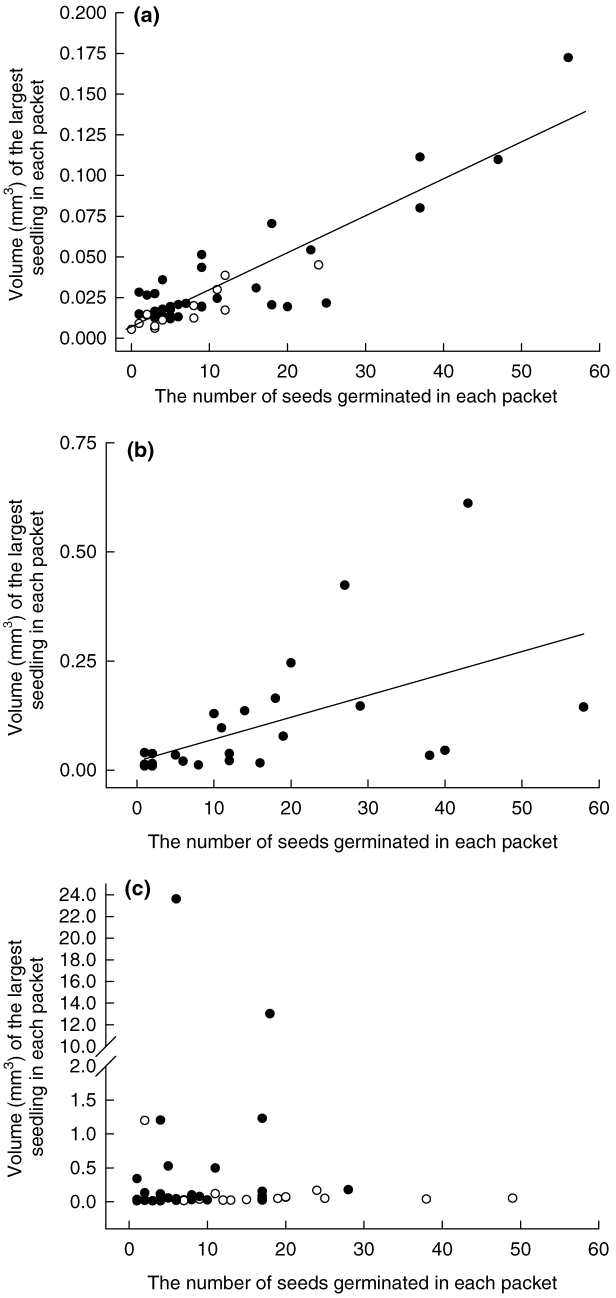

Fig. 7 Relationships between the numbers of seeds germinated in a seed packet and the volume (mm^3) of the largest seedling in the packet. (a) At the first harvest (9 months, open symbols) and second harvest (11 months, filled symbols). (b) At the third harvest (18 months). (c) At the fourth harvest (23 months, open symbols) and fifth harvest (30 months, filled symbols).

A single ITS-RFLP pattern, designated *N. nidus-avis* 1, was found in the majority of *Neottia* seedlings and adults from Galley Down. A second pattern, *N. nidus-avis* 2, was found in the two adults from Germany (Table 3). Based upon extrapolations from ITS-RFLP studies of basidiomycete fungi in the Agaricales (Gardes & Bruns, 1996b; Karen *et al.*, 1997), it is likely that *N. nidus-avis* 1 and 2 are two different fungal

Table 3 Molecular identification of fungal associates of seedlings and adult plants of *Neottia nidus-avis*

Location	Fungus/ GenBank numbers	Frequency in seedlings	Frequency in adults	Closest two GenBank 28S sequences	Match length/ per cent identity[1]	Closest two GenBank ITS + 5.8S sequences	Match length, per cent identity[2]
Galley Down, UK	*N. nidus-avis 1* AY052373 AY052375	7/8	6/11	*Sebacina dimitica* AF291364 *Sebacina aff. epigaea* AF291363	317, 95% 317, 94%	*Piriformospora indica* AF019636 *Sebacina vermifera* AF202728	170, 97% 168, 97%
Tübingen, Germany	*N. nidus-avis 2* AY052372 AY052374	–	2/2	*Sebacina dimitica* AF291364 *Sebacina aff. epigaea* AF291363	378, 98% 371, 97%	*Piriformospora indica* AF019636 *Sebacina vermifera* AF202728	168, 98% +25, 92% 172, 98%

Neottia nidus-avis 1 and 2 are the two fungal internal transcribed spacer (ITS) restriction fragment length polymorphism (RFLP) patterns obtained from *Neottia* seedlings and adults. One seedling and five adults had RFLP patterns indicative of multiple fungi (fragment sizes summed to a greater value than the complete ITS length) and there was evidence that these were contaminated by *Trichoderma* sp. which may have proliferated during the 2–4 wks between harvest and DNA extraction, and are not considered further. The most similar 28S and ITS sequences in GenBank according to the default BLAST search algorithm are provided.

[1]The number of consecutive bases providing a significant match and the percentage of those bases that were identical between the query fungus and the GenBank entry are given.

[2]The conserved 5.8S gene was the only portion of the ITS sequences that significantly matched *Piriformospora*, while a small portion of the variable ITS2 spacer region of *N. nidus-avis* 2 provided a statistically significant match to *Sebacina vermifera*.

species. Five adults and one seedling had RFLP patterns in which the fragment sizes summed to a greater value than the complete ITS length. Because these compound patterns were seen with each restriction enzyme, they suggest the presence of multiple distantly related ITS sequences in the same sample. Our favoured explanation for this is the presence of multiple fungal species. Among these, *Trichoderma* sp. appeared to be present. These fungi were considered most probably to be present as casual associates or contaminants of the seedlings and were not considered further. A less likely explanation for the compound RFLP patterns is that divergent ITS sequences occurred within a single fungus because of heterozygosity or variation among units of the ribosomal repeat. In the relatively short sequences examined here, the probability of heterozygosity differences occurring in the sequences are likely to be very low.

The 28S sequences from *N. nidus-avis* 1 and 2 were most similar to the GenBank entry for *Sebacina dimitica* from the study of Weiss & Oberwinkler (2001), there being high levels of sequence identity (95–98%) over the entire gene fragment. By contrast, BLAST searches using the ITS1 and ITS2 spacer regions of the two *Neottia* fungi did not result in statistically significant matches among GenBank entries, with the exception of a short region of 25 bases that matched *Sebacina vermifera*. The highly conserved 5.8S gene regions of the *Neottia* fungi were statistically similar to GenBank entries for many fungi. *Sebacina vermifera* and *Piriformospora indica* were most similar to the two *Neottia* fungi in the 5.8S gene region (Table 3). No ITS sequences were available for *Sebacina* species other than *S. vermifera*. We aligned the complete ITS1, 5.8S, and ITS2 sequences of *S. vermifera, P. indica* and the two *Neottia* fungi using CLUSTALW 1.7 for Macintosh (EMBL, Heidelberg, Germany) and found that the two *Neottia* fungi are quite similar to each other (9.2% sequence divergence), are moderately similar to *S. vermifera* (23% sequence divergence), and are less similar to *P. indica* (33% sequence divergence). Thus, based on both the 28S and ITS sequence data, it was concluded that the two *Neottia* fungi belong to the Sebacinaceae, and that they are more closely related to one another than to *S. vermifera*. The *Neottia* fungal sequences are available from GenBank under the accession numbers provided in Table 3.

Discussion

The study has provided the first definitive chronology for germination and development of *N. nidus-avis* from seed in the field and establishes the critical importance of a specific *Sebacina*-like fungus in the initiation of germination and subsequent development of the plant. We interpret the very low frequency of germination of *N. nidus-avis* in the plots located away from adult plants as indicating that the fungus required for germination occurs more abundantly in the plots where the adult plants were seen and is very infrequent or absent from much of the surrounding woodland where adults did not occur.

The light microscope observations suggested that germination (Stage 1a, increased translucence of seed, attributed to mobilization of stored carbon compounds) was initiated before fungal penetration. In many of these cases the distinctive fine and hyaline fungus with simple septa was seen on the testa of the seeds. This initial stage of germination occurred only in the plots in which seeds subsequently became infected. Since the plots containing adults appeared otherwise identical to those from which they were absent, it appears that the breaking of seed dormancy requires the fungal partner. The failure of the light microscope studies to detect fungal infection of seeds at this stage leads to the hypothesis that germination may be initiated by a chemical signal from the fungus. The hypothesized mechanism, which requires further investigation, would enable *N. nidus-avis* seeds to remain dormant for long periods of time before germination is initiated by its essential fungal partner(s) growing close to the seed.

In another fully myco-heterotrophic orchid, *C. trifida*, it was found that breaking of seed dormancy, again recognized by increased translucence of seeds, occurred only after infection of the suspensor region by a specific fungal partner (McKendrick *et al.*, 2000a). Growth in length and breadth of seedlings of *C. trifida* then swiftly followed even before fungal pelotons developed in the adjacent cells. In *N. nidus-avis* the breaking of dormancy (Stage 1a) and the initial infection before peloton formation (Stage 1b) led initially to very modest increases in the seed dimensions (Fig. 2). The main growth of *N. nidus-avis* occurred only following peloton formation. Recent studies on another terrestrial orchid by Yoder *et al.* (2000) have shown that infection of seeds and the development of pelotons results in greatly increased water content, and this, together with expansion of cell volumes and vacuolation (see Rasmussen, 1990), may account for much of the rapid increase in seedling volumes once fungal infection is established.

The fungus-dependent initiation of germination seen in *C. trifida* and *N. nidus-avis* contrasts with the situation described in some common initially myco-heterotrophic orchids such as *Dactylorhiza majalis* in which the initial stages of germination can occur in the absence of symbiotic fungi, even to the point of extensive cell division and the formation of rhizoids (see Rasmussen, 1995). These differences may reflect adaptations to the contrasted patterns of specificity and distribution of the fungi colonising these orchids. The two fully myco-heterotrophic species appear to depend upon a very narrow taxonomic range of fungi that have localized and restricted distributions whereas many initially myco-heterotrophic orchids have much lower fungal specificity, being normally associated with soil fungi such as species of *Rhizoctonia* which are likely to be ubiquitous in the habitats of the plants.

Neottia nidus-avis shows a remarkable ability to control the growth of its fungal symbiont. Infection commences with

penetration of the cells at the base (micropylar end) of the embryo by which it was attached to the parent plant. From here it spreads into adjacent cells in which the first pelotons are formed. It is not known whether the attachment cells provide a chemical attractant to the fungus or if the walls of these cells are the most easily penetrated by fungi. Infection later spreads towards the chalazal end of the embryo but does not enter the meristem, which continues to develop, eventually forming a shoot primordium (Fig. 1g,h). In the larger seedlings there are distinct regions of peloton-containing cells extending from the base of the seedling to a region below the apical meristem.

Germination was very patchy at both the packet and plot scales. Within seed packets in which germination occurred less than 30% of seeds typically germinated. This situation is similar to that observed in seeds of *C. trifida*, where germination ranged from 5 to 63% in different habitats (McKendrick *et al.*, 2000a). In the case of *C. trifida*, habitats that supported the highest rates of germination also provided the greatest amount of seedling development, suggesting that the abundance and activity of the fungal partner may exert strong influences upon both the extent of germination and growth of the plants. In the case of *N. nidus-avis*, at the first two harvests in Expt 1a, the rates of growth of the largest seedlings were correlated with the numbers of seeds that germinated in each seed packet, there being no evidence that large numbers of germinating seeds in a packet resulted in competition for carbon or retarded rates of plant growth (Fig. 7a,b). At the later harvests there was some evidence of 'thinning' as the largest plants were then found in packets containing less than 20 live germinated seedlings. However, since the numbers of live seedlings generally declined by the later harvests irrespective of their size a density-dependent effect on growth cannot be concluded with certainty (Fig. 7c).

The spatial variability in rates of germination and growth of seedlings in the 1 m^2 plots was very high (Fig. 5) but part of this apparent variation was due to samples being harvested at intervals over 30 months. In Expt 2, where the packets were all incubated for the same length of time, there was clearer evidence of discrete patches, often larger than 900 cm^2, apparently containing relatively high activity of the mycorrhizal fungus.

The clear evidence that germination of *N. nidus-avis* occurs at a much higher frequency in the vicinity of adult plants than in sites where they are absent is thought to reflect the patchy distribution of its fungal partner. This is likely to be the main factor causing the clumped distribution of adults often observed in the field (Fig. 5) and the strong positive relationship between rate of germination and growth of the largest seedlings (Fig. 6a).

Noel Bernard (1902) provided the first description of symbiotic germination in a small number of *N. nidus-avis* seedlings found in the field. He observed, as in the present study, that infection occurred initially through cells at the base (micropylar) end of the embryo and that the developing protocorms and adult plants lacked rhizoids. Since the age of the seeds was not known the exact chronology of development was uncertain, but his assumption that germination occurs in the spring is confirmed by the present study.

On the basis of their examination of seedlings sieved from soil in the vicinity of adult plants Fuchs & Ziegenspeck (1924) attempted to reconstruct a chronology of developmental events in *N. nidus-avis*. While their suggestion that germination took place in the year following seed dispersal is in accordance with the results presented here, the subsequent series of events were thought to take place over a far longer period of time than was observed in the seed packets. Fuchs & Ziegenspeck (1924) described the production in year 1, at the apex of the seedling axis a rosette (kreisel) of roots. However, they believed that over at least the first 5 yrs of development, this rosette died back every winter, it being replaced during each subsequent growing season by clusters of larger roots that formed progressively lower down the axis. We have found no evidence to support any such sequence of death and regeneration of seedling structures. Conversely, our results suggest that the life cycle from germination to production of the flowering spike could take place within a period of 3–5 yrs and that there is accretion of rootlets but little or no die-back.

The molecular data concerning the identities of the two *N. nidus-avis* fungi suggest that this orchid, similar to other MHP orchids including *Cephalanthera austinae* (Taylor & Bruns, 1997), *Rhizanthella gardneri* (Warcup, 1985) and several *Corallorhiza* species (Taylor & Bruns, 1999a; McKendrick *et al.*, 2000a), associates with a narrow taxonomic group of fungi. The finding that *N. nidus-avis* 1 was the predominant fungal associate of both seedlings and adults in the UK study sites agrees with a number of previous studies showing that adults and seedlings of some orchids target the same fungi (Masuhara & Katsuya, 1994; McKendrick *et al.*, 2000a).

Previous attempts to isolate and/or identify the fungal partner of *N. nidus-avis* have yielded conflicting results. The early reports by Bernard (1899) implicated a brown septate fungus with clamp connections but other reports of fungi isolated from *N. nidus-avis* suggest *Rhizoctonia*-like fungi (Wolff, 1927; Burgeff, 1936). However, the mycorrhizal status of these fungi was not proven. More recent ultrastructural studies on pelotons in *N. nidus-avis* by Barmicheva (1989) indicated that the fungus has dolipores and imperforate parenthesomes, suggesting that it was either a *Tulasnella* or *Sebacina* (Rasmussen, 1995). These observations accord with the molecular identifications reported here which suggest that the fungus is a *Sebacina*, which has affinities with *S. dimitica*.

The closely related *Sebacina vermifera* has been found as a mycorrhizal associate in a number of green Australian terrestrial orchids (Warcup, 1971, 1981; Ramsay *et al.*, 1986; Ramsay *et al.*, 1987). *Sebacina* anamorphs have been assigned to the genus *Epulorhiza* (Moore, 1987) along with anamorphs

of *Tulasnella*. *Epulorhiza* is a member of the diverse-form genus *Rhizoctonia*, which includes most fungi found as mycorrhizal associates of orchids (Smith & Read, 1997). *Epulorhiza* species have been recovered from many European and North American orchids, but most of these appear to be related to *Tulasnella* rather than *Sebacina* (Anderson, 1996), and *Sebacina* is thought to be only distantly related to *Tulasnella* (Wells, 1994). The North American MHP orchid *Hexalectris spicata* associates specifically with fungi that have quite similar ITS sequences to the two *Neottia* fungi (Taylor, 1997; D. L. Taylor, T. D. Bruns and S. A. Hodges, unpublished).

Unfortunately, the close phylogenetic affinity of the *Neottia* fungi to *Sebacina* species does not provide clear evidence of the likely sources of carbon used by the fungi to sustain the plants, as little is known about the biology and ecology of this genus. *Sebacina* has been considered to be closely related to wood-decaying *Exidia* species (Wells, 1994). However, the recent molecular systematic study of Weiss & Oberwinkler (2001) suggests that the Sebacinaceae is phylogenetically distinct from *Exidia* and from the rest of the Auriculariales. Nonetheless, the wood-decayer *Craterocolla cerasi* does appear to belong to the Sebacinaceae. A mycoparasitic lifestyle has been suggested for some *Sebacina* species (Jülich, 1983), whereas Warcup (1988) concluded that certain *Sebacina* strains formed ectomycorrhiza-like associations with tree and shrub roots. Of particular interest in the present context was his observation (Warcup, 1988) that a strain of *S. vermifera* simultaneously formed ectomycorrhiza-like infections on the autotrophic herb *Podotheca angustifolia* and shrub *Melaleuca uncinata* while initiating germination and supporting growth of achlorophyllous protocorms of an orchid in the genus *Microtis.*

There is increasing evidence that such tripartite relationships, in which fungi form mycorrhizal associations with autotrophic plants and transfer carbon from them into achlorophyllous plants that they simultaneously infect, are typical of many fully myco-heterotrophic plants, both in the Orchidaceae (Taylor *et al.*, 2001) and Monotropaceae (Cullings *et al.*, 1996). Among fully myco-heterotrophic orchids tripartite associations involving ectomycorrhiza-forming fungi have been confirmed for *Rhizanthella gardneri* (Warcup, 1985), *C. austinae* (Taylor & Bruns, 1997), *Corallorhiza maculata* and *Corallorhiza mertensiana* (Taylor & Bruns, 1999a), *Corallorhiza striata* (Taylor, 1997) and *C. trifida* (Zelmer & Currah, 1995; McKendrick *et al.*, 2000b). This type of relationship is also suspected to occur in the woodland orchids *Limodorum abortivum*, *Limodorum trabutiasnum* and *Epipogium aphyllum*, which have been reported to be infected by fungi that form clamp connections and/or dolipore septa (De Santis & Riess, 1995; Scrugli *et al.*, 1995).

Since the fine hyaline fungus was observed infecting the majority of seedlings in the present study it is reasonable to assume that this is the *Sebacina*-like fungus. The identity of the more rarely seen and coarser fungus remains uncertain. The presence of fine (2.5 μm diameter) and coarser (5.2 μm diameter) hyphae, which were believed to represent two different fungal species was reported in adult *N. nidus-avis* roots collected in Italy by De Santis & Riess (1995). Further work is required to establish whether the coarse and fine hyphae are produced by different fungal species.

Previous attempts have repeatedly failed to obtain asymbiotic germination of *N. nidus-avis* seed in the laboratory, while, with one exception (Smreciu & Currah, 1989) attempts to germinate the seeds by inoculation with fungi isolated from other orchids have also been unsuccessful. Smreciu & Currah (1989) achieved some expansion and growth of seedlings in association with *Ceratobasidium cereale* and *Rhizoctonia anaticula*. However, the limited development of the plants under these laboratory conditions, and the fact that the fungi were not isolated from the orchid or its habitat, suggests that they are unlikely to be effective symbionts of the orchid in the field. Since *in vivo* symbiotic germination can occur with lower fungal specificity than in the field (Masuhara & Katsuya, 1994; Perkins *et al.*, 1995), the previous studies are consistent with evidence presented here that *N. nidus-avis* has a high fungal specificity and is normally not associated with fungi of the *Rhizoctonia* type.

We hypothesize that the *Sebacina*-like fungus infecting *N. nidus-avis* at Galley Down may obtain its carbon primarily through mycorrhizal associations with *F. sylvatica* (the only autotrophic plant consistently present in the vicinity of *N. nidus-avis* at the site) and through similar associations with other woody hosts at other sites. This would most simply explain the rather specific association of the orchid with particular trees (Summerhayes, 1968). It is interesting to note that *Hexalectris spicata*, like *N. nidus avis*, occurs most often in ectomycorrhizal forests (Luer, 1975). The functional roles of *Sebacina*-like fungi in these forests deserve further study.

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