RESEARCH *New Phytol.* (2000), **145**, 523–537

Symbiotic germination and development of myco-heterotrophic plants in nature: ontogeny of *Corallorhiza trifida* and characterization of its mycorrhizal fungi

S. L. McKENDRICK[1], J. R. LEAKE[1], D. L. TAYLOR[2] AND D. J. READ[1]*

[1] *Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK*

[2] *Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA 93106, USA*

Received 20 May 1999; accepted 12 November 1999

SUMMARY

The processes of symbiotic germination and seedling development were analysed in the myco-heterotrophic orchid *Corallorhiza trifida*, seeds of which were buried in 'packets' either adjacent to or at varying distances from adult plants in defined communities of ectomycorrhizal tree species. Germination occurred within eight months of burial under *Betula–Alnus* and within seven months under *Salix repens*. It was always associated with penetration of the suspensor by a clamp-forming mycorrhizal fungus. Four distinct developmental stages were defined and the rates of transition through these stages were plotted. There was no evidence of a relationship between extent of germination or rate of development and the presence of naturally distributed plants of *C. trifida* at the spatial scale of 1 m. The best germination and the most rapid rate of development of *C. trifida* seedlings occurred in a *Salix repens* community located at a considerable distance from any extant *C. trifida* population. Determination of internal transcribed spacer (ITS) RFLPs and of gene sequences of the fungi involved in symbiotic germination and growth of *C. trifida*, revealed them to belong exclusively to the *Thelephora–Tomentella* complex of the Thelephoraceae. These fungi are known also to be ectomycorrhizal associates of trees. It is hypothesized that the rate of growth of the *C. trifida* seedlings is determined by the ability of the fungal symbionts to transfer carbon from their ectomycorrhizal co-associates.

Key words: symbiotic germination, myco-heterotroph, orchid, *Corallorhiza*, seedling development, mycorrhiza, Thelephoraceae.

INTRODUCTION

It is a characteristic feature of most members of the Orchidales, of some of the Ericales, and of a few of the Gentianales, that their seeds are reduced in size to such an extent that they contain virtually no storage material and an undifferentiated embryo consisting of only 4–200 cells. The form of these minute diaspores, sometimes referred to as 'dust-seeds', is remarkably uniform across these taxonomically unrelated Monocotyledonous and Dicotyledonous orders (Leake, 1994). These features, apparently selected to maximize fecundity and the effectiveness of wind dispersal, whilst minimizing the requirement for maternal investment, have resulted in a dependence, at least in the early stages of development, upon exogenous sources of C from mycorrhizal fungi. Colonization of the seed by an appropriate fungal partner is therefore a prerequisite for successful establishment in nature. The so-called 'symbiotic germination' (Burgeff, 1936) is followed by an achlorophyllous stage of development, which occupies a greater or lesser, but in any case critical, part of the life cycle of all plants of this type. Achlorophyllous plants, because of their heterotrophic dependence upon exogenously supplied C, have been referred to as 'saprophytes' but since a living fungus, rather than dead organic matter, is their source of C, these organisms are more accurately described as myco-heterotrophic plants (MHP) following Leake (1994).

All members of the Orchidales, Ericales and Gentianales which have dust seeds are, as far as is

*Author for correspondence (tel +44 114 222 4318; fax +44 114 276 0159; e-mail d.j.read@sheffield.ac.uk).

known, dependent upon symbiotic association for establishment in nature, and hence have a myco-heterotrophic phase in their lives. Some, such as members of the genera *Corallorhiza* in the Orchidales, *Monotropa* in the Ericales, and *Voyria* in the Gentianales retain their achlorophyllous condition and are hence true MHPs throughout their lives. The majority, for example members of the genera *Orchis*, *Ophrys* and *Dactylorhiza* in Orchidales, and *Pyrola* in Ericales, have a myco-heterotrophic juvenile phase followed by an autotrophic adult life in which associations with fungal symbionts are retained. They are referred to as being initially myco-heterotrophic plants (IMHP) and are thus distinguished from true autotrophs (Leake, 1994). In nature many MHPs and IMHPs grow exclusively with autotrophs of a particular mycorrhizal category but neither the physiological nor the ecological bases of these apparently obligate plant partnerships are known.

The minute size of these seeds has made it difficult both to locate and to study them in their natural environment. Consequently, our understanding of the biology of that major part of their lives which occurs before emergence above ground is limited. Thus, despite valuable demographic studies based upon emergence of a few selected species (e.g. Wells, 1981; Hutchings, 1987a,b) the nature of the processes involved in colonization by fungal symbionts in most plants with this seed habit is unexamined, the duration of the period from germination to flowering is unknown and the identity of the fungi involved in these key processes *in situ* is uncertain.

However, in recent times an increase in understanding of germination processes has been facilitated by methods enabling sequential analysis of development of seedlings following burial of seeds in natural habitats. An *in situ* method involving burial and sequential harvesting of seeds sealed in mesh 'packets' has been employed successfully by Rasmussen & Whigham (1993) who obtained up to 48% germination in the MHP *Corallorhiza odontorhiza* depending on site, and 31% in the IMHP *Goodyera*. Using a similar method Masuhara & Katsuya (1994) obtained *c*. 40% germination in the IMHP *Spiranthes sinensis*. At the same time our ability to characterize the fungi involved in the germination process has been improved by the application of molecular methods to fungal identification (White *et al*., 1990; Gardes & Bruns, 1996a,b; Taylor & Bruns, 1999a).

By combining *in situ* analysis of germination with molecular examination of the fungi sustaining the process it is possible for the first time to examine key questions of the relationships between distribution of species with 'dust seeds' and the availability of their fungal symbionts. We hypothesize that as a result of their dependence upon fungal colonization for completion of the juvenile stages of development, the pattern of distribution of adult orchids is determined by the fungi which initiate symbiotic germination.

To test this hypothesis we have employed the seed-packet method to determine the nature and chronology of the early events in the germination and growth of selected MHPs and IMHPs in the field, paying particular attention to the impact of community type upon germination and plant development. In this paper we describe some relationships between habitat and plant development in the orchidaceous MHP *Corallorhiza trifida* and provide the results of a molecular analysis of the fungi involved in its symbiotic germination.

MATERIALS AND METHODS

The work was carried out at Tentsmuir Point, Fife, Scotland (National Grid reference NO 503 263). This is an area of coastal dunes, most of which has been afforested with *Pinus* spp. but there are some areas, mostly within Tentsmuir Point National Nature Reserve (NNR), which support *Salix repens* scrub and a small area of semi-natural woodland. A population of *Corallorhiza trifida* Châtel has been known to occur here for a number of years, Smith reporting the occurrence of the orchid 'in one place near the centre' of Tentsmuir in 1905 (Smith, 1905), and is now almost exclusively confined to an area of *c*. 120 × 30 m in *Betula–Alnus* and *Pinus–Betula–Alnus* woodland at the southern boundary of the reserve. Measurements of soil characteristics in the various woodland and scrub communities at the beginning of the study showed that there were no major differences in soil pH, which was 5.1–5.9, or in organic content, loss on ignition ranging from 31–41%. A small subsidiary study was carried out at a coastal-dune site in North Wales (Newborough Warren NNR: National Grid reference SH 413 632) which, as far as is known, has never supported a population of *C. trifida* but which is floristically similar to Tentsmuir, consisting of extensive plantations of *Pinus* and of *Salix repens* scrub.

The overall aim of the experiments was to determine the chronology of symbiotic germination and seedling development of *C. trifida* in the different community types at Tentsmuir. Additional specific objectives were to determine the influence of distance from naturally occurring *Corallorhiza* plants upon germination and development of introduced seeds (Experiment one), to examine the effects of different vegetation types upon germination and growth (Experiment two), and, as far as possible, to identify the mycorrhizal fungi involved in the germination process.

Construction and deployment of seed packets

A series of seed packets was constructed from 40 × 60 mm rectangles of 53-μm nylon plankton netting

Table 1. *Developmental stages of* Corallorhiza trifida (*cf. Fig. 1*) *time taken to attain each stage after sowing under* Betula–Alnus *in September 1995* (*Expt 1*) *and under* Pinus–Betula (*PB*), Pinus–Betula–Alnus (*PBA*) *and* Salix repens (*S*) *in October 1996* (*Expt 2*)

		First observation of each developmental stage (months after sowing)			
		Habitat			
Stage	Description	*Betula–Alnus*	PB	PBA	S
0	Nongerminated seed				
1	Swelling of seed prior to rupture of testa (length $\geqslant$ 230 μm, breadth $\geqslant$ 160 μm), fungal penetration of cells at suspensor end, pelotons sometimes recorded	8	7	7	7
2	Testa ruptured, extensive development of fungal infection with abundant pelotons and starch storage cells, no rhizoids	8	7	7	7
3	Production of rhizoids and evidence of apical bud formation, rhizoids initially produced singly but later occur mainly in discrete clumps on wartlike outgrowths from the developing rhizome	15	ND	14	7
4	Protocorm branches to form coralloid rhizome, apical bud develops further, somtimes producing the first scale leaves	24	ND	ND	14

ND, stage not detected.

(Plastok Associates, Birkenhead, UK) and 50–100 seeds were placed in each. The nylon was folded once and clipped into $2 \times 2 \times 36$ mm plastic glassless slide mounts (Rasmussen & Whigham, 1993). A length of coloured nylon cord was attached to each mount to facilitate detection and removal of the packet. Seed packets were buried vertically in the soil so that the seeds lay at a depth of *c.* 8 cm, the zone of major occupancy by roots of trees and of *C. trifida* when present.

The packets were sown using strung quadrats as templates, one packet being sown in each 10-cm^2 grid square. There were thus 100 packets in each 1 m^2 quadrat and 50 in each of 0.5 m^2. Quadrats of both sizes are referred to as plots. The position of each packet in the plot was indelibly recorded on the plastic mount before insertion. The corners of each plot were marked with metal pegs and its location mapped before the quadrat was removed, using co-ordinates to prominent landscape features.

Seed was sown in each of the major community types (Table 1), most packets being deployed in the *Betula–Alnus* woodland which supported the largest naturally occurring population of *C. trifida*. Here, there were 8 1-m^2 plots (800 seed packets). These plots were dispersed throughout the area so that each of four contained adult *C. trifida* plants and the boundaries of each of the other four were 1 m from any orchid plants (Experiment one).

Packets of seed collected from the site were buried on 12–13 September 1995. A subsample of the seed was tested for viability by the tetrazolium chloride method of Van Waes & Debergh (1986) which indicated 80–85% viability. Harvests were taken after 8, 15, 20, 27 and 31 months (20 May 1996, 9 December 1996, 20 May 1997, 9 December 1997 and 20 April 1998, respectively).

A series of 0.5-m^2 plots were established in three further community types representative of those occurring at Tentsmuir: *Pinus–Betula* woodland (PB) containing *Goodyera repens*, *Pinus–Betula–Alnus* woodland (PBA) and *Salix repens* scrub (S) (Experiment two). Established *Corallorhiza* plants were only found in the PBA community. Packets were placed in the plots on 3 October 1996. There were 4 replicate 0.5-m^2 plots for the PB and PBA communities and 5 replicate plots for S. A preliminary harvest of 5 packets was taken from each in the PB and PBA communities plot after 2 months (December 1996), and from all communities after 7 months (May 1997). The main harvest took place 14 months after burial (December 1997): 12 packets per plot were taken from each of the three community types. An additional harvest was taken from the S plots in May 1998 (19 months after burial).

Fifty packets of *C. trifida* were placed in a *Salix repens* community at Newborough Warren NNR in November 1996 and harvested in December 1997.

Post-harvest analysis of germination and development

Immediately after each harvest, packets were returned to the laboratory where they were stored

moist at 5°C, and over several days they were opened and examined microscopically to detect the extent of germination and of seedling development. The time required to process the large number of samples necessitated the preservation of the contents of each packet by mounting the seedlings on a glass slide in a drop of 50% glycerol, placing a cover slip over the specimens and sealing with clear nail varnish. Seedlings too large to be mounted were examined fresh.The length and breadth of each seedling were measured with the aid of a compound microscope with an eyepiece graticule (× 40–200 magnification). Fungal colonization and stage of development were recorded. Four developmental stages were defined: Stage 1, germination had commenced, the seed expanded but the testa still intact; Stage 2, further expansion causing splitting of the testa; Stage 3, production of the first rhizoids; Stage 4, development of a branched rhizome (Table 1, Fig. 1). The percentage germination of seeds in each packet was calculated, and the volume of each seedling calculated using the equation volume (mm^3) = $(\pi lb^2)/6$ where l = length and b = breadth at the widest point (mm) (Hadley & Williamson, 1971). For the later developmental stages in which the seedlings had highly branched coralloid rhizomes, their volumes were estimated from fresh weight using a regression equation of volume against fresh weight determined for a representative sample of plants (volume (mm^3) = f. wt (mg) × 1.532 + 0.027, $r^2 = 95\%$, $n = 8$).

Differences between the percentage of seed packets in which germination occurred in plots with and without adult *Corallorhiza* (Experiment one) were assessed by ANOVA of arcsin-transformed data. Differences between the frequency of germination of packets in each of the eight plots were assessed by Chi-square.

The effect of habitats on the percentage of seed packets containing germinating seeds and the percentage of seeds germinating (Experiment two) was analysed by ANOVA and Tukey multiple-range tests of arcsin-transformed data and are presented as back-transformed means.

Isolation of a fungal symbiont of Corallorhiza trifida

To determine the characteristics of fungus forming pelotons in seedlings of *C. trifida* symbionts were isolated using aseptic techniques and cultured on 2% malt agar. Seedlings taken from packets were surface-sterilized by immersion for 10 min in a 10% solution of calcium hypochlorite. They were then either placed whole onto the agar or broken open to release individual peloton-containing cells into droplets of sterile water. Fungal hyphae, observed under the microscope to emerge from seedlings and pelotons, were excised from the medium and transferred to new plates.

Molecular identification of fungi

DNA analyses were carried out on seedlings from 14 packets from the *Salix* site, from four packets from the *Betula–Alnus* site and on two naturally occurring seedlings recovered from soil under *Betula–Alnus* at Tentsmuir. The analyses were also carried out on four adult plants: one also from the Tentsmuir *Betula–Alnus* community, one from the Northern Tyrol of Austria, one from Michigan, USA, and one from Montana, USA. In the case of seed packet samples, DNA was extracted from individual large seedlings or, where seedlings were too small to permit individual analyses, from bulked material. In total, 28 DNA samples were analysed.

Fresh or freeze-dried *C. trifida* tissue was ground in SDS buffer with a plastic pestle, extracted with an equal volume of chloroform and purified using GeneClean® (Bio 101, Inc., Carlsbad, CA, USA) glass milk as described in Taylor & Bruns (1997). The fungal internal transcribed spacer (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). A subset of samples was also amplified using ITS1F with a new primer, ITS4F (5′ GCTTTAGATGGAATTTACCA 3′) which is located near the Basidiomycete primer ITS4B (Gardes & Bruns, 1993) in the 28S gene. The fungal ITS amplicons were digested, separately, with the restriction enzymes *Hin*f I, *Alu* I and *Mbo* I and the fragments were separated on 1.5% agarose gels (High resolution Agar; Sigma Chemicals, Poole, UK) in 0.5× TBE. The primer ITS4F was used principally to produce a longer amplicon which resulted in better separation of the *Hin*f I bands in one fungal type. Neither of the primers amplified any plant product. Samples from the same site with the same ITS RFLP patterns for the three enzymes were considered to have been colonized by the same fungal species.

Using the same two primers the ITS region of a representative of each seedling and of each adult ITS RFLP type was then sequenced; ITS amplicons were purified with the Qiagen QIAquick PCR kit (Qiagen GMBH, 40724, Hilden, Germany). The ABI Big Dye terminator kit (PE Applied Biosystems, Foster City, CA, USA) was employed for cycle sequencing, which was followed by sequence separation on an ABI 310 Genetic Analyzer (PE Applied Biosystems). Additional details of the fungal ITS-RFLP approach are provided in Gardes & Bruns (1996b), Karen *et al.* (1997) and Taylor & Bruns (1999a). These workers have shown that ITS RFLP variation corresponds well with morphological species distinctions in many fungi of the Agaricales.

The sequences were subjected to a GenBank search to evaluate the taxonomic affinities of each of the fungal ITS sequences. Because fungal sequences

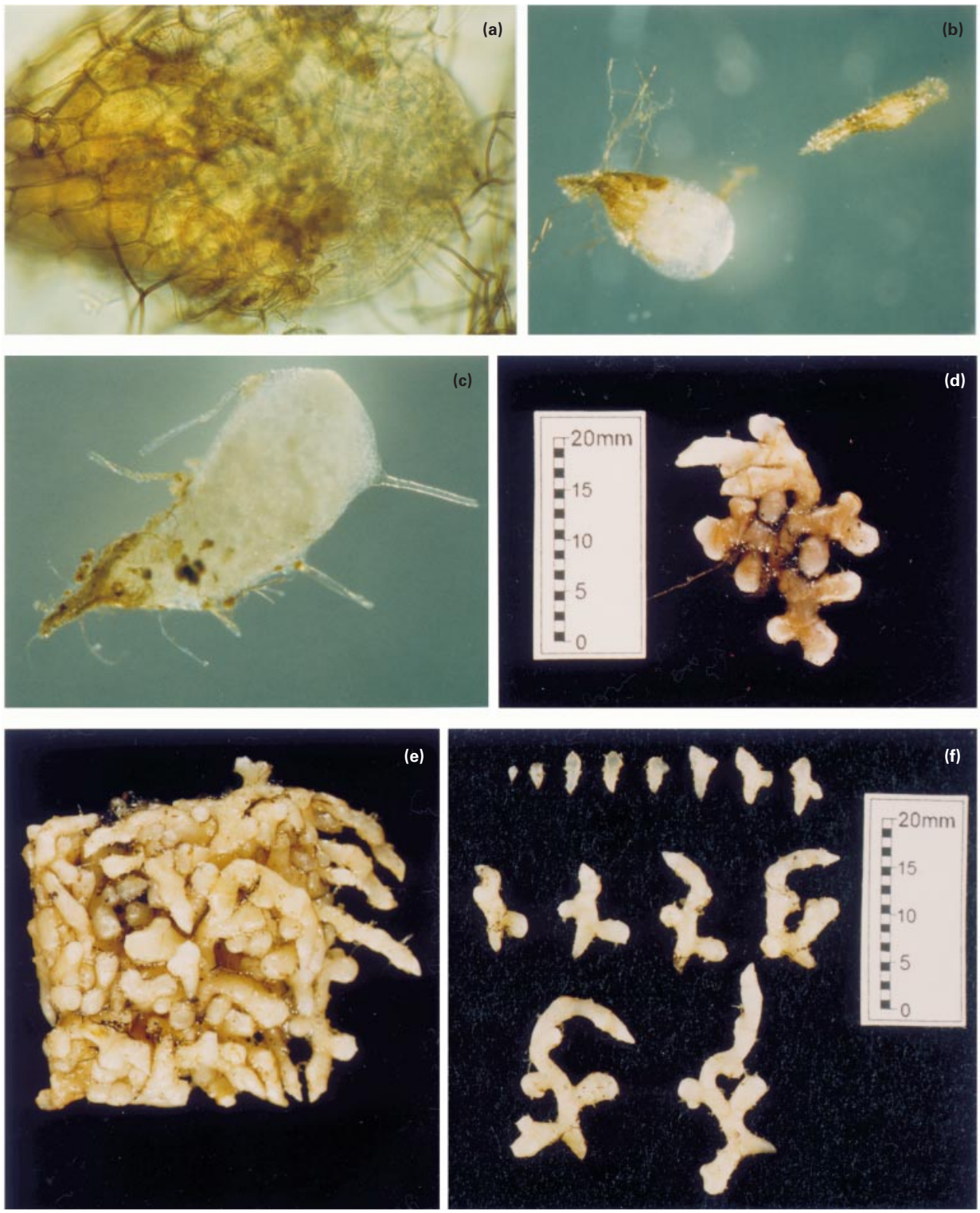

Fig. 1. Stages in development of *C. trifida* from *Betula–Alnus* woodland. (a) Stage 1: germinating seed showing initial colonization of the suspensor (right). Some hyphae of the fungus are hyaline, some melanized. (b) Stage 2: protocorm with ruptured testa, melanized hyphae of the fungal symbiont extend outwards from the protocorm (left), but no rhizoids are yet produced. An ungerminated seed can be seen to the right of the protocorm. (c) Stage 3: growth of rhizoids from the protocorm surface. (d) Stage 4: branched coralloid rhizome with shoot apex (top left). (e) Seedlings from a single packet from a *Salix repens* community, 14 months after planting. The seedlings are packed tightly together, the conformation of the group reflecting the constraints imposed by the mesh packet. (f) A selection of seedlings from the packet shown in (e), showing plantlets representative of Stages 2–4.

in the nuclear ribosomal small and large subunits are known to be highly conserved, these regions at each end of the ITS were eliminated before the search, but the short, conserved 5.8S ribosomal gene, which divides the two halves of the ITS region was included. The trimmed sequences, ranging from 500–550 bases, were submitted to the advanced search option of gapped-BLAST using default settings (Altshul *et al.*, 1997).

RESULTS

The chronology of germination and development of Corallorhiza trifida

Germination was detected by increased length, breadth (Table 1) and translucence of the seed. It was first observed 8 months after sowing under *Betula–Almus* communities in September 1995 and 7 months after sowing under *Pinus–Betula*, *Pinus–Betula–Alnus* and *Salix repens* in October 1996 (Table 1). Germination was extremely patchy and although the majority of seeds did not germinate in the course of the study, visual inspection indicated that few had deteriorated even after 31 months of burial.

Microscopical observations of subsamples of seeds from which the testa was removed confirmed that germination only occurred when cells at the suspensor end of the embryo were colonized by a peloton-forming fungus (Figs 1a, 2). The fungus

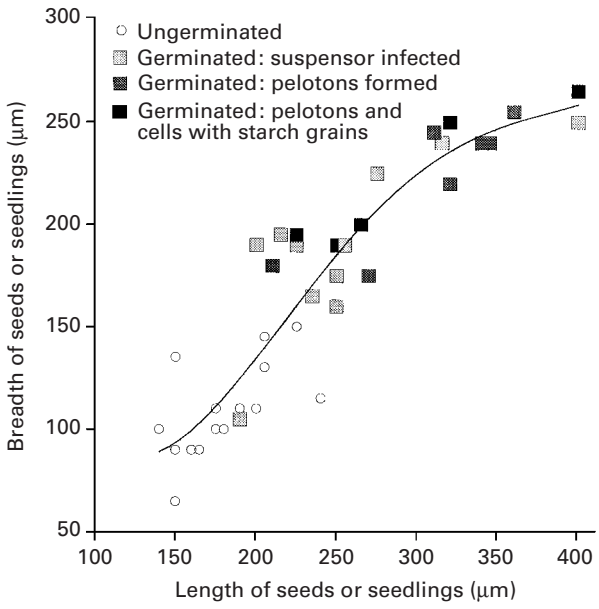

Fig. 2. The relationship between the extent of fungal colonization of seedlings and the length and breadth of ungerminated and germinated seedlings. Germination coincides with infection of the suspensor cells; the fungus then spreads to adjacent cells in which pelotons are formed. Once pelotons are formed some (uninfected) cells start to accumulate masses of starch grains.

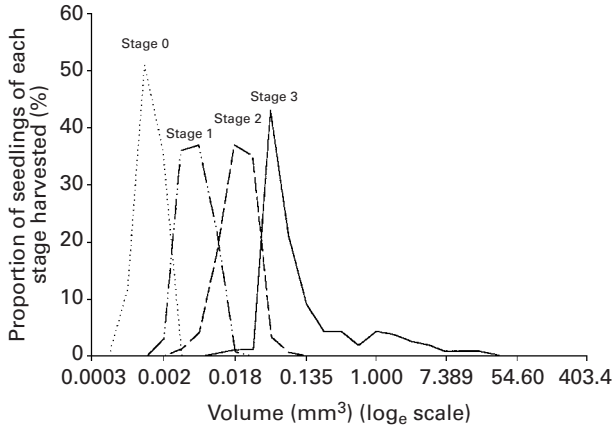

Fig. 3. Size-class distribution curves for developmental stages of *C. trifida* harvested from under *Betula–Almus* (Experiment 1). Stages: 0, ungerminated seed; 1, swollen embryo without rupture of testa; 2, protocorm with ruptured testa; 3, protocorm with rhizoids.

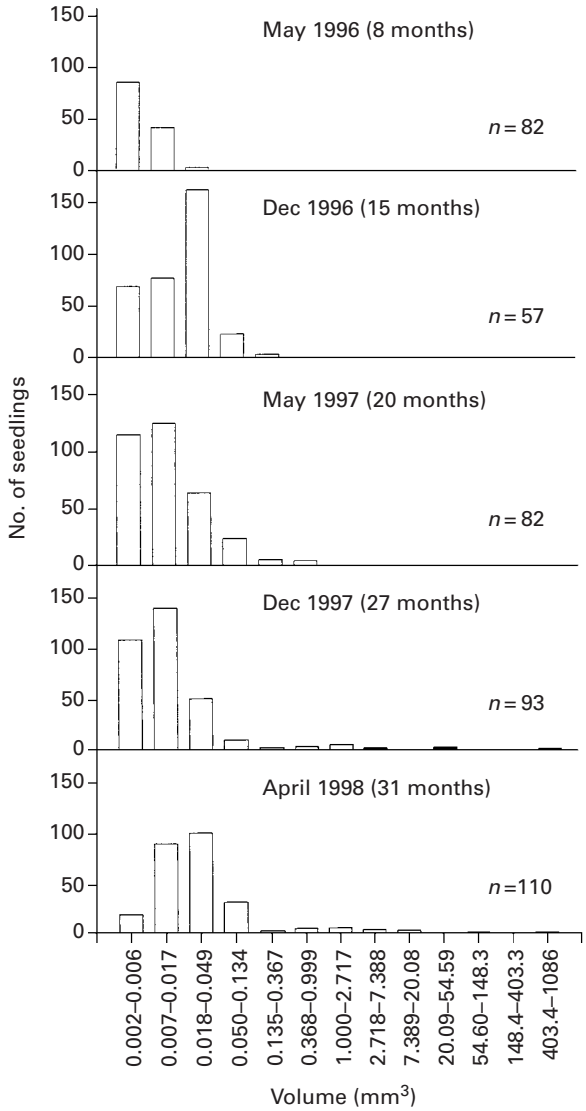

Fig. 4. The chronology of germination and growth of *Corallorhiza trifida* seedlings as indicated by size class distributions of seedling volumes at each harvest for seeds sown under *Betula–Alnus* in September 1995 (Experiment 1). n = the number of packets sampled at each harvest.

Table 2. *Effect of distance from adult plants on the germination of* Corallorhiza *seeds sown in four replicate 1-m² plots which contained adults and in four plots which were located > 1 m from the nearest adults. The packets were buried in* Betula–Alnus *woodland in September 1995 and harvested 8, 15, 20, 27 and 31 months later. Results were calculated on a plot basis and are presented as arithmetic mean values (± SE; n = 4) for plots with or without adults.*

	Germination	8 months after sowing		15–31 months after sowing	
		With adults	Without adults	With adults	Without adults
(a)	Packets per plot in which germination occurred (%)	33.5 ± 8.1	41.4 ± 14.8	57.8 ± 9.5	60.5 ± 19.5
(b)	Seeds which germinated in each packet (excluding packets with no germinating seeds) (%)	5.4 ± 1.4	7.7 ± 0.5	13.3 ± 2.2	10.8 ± 1.8
(c)	Sown seeds which germinated (%)	2.0 ± 0.8	3.1 ± 1.2	8.7 ± 1.3	8.8 ± 3.2

consistently produced clamp connections. The hyphae (diam. 3–4 μm), were initially unpigmented but progressively developed brown pigmentation with age (Fig. 1a, b). Colonization by the fungus resulted in both longitudinal and radial extension growth in the seedlings. Even before most seedlings had grown sufficiently large to split the testa (Stage 2) (Table 1) they contained fungal pelotons, and on reaching Stage 2 some cells had begun to accumulate starch grains (Fig. 2).

The fungus emerging onto agar plates from subsamples of entire surface-sterilized seedlings and individual isolated pelotons produced a hyaline mycelium which bore clamp connections. It grew slowly, with an average radial extension of 3 mm per week.

The size class distributions (relative frequency $\log_e$ volume classes) of ungerminated seeds and of plants which reached Stages 1 and 2 under *Betula–Alnus* followed distinct but somewhat overlapping normal distribution curves (Fig. 3). The size class distribution of plants which reached Stage 3 showed a pronounced skew to the right. There were insufficient plants at Stage 4 to make reliable inference about their distribution (data not shown). Despite the overlap in their distributions, it was possible to distinguish with reasonable reliability the ungerminated seeds and germinated seedlings at all stages on the basis of volume: 100% of ungerminated seeds, < 0.003 mm³; 84% of Stage 1 seedlings, 0.0031–0.0086 mm³; 66% of Stage 2, 0.0087–0.0387 mm³; 82% of Stage 3, 0.0388–54.5 mm³ (Fig. 4); 100% of Stage 4 plants, > 54.5 mm³.

Although some germination occurred 8 months after planting under *Betula–Alnus*, the size-class distributions of seedling volumes (Fig. 4) indicate that main phase of germination and growth in the first year was May–December (8–15 months), when the majority of seedlings increased in volume to 0.018–0.050 mm³. The subsequent increase in the number of plants in the 0.003–0.007 and 0.007–0.0018 mm³ volume classes at 20 months suggests that a second wave of germination occurred during the winter/spring of the second year (Fig. 4). From the samples taken at 27 and 31 months it is unclear whether any additional germination had taken place since the harvest at 20 months.

Throughout the study the majority of seeds which germinated in the *Betula–Alnus* site only grew to 0.018–0.050 mm³; however, a very small minority grew much larger. The size (volume) of the largest plants at each harvest increased progressively from 0.030 mm³ at 8 months to 0.281 mm³ at 15 months, 0.637 mm³ at 20 months, 447 mm³ at 27 months and 362 mm³ at 31 months. From a total of *c.* 15 500 seeds sown at and recovered from the *Betula–Alnus* site, 1370 germinated and only five grew to volumes > 54.5 mm³ (Stage 4) by 31 months.

All developmental stages of *C. trifida*, from ungerminated seed to the production of a branched coralloid rhizome with shoot apex, were recorded for packets of seeds buried up to 31 months in the *Betula–Alnus* woodland (Fig. 1e) and 14 months in *Salix repens* scrub (Fig. 1f). Under *Salix*, development was more rapid than under *Betula–Alnus*, some plants reaching Stage 3 within 7 months of planting (15 months for *Betula–Alnus*) and reaching Stage 4 by 14 months (24 months for *Betula–Alnus*) (Table 1). All protocorms > 1 mm long and 0.6 mm broad had rhizoids. In the larger plants rhizoids no longer occurred singly but arose in groups from wart-like projections on the developing rhizome. Most of the rhizoids contained fungal hyphae growing into or out from the plants.

The effects of the different habitats on germination and growth of *C. trifida* are reported more fully in relation to Experiment 2.

A number of the packets recovered from Newborough Warren NNR contained germinated seeds, but probably because of a severe drought during the summer of 1997, only three packets yielded living seedlings and none of the seedlings in either the dead or living categories had developed beyond Stage 1. Microscopic examination of the living seedlings revealed the presence of intracellular coils produced by a clamp bearing mycorrhizal fungus.

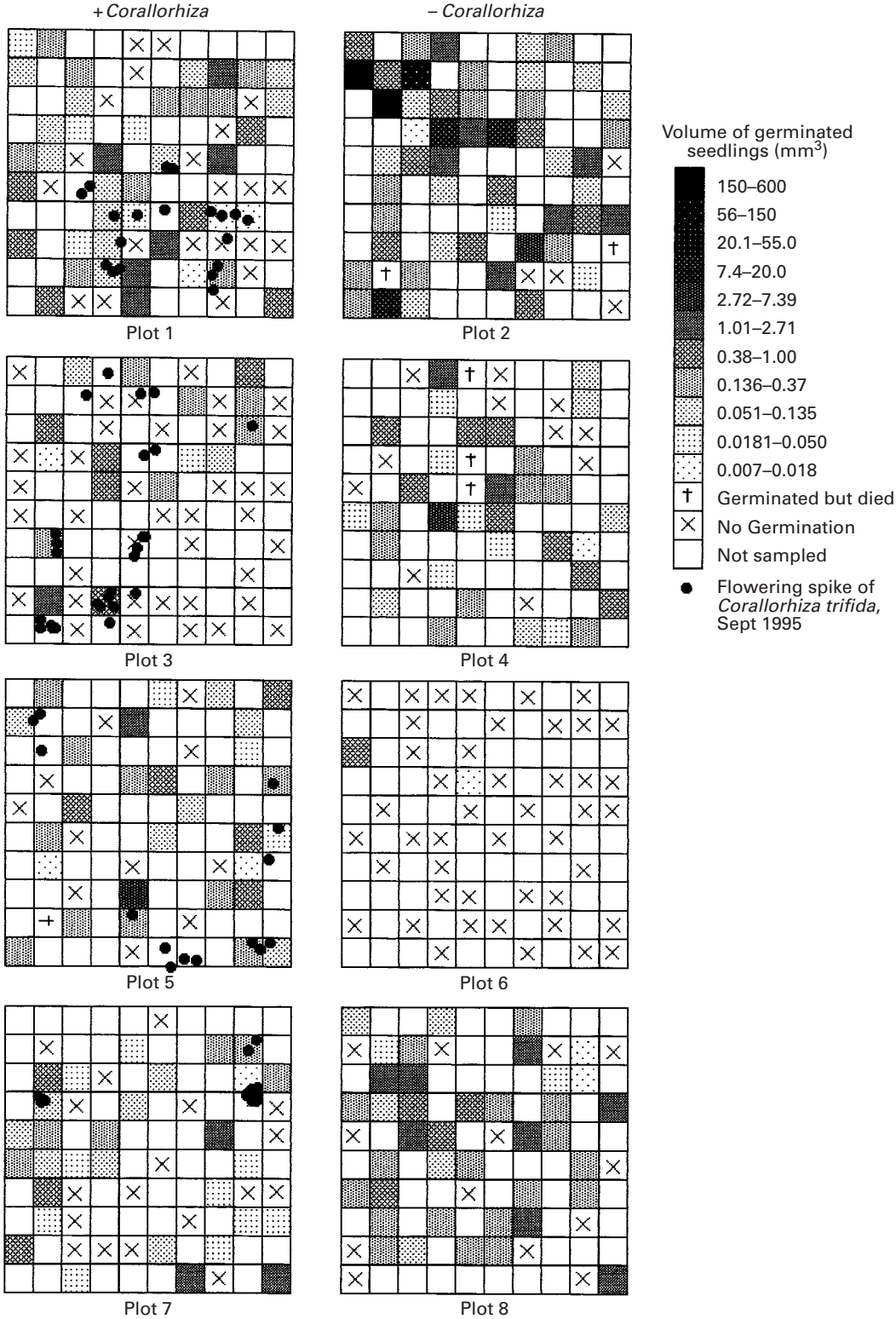

Fig. 5. The spatial distribution of seed packets in which germination occurred and the total volume of living seedlings in each packet planted in *Betula–Alnus* plots containing adult *Corallorhiza* plants (Plots 1, 3, 5, 7) and plots in which adults were not recorded (Plots 2, 4, 6, 8). The data are from all harvests.

The effect of distance from adult plants on germination and growth of Corallorhiza trifida (*Experiment one*)

In 1-m² plots there was no significant effect of the presence of adult *Corallorhiza* on the percentage of seed packets in which germination occurred, the percentage of seeds germinating in packets where germination occurred, or the overall percentage of sown seeds which germinated (Table 2). This was true of the first harvest (8 months), at which germination was only starting, and the later harvests,

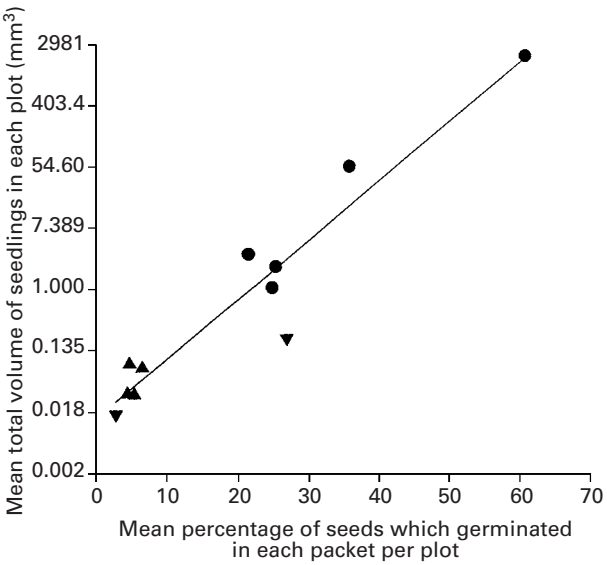

Fig. 6. The relationship between the mean total volume of germinated seedlings of *Corallorhiza trifida* in the five seed packets with greatest growth in each plot and the percentage of seed packets in which germination occurred in that plot. Harvested under *Pinus–Betula*, triangles with base uppermost; under *Pinus–Betula–Alnus*, triangles with apex uppermost; under *Salix*, circles. Values are plotted on a log scale.

at which *c*. 60% of the packets contained germinating seeds. Overall, there was a significant increase both in the percentage of seeds germinating in each packet (Table 2b) and in the total percentage of seeds which germinated (Table 2c) from the 8 month to the 15–31 month harvests (ANOVA, $P < 0.05$).

However, there were marked differences between plots in the proportion of seed packets in which germination occurred and in the extent of growth of the germinated seedlings (Figs 5, 6), in the plots without adult *Corallorhiza*, where the percentage of packets containing germinating seeds ranged from 4% in Plot 6 to 93% in Plot 2 (Fig. 5). Chi-Square analysis of the total numbers of seed packets in each plot which did or did not contain germinating seeds was carried out to test the hypothesis that there were no significant differences in frequency of packets with germinating seeds in the different plots. The analysis showed that in three of the four plots without adult *Corallorhiza* the number of seed packets in which germination occurred was significantly higher than expected (Plot 2, $\chi^2 = 24.6$, df = 1, $P < 0.001$; Plot 4, $\chi^2 = 4.7$, df = 1, $P < 0.05$; Plot 8; $\chi^2 = 4.6$, df = 1, $P < 0.05$) whereas in Plot 6 it was much lower than expected ($\chi^2 = 62.1$, df = 1, $P < 0.001$). In the plots containing adult *Corallorhiza* differences were less marked; the number of packets in which seed germination occurred in Plot 3 was significantly lower than expected ($\chi^2 = 21.4$, df = 1, $P < 0.001$) but in the remaining plots (1, 5 and 7) there were no significant differences. Overall, there were clear differences between the plots in the proportions of the sampled packets in which seed germination occurred but these could not be related to the presence of adult plants in the 1-m² plots.

Analysis of data for germination and growth of *Corallorhiza* seedlings in the immediate proximity (< 20 cm) of adult plants and at distances of > 40 cm from these plants showed no significant local effect of adults within the 1-m² plots, nor was there any relationship between the number of 10×10 cm squares in which adult plants occurred in plots and the proportion of seed packets in which germination occurred. In the majority of plots the packets in which germination occurred and the extent of seedling development showed no evidence of spatial aggregation. There were two exceptions to this: in Plot 3 none of the packets in the lower right-hand quarter of the plot contained germinating seedlings and in Plot 2 the packets with the largest seedling volume were all in the top left-hand quarter (Fig. 5).

Table 3. (*a*) *Mean percentage germination* (*arcsin back-transformed means*) *of* Corallorhiza trifida *per plot 14 months after sowing under* Pinus–Betula–Alnus (*PBA*), Pinus–Betula (*PB*) *or* Salix repens (*S*) *in October 1996. An additional sample was taken from the* Salix *community 19 months after sowing ; means sharing the same letter in a row are not significantly different Tukey test* P > *0.05*). (*b*) *Maximum percentage germination per packet after 14 months under the three communities.*

		Time after burial (months)			
		14			19
	Germination	PBA ($n = 4$)	PB ($n = 4$)	S ($n = 5$)	S ($n = 5$)
(a)	Packets in which germination occurred (%)	74.7 a	23.7 a	41.5 a	81.1 a
	Seeds which germinated in each packet (excluding packets with no germinating seeds) (%)	5.5 a	12.4 ab	31.4 bc	62.6 c
	Sown seeds which germinated (%)	3.9 a	3.8 a	12.5 a	42.5 b
(b)	Maximum germination per packet (%)	14	66	95	96

The largest seedlings were found in the plots in which the highest percentage of packets contained germinating seeds, an approximately linear relationship occurring between the proportion of seed packets with germinating seeds and the log of the total volume of seedlings in the five packets with best seedling growth in each plot (Fig. 6).

The effect of community type on germination and growth of Corallorhiza trifida (*Experiment two*)

Germination. Germination rates were highly variable within the different community types and this variability to a large extent obscured any possible differences between the habitats. The mean percentage of packets in which germination occurred varied between sites from 24% in *Pinus–Betula* to 75% in *Pinus–Betula–Alnus*, with an intermediate value of 42% at the *Salix* site (Table 3). These differences were not significant (ANOVA, $P > 0.05$) largely because of the high variability in germination in the *Pinus–Betula* habitat, where in the four subplots the proportion of packets containing germinating seeds ranged from 0–100%. Comparison between the *Pinus–Betula–Alnus* and *Salix* communities at the 14 month harvest revealed a significantly higher proportion of seed packets with germinating seeds in the former (*t*-test, $P < 0.05$).

The percentages of seeds which had germinated in the packets after 14 months differed considerably between sites, from 5.5% in the *Pinus–Betula–Alnus* habitat, 12% in the *Pinus–Betula* habitat to 31% in the *Salix* habitat (Table 3). The differences in germination between the *Pinus–Betula–Alnus* and *Salix* communities were significant (Tukey test, $P < 0.05$).

In the *Salix* community the percentage of seeds germinating in each packet doubled from 31% at 14 months to > 60% at 19 months. Although high variability meant that this change was not significant (Tukey test, $P > 0.05$), the magnitude of the increase strongly supports the view that a second wave of germination occurred in the spring of the second year. When the results were expressed as a percentage of all the sown seeds which germinated, the increases which occurred between 14 months (12.5 %) and 19 months (42.5 %) in the *Salix* plots were highly significant. There were, however, no differences between the habitats at 14 months, although germination in the *Salix* community was three times that in the *Pinus–Betula–Alnus* and *Pinus–Betula* habitats.

There were also marked differences between the maximum percentage of seeds germinating in individual packets in the three communities, ranging from only 14% in the *Pinus–Betula–Alnus* to 95–96% in the *Salix* plots (Table 3).

Compared with the rates of germination recorded for packets sampled 15–31 months after burial in the *Betula–Alnus* woodland in Experiment 1, those of the *Salix* community were greater, with higher proportions of packets with germinating seeds, and much higher rates of germination of seeds in those packets (mean percentage of seeds germinating 10.8–13.3% (Table 2b) and 62.2% (Table 3), respectively).

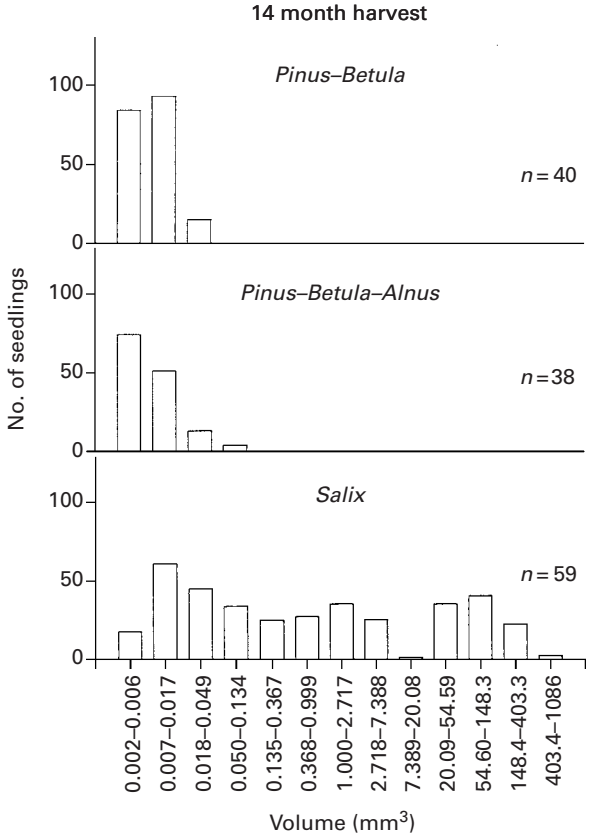

Fig. 7. Size class distribution curves of seedlings of *Corallorhiza trifida* harvested under *Pinus–Betula–Alnus*, *Pinus–Betula* and *Salix* (Experiment 2). Seeds were sown in October 1996 and harvested 14 months later in December 1997. The class intervals are in mm^3 on increments of one unit on $\log_e$ scale. n = number of packets harvested in each habitat.

Growth of seedlings. There were clear differences in the extent of growth of *Corallorhiza* seedlings between the *Pinus–Betula*, *Pinus–Betula–Alnus* and *Salix* communities 14 months after planting (Fig. 7). The most striking result was the high proportion (57% of 150 seedlings) in the *Salix* community of volumes of 0.14–500 mm^3; none of the seedlings in the other two communities attained volumes > 0.14 mm^3. The pattern of size class distribution of plants in the *Salix* community differed markedly from those of the other two communities, which appeared to approximate to the Poisson distribution, the seedlings in the smallest size classes occurring with the highest frequency. In the *Salix* community there was a similar frequency of plants throughout the entire range of $\log_e$ volume classes (Fig. 7).

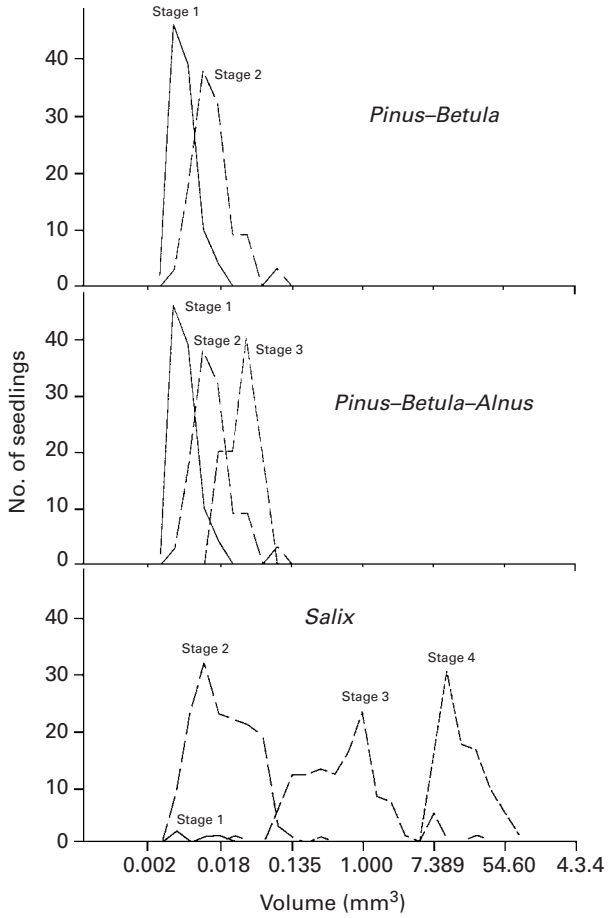

Fig. 8. Size class distribution curves for each developmental stage of *Corallorhiza trifida* harvested under *Pinus–Betula–Alnus*, *Pinus–Betula* and *Salix* communities (Experiment 2). Developmental stages: 1, swollen embryo without rupture of seed coat; 2, protocorm with ruptured testa; 3, protocorm with rhizoids; 4, branched, coralloid rhizome. The size classes are in mm^3 on increments of 0.5 units on $\log_e$ scale.

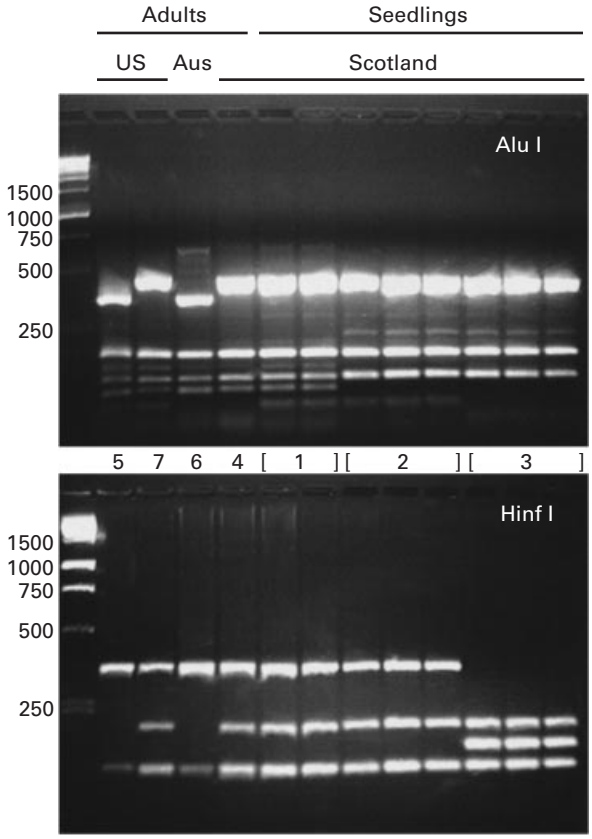

Fig. 10. Fungal ITS-RFLP patterns observed in adults and seedlings of *Corallorhiza trifida*. Upper panel, fragments obtained after restriction digestion with *Alu* 1. Lower panel, samples in the same order after restriction digestion with *Hinf*I. Fungal ITS amplicons were generated using PCR and the primers ITS1F and ITS4F. Geographical origins of samples: USA (US); Austria (Aus). The fungal code number for each ITS-RFLP lane shown between the panels refers to *C.trif* numbers used in Table 4 (e.g. Fungus 5 = *C.trif5*).

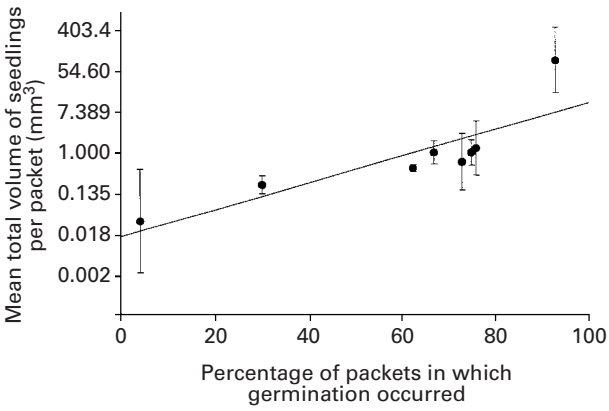

Fig. 9. Relationship between the mean percentage of seeds of *Corallorhiza trifida* which germinated in each packet (excluding those in which no germination occurred) and the mean volume of seedlings germinated per packet after 14 months in *Pinus–Betula–Alnus*, *Pinus–Betula*, and *Salix repens* communities (Experiment 2). Standard error bars are shown.

The differences in growth of seedlings in the *Salix* community were, most surprisingly, also reflected in the relationships between volume and developmental stage of the seedlings. Whereas the size class distributions of seedlings in Stages 1–3 were almost identical in the *Betula-Alnus* community in Experiment one (Fig. 4), and in the *Pinus–Betula* and *Pinus–Betula–Alnus* communities in Experiment two (Fig. 8), the seedlings in the *Salix* community were much larger at the equivalent stages of development.

The higher rate of germination seen in the *Salix* than in the other communities was correlated with a higher rate of growth of the *Corallorhiza* seedlings (Fig. 9).

Molecular identification of fungal symbionts

Among the 24 *C. trifida* seedling DNA extracts analysed, there were three distinct fungal ITS RFLP patterns, referred to as *C. trif* 1, *C. trif* 2, *C. trif* 3

Table 4. *Molecular characterization of mycorrhizal fungi from seedlings* (C. trif *1–3*) *and adults* (C. trif *4–7*) *of* Corallorhiza trifida

Fungus	GenBank no.	Geographic distribution	No. of records of fungus at *Salix* sites	No. of records of fungus at *Betula–Alnus* site	Closest GenBank sequence
Seedlings					
C. trif 1	AF184743	Scotland (Tentsmuir)	11	0	*Tomentella* sp. 'LT56' U83482
C. trif 2	AF184745	Scotland (Tentsmuir)	5	0	*Tomentella* sp. 'LT56' U83482
C. trif 3	AF184742	Scotland (Tentsmuir)	2	7 (5+2)	Thelephoraceae sp. 'Taylor #11' U83475
Adults					
C. trif 4	AF184744	Scotland (Tentsmuir)	–	1	*Tomentella* sp. 'LT56' U83482
C. trif 5	AF184747	Montana, USA	–	–	*Tomentella* sp. 'LT56' U83482
C. trif 6	AF184748	Austrian Alps	–	–	*Tomentella* sp. 'LT97' U92537
C. trif 7	AF184746	Michigan, USA	–	–	Thelephoraceae sp. 'Taylor #9' U83471

(Fig. 10, Table 4). These fungi were found in both the extremely small Stage 2 seedlings and in some of the largest Stage 4 plants. Clearly in the case of the small seedlings, which were bulked, it was not possible to determine whether more than one of the fungi was present in any single seedling. However, one of the larger seedlings, which was analysed individually did contain both *C. trif* 1 and 2. Whereas all three of the fungi were recorded in samples from the *Salix* site, only *C. trif* 3 was found at the *Betula–Alnus* site. It was recovered from seedlings in a packet and the two free-living seedlings analysed.

The ITS RFLP patterns indicated that the fungi represented different species within the thelephoroid complex of basidiomycete fungi. Previous analysis using sequence comparison (Taylor & Bruns, 1997) has confirmed that different ITS RFLP patterns of the kind seen here enable 'morpho species' of thelephoroid fungi to be separated.

Further confirmation of the identity of the isolates was obtained by sequencing. Use of the primers ITS1F and ITS4 in separate cycle sequencing reactions resulted in two directional sequences with 80–100% overlap, providing unambiguous sequences for the complete ITS1 5.8S and ITS2 regions for all fungal taxa except *C. trif* 1. In this case sequence was obtained in only one direction because of Taq polymerase stutter following a mononucleotide repeat. The sequences from *C. trif* 1, *C. trif* 2 and *C. trif* 3 together with those from each of the four adult plants sampled, were deposited in GenBank (accessions AF184742–AF184748 see Table 4) and subjected to a gapped-BLAST search. This search revealed that in every case the 28 most similar sequences were ITS regions belonging to fungi in the Thelephoraceae. The extent of similarity between particular *C. trifida* fungi and other confirmed members of the Thelephoraceae was often high. In the case of *C. trif* 2, for example, there was a 94% sequence identity with the sequence from a *Tomentella* fruit body (LT56) over an uninterrupted span of 450 bases. *C. trif* 1, 4 and 5 were also most similar to the LT56 GenBank entry despite the fact that their RFLP patterns differed somewhat (Fig. 10). This suggests a relatively close relationship among the taxa *C. trif* 1, 2, 4 and 5 within the Thelephoraceae.

There were differences between ITS RFLP patterns and ITS sequence relationships in the USA and UK samples. The fungus associated with an adult plant from Michigan, USA had ITS RFLP patterns for *Hin*f1, *Alu*1 and *Mbo*1 that matched those of *C. trif* 1 from Scotland, UK (Fig. 10). However the ITS sequences were sufficiently divergent to be attributed to different thelephoroid species according to GenBank (Table 4). *C. trif* 3 was distant from the other types in both sequence and GenBank entry but still corresponded more closely with other thelephoroid accessions than with any other group of fungi.

DISCUSSION

This study has established for the first time a definite chronology for the developmental stages of the plant and has shown that this chronology can differ between habitats. The fastest-growing plants, which occurred in the *Salix* community produced a protocorm with rhizoids (Stage 3) only 7 months after burial in October 1996, and reached Stage 4 after a total of 14 months (Table 1). Under *Betula–Alnus* and *Pinus–Betula–Alnus*, plants reached Stage 3 by 14–15 months after burial, and some in the *Betula–Alnus* community reached Stage 4 by 24 months. Reinke (1873) suggested that it took

24 months for plants to reach what we have defined as Stage 4 while Fuchs & Zeigenspeck (1924) believed that plants reached what we have defined as Stage 3 only after 11 months, 35 months being required to reach Stage 4. Clearly, the chronology of development at the *Salix* site was much shorter than these estimates, many of the largest plants having formed shoot buds after 14 months. It is likely that they would have flowered within a further 12 months if they had not been constrained by the seed packets.

The experiments also established the seasonality of germination. The absence of germination at the December harvest, 4 months after autumn sowing, indicates that germination occurs predominantly in the spring/summer of both the first and second years after release of seed. Previous assumptions (Fuchs & Ziegenspeck, 1924, 1927; Weber, 1981) that germination occurred in autumn were based upon the discovery of what were referred to as 'hirsute protocorms' indicating the presence of prolific rhizoid formation. However, such a stage of development, equivalent to Stage 3 in the present study, would be reached only some months after germination and its presence should not therefore be taken to indicate recent occurrence of early stages. One possible basis for the delay in germination until spring might be the requirement for a chilling period to break dormancy. Germination in the related species *C. odontorhiza* was shown by Rasmussen (1995) to be significantly increased by exposure of seeds to 5°C for 9 wk. In that species, seed packets failed to yield seedlings before June, approx. 7 months after autumn sowing (Rasmussen & Whigham, 1993).

The clamp-bearing fungi involved in the germination of *C. trifida* were morphologically similar to those observed in pelotons of *C. trifida* by Zelmer & Currah (1995) and Scrugli *et al.* (1995). Scrugli *et al.* (1995) describe pelotons in mature rhizomes of *C. trifida* formed by what are described as 'two morphologically distinct endophytes'. One, similar to that seen in the present study had broad clamped hyphae which were either hyaline or reddish-brown in colour. The second type produced pelotons made of 'thin hyphae' and were located in the innermost cortex of *C. trifida*. We have seen such structures but have interpreted them as representing stages in the digestion of the broad type.

As in the case of other orchids studied in their natural environments it appears that *C. trifida* shows specialized compatibility with a narrow taxonomic group of fungi. It is shown here that this specificity applies from the earliest stages of seed germination through adulthood and flowering. Ontogenetic consistency in specificity was also demonstrated by Masuhara & Katsuya (1994) in the IMHP orchid *Spiranthes sinensis*, which associates with a particular *Rhizoctonia* species.

It is likely to be of particular functional significance that, as in the recently reported case of the MHP *Cephalanthera austinae* (Taylor & Bruns, 1997), *C. trifida* associates with fungi in the Thelophoraceae. This is a family which, as far as is known, is otherwise exclusively of ectomycorrhizal habit. The constituent species are generally of low specificity, the universally distributed *Thelephora terrestris*, for example, being found as an ectomycorrhizal associate of *Pinus*, *Betula* and *Salix*. The observation that *C. austinae* formed orchid endomycorrhiza with thelephoroid species which were simultaneously in ectomycorrhizal symbiosis with surrounding conifers led Taylor & Bruns (1997) to hypothesize that such 'cheating' by mycoheterotrophs would provide them with access to C fixed by the autotroph. This hypothesis is tested experimentally using *C. trifida* seedlings by McKendrick *et al.* (2000).

Thelephoroid fungi appear to be dominant components of a variety of ectomycorrhizal communities, at least in the western USA (Taylor & Bruns, 1999b, and references therein). However, the occurrence of particular thelephoroid species varied considerably among the sites so far sampled. Therefore, if only certain species are suitable partners for *C. trifida*, we would predict a highly patchy distribution of the orchid related to the patchiness of particular thelephoroid fungi. Some evidence supporting this view is provided by the current study.

Although it appears that *C. trifida* is compatible with several thelephoroid species (or genotypes) the extent of its specificity relative to the range of thelephoroid fungi present at any particular site is not yet known. It is possible that seedling growth under *Betula–Alnus* was slower than that under *Salix* because the fungus *C. trif* 3 which was the only symbiont found in the former community was less physiologically compatible with the orchid than *C. trif* 1 and 2 identified at the *Salix* site. Alternatively, the latter fungi might have occupied a greater proportion of the *Salix* root system than did *C. trif* 3 on *Betula–Alnus* and so have had access to more C from the autotrophs.

It is possible that primer bias could mean that other fungal occupants of the root were overlooked. However, from the earliest stage of germination, the pelotons are of uniform appearance in the seedlings and they yielded isolates which, in culture, had consistent characteristics. This suggests, in accordance with the molecular information, that closely related fungi are involved in the critical events associated with establishment of the plant. This interpretation is consistent with earlier studies of adult *C. trifida* plants (Burgeff, 1936) but does not exclude the possibility, even the likelihood, that there are other more casual occupants of the mature rhizome.

The presence or absence of appropriate fungal symbionts might determine the ability of the orchid

to germinate at the regional scale. When Rasmussen & Whigham (1998) buried seed of *C. odontorhiza* in a forest of unspecified tree composition in which this orchid did not occur naturally, they obtained no germination. The forest did, however, support populations of the autotrophic orchid genera *Liparis*, *Galearis*, and *Tipularia*. In such cases it can be hypothesized that the myco-heterotroph failed to germinate because the trees were not of ectomycorrhizal habit or supported inappropriate ectomycorrhizal fungal populations. The autotrophic orchids, by contrast, having a requirement for widely distributed soil fungus of the *Rhizoctonia* type would not be constrained in this way.

The failure of *C. trifida* to produce adult plants in sites supporting *Salix* at both Newborough and Tentsmuir, although a fungal symbiont capable of initiating germination was present, demonstrates that factors other than fungal availability can contribute to the observed pattern of orchid distribution. Amongst these, the effectiveness of seed dispersal is a prime candidate for investigation.

What emerges from this study is that where dispersal of *C. trifida* seed is adequate to bring propagules into a site, the chances of their achieving germination are optimized by selection of widely distributed ectomycorrhizal generalists as symbionts.

Most of the hypotheses already described, concerning the functional basis of the relationship between *Corallorhiza trifida* and its fungal partners, are contingent upon the epiparasitism of the orchid enabling C flow from the ectomycorrhizal co-host. In McKendrick *et al.* (2000) we provide experimental evidence to support this hypothesis.

ACKNOWLEDGEMENTS

We thank NERC for financial support (GR3/10062) and Scottish Natural Heritage for permission to carry out the study at Tentsmuir NNR. Irene Johnson assisted with the analysis of seed germination and Tom Cunningham provided logistical support at the field site. Thanks are also due to Wilhelm Sauer and Jan Schlauer who supplied the *C. trifida* sample from Austria.

REFERENCES

Altshul SF, Madden TL, Schäffer AA, Zhang Z, Zheng Z. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

Burgeff H. 1936. *Samenkeimung der Orchideen*. Jena, Germany: Gustav Fischer.

Fuchs A, Ziegenspeck H. 1924. Aus der Monographie des *Orchis traunsteineri* Saut. III. Entwicklungsgeschichte einiger deutscher Orchideen. *Botanisches Archiv* **5**: 120–132.

Fuchs A, Ziegenspeck H. 1927. Entwicklungsgechichte der Axen der einheimischen Orchideen und ihre Physiologie und Biologie. III. *Botanisches Archiv* **18**: 378–475.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Gardes M, Bruns TD. 1996a. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. *Canadian Journal of Botany* **74**: 1572–1583.

Gardes M, Bruns TD. 1996b. ITS-RFLP matching for identification of fungi. In: Clapp JP, ed. *Methods in molecular biology*, 50**.** *Species diagnostics protocol : PCR and other nucleic acid methods*. NJ, USA: Humana Press Inc., 177–186.

Hadley G, Williamson B. 1971. Analysis of the post-infection growth stimulus in orchid mycorrhiza. *New Phytologist* **70**: 445–455.

Hutchings MJ. 1978a. The population biology of the early spider orchid, *Ophrys sphegodes* Mill. I. A demographic study from 1975 to 1984. *Journal of Ecology* **75**: 711–727.

Hutchings MJ. 1978b. The population biology of the early spider orchid. *Ophrys sphegodes* Mill. II. Temporal patterns in behaviour. *Journal of Ecology* **75**: 729–742.

Karen O, Hogberg N, Dahlberg A, Jonsson L, Nylund J-E. 1997. Inter- and intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. *New Phytologist* **136**: 313–325.

Leake JR. 1994. The biology of myco-heterotrophic ('saprophytic') plants. Tansley Review No. 69. *New Phytologist* **127**: 171–216.

Masuhara G, Katsuya K. 1994. *In situ* and *in vitro* specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon) Ames. Var. *amoena* (M. Bieberstein) Hara (Orchidaceae). *New Phytologist* **127**: 711–718.

McKendrick S, Leake JR, Read DJ. 2000. Symbiotic germination and development of myco-heterotrophic plants in nature: transfer of carbon from ectomycorrhizal *Salix repens* and *Betula pendula* to the orchid *Corallorhiza trifida* through shared hyphal connections. *New Phytologist* **145**: 539–548.

Rasmussen HN. 1995. *Terrestrial orchids – from seed to mycotrophic plant*. Cambridge, UK: Cambridge University Press.

Rasmussen HN, Whigham D. 1993. Seed ecology of dust seeds *in situ*: a new technique and its application to terrestrial orchids. *American Journal of Botany* **80**: 1374–1378.

Rasmussen HN, Whigham DF. 1998. The underground phase – a special challenge in studies of terrestrial orchid populations. *Botanical Journal of the Linnean Society* **126**: 49–64.

Reinke J. 1873. Zur Kenntniss des Rhizoms von *Corallorhiza* und *Epipogon*. *Flora* **56**: 145–152, 161–167, 177–184, 209–224.

Smith WG. 1905. Botanical survey of Scotland, III & IV – Forfar & Fife. *The Scottish Geographical Magazine* **21**: 57–83.

Scrugli A, Cogoni A, Riess S. 1995. Endofiti micorrizici di orchidee 'aclorofillische', *Corallorhiza trifida* Chatelain ed *Epipogium aphyllum* Swartz, analizzati al microscopio ottico e al microscopio laser confocale. *Caesiana* **5**: 29–38.

Taylor DL, Bruns TD. 1997. Independent, specialised invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. *Proceedings of the National Academy of Sciences, USA* **94**: 4510–4515.

Taylor DL, Bruns TD. 1999a. Population, habitat and genetic correlates of mycorrhizal specialization in the 'cheating' orchids *Corallorhiza maculata* and *C. mertensiana*. *Molecular Ecology* **8**: 1719–1732.

Taylor DL, Bruns TD. 1999b. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. *Molecular Ecology* **8**: 1837–1850.

Van Waes J, Debergh PC. 1986. Adaptation of the tetrazolium method for testing seed viability and scanning electron microscopy of some Western European orchids. *Physiologia Plantarum* **66**: 435–442.

Weber HC. 1981. Orchideen auf dem Weg zum Parasitismus? Über die Möglichkeit einer phylogenetischen Umkonstruktion der Infektionsorgane von *Corallorhiza trifida* Chat. (Orchidaceae) zu Kontaktorganen parasitischer Blütenpflanzen. *Bericht der Deutschen Botanischen Gesellschaft* **94**: 275–286.

Wells TCE. 1981. Population ecology of terrestrial orchids. In:

Synge H, ed. *The biological aspects of rare plant conservation*. Chichester, UK: J. Wiley, 281–295.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*, eds. San Diego, CA, USA: Academic Press Inc., 315–322.

Zelmer CD, Currah RS. 1995. Evidence for a fungal liaison between *Corallorhiza trifida* (Orchidaceae) and *Pinus contorta* (Pinaceae). *Canadian Journal of Botany* **73**: 862–866.