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## **Soils in transition: dynamics and functioning of fungi**

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## Chapter 6

### Possible mechanism for spontaneous establishment of *Calluna vulgaris* in a recently abandoned agricultural field



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**Abstract**

In Western Europe, arable lands have been abandoned to increase the area of nature, such as *Calluna vulgaris*-dominated heathlands. However, the growth conditions, e.g. nutrient availability and lack of a phenolics-rich organic layer, on ex-arable sandy soils differ markedly from those of heathland and will favor fast-growing plant species. Succession towards *Calluna*-dominated heathland is expected to take decades, unless intensive restoration management is applied. Here we report a possible mechanism to explain the occurrence of *Calluna* patches (0.7 – 2.0 m dia) in a 10 year abandoned agricultural field within a dominant vegetation of grasses and forbs. All roots sampled from the *Calluna* patches were colonized by ericoid mycorrhizal (ERM) - and other endomycorrhizal fungi. Both nitrogen mineralization of soil organic N and potential nitrogen mineralization (arginine ammonification) were much lower in soil under *Calluna* patches than in the rest of the ex-arable soil, although other soil characteristics did not differ. The nitrogen to phosphorus ratio in *Calluna* shoots was much greater than that in shoots of grasses and forbs, indicating that the latter were more N limited. The results indicate that the association with ERM fungi is probably providing the host competitive superiority for nitrogen even in a soil with low organic matter content. Our results suggest that the conversion from arable land into heathland may be accomplished by the immediate establishment of *Calluna* seedlings and ericoid mycorrhizal inoculum when agricultural activities are stopped. This needs to be tested in controlled experiments.

## Introduction

In Western Europe arable land has been taken out of production for a long time. Within the context of creating nature on abandoned lands, policy makers and nature managers often aim to create dry heathlands on ex-arable sandy soils. However, the growing conditions for the dominant heathland species, *Calluna vulgaris* (L.) Hull (hereafter referred to as *Calluna*), on ex-arable land differ markedly from those in mature heathlands. Most notable differences are the absence of an organic layer, the relatively high pH and the high nutrient availability, in particular phosphate, in ex-arable soils (Van der Wal et al., 2006b). A soil pH of 5 or higher and high levels of extractable phosphorus favor fast-growing plant species and soon after abandonment, arable weeds and pioneer species dominate the vegetation (Pywell et al., 1994; Kardol et al., 2005). These species will outcompete, overgrow or prevent establishment of slow-growing plant species with nutrient-conserving strategies, such as *Calluna* plants (Aerts, 1999). Therefore, spontaneous establishment of *Calluna* is not likely on these soils and specific treatments, such as reduction of fertility or introduction of heathland propagules, have to be applied in order to create conditions appropriate for development of a *Calluna* vegetation (Aerts et al., 1995; Pywell et al., 1995; Owen and Marrs, 2000; Allison and Ausden, 2004; Lawson et al., 2004).

In a previous study on fungal biomass development in a chronosequence of ex-arable land, we found several patches of healthy *Calluna* plants in a 10 year-old abandoned site within a vegetation dominated by weeds (Van der Wal et al., 2006b). No restoration techniques were applied on this site. The presence of *Calluna* is surprising as the prevailing soil conditions, as mentioned above, would favor the fast-growing plant species and suppress the spontaneous establishment of the slow-growing *Calluna* plants. The size of the *Calluna* patches (0.70 to 1.20 m diameter) in this ex-arable field indicated that they probably established soon after abandonment and have been able to maintain and extend among the ruderal weedy species and grasses.

The aim of this study was a hypothesis-generating field investigation. Factors that may allow spontaneous establishment of *Calluna vulgaris* plants in a recently abandoned agricultural field were determined. Ericoid mycorrhizal (ERM) fungi are known to provide their host with organic nitrogen (e.g. Bending and Read, 1997) and this association may be beneficial as net nitrogen mineralization is low in abandoned arable soils (Marrs, 1993; Malý et al., 2000; Van der Wal et al., 2006b). Therefore, we determined whether the *Calluna* plants on the ex-arable land were associated with ERM fungi. The association of ERM fungi with *Calluna* plants together with production/exudation of polyphenolic rich organic compounds can have a strong influence on nitrogen mineralization in such a way that *Calluna* plants may

be better able to exploit the organic nitrogen pool than grasses and forbs (Read, 1991). Therefore, we determined parameters related to organic matter quality and nitrogen availability both within and outside *Calluna* patches.

## Materials and methods

The abandoned agricultural field site (cultivated from 1850 till 1994), named Plantage Willem III, is located in the central part of the Netherlands (°N 51.98; °E 5.52) and has been described previously as part of a chronosequence study (Van der Wal et al., 2006b). Several patches of vital *Calluna* plants are distributed randomly over the field (about 30 patches in an area of 5 ha). In August 2005, we sampled 50 soil cores (3.5 cm diameter and 10 cm deep) within 10 *Calluna* patches chosen randomly (diameter patch > 70 cm), and soil cores (5) were bulked per patch. These soil samples are hereafter referred to as ‘*Calluna* ex-arable soil’. Another 500 g of soil was collected with a shovel within each patch to collect *Calluna* roots. In addition, at least 25 soil cores were randomly collected in two 30 x 30 m plots in the ex-arable field. Soil cores were bulked per plot. These soil samples are hereafter referred to as ‘non-*Calluna* ex-arable soil’. In a heathland that is situated in the near vicinity of the ex-arable field, soil was randomly collected in a 30 x 30 m plot and we separated the mineral soil (3-13 cm) and the organic layer (0-3 cm). Here, we also sampled another 500 g from 10 patches to collect *Calluna* roots. In order to determine the limiting nutrient for plant growth (N:P ratio, Koerselman and Meuleman, 1996), plant shoot material was sampled from 4 *Calluna vulgaris* patches and from a dominant grass (4 plants of *Holcus lanatus* L.) and a herb (4 plants of *Hypochaeris radicata* L.) that was found surrounding these patches.

Root samples were stained according to Massicotte et al. (2005) and observed using differential interference contrast microscopy with an Olympus BH2 light microscope to determine presence of fungal endophytes. Cleaned roots were analyzed for ergosterol content (extracted as described by De Ridder-Duine et al., 2006) to obtain an estimate of ericoid mycorrhizal fungal biomass (Olsrud et al., 2004).

To identify ERM species, DNA was isolated from soil samples and sequenced using PCR-DGGE analyses as described by Van der Wal et al. (2006a). Similarities between obtained sequences were compared with nucleotide sequences available in GenBank by using the nucleotide BLAST (blastn) program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Total carbon and nitrogen of soil was measured with a Carlo-Erba NA1500 C/N-analyzer after in situ HCl acidification procedure (Nieuwenhuize et al., 1994). Total phenolics were determined in a labile organic C pool (extraction by 5N H<sub>2</sub>SO<sub>4</sub>) as described by Rovira

and Vallejo (2002). Extraction in 0.5 M NaHCO<sub>3</sub> (P-Olsen) was used to have an indication of potentially available P. Concentration of total N and total P in plant tissue were measured by digestion of samples with a mixture of H<sub>2</sub>SO<sub>4</sub>-Se and salicylic acid (Novozamsky et al., 1984). Net nitrogen mineralization was determined as the accumulation of mineral N in field-moist soil during incubation for 3 months at 4°C and for 3 weeks at 20 °C. Soil mineral N was extracted as described by Van der Wal et al. (2006a). Ammonification of arginine (Arg) was used as an estimate for microbial activity potential (Alef and Kleiner, 1986). Protein binding capacity of the soil was determined by mixing 1 g fresh soil with 2 ml of a 400 µg ml<sup>-1</sup> milliQ ultrapure water solution of bovine serum albumin (BSA, Sigma). After incubation for 16 h at 4°C, samples were centrifuged at 3800 x g for 5 min and the quantity of protein in 125 µl supernatant was measured colorimetrically (Bradford, 1976). The amount of fixed protein was calculated from the difference between added and recovered protein.

Bacterial numbers were enumerated as described by Van der Wal et al. (2006a). Bacterial growth on arginine medium (pH 6.5) (1.0 g l<sup>-1</sup> arginine, 0.25 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.1 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.04 g l<sup>-1</sup> MgSO<sub>4</sub>\*7H<sub>2</sub>O, 0.02 g l<sup>-1</sup> CaCl<sub>2</sub>\*2H<sub>2</sub>O and 0.05 g l<sup>-1</sup> (Bacto) yeast extract) was determined to indicate possible differences in the ability of bacteria to grow on arginine collected from soil under and outside *Calluna* patches. After 0, 17, 25, 41 and 49 hours of incubation, the bacterial density was measured spectrophotometrically at 600 nm using a micro-plate-reader (Synergy<sup>TM</sup> HT, BIO-TEK).

## Results and discussion

Light microscopy revealed colonization of *Calluna* roots by fungal endophytes in both the ex-arable field and the heathland. Roots from both field sites contained a similar amount ( $P > 0.98$ ) of ergosterol namely  $93.90 \pm 27.83$  mg ergosterol kg<sup>-1</sup> dry roots in the ex-arable field and  $93.63 \pm 44.15$  mg ergosterol kg<sup>-1</sup> dry roots in the heathland. Ergosterol content in soil collected under *Calluna* patches was significantly greater than that of the rest of the ex-arable soil (Table 1). Dominant bands that were only present in the soil under *Calluna* patches and absent in the rest of the soil revealed, after sequencing,  $\geq 98\%$  homology to species in the order *Heliales*, namely the genera *Phialocephala* and *Rhizoscyphus* (formerly *Hymenoscyphus*) (Table 2). *Phialocephala fortinii* has been isolated from *Calluna* (Jumpponen and Trappe, 1998) and has been shown to influence its growth positively (Zijlstra et al., 2005). The mycorrhizal status of *Scytalidium lignicola* is not yet clear, however, it is phylogenetically related to *Rhizoscyphus ericae*, which does form ericoid mycorrhizae with plants in the families *Ericaceae* and *Epacridaceae* (Smith and Read, 1997; Hambleton and

Sigler, 2005). Bands present in the rest of the ex-arable soil were identified as *Marasmius oreades*, which is a common saprotrophic basidiomycete in grasslands. Hence, it seems evident that the *Calluna* roots in the ex-arable land were associated with endomycorrhizal fungi, and that the grasses and forbs did not have such an association. This association may have been essential for the establishment and expansion of *Calluna* in the ex-arable site. A closer look at the nutrient pool of the ex-arable sandy soil indicated that the potentially available phosphate level was rather high (Table 1, > 45 mg Olsen P kg<sup>-1</sup> soil, Sharpley and Withers, 1994), making competition between plants for phosphate unlikely. Available nitrogen levels of ex-arable sandy soils can, however, drop rapidly after abandonment which is due to leaching of nitrate and low net N mineralization rates as a result of low organic matter content (Marrs, 1993; Malý et al., 2000; Van der Wal et al., 2006b). In the ex-arable soil, plant tissue of *Calluna vulgaris* had the highest N: P ratio ( $9.26 \pm 0.60$ ), followed by the grass *Holcus lanatus* ( $5.30 \pm 0.54$ ) and the herb *Hypochaeris radicata* ( $3.35 \pm 0.45$ ,  $P < 0.05$ ). The N to P shoot ratio of < 10 of the grass and the herb in the ex-arable field implies that nitrogen is the limiting nutrient (Koerselman and Meuleman, 1996; Pegtel et al., 1996). Hence, associations of plants with N<sub>2</sub>-fixing microorganisms or mycorrhizal species that are especially specialized in the efficient take-up of nitrogen could have a selective advantage under such conditions. Ericoid mycorrhizal fungi can gain access to organic N sources, such as proteins and peptides, and provide their host with nitrogen (Bending and Read, 1996a,b; Bending and Read, 1997). Aerts and Chapin (2000) suggested that a N: P shoot ratio of about 10 indicates optimal conditions for plant growth and, with regard to this value, the *Calluna* plants in the ex-arable field have only a minor limitation of N, hypothetically due to the association with ERM fungi. Arbuscular mycorrhizal fungi are especially known for the improvement of the phosphorus nutrition of their host plant, but the successful contribution of arbuscular mycorrhizal fungi to the N nutrition of plants is still under discussion as the extra contribution of nitrogen transported via hyphae to the plant is small when mycorrhizal plant are compared to non-mycorrhizal control plants (Hawkins et al., 2000). Therefore, only plants which can form an association with endomycorrhizal fungi which provide them with nitrogen may have a nutritional benefit. In this field, it appeared that only *Calluna* plants had these associations.

The polyphenolic rich organic layer and the association with ERM fungi possibly play an important role for maintaining dominance of *Calluna* plants in mature heathlands (Read et al., 2004). This layer is, however, lacking in the ex-arable field. Yet, ERM fungi were present and net nitrogen mineralization and ammonification of arginine were both significantly lower in soil under *Calluna* patches than in the rest of the ex-arable field (Table 1); this points to a nitrogen conservation mechanism similar to that in mature heathland. However, unlike mature

**Table 1:** Soil characteristics, percentage bound BSA (= protein-binding capacity), ergosterol content, net N-mineralization, Arginine (Arg) ammonification (= potential N-mineralization) and bacterial properties (numbers and growth on arginine) of the fields under study (0-10 cm ex-arable land and 3-13 cm heathland).

Soil	Former crop	pH	% organic matter	C:N ratio	P Olsen mg kg <sup>-1</sup>	phenolics C kg <sup>-1</sup>	% bound BSA	Ergosterol mg kg <sup>-1</sup>	N-mineralization mg N kg <sup>-1</sup>	Arg ammonification mg N kg <sup>-1</sup>	Bacterial numbers	Bacterial growth on arginine (mOD)
<i>Calluna</i> ex-arable field	Tobacco, barley, rye	5.6	4.1	25.1	66.0	1.26	35.7	3.9*	0.9*	16.6*	3.6E+08	0.20
Non- <i>Calluna</i> ex-arable field	Tobacco, barley, rye	5.7	3.9	24.5	62.3	1.26	69.2	1.6	8.3	20.0	5.6E+08	0.22
Heathland	-	3.6	5.5	39.6	1.1	0.94	100	3.7	-1.0	0.3	1.6E+08	not measured

One-way ANOVA and one sample t test was performed to test for significant differences ( $P < 0.05$ ) between means of variables in *Calluna* ex-arable soil and non-*Calluna* ex-arable soil and between these two soils and the heathland soil. Welch's test was used to test difference between two groups with unequal group sizes. Asterisks denote significant differences between *Calluna* ex-arable soil and non-*Calluna* ex-arable soil.

**Table 2:** List of fungal 18S ribosomal DNA sequences isolated from *Calluna* ex-arable soil and non-*Calluna* ex-arable soil.

Soil	Genbank accession no.	% similarity to Genbank sequences	Species according to Blast search
<i>Calluna</i> ex-arable field	EF535224	99	<i>Phialocephala fortinii</i>
	EF535225	99	<i>Rhizoscyphus ericae</i>
	EF535228	99	<i>Phialocephala sphaeroides</i>
	EF535226	97	<i>Scytalidium lignicola</i>
Non- <i>Calluna</i> ex-arable field	EF535227	100	<i>Marasmius oreades</i>



heathlands, the low nitrogen mineralization is not due to the formation of resistant complexes of protein with phenolic compounds originating from *Calluna* plants, since the protein binding capacity of soil under *Calluna* was not different from that of the rest of the ex-arable soil (Table 1). In addition, the low nitrogen mineralization can also not be explained by different soil characteristics or different bacterial properties (Table 1). Therefore, the most likely explanation for the reduction of N-mineralizing microbial activity in soil from *Calluna*-patches is the presence of toxins produced by the plant and/or the fungus (Jalal and Read, 1983). The lower nitrogen mineralization in soil under *Calluna* and the possible presence of phytotoxic compounds are likely to have a negative impact on weeds, since less available nitrogen is present and seed germination may be inhibited.

Interestingly, the presence of high amounts of phosphate did not reduce the colonization of *Calluna* and ERM fungi, although they are normally growing in environments with low nutrient-availability (Read, 1991), and it is often suggested that their establishment on ex-arable sites requires a reduction of soil fertility (Marrs, 1993; Pywell et al., 1994). This suggests that a high concentration of soil phosphate is not posing a constraint to the colonization of *Calluna*. Soil stripping is often used as a restoration technique to achieve this goal (Aerts et al., 1995; Snow and Marrs, 1997). However, our study suggests this may not be always needed. In addition, *Calluna* plants are normally associated with acidic soils and soil acidification has been used to restore heathlands (Owen and Marrs, 2000; Lawson et al., 2004). Soil acidification under *Calluna* patches can, however, be ruled out as possible mechanism to explain their success here, since the soil pH was not significantly lower than in the surrounding area. Our results may indicate that the immediate availability of a seed inoculum and establishment of the symbiotic relation is sufficient to reach a *Calluna* dominated heathland on abandoned arable soils. However, this should be tested in controlled field trials and may not be applicable to all situations (e.g. see Owen and Marrs, 2000). The importance of sufficient seed sources of the desired plant species to restore a degraded system is recognized in many studies (e.g. Muller et al., 1998; Bakker et al., 2004; Walker et al., 2004). Mycorrhizal infection is not systemic and cells of roots have to be colonized by ERM fungi present in the soil (Harley and Smith, 1983). Therefore, introducing a fungal spore inoculum of ERM fungi may be important to establish the infection, but this is until now hardly acknowledged. Positive feedbacks between the host plant and the mycorrhizal fungi could have created a situation for *Calluna* to maintain in an unexpected environment, a formerly intensively managed arable land.

During the last few decades, Dutch *Calluna* heathlands have largely been replaced by grassland species and this is generally thought to be due to increased atmospheric deposition of ammonium and lack of management (Berendse and Aerts, 1984; Heil and Bruggink, 1987).

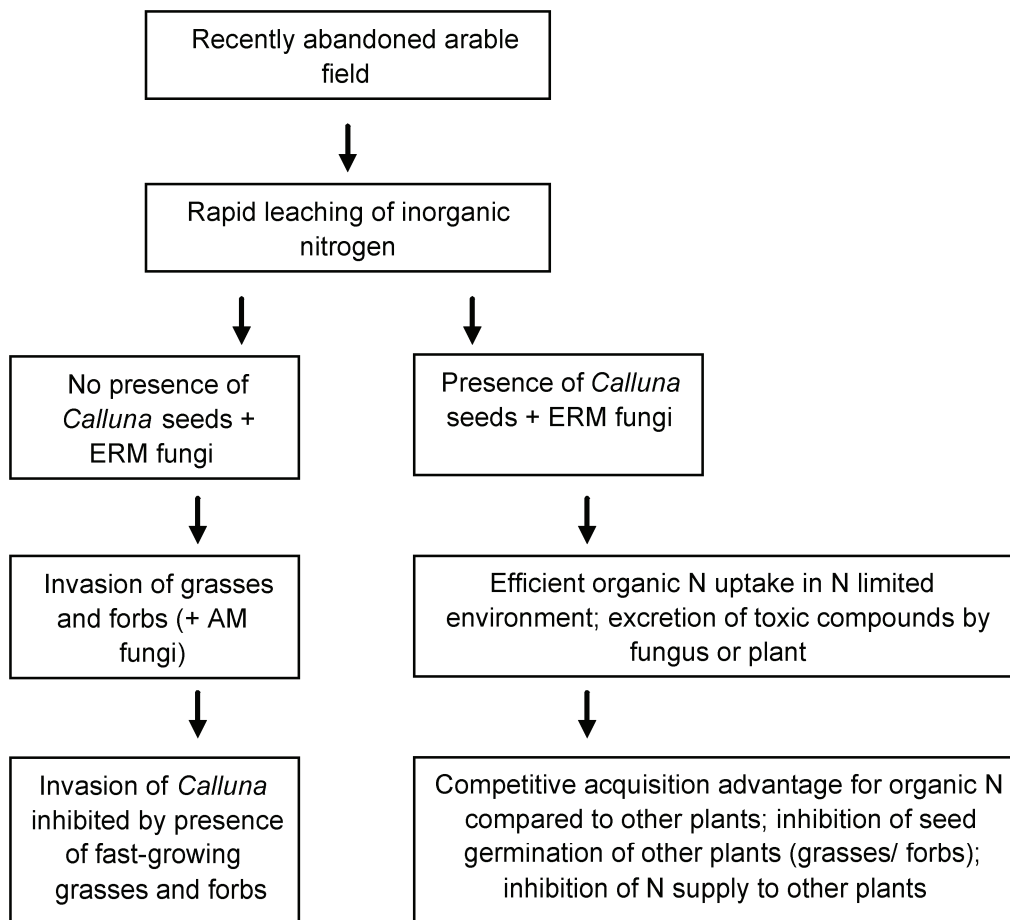
Surprisingly, in this field an opposite situation occurred; several patches of *Calluna* plants were growing within a dominant vegetation of grasses and forbs. We did not observe *Calluna* patches in other recently abandoned field. This appears to indicate that the appropriate conditions for spontaneous establishment of *Calluna* are rarely met.

## Conclusions

In summary, we hypothesize that *Calluna* plants in a recently abandoned arable field can maintain themselves between ruderal plants because of the association with (ericoid) mycorrhizal fungi that 1) offer a nutritional benefit for organic nitrogen, and/or 2) inhibit nitrogen mineralization by releasing toxins and/or 3) inhibit seed germination of other non-ericaceous plants by releasing phytotoxins (Fig. 1). It is important to mention that the time of colonization might be crucial for their successful establishment. Lawson et al. (2004) suggested that the absence of competition from ruderal plant species enhances the successful establishment of *Calluna* plants on ex-arable land. In this ex-arable field, the size of the *Calluna* patches (from 0.70 up to 1.20 m) suggests that they colonized the field soon after abandonment. We did not observe any seedlings of *Calluna* plants in the field, which may indicate that, once the ruderal plant species colonize the rest of the field, the establishment of new *Calluna* seedlings is not taken place anymore. Although spontaneous establishment of *Calluna* in recently abandoned arable lands appears to be rare, it may be used to develop novel management strategies to create *Calluna*-dominated heathlands on ex-arable land.

### Implications for Practice

- Immediately after abandonment of agricultural fields, the introduction of *Calluna* seeds or seedlings may result in a rapid restoration of heathland.
- The simultaneous introduction of ERM fungi may be a prerequisite for successful establishment of *Calluna* on ex-arable land. Soil of a mature heathland may be used to introduce both seed and ERM fungi.



**Figure 1:** Conceptual model for the establishment and maintenance of *Calluna* between ruderal plants in ex-arable fields.

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