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Growth release of stunted black spruce (*Picea mariana*) in *Kalmia* heath: The role of ectomycorrhizal fungi and near-ground microclimate

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Abstract

Naturally regenerating and planted black spruce (*Picea mariana* [Mill.] BSP) in post-fire landscapes in eastern Canada often exhibit stunted growth in the presence of ericaceous shrubs such as *Kalmia angustifolia* (L.). After a period of stunted growth, some seedlings experience a growth release exhibiting growth rates closer to normally growing seedlings. We hypothesized that an increase in colonization of root tips by ectomycorrhizal (ECM) fungi is responsible for this release and that the percentage of root tips colonized by ECM fungi would be higher on seedlings which had a released or normally growing neighbour within close proximity. We quantified ECM fungi diversity and abundance from 255 soil cores from stunted, released, and normally growing black spruce seedlings sampled in two *Kalmia* dominated sites in Newfoundland. Growth and microsite characteristics around each seedling were also measured. We found that normal and released seedlings had significantly higher proportions of ECM fungi root tips than stunted seedlings supporting our final hypothesis but there was no significant difference in distance between neighbours. Soil chemical properties are thought to inhibit the vegetative spread of ECM fungi species in this particular system and are identified as an important topic for further research.

18 Introduction

19 In thick ericaceous heaths conifer seedlings/saplings often experience stunted growth
20 referred to as ‘growth check’. In Sweden, extracts of *Empetrum hermaphroditum* L. litter have
21 been shown to interfere with the regeneration of *Pinus sylvestris* L. seedlings by reduced root
22 development, mycorrhizal colonization and N uptake (Nilsson *et al.* 1993). In western Canada,
23 *Gaultheria shallon* Purch has been reported to suppress conifer growth in clearcuts by out-
24 competing the tree seedlings for N and P (Messier 1993). Similarly, black spruce (*Picea mariana*
25 Mill. BSP) growth inhibition in the presence of ericaceous shrubs such as *Kalmia angustifolia* L.
26 (hereafter referred to as *Kalmia*) and Labrador tea (*Rhododendron groenlandicum* Oeder Kron et
27 Judd) has been reported from eastern and mid-Canada (Mallik 2001; Inderjit and Mallik 1996).
28 This phenomenon of tree seedling growth stunting is generally concomitant with a rapid growth
29 of the understory ericaceous plants after removal of canopy trees by forest fires or logging. The
30 post-disturbance dominance of ericaceous shrubs and poor tree seedling regeneration following
31 disturbances that remove the overstory canopy yet leave the soil relatively undisturbed may
32 cause vegetation shift from forest to heath (Mallik 2001). *Kalmia* exhibits vigorous vegetative
33 regrowth from basal sprouting and rhizomes; rhizomes may grow up to one metre in length in a
34 single growing season (Mallik 1993) and *Kalmia* can quickly dominate any suitable seedbed for
35 black spruce regeneration within the first four years after a fire (Mallik *et al.* 2010). Siegwart-
36 Collier and Mallik (2010) reported that successful germination and establishment of black spruce
37 seedlings require exposed mineral soil or thin post-fire residual organic matter up to 2 cm,
38 conditions that are rare after low intensity forest fires or clearcuts.

39 This lack of suitable seedbed is reflected in the drastic reduction of black spruce stem
40 densities in *Kalmia* heaths. In a study conducted in eight post-fire heaths, the mean stem density

of black spruce was found to be only 314 stems ha⁻¹, much lower than the 3000 stems ha⁻¹ required for canopy closure (Kravchenko 2012). However, some scattered black spruce seedlings can be found in post-fire *Kalmia* heaths with variable growth but a large majority of seedlings exhibit stunted growth. Krause *et al.* (2009) reported that the leader growth of juvenile black spruce in naturally regenerating post-fire stands is an increase of at least 10 cm per year and this can be used as a benchmark for the growth rate of normally growing trees. This growth rate of black spruce is not commonly observed in a *Kalmia* heath. However, over time some previously stunted black spruce seedlings may achieve this growth rate, a phenomenon call ‘growth release’. Past studies attributed various abiotic and biotic factors to inadequate black spruce regeneration and poor growth in *Kalmia* heath such as limitation of suitable seedbeds (Mallik *et al.* 2010), competition for limited soil N and P (Yamasaki *et al.* 2002) and allelopathy (Zhu and Mallik 1994). However, the mechanism of growth release of stunted black spruce in *Kalmia* heath remains unknown. It has been proposed that interactions with symbiotic fungi may be a driving force behind the growth release (Johnson *et al.* 2006).

Plenchette *et al.* (1983) have shown that in nutrient poor systems, the removal of mycorrhizal communities resulted in stunted growth of 16 different species and this growth check was corrected by re-inoculation with fungi. It has been shown that some ECM fungal species may be inhibited by *Kalmia* allelochemicals (Mallik and Zhu 1993; Yamasaki *et al.* 1998) leading to a deprivation of nutrients to the host, while the growth of other ECM fungi species may be stimulated as they utilize the phenolics as a carbon source (Zeng and Mallik 2006). The metabolizing of phenolics by ECM fungi reduces their concentration in the soil and lessens the inhibitory effect they have on black spruce root growth. The degree to which ECM fungi play a role in black spruce growth release in *Kalmia* dominated heaths is unknown.

Vegetative growth by mycelia can be classified into three broad categories: short, medium and long-range exploration mycelia, based on how far from the inoculum source a particular species may extend their mycelia to capture nutrients (Agerer 2001) and to colonize new root tips (Peay *et al.* 2011). The distribution and composition of ECM fungi communities depend on both biotic and abiotic factors (Nikonov *et al.* 2006). Other studies have shown that composition and abundance of ECM fungi are dependent on the abundance of host plants (Piotrowski *et al.* 2008) and their root density (Peay *et al.* 2011). Because host plants influence microsite soil chemistry and provide habitat for ECM fungi, their growth and distribution in the system should reflect the composition and distribution of local ECM fungi (Aponte *et al.* 2013). Based on the dendrochronology of black spruce seedlings in eight post-fire *Kalmia* heaths in Newfoundland, Kravchenko (2012) identified three distinct growth forms of black spruce: stunted, released and normal. From a positive correlation between the seedling height and microsite organic matter depth he speculated that with time increased ECM fungi abundance in the organic matter might help initiate the growth release of black spruce seedlings.

The benefits imparted by an ECM symbiotic relationship are numerous and this study aimed to determine if they were sufficient to initiate a growth release in stunted seedlings. Because the abundance and density of any particular ECM fungi species depends partly on its ability to disperse through soil from host to host, we assumed that a spatial pattern should exist among trees showing stunted, released and normal growth that reflects the degree to which their roots have been colonized. We hypothesised that i) a maximum lateral distance exists between inoculum source and new host (i.e. neighbouring seedlings) over which the local ECM fungi can spread to initiate a growth release, ii) soil microsite variables around normal and released seedlings would be more favorable to black spruce seedling growth and the spread of ECM

fungi, and iii) ECM fungi abundance will be higher in normal and released seedlings than stunted ones because their presence help the host seedlings acquire limited essential nutrients.

Materials and Methods

Study area and sites

We conducted this study in Terra Nova National Park in the Central Newfoundland ecoregion of the Boreal Shield ecozone. Here average January and July temperatures vary from -14° to -10 °C and 11° to 15 °C respectively (Natural Resource Canada 2011). The area receives 801 to 1200 mm average annual precipitation, of which approximately 30% is snowfall. The soils of the area are nutrient poor podzolic gravel-loam (Agriculture and Agri-Food Canada 1998). The study sites were 18 yr-old Spracklin Road (SR) burn (GPS 48.5577502 -54.0311050) and 31 yr-old Charlottetown (CT) burn (GPS 48.4508143 - 54.0056102) at 107 m a.s.l. Previous to fire, both sites were dominated by black spruce with *Kalmia* as the main understory species. Bloom (2001) determined fire severity indices (pre-fire drought code, burn days) were similar in both sites: 446 for SR burn and 412 for CT burn (Table 1). After fire both sites turned into *Kalmia* heath interspersed with black spruce and larch (*Larix laricina* Koch) seedlings and saplings. Other ericaceous shrubs such as *Rhododendron canadense* L. and *R. groenlandicum* were subordinate to *Kalmia*. Ground vegetation was dominated by pleurocarpous mosses such as *Pleurozium schreberi* Brid. Mitt. and *Dicranum polysetum* Swartz, and fruticose lichens such as *Clintonia borealis* Aiton. Raf. and *Cladina rangiferina* L. Both study sites were naturally regenerating after fire and experiencing the same climatic conditions as they are geographically close to one another. The sites experienced no other disturbance since the last fire and varied

only in time since last fire and organic matter depth (Table 1). Stunted and released seedlings were present in both sites.

Pre- and post-fire black spruce stem density

The black spruce stem densities for each site were recorded as well as those of other trees, which may act as alternate hosts for ECM fungi. Pre-fire black spruce stem densities were obtained from L. Siegwart-Collier based on densities in adjacent unburned stands (unpublished data). Post-fire stem densities were obtained by counting stems in a 100 m² area around each focal seedling/sapling (described below) and converting to stems ha⁻¹. Black spruce seedlings of same age can achieve different size because of growth differences, larger ones are saplings but hereafter for simplicity we use seedlings to refer both seedlings and saplings.

Seedling growth measurement

We measured 22 + 9 stunted, 19 + 7 released, and 2 + 11 normally grown black spruce seedlings from the Charlottetown (CT) and Spracklin Road (SR) study sites respectively. From each seedling we obtained a stem disc at the ground level to measure annual ring growth increments. In the laboratory we used WinDENDRO software (Regent Instruments, Canada) to calculate the annual ring widths. Mean ring width of the previous five years was taken to account for variations in year-to-year climate, which may have affected growth. Stem discs were also used to assess the age of each tree. After the mycorrhizal and non-mycorrhizal root counting the root samples were placed in an oven at 103 °C for 24 hrs and mean oven dry weight of root biomass was recorded for each seedling.

Seedling microsite characteristics

We measured microsite environmental conditions around the focal seedlings in a 1 m x 1 m quadrat to determine if the site conditions around the seedlings are associated with colonization of ECM fungi and the potential release of the seedlings. We recorded % cover of *Kalmia* and *R. canadense* because they were the most dominant plants in the community. We measured soil temperature, soil moisture and conductivity, 48 hrs after rainfall using a W.E.T. Sensor HH2 moisture metre (Delta-T Devices, Cambridge, England) at three locations along a diagonal line spanning the corners of the plot. This sensor measures the three parameters of the soil in direct contact with the probes, to a depth of 7 cm. Soil pH was also measured in three locations in 1:1 soil-water ratio using a portable pH metre (Spectrum Technologies Inc., U.S.A.). We measured organic matter depth at the time of soil core extraction (described below) to a maximum depth of 35 cm.

Extraction of mycorrhizal and non-mycorrhizal roots

Three soil cores were collected from under each tree's canopy using a 40 cm long, 5.5 cm diameter Eijkelkamp split tube sampler (Hoskin Scientific, Burlington, Ontario). Cores were obtained as far down as mineral soil if possible. Each soil core was placed in a labelled re-sealable plastic bag. In the laboratory the cores were soaked for 24 hrs to facilitate separation of roots from the soil mass. Then black spruce roots were extracted by gently washing and rubbing the soil cores under slow running water over a sieve and then placed in another re-sealable bag. Any remaining rhizosphere soil and unrelated roots were removed under a dissection microscope at 25× magnification. The black spruce root samples were kept at 4 °C until identification of ECM fungi species.

Identity, diversity and abundance of ECM fungi.

The root samples were placed in a Petri-dish and covered with water to a depth of ~1 cm and examined using a dissection microscope at 25× and 40× magnification. Initial identification was performed using morphological characteristics including colour, ramification, presence and structure of rhizomorphs and emanating hyphae, appearance of the mantle, autofluorescence under UV light, and the detection of a chemical colour change in the presence of aqueous FeSO_4 and/or a 10% KOH aqueous solution. This process resulted in the assignment of ECMs to nine morphotype groups, labelled A-J (letter E was assigned to uncolonized root tips as a control). Once identification of the morphotype groups was complete, we counted the mycorrhizal and non-mycorrhizal root tips systematically for each root from left to right of the dish. We recorded the mean number of mycorrhizal and non-mycorrhizal root tips from three soil cores around each seedling.

A minimum of three samples of each identified morphotype was collected for molecular identification. Fungal DNA was extracted from the root tips using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). DNA amplification and sequencing was performed in the Paleo-DNA lab at Lakehead University. The internal transcribed spacer 2 (ITS2) region was chosen as the target for amplification for both the soil and root tip DNA samples. Of the published primers targeting the fungal ITS region, none were outstanding, or outperformed the others, so custom forward (5' - CCTTGGTATTCCGAGGAGC - 3') and reverse (5' - CGTGTC AAAGACGGGATT - 3') primers were designed at the Paleo-DNA lab of Lakehead University. The optimal annealing temperature of 60°C was determined for the primers by running a gradient PCR. For all samples, the PCR amplification mixture included: 3 µl DNA template, 2.5 nmol of each primer, 12.5 µl AccustartTM II PCR SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), and distilled deionized water for a total volume of 25 µl. The

Accustart mix includes Taq DNA polymerase for a 'hot start' PCR. The thermocycling program for the Eppendorf Mastercycler thermocycler included an initial denaturation step at 96 °C for 2 minutes, followed by 50 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min, with the lid temperature held at 105 °C throughout. Successful amplification was confirmed in 2 % agarose gels stained with ethidium bromide. Soil samples that failed to amplify were subjected to an additional silica bead/guanidine thiocyanate purification protocol before repeating the PCR protocol.

Amplified DNA products were purified with the QIAquick PCR Purification Kit (Qiagen), and the ExoSAP-IT PCR Product Cleanup (Affymetrix, Santa Clara, CA, USA) for root tip DNA. An aliquot of 3 µl purified DNA template was labeled with BigDye Terminator Cycle v3.1 Sequencing Kit, and sequenced on a 16-capillary ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). All sequences were visually inspected and machine errors manually edited with SEQUENCHER software (v5.3; Gene Codes Corporation, Ann Arbor, MI, USA) before carrying out BLASTn searches on the NCBI and UNITE online sequence databases (Appendix I). Multiple alignments were carried out using MAFFT v.7.211 (Katoh 2013) with default options, and viewed with Seaview v4.5.4 (Guoy *et al.* 2010), to verify that the ITS2 region was being amplified by confirming the presence of the adjacent highly conserved 5.8S region in each sequence.

Spatial pattern of stunted, released and normal seedlings

We used a randomized complete block design to select the specimen seedlings for this study with the two sites as blocks. Within each site we placed three 150 m transects at random bearings at least 25 m between the two adjacent transects. At every 15 m along each transect we

sought a black spruce seedling within a 10 m radius; that black spruce seedling was selected as the focal seedling. If no black spruce was found at a point the process continued on to the next 15 m of the transect until 10 focal black spruce seedlings were selected per transect. Each focal black spruce seedling was assigned a growth type (normal, released or stunted) based on its appearance (height, inter-nodal distance and length of current year's leader; (Krause *et al.* 2009)). In a 5.6 m radius around each focal seedling, all other black spruce seedlings were considered as neighbours and assigned growth type; the distance from the focal seedling was measured and their growth characteristics recorded. The presence of balsam fir (*Abies balsamea* L.) and birch (*Betula spp.*) as neighbours was avoided in this assessment because many of them had been browsed heavily by moose (*Alces alces* L.) and above ground herbivory would affect below ground growth and therefore the magnitude of colonization by ECMs (Piippo *et al.* 2011). Therefore, the presence of their roots in soil cores was deemed to be unlikely. We initially considered *L. laricina* seedlings as they may act as potential alternate hosts for ECM fungi. However, the morphological distinction of roots belonging to *P. mariana* and *L. laricina* was difficult and therefore all spruce focal trees that had a larch neighbour were removed from the sample set. This deletion of soil cores from the dataset was performed only after we had detected the presence of host specific ECM fungi associated with *L. laricina*.

Statistical analysis

We used one-way ANOVA on the distance between neighbours, their mean colonization and the time interval between release events for the focal seedling and its neighbour, followed by a Tukey *post hoc* test to determine if there was a significant difference between the four seedling growth relationship types (normal-released, released-released, released-stunted, stunted-stunted). Separate ANOVAs were performed followed by Tukey *post hoc* tests to determine if any

significant difference existed among the three black spruce seedling growth types (normal, released and stunted) in terms of percentage of mycorrhizal root tips, annual ring width, oven dry weight of root mass extracted and microsite variables (organic matter depth, soil pH, moisture, and temperature). Square root transformations were performed to meet assumptions of normality for annual ring width and mycorrhizal root tips. All statistical tests were performed using R version 2.15.3 (R Core Team, 2013).

Results

Pre- and post-fire black spruce stem densities

Black spruce stem densities in both sites dropped considerably after fire (Table 1). The SR site had lower overall black spruce stem density but a higher proportion of normal and released seedlings than the CT site (Figure 1).

Seedling growth measurement

For the SR site, normal and released seedlings had significantly higher annual ring widths than stunted seedlings ($p = 0.01$, 0.004 respectively) and the same held true for the CT site ($p = 0.04$, 4.5×10^{-4} respectively). Significant difference in annual ring width was found between normal and released seedlings in the SR site, but not the CT site (Table 2). When the growth types are compared between the sites, released seedlings in the CT site had a significantly higher annual ring width than released seedling in the SR site ($p = 0.01$). Correlation between annual ring width and seedling growth type justified assignment of growth type based on physical

appearance of the seedling (Pearson correlation coefficient, $r = 0.455$). We found no significant difference in age or root dry weights among the seedling growth types (Table 2).

Seedling growth, ECM colonization and microsite characteristics

Within each site, microsite characteristics varied little among normal, stunted and released seedlings. Organic matter depth, and soil pH, moisture, and temperature were similar in the area surrounding each seedling (Table 2). There was also no significant difference in % cover of *Kalmia* among the seedling growth types for either site. Normal seedlings had significantly higher ECM fungi colonization ($p = 3.8 \times 10^{-5}$) and annual ring width ($p = 7.6 \times 10^{-4}$) than stunted seedlings. The same trend held for released seedlings having higher ECM colonization ($p = 1.2 \times 10^{-6}$) and annual ring width ($p = 2.3 \times 10^{-6}$) than stunted seedlings (Table 2). Both normal and released seedlings of the CT site had higher ECM fungi colonization than the normal and released seedlings in the SR site ($p = 0.03$) (Table 2).

Identity, diversity and abundance of ECM

We analysed ECM fungi root tips for 13 normal, 26 released, 31 stunted black spruce seedlings for a total of 17 550 fungal root tips. Due to the cryptic nature of ECMs, the assignment of morphotypes to groups labelled A and B included a total of 5 species of the genus *Cortinarius*. Samples included in morphotype groups H and I encompassed two species of the *Lactarius* genus. Therefore, root tip counts for those morphotype groups were combined and all analyses were performed at the genus level. One morphotype was identified as *Suillus cavipes* which is thought to exclusively form on the roots of *L. laricina*, and very occasionally *Pinus sylvestris* L. (Finlay 1989). Therefore all soil cores containing that morphotype were removed from the data set so as to not include *L. laricina* roots in the analysis. The genus *Leccinum* is

thought to be composed of mostly host-specific species that form associations with trees mainly belonging to Betulaceae, Salicaceae, and Fagaceae, although *Leccinum piceinum*, was reported to form associations with *Picea* spp. (den Bakker *et al.* 2004). Our study sites contained little or no *Populus* sp. and *Betula* sp. and so the presence of their roots in our soil cores is very unlikely. Abundances of all genera showed no clear trend among the growth sites, or within growth types between sites. The wide range of ECM fungi colonization observed in individual seedlings made the differences non-significant (Table 4). There was a significant difference in which genera were most abundant between sites, with *Cortinarius* being most abundant in normal seedlings in the younger SR site and *Tylospora* being most abundant in released seedlings in the older CT site.

Spatial patterns of stunted, released and normal seedlings

The selection of a released neighbour within 5.6 m of the focal seedling resulted in 33 focal seedling-neighbour relationships with the other 17 focal seedlings lacking a black spruce neighbour, reflecting the poor recruitment of black spruce in the post-fire *Kalmia* heath. Due to low sample sizes within sites, samples were pooled for both sites. Seedlings in normal-released pairs had significantly higher colonization rates than those in a stunted-stunted pair (Table 3). When considering the distance separating two individuals in a growth type pair, we found no significant difference among any growth type pair ($p = 0.47$) (Table 3). We did however find a significant difference between the growth type pairs in the years between release events. We considered only two growth type relationships (released - released and released - stunted) for this comparison because normal seedlings would not experience release and there are no release events to consider in a stunted - stunted pair. For a released - released pair, both release events occurred within approximately two years of each other; in a released - stunted pair, we observed

a significantly higher time interval ($p = 0.03$) in which the second release event has yet to occur
Mean organic matter depth did not vary significantly between relationship pairs (Table 3).

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Discussion

Growth release of black spruce seedlings observed in *Kalmia* heath appears to be linked to
the percentage of ECM fungi colonization of their root system, which supports our third and
main hypothesis (Table 2). As predicted, the normal seedlings had a higher percentage of their
root tips colonized by at least one type of ECM and this trend holds for the released seedlings as
well. Higher levels of ECM fungal colonization are associated with favourable seedling growth
and this is evident in the annual ring widths of normal and released seedlings having wider
annual rings (Table 2).

As reported in Siegwart-Collier and Mallik (2010) post-fire residual organic matter (ROM)
creates a seedbed filter and the strength of the filter is dependent on its thickness. The SR site is
characterized by lower OM depth (5 cm) compared to the CT site (15 cm). Black spruce seeds
germinating in the SR site would have experienced better initial growing conditions than those in
the CT site with thicker OM. This is reflected in the higher proportion of stunted seedlings in the
CT site (Fig. 1), which can be interpreted as a lower number of germination safe sites (Harper
1977). One can conclude from this that overall black spruce growth would be better in an area
that has a more suitable seedbed for germination and lower amounts of allelopathic phenolics
(i.e., lower OM depth and lower *Kalmia* litter) however this is not the case here. The CT site had
higher OM depth and *Kalmia* cover than SR site (15 cm vs. 5 cm and 44% vs. 30% respectively;
Table 1), which are conditions that are less favourable for black spruce seedling growth.
However, this site also possesses characteristics that favour the growth and colonization of ECM

fungi, such as higher densities of potential hosts (black spruce seedlings), thicker OM allowing for niche differentiation among ECM species (Bahram *et al.* 2015) and a longer time since fire. The normal and released seedlings of the CT site, although lower in numbers (Fig. 1), possess higher rates of colonization than those in the SR site, and also exhibit similar if not significantly higher annual growth increments (Table 2). So while conditions in the CT site may be more severe for black spruce in the beginning, if a high percentage of their roots are colonized by ECM fungi, the characteristics of the site (which favour ECM fungi development) determine that the benefits imparted by ECM fungi are greater than the inhibition imposed by *Kalmia* phenolics.

Another interpretation that leads to a similar conclusion is that lower colonization rates in a site which has more favourable growing conditions is that the host seedlings are resisting infection by fungal mycelia, as this has been shown in many systems. It has been shown that carbohydrate allocation to fungal symbionts might be regulated by the host based on nutrient availability (Wright *et al.* 2000; Nehls *et al.* 2007). Trees growing in harsh conditions may allocate more photosynthates to symbionts and they are rewarded with better growth because of increased ECM fungi colonization. Those growing in less stressful conditions may not supply as much carbohydrate to their mycorrhizae; a trade-off between photosynthate retention and soil nutrient uptake that results in lower growth rates. Regardless of the events leading to colonization, we observe better seedling growth in conditions which should be more inhibiting, but only in seedlings which experience high ECM fungi colonization rates (> 50%), leading us to conclude that the benefits imparted to black spruce by ECM fungi in a *Kalmia* dominated heath are indeed positive but that the magnitude of the benefit is site dependent. It is important to note that the improved growth of individual black spruce through ECM fungal colonization does not wholly solve the problem of poor recruitment in *Kalmia* heaths. Even if every seedling

experienced a release event, stem densities would still be insufficient for canopy closure, a fact that is related to safe site limitations immediately after fire (Kravchenko 2012).

Despite the evidence that ECM fungi colonization is a driving force in the growth of seedlings in heaths, there was no significant difference in the distance that separated two individuals of a growth type pair (Table 3). The first hypothesis that a pattern exists among black spruce seedlings of the three growth types (normal, released and stunted) and should be reflected in their ECM fungi community was not supported. Therefore, we cannot conclude that proximity to an inoculum source alone drives colonization of ECM fungi in black spruce seedlings in a *Kalmia* dominated heath. This is contrary to other studies showing that neighbouring trees or shrubs act as sources of inoculum and increases the chance of colonization of an individual by ECM fungi (Cline *et al.* 2005; Hubert and Gehring 2008; Bai *et al.* 2009).

One could speculate that the seedlings in a released - released relationship once existed as a released - stunted relationship and that the distance between them was eventually covered by ECM fungi to initiate the release of the stunted seedling. This is not an unreasonable assumption as multiple studies have shown how mycorrhizal species richness and abundance increases with time following a disturbance (e.g. Smith *et al.* 2002), including this study where the seedlings in the older site had higher percentages of root tips being mycorrhizal (Table 2). The similarity in the mean distances between neighbours of any relationship (Table 3) could suggest that either i) time is a factor and that stunted seedlings within an approximate 5.6 m radius are more likely to experience a release event in the near future or that ii) some other factor(s) exist that inhibit the colonization of a stunted seedling despite its proximity to a released neighbour. When considering the time interval between the release events (Table 3), the release events between released - released pair occurred within a shorter time frame than a released - stunted pair. For

released - stunted pairs, assuming that the time interval between the release events is open-ended, we see a mean time of approximately four years in which a second release event has yet to occur. The similarity in distance between individuals, accompanied by dissimilarity in time between release events, lends more weight to the argument that microsite conditions, rather than time, are exerting an influence on ECM fungi dispersal. Microsite conditions may create this time lag by slowing or inhibiting the spread of fungal mycelia.

Root mass potential of host seedlings influences ECM colonization because of varying strategies of vegetative growth in ECM fungi. Those with contact or short exploration types, *sensu* Agerer (2001) (i.e. limited vegetative spread), are restricted to areas where root density is high whereas the ones with extensive hyphal spread are better able to disperse from the source of inoculum to colonize other seedlings. This is an important trait as above ground black spruce stem density of the study areas dropped from 5100 and 3240 stems ha⁻¹ (L. Siegwart-Collier, unpublished data) to 188 and 273 stems ha⁻¹ measured 18 and 31 yrs after fire respectively in the two sites (Fig. 1), meaning that host root availability for ECM fungi colonization also dropped. Four out of six genera identified are described as being medium (*Cortinarius*, *Piloderma*, *Tomentellopsis*) - or long-range (*Leccinum*) exploration type, the remaining two (*Tylospora* and *Lactarius*) are described as being contact or short distance exploration types (Agerer 2001). Long range foraging and reproduction may reflect an ideal strategy when hosts are sparse and separated by long distances. Indeed, *Lactarius* ECM were only found on trees with a neighbour within the 5.6 m radius. Yet consideration must be given to the lack of a spatial pattern of colonization rates among neighbouring seedlings reported, suggesting that even if a host is close by, dispersal may still be inhibited.

Therefore, soil environment becomes the determining factor of whether or not mycelia of ECM fungi can spread to colonize a new host should a potential host be present within the theoretical range of ECM fungi mycelia, which can be up to several decimetres (Agerer 2006). The second hypothesis that microsite conditions influenced the abundance of ECM fungi root tips was also not supported because we found no significant difference in the soil variables measured around the seedlings (Table 2). ECM fungal vegetative growth is dependent on factors such as nutrient availability and pH (Wallander *et al.* 1999), and concentration of allelochemicals (Mallik and Zhu 1993). Toljander *et al.* (2006) showed that plant litter chemistry significantly influences the distribution of ECM fungi in soil and Joannis *et al.* (2008) showed that *Kalmia* litter increased the amounts of nitrogen sequestered as tannin-protein complexes, making nitrogen more readily available to its own ericoid mycorrhizae and less to those ECM fungi associated with black spruce. Yamasaki *et al.* (1998) showed that *Kalmia* phenolics may inhibit the colonization of ECM fungi in black spruce and this restriction by *Kalmia* litter may make it difficult or even impossible for ECM fungi to expand their mycelia and colonize new hosts. Conversely, Zeng and Mallik (2006) showed that the growth of the ECM *Paxillus involutus* was stimulated by the presence of *Kalmia* phenolics, highlighting a contrasting response among ECM fungal groups to the phenolic acids found in the soil.

Although genera identified in this study were not among those used in studies examining ECM growth in the presence of *Kalmia*, *Cortinarius* and *Piloderma* are among taxa listed as being able to utilize proteins as a source of N in nitrogen poor conditions (Lilleskov *et al.* 2002). This would provide a significant competitive advantage if they were able to access sequestered organic nitrogen in the form of protein-tannin complexes. Also, it has been suggested by Clemmensen *et al.* (2015) that ECM fungi producing rhizomorphs (i.e. medium- and long-range

exploration types) such as *Cortinarius* and *Piloderma* use N more efficiently as they are able to recycle their mycelial necromass. The results of this study support this idea as *Cortinarius* and *Piloderma* are the two most abundant ECM on all three growth types whereas *Lactarius*, characterized by a lack of rhizomorphs and by the inability to use protein or its necromass as an N source, is the least abundant (Table 4). For this reason, it is thought that microsite characteristics not accounted for (phenolic content, nutrient status) might influence the vegetative growth of ECM fungi and the abundance of ECM fungal root tips in black spruce. The high phenolic content and low nutrient levels of *Kalmia* heath soils may be acting as a filter, and thus be responsible for the relatively low ECM species richness found in our study compared to studies conducted in other boreal systems (Robertson *et al.* 2006). Alternatively, it could be a result of low overstory tree species diversity, which has been shown to influence ECM fungal diversity (Kernaghan *et al.* 2003). Since we used a custom designed primer, it is possible that it did not amplify ascomycetes ITS regions, explaining their absence in the data. However, considering that ascomycetes are reported as being low in abundance despite their common occurrence (Robertson *et al.* 2006; Kernaghan *et al.* 2003), we may assume that their contribution to growth release is not as important as basidiomycetes. Nonetheless, we acknowledged this shortcoming and suggest that the use of the universal primer ITS1-F (Gardes and Bruns 1993), instead of our custom designed primer, might have detected ascomycetes sequences in some of our root tip samples.

Conclusions

The dominance of *Kalmia* following low severity fires has a direct negative effect on natural regeneration of black spruce. Regardless of the specific mechanisms of inhibition involved, the environmental conditions of a *Kalmia* dominated heaths result in low black spruce recruitment and the majority of the recruited seedlings exhibit stunted growth at some point in their lives. We found evidences that support the view that black spruce seedling growth release is associated with higher ECM fungi colonization. An abundance-distribution pattern of the ectomycorrhizal community in relation to black spruce growth could not be detected. However, importance of ECM fungi to black spruce in *Kalmia* heaths is a significant factor. Soil properties which limit or promote ECM fungi vegetative spread should be studied in depth on a per species basis to identify which ECM fungi are best suited to ericaceous soils. This will in turn facilitate the restoration of black spruce forests in *Kalmia* heaths.

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Figure Caption

Fig. 1. Stem density of normal, released, stunted (stressed) and total black spruce seedlings recorded in Spracklin road (SR) and Charlottetown (CT) study sites.

Draft

Table 1. Fire history, pre- and post-fire vegetation and soil characteristics of the study sites in Spracklin Road and Charlottetown, Newfoundland.

	Site	
	Spracklin Road	Charlottetown
Time since fire (yrs)	18	31
Fire severity index	466	412
<i>P. mariana</i> stem density (stems ha ⁻¹)		
Pre-fire	5100	3240
Post-fire	188	273
<i>Kalmia</i> cover (%)*	30 ± 18 ^a	44 ± 17 ^b
Organic matter depth (cm)*	5 ± 3 ^a	15 ± 7 ^b
Soil pH (H ₂ O)*	2.9 ± 0.4	2.9 ± 0.3
Soil moisture (% vol)*	13.1 ± 5.5 ^a	27.9 ± 6.7 ^b
Soil temperature (°C)*	21.7 ± 1.1 ^a	18.8 ± 1.7 ^b

*Values are mean ± 1 S.D of measurements taken at the base of sampled seedlings during the month of July. Soil pH, moisture and temperature measured at a depth of 7 cm. Sample sizes for soil parameters were *n* = 55 (SR) and *n* = 43 (CT)

Table 2. Growth measurements, ECM colonization and microsite variables around normal, released and stunted black spruce seedlings in Spracklin Road and Charlottetown study sites.

Values are reported as mean $\pm$ 1 S.D.

Variable	Spracklin Road			Charlottetown		
	Normal	Released	Stunted	Normal	Released	Stunted
	(n = 11)	(n = 7)	(n = 9)	(n = 2)	(n = 19)	(n = 22)
Annual ring width (mm)	1.8 $\pm$ 0.5 ^a	0.9 $\pm$ 0.3 ^{b†}	0.5 $\pm$ 0.1 ^c	1.8 $\pm$ 0.5 ^a	1.4 $\pm$ 0.4 ^a	0.7 $\pm$ 0.6 ^b
Age (yrs)	11 $\pm$ 2	11 $\pm$ 2	11 $\pm$ 3	20 $\pm$ 5	20 $\pm$ 5	17 $\pm$ 4
Oven dry weight of extracted roots (g)	1.7 $\pm$ 3.0	1.7 $\pm$ 2.0	1.3 $\pm$ 1.5	0.10 $\pm$ 0.01	0.9 $\pm$ 0.7	0.3 $\pm$ 0.4
ECM colonization (%)	54 $\pm$ 9 ^{a†}	52 $\pm$ 13 ^{a†}	41 $\pm$ 11 ^b	69 $\pm$ 2 ^a	63 $\pm$ 12 ^a	38 $\pm$ 13 ^b
Kalmia cover (%)*	21 $\pm$ 13	24 $\pm$ 18	28 $\pm$ 24	65 $\pm$ 35	38 $\pm$ 13	46 $\pm$ 15
Organic matter depth (cm)*	5.0 $\pm$ 3.3	4.2 $\pm$ 2.5	4.4 $\pm$ 3.5	24.3 $\pm$ 12.3	14.3 $\pm$ 5.5	15.3 $\pm$ 8.2
Soil pH (H ₂ O)*	2.9 $\pm$ 0.5	2.9 $\pm$ 0.3	2.9 $\pm$ 0.5	2.9 $\pm$ 0.1	3.0 $\pm$ 0.1	2.9 $\pm$ 0.4
Soil moisture (% vol)*	10.6 $\pm$ 71.9	13.3 $\pm$ 4.0	15.4 $\pm$ 7.9	26.6 $\pm$ 5.7	26.1 $\pm$ 6.4	30.4 $\pm$ 6.7
Soil temperature (°C)*	21.6 $\pm$ 1.0	21.4 $\pm$ 1.0	22.1 $\pm$ 1.1	18.2 $\pm$ 1.8	18.7 $\pm$ 1.9	19.0 $\pm$ 1.7

Unlike letters indicate significant difference within sites among the three growth types ($p < 0.05$).

† Represents significant difference between sites for individual growth types ($p < 0.05$);

*Organic matter depth, soil pH, soil moisture and soil temperature were measured just before soil core extraction during the month of July. Soil pH, moisture and temperature measured at a depth of 7 cm. Sample sizes for soil parameters were $n = 55$ (SR) and $n = 43$ (CT).

Table 3. Variables among neighbours used to examine spatial patterns in black spruce growth types. Growth type pairs are pooled between the two sites ($n = 33$). Values are reported as mean ± 1 S.D.

	Normal –	Released –	Released –	Stunted –
Variable	Released	Released	Stunted	Stunted
ECM colonization (%)	57 $\pm$ 18 ^a	52 $\pm$ 11 ^{ab}	48 $\pm$ 8 ^{ab}	40 $\pm$ 6 ^b
Distance between neighbours (m)	2.3 $\pm$ 0.9	3.8 $\pm$ 1.1	3.6 $\pm$ 1.6	3.3 $\pm$ 1.4
Years between release events	-	1.67 $\pm$ 1.73 ^a	3.50 $\pm$ 1.91 ^b	-
Organic matter depth (cm)	9 $\pm$ 5	7 $\pm$ 5	7 $\pm$ 4	4 $\pm$ 3

Unlike letter(s) in each line denote a significant difference among growth type pairs ($p < 0.05$).

Table 4. Percentage of ECM root tips to total root tips counted (ECM + uncolonized) in normal, released and stunted black spruce seedlings.

ECM genera	Spracklin Road			Charlottetown		
	Normal	Released	Stunted	Normal	Released	Stunted
<i>Cortinarius</i>	35.8 ±	20.0 ±	17.3 ±	0.7 ± 1.0	21.2 ±	8.4 ± 8.5
	13.2 ^{a†}	20.6 ^{ab}	16.3 ^b		24.5	
<i>Piloderma</i>	4.6 ± 6.7	12.7 ±	9.2 ±	8.6 ± 4.1	4.8 ± 7.0	8.5 ± 12.6
		15.2	14.3			
<i>Tylospora</i>	4.3 ± 6.2	4.6 ± 5.9 [†]	2.1 ±	28.1 ± 36.1	21.0 ±	9.1 ± 8.4
			4.5		16.9	
<i>Tomentellopsis</i>	9.5 ± 8.9	9.8 ±	8.4 ±	23.3 ± 32.2	14.6 ±	9.0 ± 9.5
		11.0	10.9		15.7	
<i>Leccinum</i>	0	4.5 ±	3.8 ±	8.5 ± 12.0	0.8 ± 1.0	2.3 ± 6.1
		17.3	13.3			
<i>Lactarius</i>	0	1.5 ± 5.9	0	0	0.3 ± 1.0	0.8 ± 2.0
Uncolonized	45.8 ±	47.0 ±	59.2 ±	30.8 ± 2.2 ^a	37.3 ±	62.4 ± 12.9 ^b
	9.3 ^{a†}	13.2 ^{a†}	11.4 ^b		11.8 ^a	

Unlike letters indicate significant difference within sites among the three growth types ($p < 0.05$).

† Represents significant difference value between sites for individual growth types ($p < 0.05$).

Fig. 1

Appendix I

Identification of ECM morphotypes via DNA sequencing and NCBI/UNITE database matches.

Morphotype	Species	Sequence length	GenBank accession #	Identity (%)	E-value
A	<i>Cortinarius laetus</i>	383	FJ157034	98	0
	<i>Cortinarius sp. 1</i>	375	HM044581, JN544449, FR852018	99	0
B	<i>Cortinarius brunneotinctus</i>	417	HQ604691	99	0
	<i>Cortinarius cinnamomeus</i>	386	FJ157025	99	0
	<i>Cortinarius sp. 1</i>	329	HM044581, JN544449, FR852018	99	2.00E-164
	<i>Cortinarius sp. 2</i>	380	DQ481782	97	6.00E-180
C	<i>Piloderma sphaerosporum</i>	432	JQ711875	99	0
D	<i>Tylospora fibrillosa</i>	325	JQ712008	99	3.00E-163
F	<i>Tomentellopsis submollis</i>	451	AY641459, AM086447, AJ893354	99	0
G	<i>Leccinum sp.</i>	431	HQ433154, KC552011	97	0
H	<i>Suillus cavipes</i>	455	JF899572	98	0
I	<i>Lactarius sp.1</i>	333	AF506411	99	3.00E-163
J	<i>Lactarius sp.1</i>	379	AF506411	99	0
	<i>Lactarius sp.2</i>	405	DQ481782	97	0