

Article

# Pathogenicity of *Neonectria fuckeliana* on Norway Spruce Clones in Sweden and Potential Management Strategies

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**Abstract:** The fungus *Neonectria fuckeliana* has become an increasing problem on Norway spruce (*Picea abies*) in the Nordic countries during recent years. Canker wounds caused by the pathogen reduce timber quality and top-dieback is a problem for the Christmas tree industry. In this study, four inoculation trials were conducted to examine the ability of *N. fuckeliana* to cause disease on young Norway spruce plants and determine how different wound types would affect the occurrence and severity of the disease. Symptom development after 8–11 months was mainly mild and lesion lengths under bark were generally minor. However, *N. fuckeliana* could still be reisolated and/or molecularly detected. Slow disease development is in line with older studies describing *N. fuckeliana* as a weak pathogen. However, the results do not explain the serious increased damage by *N. fuckeliana* registered in Nordic forests and Christmas tree plantations. Potential management implications, such as shearing Christmas trees during periods of low inoculum pressure, cleaning secateurs between trees, and removal and burning of diseased branches and trees to avoid inoculum transfer and to keep disease pressure low, are based on experiments presented here and experiences with related pathogens.

**Keywords:** *Picea abies*; Nordic countries; canker; microconidia; inoculation; lesion length; infection

## 1. Introduction

Neonectria canker, caused by the fungus *Neonectria fuckeliana* (C. Booth) Castl. & Rossman, is a disease of Norway spruce (*Picea abies* (L.) Karst.) and other spruce species. For the Nordic forestry sector, Norway spruce is of great economic and ecological importance. Norway spruce is also the dominant Christmas tree species in Sweden, accounting for more than 50% of sales. Neonectria canker can diminish the value of Norway spruce by causing stem defects of timber trees or top-dieback of several branch whorls (Figure 1), the latter being especially destructive in Christmas tree fields [1]. In addition to dark canker wounds and dying tops (Figure 1), symptoms caused by *N. fuckeliana* on Norway spruce often include heavy resin-flow [2,3]. Affected trees may become more vulnerable to insect pests and decay fungi, as well as susceptible to breakage by wind, snow, and ice. Infections may also result in slower growth. Mortality of young trees may occur but, more commonly, the terminal leader shoots along with the top 3–4 whorls die [1].



**Figure 1.** Damage caused by *Neonectria fuckeliana* on Norway spruce (*Picea abies*) in the Nordic countries: dark necrotic canker wounds on trees in a Norwegian forest stand (**left** and **middle**, red arrows point towards clusters of perithecia of *N. fuckeliana* in or around the canker wound), and top-dieback including several branch whorls in a Swedish Christmas tree field (**right**). Photos: Martin Pettersson.

Signs of the fungus are globose, red fruiting bodies (perithecia) on the bark of dead stems, branches, and in and around canker wounds, mainly on larger dying or dead trees. Despite the small size (300–400  $\mu\text{m}$  diam.), they are visible to the naked eye. *Neonectria fuckeliana* has three spore states for colonizing new host trees [4–6], where the ascospores (sexual state) are produced inside the perithecia are likely the major dispersal propagules [4,6]. Under humid conditions, ascospores are forcibly ejected through a small opening (ostiole) on the top of the perithecia and distributed by wind. Asexual conidial pustules (sporodochia) are rarely seen in nature and the significance of this state in the life cycle is not fully understood. Two types of conidia are produced on the sporodochia: microconidia (*Acremonium* state) and macroconidia (*Cylindrocarpon* state), the former is massively produced in culture. Observations from related fungal canker pathogens, for example, *Neonectria ditissima* (Tul. & C. Tul.) Samuels & Rossman have shown that both macro- and microconidia are important for short-distance dispersal [7].

The main infection pathways are via openings such as pruning wounds, dead branches, branch stubs, or cracks due to wind or frost [4–6]. The infection rate of *N. fuckeliana* increases when large numbers of ascospores or conidia land on fresh wounds under moist weather conditions with temperatures between 15–25 °C [4,8]. For radiata pine (*Pinus radiata* D. Don) stands in New Zealand, where *N. fuckeliana* is an aggressive pathogen, successful infection by *N. fuckeliana* depends on the time of year that the trees are pruned [9] and on host genetics [10]. *Neonectria fuckeliana* has previously not behaved as an aggressive pathogen on Norway spruce in the Northern Hemisphere. In older European studies conducted on larger Norway spruce trees, *N. fuckeliana* was regarded as a weak pathogen or a saprophyte [5,11–13]. However, the current situation with *N. fuckeliana* in Finland [2,3], the other Nordic countries [14], and in Northern Ireland (Richard O’Hanlon personal communication) [15] indicates that changes have taken place. In the Nordic countries, disease incidence of *Neonectria* canker has increased in some regions during the last ten years [1–3,16]. Possible explanations for this increase include mild winters and wet growing seasons, which provide better living and dispersal conditions for the fungus. With global warming and current climate change projections, the increase in disease pressure may intensify [17]. In Eastern Finland, *N. fuckeliana* has infected stands of young Norway spruce plantations (5–30 years old) leading to reduced timber quality due to canker wounds and significant mortality [2,3]. In a provenance trial, Lilja et al. (2012) found that 13% and 37% of Finnish and Polish provenances, respectively, had dying tops and blackened wounds caused by this fungus [2]. *Neonectria fuckeliana* has also been reported in young spruce stands and Christmas tree fields in Sweden, Denmark, and Norway [1,14,16,18]. Since 2012 in Northern Ireland, *N. fuckeliana* has been implicated in the disease and mortality of Sitka spruce (*Picea sitchensis* (Bong.) Carr.), an important tree species in the region, at several sites spread across the entire region (Richard O’Hanlon personal communication) [15].

The same changes have been observed in Norway with other ascomycetes that have been present in the country for decades, for example, *N. neomacrospora* [19] and *Delphinella abietis* [20]. Both pathogens have caused epidemics on fir (*Abies* spp.) during the last ten years, which can be linked to increasing temperature and precipitation [18,21].

*Neonectria fuckeliana* has not been extensively studied, and there are gaps in knowledge concerning its basic biology, pathogenicity, and infection mechanisms. Even the taxonomic status of this species has changed several times [22,23] and it has recently been suggested that it belongs to a completely new genus [24]. However, infection studies where Norway spruce seedlings were inoculated with *N. fuckeliana* using a map pin technique [25] resulted in canker wounds, resin flow, and shoot dieback, similar to observations of infected Norway spruce trees in the field [1]. The study suggested that further investigation into the pathogenicity of *N. fuckeliana* was needed to be able to justify preventive or sanitary actions.

The primary objective of this study was to determine the ability of *N. fuckeliana* to cause disease on younger Norway spruce rooted cuttings in order to better understand and manage *N. fuckeliana* infections in younger Norway spruce plantations. Four specific aims were outlined: (1) Determine how different wound types would impact the occurrence and severity of infections on three-year-old Norway spruce cuttings and seven-year-old Norway spruce trees; (2) Describe symptom development after inoculation with microconidia; (3) determine if symptom development of the cuttings correlated with field observations of top-dieback; and (4) estimate the potential threat to young Norway spruce plantations and discuss potential management implications.

## 2. Materials and Methods

### 2.1. Plant Material and Location

**A pilot inoculation study** was undertaken to examine the usefulness of microconidia as an inoculation source for young Norway spruce plants. A total of 32 two-year-old seedlings were used. The inoculation study was carried out in a growth chamber at NIBIO, Norway, with 65% relative humidity (RH), a temperature of 20 °C, and 18 h daylight (dark from 21:00–03:00). The seedlings were regularly watered.

**A greenhouse inoculation study (the main experiment)** was undertaken to examine the susceptibility to *N. fuckeliana* of actively growing and dormant Norway spruce cuttings, and the importance of wound type. Active and dormant three-year-old cuttings were inoculated when they were approximately 44 and 37 cm tall, respectively. The experiment was conducted at Skogforsk, Sweden.

In March 2016, 500 dormant cuttings were placed into a freezer (−4 °C) to maintain dormancy, while 500 were placed into a greenhouse to start a third growing season. The cuttings in the freezer were transferred to a refrigerator for acclimatization by the time the cuttings in the greenhouse started to break bud in early May. When all the actively growing cuttings had shoots that were at least 5 cm long (end of May), the dormant cuttings were moved from the cooler to the greenhouse. Both the actively growing and dormant cuttings were re-potted into potting trays (inside measurement 50 × 29 × 9 cm) with sphagnum containing eight cuttings each (in mid-May and end of May, respectively). A total of 120 potting trays (960 cuttings), were distributed onto five benches (24 potting trays per bench).

The cuttings were regularly watered and the temperature in the greenhouse followed outdoor temperature fluctuation patterns. However, the temperature in the greenhouse was kept above 0 °C. The temperature was continuously measured with three HOBO water temperature Pro V2 data loggers (Onset Company, Bourne, MA, USA) hanging above the cuttings in the greenhouse.

**A field study** employing seven-year-old Norway spruce trees was undertaken to test whether stem infections would result in similar top-dieback symptoms as observed on Norway spruce in Finnish and Norwegian forest stands and in Swedish Christmas tree fields [1–3,14]. The trees were planted in rows, with different families in succession. A total of 180 plants from twelve different families grown in the edge rows were used for the study.

### 2.2. Inoculum Preparation

Three *N. fuckeliana* isolates collected from infected Norway spruce trees were used for the inoculation studies (Table 1). All isolates were identified based on morphology and sequencing

of the internal transcribed spacer (ITS) region of ribosomal DNA using ITS1 and ITS5 primers [26]. Pathogenicity of isolate no. 250605 and 250606 had not been tested before, but isolate no. 250471 had proven to be pathogenic to Norway spruce in a previous inoculation study [1].

**Table 1.** *Neonectria fuckeliana* isolates from Norway spruce (*Picea abies*) used for inoculation studies of young Norway spruce plants.

Isolate No.	GenBank Accession No.	Location *	Collected
250471	KT350495	Sösådala, Skåne	2015
250605	MF593144	Knutstorp, Skåne	2016
250606	MF593145	Illestorp, Skåne	2016

\* Municipality and county in Sweden.

**For the pilot inoculation study**, one 14-day-old Potato Dextrose Agar (PDA) culture for each of the three isolates in Table 1 was used.

**For the greenhouse study**, isolates no. 250471 and no. 250605 were used for inoculation. Both isolates were grown on poor Difco™ PDA medium enhanced with needles and wood chips of Norway spruce (Poor-PDA<sup>+</sup>) to stimulate microconidial production. The medium was prepared by suspending 13 g of commercial PDA powder in 1 L of deionized water, adding 75 needles from one living Norway spruce branch, plus 15 cut branch segments (0.5–1.0 cm) of one living and one dead Norway spruce branch. The suspension was heated with frequent agitation, and boiled for one minute before autoclaving at 121 °C for 15 min.

For each isolate, a microconidial spore suspension was prepared by pouring 20 mL deionized water onto two 19-day-old cultures grown on Poor-PDA<sup>+</sup>. They were swirled gently for 30 s and the microconidial spore suspensions were poured into separate beakers. The number of microconidia was counted using a Fuchs-Rosenthal hemocytometer under a Leica DMLB compound microscope. The spore concentration was adjusted to approximately  $5 \times 10^5$  conidia/mL (5000 spores per 10 µL). To determine germination of the conidial inoculum before and after inoculation, a serial dilution was prepared and 10 µL aliquots (three replicates per dilution) were plated and streaked out onto PDA Petri plates using a sterile spatula. Plates were incubated at room temperature and colonies were counted after 4 days.

**For the field study**, 14-day-old PDA cultures of isolate no. 250471 and no. 250605 (Table 1) were used as inoculums.

### 2.3. Experimental Design

**For the pilot inoculation study**, the seedlings were organized in four rows in a plug tray. Three rows (one for each isolate) with nine seedlings and one row of five control seedlings.

**For the greenhouse study**, a randomized split-block design of four blocks (benches) was used. Each block was vertically split into actively growing and dormant cuttings (main plot factor) and horizontally split into isolate no. 250471 or isolate no. 250605 (main plot factor) (i.e. inoculation with spore suspension of *N. fuckeliana* from isolate no. 250471 or no. 250605). Each main plot contained four different treatments (subplot factors) with 12 replicate cuttings for each treatment and block.

**For the field study**, ten Norway spruce families were inoculated with isolate no. 250471 and two families were inoculated with isolate no. 250605. For each family, 15 trees were selected; ten trees were randomly inoculated and the remaining five trees were controls.

### 2.4. Inoculation Trials

Four inoculation trials (2.4.1.–2.4.4.) were performed with the following number of plants: 32, 960, 480, and 180, respectively. In the same order, the number of seedlings inoculated with *N. fuckeliana* was 27, 768, 384, and 120. The remaining seedlings in each of the four trials were used as controls, that is, inoculated with water.

#### 2.4.1. Pilot Study, First Inoculation Trial

In the pilot study, nine two-year-old seedlings were used per isolate. The microconidial solution for inoculations was prepared by carefully pipetting up and down 15  $\mu$ L of water on the 14-days-old *N. fuckeliana* culture and placing the droplet onto a 1 cm artificial stem wound made with a scalpel to reveal the sapwood. The five control seedlings received water instead of spore suspension. Wounds were left open (not covered).

#### 2.4.2. Greenhouse Study Part I, Second Inoculation Trial

Four different treatments were applied to the terminal leader of both actively growing and dormant cuttings; (1) shoot-topped treatment: approximately 2 cm of the terminal leader was cut using sterilized secateurs; (2) shoot-wounded treatment: 5–10 mm of the bark and cambium in the middle of the terminal leader was removed using a sterilized knife; (3) needles-removed treatment: ten needles were removed from one area in the middle of the terminal leader; and (4) non-wounded treatment: the terminal leader was left intact. Tools were sterilized between trees by dipping them in 70% ethanol and drying with paper towels.

Each treatment was inoculated with spore suspension within 1 min after wounding. A micropipette was used for applying 10  $\mu$ L of suspension to each wound of the shoot-topped and shoot-wounded treatments. The needles-removed and the non-wounded treatments were sprayed with 1 mL of spore suspension per plant.

All cuttings were inoculated over three consecutive days from 31 May to 2 June 2016. Before inoculation, a resealable plastic bag with the bottom cut open was fitted over the terminal leader and the top whorl of the cutting, and tightened around the stem at the internode between two whorls using cable ties (2.5  $\times$  100 mm) according to a method by Thomsen et al. (personal communication) [27]. The resealable front of the bag was opened and the treatment was executed before resealing the bag. For the needles-removed and the non-wounded treatments, the plastic bag enabled spraying suspension onto the terminal leader without contaminating other plants. The shoot-topped and shoot-wounded treatments received a small piece of moist cotton inside the bag before resealing to give a sufficient amount of humidity to all treatments. After only a few hours, a moist film could be seen on the inside of the plastic bag. The bags were kept on for 5 days to enable germination of the microconidia.

The control cuttings received the same wound treatments, but were inoculated with sterile water, that is, mock inoculation, instead of the *N. fuckeliana* spore suspension. Half of the control cuttings were covered with plastic bags while the other half were uncovered to control for the effects of the plastic bags, that is, increased moisture and fluctuations in temperature.

#### 2.4.3. Greenhouse Study Part II, Third Inoculation Trial

When there were no symptoms present on the cuttings in the greenhouse study part I (second inoculation test) after more than two months the third inoculation trial was conducted on all the cuttings in two of the blocks (benches 1 and 3). The shoots of the cuttings that were actively growing by the start of the greenhouse study part I had finished elongation, hardened, and set buds, while those that were dormant at that time were now actively growing and not fully elongated.

The largest lateral branch leader was chosen to receive the same treatment as its terminal leader during the previous inoculation. This branch was marked with tape. Plastic bags were not used during the third inoculation due to the risk of another heatwave. The foliage of the cuttings was kept moist by using a hose with mist spray nozzles once an hour during the day for 5 days after inoculation.

#### 2.4.4. Field Study, Fourth Inoculation Trial

In a field study, 180 seven-year-old Norway spruce plants were inoculated with *N. fuckeliana* on the 16th of July 2016. Ten families were inoculated with the isolate no. 250471 and two families inoculated with the isolate no. 250605. Of the 15 trees for each family, ten trees were randomly selected

and inoculated with mycelial plugs of *N. fuckeliana*, and the remaining five trees were inoculated with clean PDA plugs as controls.

The trees were wounded prior to inoculation by making shallow holes (10–15 mm deep) in the sapwood of the stem, located 1–3 cm above the third branch whorl (counted from the top). The holes were made by using a cordless power drill with an 8 mm drill bit. The plug (with or without mycelium) was placed into the hole with the surface facing the center of the tree. Mycelial plugs were used because they are a fast and secure way of initiating an infection. A moist cotton tuft was used to seal the wound. The cotton plug was kept moist during the first 5 days by spraying it with water once a day, and left in the inoculation site throughout the experimental period.

### 2.5. Symptom Evaluation

The appearance and development of plant symptoms (such as visible depression around the inoculation point, stem canker formation, color loss of needles, brown needles, wilting of shoots, necrosis, and top-dieback), and presence/absence of pathogen signs (such as perithecia and/or sporodochia) were monitored monthly.

Final symptom evaluation and harvesting of the inoculation trials occurred after two months for the pilot study (first inoculation trial), ten months for the greenhouse study part I (the second inoculation trial), eight months for the greenhouse study part II (third inoculation trial), and eleven months for the field study (fourth inoculation trial).

For each plant, notes of visible plant symptoms and pathogen signs were taken, and the lesion length under bark (mm) was measured after removal of a thin layer of bark to reveal the cambium. The length of any visible lesion was measured from the lower wound edge towards the stem/root of the plant.

For the pilot study, all seedlings were sampled by removing the needles around the inoculation point, scraping the bark with a scalpel to reveal the sapwood at the wound area, and excising that stem segment for reisolation.

All the cuttings from the greenhouse study part I and II were cut using sterilized secateurs, and needles were stripped from the inoculated leader. A knife was used to remove the bark and cambium to reveal the sapwood at the inoculation point. The inoculation point and at least 1 cm below and above, or any visible depressions around the inoculation point, were removed under aseptic conditions, placed into 1.5 mL micro centrifuge tubes, and stored into a  $-20^{\circ}\text{C}$  freezer to secure material for later testing if necessary. However, for samples selected for investigating pathogen survival by isolation to culture, the stem/branch segments were split in half and placed into two different 1.5 mL micro-centrifuge tubes. One of the tubes was stored in a  $-20^{\circ}\text{C}$  freezer, while the other was used directly for reisolation.

For the field study, a 20 cm stem segment with the inoculation wound in the middle was cut, wrapped in paper, put into a marked plastic bag and transported in a cooler to the laboratory. In the laboratory, the samples were longitudinally split at the inoculation point to reveal any discoloration and the lesion length under bark was measured. One half of each sample was stored in the freezer while the other half was used for reisolation onto PDA and incubation in a moist chamber to observe any development of typical *N. fuckeliana* signs.

### 2.6. Reisolation from Inoculated Plants

Stem segments from the inoculation points were dissected into 5–10 mm segments, surface sterilized for 10 s in 70% ethanol followed by 90 s in 0.5% NaOCl and plated on PDA in 9 cm Petri dishes. The plates were incubated at room temperature in the dark and monitored daily for growth of *N. fuckeliana* or until the plates were overgrown by other fungi or bacteria (7–21 days).

#### 2.6.1. Reisolation from the Pilot Study, First Inoculation Trial

After two months, all seedlings were harvested and investigated for pathogen survival. Identification of *N. fuckeliana* was based on morphological observations of the resulting cultures.

### 2.6.2. Reisolation and Molecular Detection from the Greenhouse Study Part I and II, Second and Third Inoculation Trial

From the greenhouse study part I, 128 out of 768 inoculated cuttings and 64 out of 192 control cuttings were investigated for the presence of *N. fuckeliana* through culture-based and molecular-based identification techniques described below. Specifically, one row from both active and dormant cuttings for each of the four benches with inoculated cuttings, and two rows from both active and dormant cuttings from the control bench were examined.

From the greenhouse study part II, a total of 64 out of 384 inoculated cuttings and 32 out of 96 control cuttings were investigated for presence of *N. fuckeliana* following the same methods as for the greenhouse study part I.

### 2.6.3. Reisolation and Molecular Detection from the Field Study, Fourth Inoculation Trial

For the field study, 36 trees (three randomized trees per family, two inoculated, and one control) were investigated for the presence of *N. fuckeliana* through reisolation and incubation. Twelve of these plants (six inoculated and six control) were tested molecularly. A wood chip (1–2 cm) was removed under sterile conditions from the zone between the dead and alive tissue. The wood chip was taken from the longitudinal area of the depression on the side with the longest sunken area, either above or below the inoculation point. The wood chip was surface sterilized and plated onto PDA. After the wood chip was removed, the rest of the sample was incubated for 14 days in a moist chamber to determine if sporulation would take place. Each sample was inspected under the dissection microscope and a sterilized needle was used to carefully transfer microconidia from the wood sample to PDA supplemented with 0.5 mg/mL of streptomycin sulfate (PDAS).

## 2.7. Identification

### 2.7.1. Culture-Based Identification

Identification of *N. fuckeliana* was based on morphological observations of the cultures on PDA and spore characteristics described in earlier work [1].

### 2.7.2. Molecular-Based Identification

The samples that were stored in the freezer were analyzed for the presence of *N. fuckeliana* DNA. They were assessed by using a *N. fuckeliana* Taqman real-time PCR-based test developed by Pettersson et al. (2017) [28]. Each stem/branch segment was ground into a fine powder in liquid nitrogen using a mortar and a pestle. The DNeasy® Plant Mini Kit was used to extract DNA from 0.1 g of the homogenized samples. The Taqman assays with Nf-fw1/Nf-rw1 primer pair, were used to amplify any extracted *N. fuckeliana* DNA. The reactions were performed in 20 µL reaction mixtures containing 0.6 µL of each primer, 1.6 µL of probe (2.5 µM), 10 µL of SsoAdvanced Universal Probes Supermix (2×), 5.2 µL of mQ H<sub>2</sub>O, and 2 µL of genomic DNA (1/10 diluted after extraction), and run on 0.2 mL 96-well PCR plates. The samples were amplified and analyzed with Thermocycler CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA) under the following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 66 °C for 30 s. All reactions had two technical replicates. For each 96-well plate, two negative controls containing sterile mQ water were included. Results were analyzed with Bio-Rad CFX Manager software v2.0.

## 2.8. Statistical Analysis

Data analyses for the greenhouse study part I and II were performed using the statistical software SAS Enterprise Guide 7.11 HF3 (SAS Institute, Cary, NC, USA).

Data for the sum for the sub-plots of lesion length under bark, that were normally distributed and had homogeneous variances, were subjected to an ANOVA for criss-cross (or split block) design using PROC GLIMMIX. Non-normally distributed data was transformed with log-transformation. The same

analyses were run with the non-wounded and the needles-removed treatments were excluded (due to the low number of symptoms observed). The Tukey's honestly significant difference (HSD) test was used to identify the significant differences ( $p \leq 0.05$ ) among treatment means.

The molecular-based identification data was analyzed using PROC GLIMMIX with binominal distribution and logit link for the criss-cross design.

The culture-based identification data was not statistically analyzed due to low number of isolates retrieved.

### 3. Results

#### 3.1. Results from the Inoculation Trials

##### 3.1.1. Results from the Pilot Study, First Inoculation Trial

All 27 microconidial inoculated seedlings had swollen, non-healing wounds with resin flow (Figure 2). Six seedlings had necrotic cambium all the way around the wound, but the needles in the top were still green although losing color. All nine seedlings per isolate had *N. fuckeliana* outgrowth on PDA. The wounds on the five control seedlings were healing and no *N. fuckeliana* outgrowth was detected on PDA.



**Figure 2.** Symptoms observed in the inoculation trials with *N. fuckeliana* on Norway spruce (*Picea abies*): **Top row** = pilot study (the first inoculation trial) using microconidia from isolate no. 250603 (left), isolate no. 250605 (middle), and isolate no. 250603 (right), resulting in dark necrotic canker wounds with resin production. Photographed during assessment in 2016, 2 months after inoculation. **Second row** = greenhouse study part I (the second inoculation trial) where four different treatments (shoot-topped [left], shoot-wounded [middle], needles-removed [right], and non-wounded) were applied to the terminal leader of actively growing and dormant cuttings. Photographed during assessment in 2017, 10 months after inoculation. **Third row** = greenhouse study part II (the third inoculation trial) where the biggest branch shoot was inoculated using the same four treatments as in the second inoculation trial. Photographed during assessment in 2017, 8 months after inoculation. **Fourth row** = field study (the fourth inoculation trial) where 7-year-old Norway spruce plants were inoculated with mycelial plugs of *N. fuckeliana* in the stem. Photographed during assessment in 2017, 11 months after inoculation. Photos: Martin Pettersson.



### 3.1.2. Results from the Greenhouse Study Part I and II, Second and Third Inoculation Trial

In the greenhouse study part I, a small percentage of the cuttings developed disease symptoms, such as non-healing wounds with resin flow, and they all belonged to the shoot-topped and shoot-wounded treatment (Figure 2). The needles-removed and non-wounded treatment did not develop any disease symptoms. None of the control seedlings developed any disease symptoms and the wounds healed. There was a statistically significant difference among treatments for lesion length under bark ( $p < 0.0001$ ) and a significant growth stage treatment effect ( $p = 0.0006$ ). When the needles-removed and the non-wounded treatments were removed from the statistical analyses, lesion length under bark was significantly different ( $p < 0.0001$ ) for dormant shoot-topped versus dormant shoot-wounded treatments (Table 2). Furthermore, dormant and active growing plants were significantly different for shoot-topped ( $p = 0.0013$ ), but not for shoot-wounded. For lesion length under bark, there were no significant differences among growth stages or isolates, and no significant interaction was found.

**Table 2.** *Neonectria fuckeliana* detection from 128 terminal leader and 64 branch cuttings of Norway spruce inoculated with a microconidial suspension, and 64 control cuttings (inoculated with water). The treatments included were shoot-topped, shoot-wounded, needles-removed, and non-wounded.

		Terminal Leader Shoot (Greenhouse Study Part I)					
		Lesion Length under Bark			Detection		
Growth Stage	Treatment	Min–Mean–Max (mm) *	Std (n) (mm) *	(%) **	Tukey ***	Reisolation (%)	PCR (%)
Active	needles-removed	1–2.4–5	1.7 (5)	5	-	19	94
	non-wounded	-	-	0	-	0	19
	shoot-topped	1–3.0–8	2.0 (19)	20	B	38	88
	shoot-wounded	2–7.4–19	6.2 (8)	8	BC	25	94
Dormant	needles-removed	-	-	0	-	0	13
	non-wounded	-	-	0	-	0	6
	shoot-topped	1–7.3–65	10.2 (54)	56	A	0	44
	shoot-wounded	1–1.5–2	0.7 (2)	2	C	6	69
<b>All</b>		<b>1–5.9–65</b>	<b>8.5 (88)</b>	<b>11</b>	<b>-</b>	<b>11</b>	<b>53</b>
		Biggest Branch Shoot (Greenhouse Study Part II) ****					
		Lesion Length under Bark			Detection		
Growth Stage	Treatment	Min–Mean–Max (mm) *	Std (n) (mm) *	(%) **	Tukey ***	Reisolation (%)	PCR (%)
Active	needles-removed	1–4.5–8	5.0 (2)	2	-	13	88
	non-wounded	-	-	0	-	0	75
	shoot-topped	2–13.9–52	8.4 (94)	98	A	50	100
	shoot-wounded	1–12.1–51	10.7 (88)	92	A	44	100
<b>All</b>		<b>1–12.9–52</b>	<b>9.6 (184)</b>	<b>48</b>	<b>-</b>	<b>27</b>	<b>91</b>

\* The minimum, mean, maximum, standard deviation and sample size for lesion length under bark measured in millimeters were based on plants with a measurable lesion length under bark (that is, excluding all the plants with zero values from the analyses); \*\* Percent of plants that had a measurable lesion length under bark; \*\*\* In the Tukey Grouping analyses for lesion length under bark for the greenhouse study part I and II, the needles-removed and non-wounded treatments were excluded. The Tukey Grouping analyses were based on all the plants (that is both the plants with and without a measurable lesion length under bark was included in the analyses); \*\*\*\* At the time of the greenhouse study part II, all the cuttings were actively growing.

In the greenhouse study part II, more cuttings than in the greenhouse study part I developed disease symptoms, such as non-healing wounds with resin flow, and they all belonged to the shoot-topped and shoot-wounded treatment (Figure 2). Again, the needles-removed and non-wounded treatment did not develop any disease symptoms. None of the control seedlings developed any disease symptoms and the wounds healed. There was a statistically significant difference among treatments for lesion length under bark ( $p < 0.0001$ ). When needles-removed and non-wounded treatments were removed from the statistical analyses, the lesion length under the bark was not significantly different between the shoot-topped and shoot-wounded treatments (Table 2). For lesion length under bark,

there was no statistically significant difference among growth stage, isolate, and no interaction effects were significant.

For the molecular identification, there was no statistically significant difference for any of the factors. For the reisolation data, there were not enough isolates to make meaningful data analyses.

The wounds of the control cuttings, with or without plastic bags, inoculated with sterile water, had healed smoothly after inoculation of the terminal leader and the biggest branch shoot. No *N. fuckeliana* outgrowth was detected on PDA and *N. fuckeliana* could not be detected molecularly.

In the germination tests, mycelia emerged from the microconidia on all the PDA plates.

### 3.1.3. Results from the Field Study, Fourth Inoculation Trial

In the field study, a small percentage of the plants developed disease symptoms, such as slightly sunken areas around the wound and resin flow (Figure 2). However, this also appeared for a few of the non-inoculated controls. There was a statistically significant difference ( $p < 0.0001$ ) in total lesion length under bark between inoculated and control trees across both isolates. The average lesion length under bark was 53.3 mm for the inoculated trees and 41.2 mm for the control trees. There were also significant differences ( $p < 0.0001$ ) and ( $p = 0.001$ ) in lesion length under bark between families for the inoculated and control trees, respectively. However, all 24 inoculated trees and 11 out of 12 control trees had *N. fuckeliana* growing out on PDA. For the incubated samples, no sporodochia could be seen within the 14-day incubation period. However, microconidia could be seen under the dissection microscope for 22 out of 24 inoculated plants and 11 out of 12 control plants. These microconidia germinated to form cultures on PDAS.

*Neonectria fuckeliana* could be molecularly detected in the wood tissue in 6 out of 6 inoculated plants and 6 out of 6 control plants.

### 3.2. The Temperature in the Greenhouse Study Part I and II

On average over the whole year, the temperature in the greenhouse was 13.5 °C, but right after the greenhouse study part I started in June, a heat wave brought the temperature to above 30 °C for several (2–10) hours per day during the 5-day incubation time.

After the greenhouse study part II started, the greenhouse was approximately 4 °C cooler than during the inoculations in June.

The monthly average, standard deviation, maximum and minimum temperature can be seen in Appendix A Table A1. There is a significant difference ( $p < 0.0001$ ) in mean temperature between the 8-day period of the greenhouse study part I (24.5 °C) and the greenhouse study part II (20.9 °C) (Appendix A Table A2).

## 4. Discussion

*Neonectria* canker on Norway spruce caused by *N. fuckeliana* has become an increasing problem in the Nordic countries during recent years [1–3,14,16,29,30]. Factors influencing the pathogenicity of *N. fuckeliana* on Norway spruce are currently unclear. However, these studies demonstrated that:

- (1) *N. fuckeliana* infections can take place during different growth stages (i.e., dormant and active). This was evident from the greenhouse study part I, where no clear difference could be found for lesion length under bark between dormant and active cuttings for all treatments. However, the fungus was more frequently reisolated and detected molecularly from active cuttings. The lower detection frequency from dormant cuttings was surprising, since dormant Norway spruce does not have the same immediate defense reaction to wounding as actively growing trees [31]. It is unclear what caused the difference in detection frequency, but there was a clear difference between age and character of the inoculated tissue for the dormant and active cuttings. The inoculated shoots of the dormant cuttings were one year old, woody and brown, whereas the shoots of active cuttings were a few months old and had softer greenish juvenile tissue (semi-woody).

- (2) *Neonectria fuckeliana* needs an open wound as an entry point when inoculated with microconidia. This was clear from the greenhouse study parts I and II. None of the non-wounded cuttings developed symptoms and the fungus could not be reisolated; whereas the opposite was true for the wounded cuttings.
- (3) Treatments with larger wounds, such as the shoot-topped and shoot-wounded treatments had larger lesion length under bark and resulted in higher reisolation frequencies. This was clear from the greenhouse study parts I and II. *Neonectria fuckeliana* was seldom or not at all reisolated from the needles-removed and non-wounded treatment, respectively. However, the shallow damage inflicted by the needles-removed treatment proved to be a large enough entry point for *N. fuckeliana* to cause an infection on the active cuttings. In contrast, none of the dormant cuttings with needles-removed were positive for *N. fuckeliana* reisolation. Therefore, it seems that removing needles from young actively growing shoots is more serious in terms of pathogen attack than removing needles from older and dormant shoots.
- (4) It was evident that wound treatments of control cuttings inoculated with water healed smoothly, whereas wounds of microconidia inoculated cuttings healed slower or not at all. Therefore, we can conclude that some microconidia survived the high temperatures that occurred right after the inoculation of the greenhouse study part I. However, it is likely that the microconidia germination was abnormal or negatively affected by the heatwave as fewer cuttings were positive for *N. fuckeliana* reisolation and molecular detection compared to the greenhouse study part II. The larger lesions in combination with the higher detection of *N. fuckeliana* in the greenhouse study part II, suggests that the lower temperatures at that time had a positive influence on the survival of the microconidia. It also seems likely that the incubation technique with plastic bags was redundant or even had a negative impact as temperatures inside the plastic bags may have exceeded the greenhouse temperatures.

However, of the factors influencing the pathogenicity of *N. fuckeliana* on Norway spruce, much still remains unknown and several inconsistencies are evident from this study. These include latent period, temperature, microconidia inoculation technique, and the “background” inoculum from the environment (natural infection).

It seems evident from our experiments that *N. fuckeliana* can remain in a latent infection state for a long time before disease symptoms emerge. Latent infections are also reported for *N. ditissima* [32–34] and the latent period varies with wound size and spore concentration. Larger wounds and higher spore concentration shorten the latent period. This may be the reason for the slower disease development in the greenhouse study part I and II compared to the pilot study. It is unknown how much more concentrated the undiluted spore suspension for the pilot study was. Judging from the spore concentration preparation of the greenhouse inoculation study (using approximately  $5 \times 10^5$  conidia/mL), it was two to three times higher. For the field trial, mycelial plugs containing *N. fuckeliana* hyphae with microconidia were used to give a fast and secure way of establishing an infection without risking inoculum failure due to climatic conditions. However, as in the greenhouse study, none of the inoculated trees developed the top-dieback as observed in the pilot study, or seen in Norway spruce stands in Finland [2,3] and Christmas tree fields in Sweden [1].

Temperatures at the time of inoculation and over the summer periodically exceeded 25 °C. Temperatures over 25 °C have been proven to negatively affect spore germination, resulting in abnormal germination [4] and slower mycelial growth [1]. For the pilot study, the temperature was continually kept at 20 °C, which is in the range of favored growth temperature for the fungus. This may be another explanation for the slower disease development in the greenhouse study part I and II compared to the pilot study.

Microconidia may not be the optimal spore type for inoculation. In New Zealand, inoculation trials with *radiata* pine using *N. fuckeliana* microconidia and ascospores showed that both were highly infectious and there was no significant difference in infection level between the two spore types [8]. Based on this, we chose microconidia for our studies since they are readily produced in

culture. However, the inoculation technique of applying microconidia spore suspension onto artificial wounds may not have been optimal because resin produced by the cuttings may have hindered spore germination (Figure 2). Larger wounds such as bark stripping by deer or tree felling damage from forest management actions do not seal as fast as the artificially created wounds on the cuttings. This may be one of the reasons for the lower amount of disease development in our greenhouse inoculation trials (resin flow sealed the wounds). From our greenhouse inoculation trials with low reisolation frequencies, the potential of *N. fuckeliana* to cause disease in natural settings may easily be underestimated. This is obvious from the results from our outdoor inoculation trial where the background level of *N. fuckeliana* infections must have been high. However, one also has to bear in mind that *N. fuckeliana* has coexisted with Norway spruce through generations in the Nordic countries; thus, the host probably harbor a certain degree of resistance towards the pathogen. In New Zealand, on the other hand, *N. fuckeliana* is an alien, invasive species with great impact on radiata pine, a host that has had no chance to build up resistance.

For the field trial, there were almost no difference between inoculated and control trees regarding reisolation and molecular detection of *N. fuckeliana*. There are two possible explanations for this. The trees could have been contaminated with *N. fuckeliana* before the trial began or they could have become contaminated by natural inoculum at the time of wounding. Since the trial was conducted outdoors, some background level of *N. fuckeliana* infections could be expected [8]. Latent natural infections are widely reported in literature where *N. fuckeliana* has been isolated from wounded and healthy looking stems of Norway spruce [5,12,13,35]. Furthermore, a light rain occurred during part of the inoculation process and the temperature was under 20 °C. Mild and wet weather conditions are favorable for pathogen sporulation, dispersal, and infection of *N. ditissima* [33,36,37] and likely also for *N. fuckeliana*. If the trees in the field trial received the same background inoculum level, then the larger lesion length under bark (approximately 12 mm or 46%) for the inoculated trees was likely due to the additional inoculum of *N. fuckeliana* mycelial plugs. This suggests that the inoculation was probably successful despite the ambient inoculum. There were also significant family differences for lesion lengths under bark. Therefore, screening Norway spruce material in breeding programs may be helpful in determining sensitivity towards *N. fuckeliana*.

The role of *N. fuckeliana* as a wound pathogen is supported by literature [4–6]. However, the relatively slow disease development seen in this study questions the role it has in the top-dieback of Norway spruce. Previous research done in Sweden, Norway, Denmark and Finland shows that *N. fuckeliana* has been found in fields and forests, and seems to be a potential threat to Norway spruce [1–3,14,16,29,30]. There may be large differences between isolates given that sexual outcrossing is common for *N. fuckeliana* [6]. Another explanation may be the effect climate changes may have. *Delphinella abietis* is an example from Scandinavia where an epidemic outbreak was related to increased temperature and precipitation [18]. Furthermore, damage by *N. neomacrospora* on fir has dramatically increased during the last years in Scandinavia [38,39]. It is suggested that the increase is due to milder winters and wetter growing seasons, which benefit dissemination and infection success of *N. neomacrospora*. *Neonectria fuckeliana* also seems to have increased during the last decade in the Nordic countries [1–3,14,16,29,30]. Damage by *N. neomacrospora* and *N. fuckeliana* may therefore continue to increase due to global warming and climate change projections [17].

Our inoculation trials have revealed some complexity in doing inoculation experiments with *N. fuckeliana*. The fungus was difficult to reisolate onto PDA. Even though the infected woody tissues were surface sterilized before being plated out, other fungi such as *Sydowia polyspora*, *Fusarium* sp., *Alternaria* sp., and *Penicillium* sp. were commonly isolated and morphologically identified. *Neonectria ditissima* has also been found difficult to isolate [40]. Hence, a more selective media for *N. fuckeliana* would be useful. Even though the molecular identification is much more sensitive, it needs to be complemented with isolates to prove that the fungus is alive inside the wood tissue. Furthermore, no statistically significant difference for the molecular identification for any of the factors could be found. This was likely due to the limited number of samples analyzed (one sixth

or 192 inoculated cuttings) due to limited resources. A difference might have been found between the shoot-topped and non-wounded treatments if the molecular sample size had been larger for the greenhouse inoculation trial.

The effect of temperature and wetness duration on *N. fuckeliana* infections needs to be clarified. In our experiments, the disease was more severe in the pilot study even though the plants were not kept moist after inoculation. The greenhouse inoculation study parts I and II, in which the plants were kept moist and at higher temperature than in the pilot study for several days, less severe disease symptoms developed and even at a slower pace. It is possible that a higher dose than  $5 \times 10^5$  conidia/mL would be preferred as a higher spore concentration would shorten the latent period [32]. We also lack information about the epidemiology of the fungus. This includes how the inoculum pressure varies with climatic conditions throughout the year and how the infection success varies with wound age. However, it is clear from our inoculation trials that even small wounds such as needle scars are sufficient for *N. fuckeliana* infections. Hence, growers who annually shear their trees to make a denser Christmas tree are at higher risk of getting *N. fuckeliana* contaminations through natural inoculum. Therefore, implementation of proper management strategies may become necessary to avoid future top-dieback in Christmas tree fields.

A key element in keeping any crop healthy is removal of inoculum sources to keep the disease pressure low. Even though we have not yet observed perithecia developing on the Christmas trees with top-dieback, we strongly recommend that diseased trees are removed and burnt. We argue against the practice of piling up removed trees next to the fields or discarding the diseased trees in the nearby forests. Such management may result in a buildup of inoculum that could spread and infect nearby forests and Christmas trees. Often sections of Christmas tree plantations are the same species (monocultures) planted in dense straight rows. Good sanitation practices are important as such an environment could become an incubation chamber for diseases to multiply. Diseases may enter from a forest to a Christmas tree plantation, and later re-enter to the forest or vice versa. Therefore, if a Norway spruce Christmas tree field has top-dieback caused by *N. fuckeliana* and is located close to a Norway spruce forest, it may be important to manage the disease in both in the Christmas tree field and the nearby forest. Furthermore, good hygiene practices, such as cleaning tools between pruning different trees and especially between fields, are important to avoid inoculum transfer.

Future research can include a standardized inoculation method for comparing aggressiveness of different isolates of the fungus on different tree provenances, or preferably on cuttings (clones) to avoid differences in susceptibility between individuals within a provenance. Other studies examining the growth rate of inoculated and non-inoculated Norway spruce plants under different temperature regimes are needed to determine growth loss due to *N. fuckeliana* infections and clarify to role of temperature on symptom development. To come up with better management tactics both for forestry and Christmas tree production, such as thinning operations and shearing during periods with low inoculum pressure, we need to learn more about the life-cycle of *N. fuckeliana*. This means that future research also needs to include factors such as variation in spore dissemination thought the year, in order to reduce the probability of infection from this fungus. Under natural conditions, the trees are exposed to a number of pests and diseases that may influence the damage potential of *N. fuckeliana*; thus, further research on such interactions are needed.

## 5. Conclusions

*Neonectria fuckeliana* needs an open wound as an entry point for causing disease on Norway spruce. Data from our inoculation trials showed that larger wound treatments resulted in larger lesion length under bark as well as higher detection frequencies of the fungus. Infections took place in both actively growing and dormant Norway spruce cuttings. The ability of *N. fuckeliana* to cause infection on inoculated Norway spruce is clearly dependent upon the technique used. Further inoculation studies are necessary to create a standardized inoculation method for reliable comparison of different *N. fuckeliana* isolates.

Symptom development of the cuttings in this study was generally minor. This does not correlate well with the recent observations in Sweden, Finland, and Norway, where *N. fuckeliana* has caused cankers, heavy resin flow, and top-dieback on young Norway spruce, suggesting it has become a more aggressive pathogen. This study is more in line with older studies describing *N. fuckeliana* as a weak pathogen on Norway spruce. Thus, further research into the epidemiology and pathogenicity of the fungus is needed to increase the understanding of why *Neonectria* canker has become an increasing problem on Norway spruce in the Nordic countries during recent years.

Proper sanitation practices in Christmas tree plantations and new management strategies in young spruce forest stands are needed to prevent and control the disease.

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**Author Contributions:** Martin Pettersson initiated, designed, and carried out the inoculation trials; followed by evaluation, reisolation, molecular detection, and writing of this manuscript; all in close collaboration with the coauthors.

**Conflicts of Interest:** We declare no conflicts of interest.

## Appendix A

**Table A1.** Monthly average, standard deviation, minimum and maximum temperature fluctuation patterns for day and night from April 2016–March 2017 in the greenhouse where the second and third inoculation trials took place.

Month, Year	Temperature (°C) *			
	Average	StdDev	Max	Min
April, 2016 Day	11.6	3.5	25.8	4.5
Night	8.7	3.0	21.8	5.0
May, 2016 Day	22.7	7.5	41.1	6.6
Night	15.1	6.1	39.8	6.9
June, 2016 Day	24.9	8.0	52.5	8.6
Night	16.4	6.3	46.2	6.5
July, 2016 Day	25.5	7.9	51.1	10.3
Night	17.1	5.9	44.2	8.9
August, 2016 Day	24.1	7.0	44.6	10.2
Night	16.2	5.8	39.7	8.8
September, 2016 Day	21.4	7.3	42.6	7.0
Night	15.0	5.8	37.3	7.1
October, 2016 Day	10.6	4.4	34.7	3.7
Night	8.7	3.1	29.1	4.1
November, 2016 Day	6.8	2.2	15.3	3.3
Night	6.0	2.1	13.8	3.5
December, 2016 Day	7.0	1.6	12.6	3.8
Night	6.7	1.6	12.1	3.9
January, 2017 Day	6.0	1.5	12.4	3.6
Night	5.6	1.3	11.0	3.7
February, 2017 Day	8.1	4.0	25.0	3.8
Night	6.6	3.0	21.7	3.8
March, 2017 Day	10.2	5.2	30.5	4.1
Night	7.2	3.9	27.5	4.5

\* Measured by three HOBO water temperature Pro V2 data loggers (Onset Company, Bourne, MA, USA).

**Table A2.** Daily average, standard deviation, minimum and maximum temperature fluctuation patterns for day and night for the 8-day-period following the second inoculation trial and the third inoculation trial.

Temperature (°C) *									
Second Inoculation Trial					Third Inoculation Trial				
Date:	Mean	Stdev	Max	Min	Date:	Mean	Stdev	Max	Min
31 May Day	24.1	3.7	32.3	17.6	16 Aug Day	20.8	5.4	33.6	11.8
Night	18.5	1.8	22.6	16.7	Night	13.5	5.3	30.6	10.8
1 June Day	28.8	6.3	37.0	18.4	17 Aug Day	23.8	6.2	33.7	11.0
Night	20.7	5.8	35.8	17.6	Night	14.7	5.7	29.0	10.0
2 June Day	30.2	7.0	44.2	18.6	18 Aug Day	21.2	4.5	31.8	13.1
Night	20.7	6.0	37.3	17.5	Night	15.3	3.8	25.3	11.7
3 June Day	29.3	7.0	40.3	17.2	19 Aug Day	24.3	6.6	42.2	13.5
Night	19.3	6.7	37.2	16.0	Night	16.6	4.8	29.2	14.0
4 June Day	31.7	11.4	51.8	15.5	20 Aug Day	21.3	4.1	28.9	13.7
Night	18.8	9.9	45.7	13.7	Night	16.8	4.1	27.8	13.9
5 June Day	27.0	7.3	42.5	13.0	21 Aug Day	26.9	7.7	42.6	16.2
Night	15.9	6.4	34.8	12.0	Night	17.2	4.7	31.8	13.9
6 June Day	24.6	7.0	36.8	12.7	22 Aug Day	23.3	5.9	36.2	13.1
Night	15.1	6.2	31.5	10.4	Night	16.5	5.3	31.0	12.7
7 June Day	26.4	7.6	39.9	10.3	23 Aug Day	24.7	5.6	37.7	15.4
Night	14.9	7.0	32.1	9.5	Night	17.3	4.9	31.6	12.9
Average	24.5	8.3	51.8	9.5	Average	20.9	6.5	42.6	10.0

\* Measured by three HOBO water temperature Pro V2 data loggers (Onset Company, Bourne, MA, USA).

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