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The efficacy of *Chondrostereum purpureum* as a biological control agent

A comparative analysis of the decay fungus (*Chondrostereum purpureum*), a chemical herbicide and mechanical cutting to control sprouting of broad-leaved tree species.

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In forestry, manual control of broad-leaved trees is tedious and costly. To reduce costs, chemicals have been applied to keep these species in control. However, some chemicals are not recommended to use because of possibly adverse effects on the environment. Instead of chemicals, biological alternatives, such as a fungus, *Chondrostereum purpureum*, might be used to prevent sprouting. *C. purpureum* is a common decay fungus in Finland; it has been investigated at Metla, to find out whether it could be used as a biological control agent against sprouting of broad-leaved tree species.

This thesis project is related to the Metla research project. The aim of the thesis project is to investigate, how efficiently *C. purpureum* prevents sprouting of broad-leaved tree species such as birch, aspen, rowan and willow when compared to chemical treatment and mechanical cutting only, and whether *C. purpureum* is able to penetrate into the roots of these broad-leaved trees.

In the field, freshly cut weed trees were inoculated with *C. purpureum* or Biomax (a glyphosate containing herbicide), to compare their efficacy to the traditional way of sprout control i.e., mechanical cutting. Altogether 483 saplings (birch, aspen, rowan and willow) were treated in southern Finland. After three months, the mortality, the number and height of stumps sprouts of the saplings were investigated, and 124 saplings were excavated for further analysis in the laboratory. The occurrence of *C. purpureum* was investigated in wooden samples taken from inoculated stumps and connected roots. The data was analyzed using generalized linear mixed models.

After three months, the mortality in fungus treatment was exceptionally high within birch saplings, and did not differ significantly from that of herbicide treatment. However, significant effects caused by herbicide treatment were observed on all tree species. The fungus, *C. purpureum*, was successfully isolated from root samples of birch stumps. On the basis of the results of earlier research, three months is not a long enough exposure time for *C. purpureum* to fully unfold its biological control potential.

Keywords	sustainability, forestry, environment, biological control agent, herbicide

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Abbreviations

GDP	Gross domestic product
$g_1()$	First transformation formula
$g_2()$	Second transformation formula
lme4	Linear mixed-effects models using Eigen and S4 a package of R statistical language created by Bates et al. 2014
p	Probability value [0-1]
PCR	Polymerase chain reaction
PPMCC	Pearson product-moment correlation coefficient
p-value	Value indicating the probability for the error to refuse the null hypothesis even though it is true [0-1]
y_i	The i'th element of the response variable *y*
x_i	The i'th element of the explanatory variable *x*
$\bar{x}$	Sample mean value
$(1\|Area)$	The random variable Area indicated as random factor
β	Model coefficients i.e., slopes of each variable and intercept
ε	Experimental error i.e., the sum of all random errors occurring during the experiment
λ_i	Mean value of the elements of the Poisson distributed response variable
μ	Population mean value

Glossary

Basidiomycota	Mushroom producing fungi, developing fruiting bodies (basidia) producing spores on the gills under their cap (Campbell and Reece, 2009).
Basidiospores	Reproductive spores produced by basidiomycete fungi (Campbell and Reece, 2009).
EPSP	5-enolpyruvylshikimate-3-phosphate, an enzyme participating in biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan (Dill et al., 2010).
Lignicolous fungi	Wood decaying fungi (Campbell and Reece, 2009).
Phenylalanine	An essential amino acid and building block of protein (Kessel and Ben-Tal, 2011).
Phosphoenolpyruvic acid	A chemical compound in plants involved in biosynthesis of various aromatic compounds and carbon fixation pathway (Sigmaaldrich.com, 2014).
Sapling	A young tree
Shikimate pathway	The shikimate pathway links metabolism of carbohydrates to biosynthesis of aromatic compounds in plants, fungi, bacteria and algae (Campbell and Reece, 2009).
Sprouts	New branches growing from a sapling after cutting.

Henry's Law Constant	A substance specific constant, describing the solubility of the substance in water under constant temperature (Young et al., 2004).
Tryptophan	An essential amino acid and building block of protein (Kessel and Ben-Tal, 2011).
Tyrosine	A non-essential amino acid and building block of protein (Kessel and Ben-Tal, 2011).
Xylem	Tissue found in vascular plants that transports water and minerals (Campbell and Reece, 2009).

1 Introduction

The goal of this thesis was to investigate the efficacy of a decay fungus *Chondrostereum purpureum* as a biological control agent compared to the treatment with a herbicide (Biomax), used to prevent sprouting of broad-leaved trees, and manual cutting (a control treatment). Because of its wood decaying properties, *C. purpureum* has been found to be a suitable biological control agent for several broad-leaved trees (Harper et al., 1999). Specifically, I studied the effect of *C. purpureum* on those broad-leaved trees which are the most abundant weed species in conifer regeneration areas in boreal forests, namely birch (*Betula pendula* and *Betula pubescens*), aspen (*Populus tremula*), rowan (*Sorbus aucuparia*) and willow (*Salix caprea*). Furthermore, it was investigated whether *C. purpureum* is able to penetrate into the roots of inoculated stumps of the tree species.

On 28 April 2014, this experiment was established in eight different forest areas in southern Finland. Three of the areas are situated in Lapinjärvi, four in Liljendal, and one in Kuggom, all belonging to Uusimaa.

1.1 Weed control in Finnish forestry

In Finnish forests, broad-leaved trees, such as birch, aspen, rowan and willow are often considered as weeds because of their abundant and fast growth. They compete with conifer species for water, light and nutrients, and therefore significantly delay their growth (Hytönen and Jylhä, 2008). Weed control is defined as preventing and hindering unwanted plant species from growing (Baker, Helms and Theodore, 1979). However, in practice it is a costly and time consuming process since it is usually carried out mechanically (i.e., cutting only, without any further treatment on stumps) even once or twice a year, due to the capability of broad-leaved trees to quickly resprout after cutting. Especially along highways, railroads and under electricity cables, it is important to regularly carry out weed control operations in order to prevent security hazards (Vartiamäki, 2009). The highest costs paid for sprout control are in regeneration areas of conifers, i.e., Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*). To decrease the amount of time and costs invested in control operations, sustainable alternatives to annual cuttings are developed. Since the use of large scale herbicides is restricted for Finnish forestry in areas where the ground water is close to the surface

(FSC Standard for Finland, 2010), the focus of interest has recently shifted towards biological control agents (Vartiamäki, 2009).

1.2 Chondrostereum purpureum

Chondrostereum purpureum (Pers. Ex Fr.) Pouzar is a lignicolous, white rot fungus, belonging to the lignicolous basidiomycete fungi (Dye 1974; Vartiamäki et al. 2008). The fungus occurs naturally in temperate and boreal vegetation zones of the Northern and Southern Hemispheres (Vartiamäki, 2009). *C. purpureum* has been found to stimulate silver-leaf disease in fruit trees such as apple (*Malus* Mill), pear (*Pyrus* L.), plum (*Prunus* L.) (Brooks & Moore 1926). It penetrates freshly wounded stumps of broad-leaved trees and decays wood, which hinders affected trees from forming new sprouts from the stump. In case new sprouts occur after fungal infection, the sprouts tend to decay and stop growing after certain exposure time depending on tree species (Rayner & Boddy, 1986). The fungus grows and spreads through the whole stump and occludes xylem vessels, thus causing mortality of a tree. (Setliff & Wade 1973, Bishop 1979). Naturally, *C. purpureum* spreads via airborne basidiospores, which it produces to colonize new plants, in case the current host plant is no longer satisfying or its environment has changed unbeneficial for the fungus (see Figure 1). When two spores meet, they tend to form a new individual by sexual reproduction (Rayner, 1977; Wall, 1991; Wall, 1994).

Figure 1: *C. purpureum* growth on a stump surface. This picture was taken by Tom Smidth, 2008.

At the Finnish Forest Research Institute (Metla) fungal individuals (strains) of *C. purpureum* have been paired in order to find an effective strain for sprout control. The best strain tested in field experiments was named “R5”. Even though R5 is able to produce fruiting bodies and thus produce spores, it is not able to spread as such in the nature, outside of inoculated stumps, since its spores pair with naturally occurring spores of *C. purpureum* strains, which form new strains different from the strain R5. The strain R5 is used in the field experiment of the present work. Formulations of different biological control products using *C. purpureum* have been developed in Finland and Canada, but they are not yet commercially available. Furthermore, there are several different types of equipment in development to spread the fungus; however, in this study, back-pack type equipment was used.

According to the Environmental Protection Agency of the United States, *C. purpureum* does not cause any adversary effects to the environment if applied in areas where the fungus occurs naturally. No negative effects of *C. purpureum* could be observed in test rabbits which were injected orally and dermally. Neither was there any irritation of the eyes observed after instilling the fungus to their eyes. Since the fungus is not able to survive in temperatures above 36.5 °C no negative effects on humans are expected nor have they been reported. *C. purpureum* is only able to infect wounded trees, and therefore the risk of damaging non-target trees is rather small. However, in case of strong winds during inoculation, it has been observed that naturally wounded trees close to

the place of application, developed fungal infection. (Environmental Protection Agency, 2014). Yet, the fungal spores are abundant in the air since it occurs naturally in Finland.

There are also other fungi used as biological weed control agents, among others *Colletotrichum coccodes* used in the United States and Canada, as well as *Colletotrichum gloeosporioides f. sp cuscutae* used in China. However, these fungi are applied in agriculture to protect crops or soybeans from weeds, rather than in forestry (Butt and Copping, 2000).

1.3 Glyphosate

Glyphosate was invented by the Swiss chemist Henri Martin in 1950; however, the pharmaceutical company (Cilag) Martin was working for did not patent its newly discovered substance for herbicidal use. Its herbicidal properties were discovered in 1970 by John E. Franz of Monsanto Company, which issued the patent of glyphosate for herbicidal use (Franz, J.E et al, 1997). Glyphosate was commercially introduced in 1974 by Monsanto, under the trade name “RoundUp” and experienced its breakthrough as its price dropped significantly since it became a generic compound (Duke and Powles, 2008). After glyphosate resistant crops were developed genetically and introduced to agriculture in 1996, it became the most used herbicide in the world (Dill et al., 2010).

Glyphosate [*N*-(phosphonomethyl)glycine] belonging to the group of phosphoric acids, is a non-selective (not limited to a specific range of plants), biologically active main component in many of the most popular herbicides.

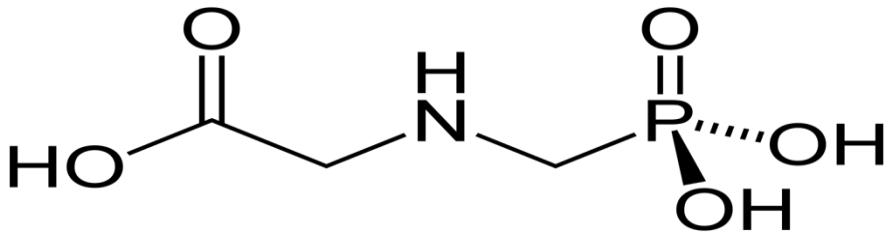

Figure 2: Structure of the [*N*-(phosphonomethyl)glycine] molecule (Mills, 2006).

Glyphosate interrupts the synthesis of aromatic amino acids such as phenylalanine, tyrosine and tryptophan by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase from performing as a biocatalyst in the shikimate pathway. The inhibi-

tion happens because of the chemical similarity of glyphosate with phosphoenolpyruvic acid, which is the common substrate of EPSP (Dill et al., 2010).

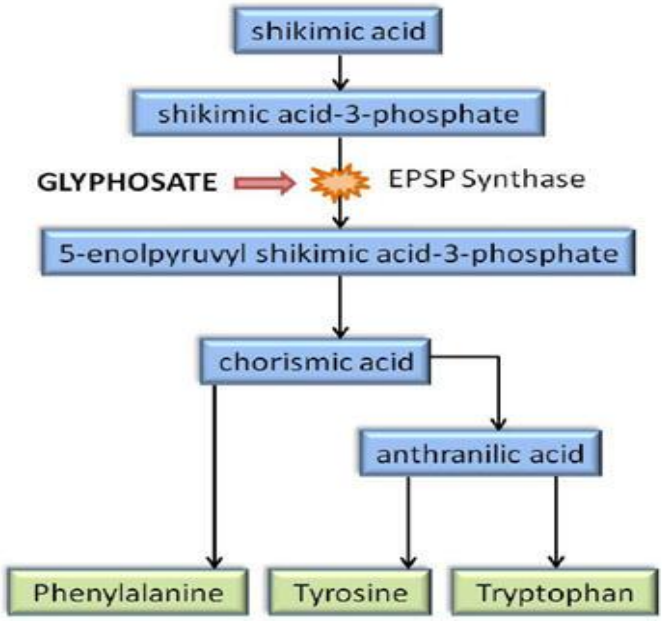

Figure 3: Glyphosate interrupting the synthesis of aromatic amino acids by inhibiting the EPSP enzyme (Pioneer, 2013).

Due to its low vapour pressure glyphosate is considered as non-volatile (Franz *et al,* 1997). Glyphosate is more likely to partition in water rather than air, because of its low Henry's Law Constant. However, strong winds tend to carry the glyphosate molecules from target plants to non-target plants, which cause injuries to non-target plants and eventually damages the nearby vegetation. Glyphosate is most likely carried away by winds during its release. Compared to other commercially available herbicides, glyphosate exhibits less toxicity towards the environment and wildlife because of its reduced half-life due to fast oxidation and immobility in soil (Schuette, 2014).

Depending on the scale of the target areas, glyphosate containing herbicides are sprayed by using a wide variety of different equipment, ranging from simple back-pack sprayers to target weed individually, to machines equipped with spraying mechanisms, to cover large areas (United States Environmental Protection Agency, 2001).

1.4 The site of research

The field and laboratory work for this thesis was done in the Forest Pathology department of the Finnish Forest Research Institute - Metla (Metsäntutkimuslaitos) and is part of the *C. purpureum* research project. Metla is a research institution established in 1917. Its main services are providing forestry related knowledge and solutions to its clients, and generating information through research. There are Metla units spread all over Finland. Currently 750 employers are working at Metla. Altogether 370 are researchers, which makes Metla the largest research institution specialized on forestry in the world. Most of its funding (70%) is provided by the Ministry of Agriculture and Forest of Finland, the remaining 30% consists of funding from different ministries and outside parties (Metla.fi, 2014).

1.5 Hypotheses

In order to compare the efficacy of C*. purpureum* (i.e., the performance to cause mortality and prevent sprouting) with the chemical herbicide Biomax, and the mechanical cutting (control treatment), the following hypothesis was tested.

1. The treatment efficacies are not equal (1):

$$H_1: \mu_{Fungal} \neq \mu_{Control} \neq \mu_{Glyphosate} \quad (1)$$

In order to study whether *C. purpureum* is able to penetrate into the roots of inoculated saplings, the following hypothesis was tested:

2. The amount of root samples containing *C. purpureum* R5 is more than zero (2):

$$H_2: \mu_{roots} > 0 \quad (2)$$

2 Materials and methods

2.1 Establishment of the field treatments

Three different treatments were tested to reveal sprout control efficacy (Table 1):

1 Fungus (*C. purpureum*) treatment: saplings are cut and treated immediately with the fungal inoculum including *C. purpureum*

2 Herbicide (Biomax) treatment: saplings are cut and treated immediately with glyphosate containing Biomax

3 Control treatment (cutting only)

Table 1: Description of different treatments

	Fungus	**Herbicide**	**Control**
Properties	*C. purpureum* is a wood decaying fungus used in a biocontrol product under development	Biomax is a herbicide consisting of 41 % glyphosate	Mechanical cutting, the traditional treatment to prevent sprouting
Preparation	Hyphae pieces of *C. purpureum* are mixed with nutrients and diluted with tap water (1:10)	Biomax is diluted with tap water (1:10)	
Application	Saplings are cut and an inoculum is spread to freshly cut stump surfaces	Saplings are cut and Biomax dilution is sprayed on stump surfaces	Saplings are cut only
Benefits	Environmentally neutral if applied in areas of natural occurrence	High and fast efficacy	
Adverse effects		Possible environmental adversary effects. The use of this chemical is restricted by recommendations	Time consuming Expensive Has to be repeated frequently

The treatments were tested in eight different regeneration areas (young forests) with plenty of naturally growing broad-leaved saplings, i.e., birch, aspen, rowan and willow. All areas were rather similar in terms of vegetation and soil composition except for area 8 belonging to Kuggom; this area was rocky and comparably sparsely covered with

vegetation. Saplings belonging to the herbicide and control treatments were investigated in sample plots; thus in each area, at least two sample plots were established. Saplings belonging to the fungus treatment were chosen separately and scattered from each other in order to prevent possible effects of excavation on fungus treated saplings (some of the saplings were excavated for further investigations in the laboratory). Before the treatment phase could be started, enough saplings per species, treatment and area had to be found and marked (see Table 2).

Table 2: The number of saplings treated per area and tree species in each treatment. Forest areas in which the experiments were established are coded as [1-8] in the area column.

Treatment	Area	Birch	Aspen	Rowan	Willow
Fungus Total: 174	1	6	6	3	6
	2	6	1	3	6
	3	6	0	6	6
	4	6	6	9	4
	5	6	2	7	6
	6	5	10	7	6
	7	6	6	6	6
	8	6	6	6	3
	Total:	**47**	**37**	**47**	**43**
Herbicide Total: 163	1	5	2	3	6
	2	6	0	0	0
	3	3	0	1	6
	4	6	2	4	6
	5	6	0	10	6
	6	6	10	6	6
	7	7	12	7	7
	8	6	12	6	6
	Total:	**45**	**38**	**37**	**43**
Control Total: 146	1	6	0	6	0
	2	6	4	0	6
	3	6	0	0	5
	4	6	6	6	1
	5	6	2	6	6
	6	0	8	5	2
	7	6	7	7	9
	8	6	6	6	6
	Total:	**42**	**33**	**36**	**35**

In each of the eight investigated areas, about six saplings per tree species, which corresponds to altogether 174 saplings, were marked to be cut and subjected to the fungal treatment. The saplings were cut with a brush saw, (Husqvarna) and freshly cut stumps

were sprayed immediately after cutting with the liquid (diluted with tap water in the ratio 1:10) containing fungal mycelia of R5. The liquid was supplemented with blue colour to distinguish the treated stumps visually from untreated stumps and to ensure that the liquid is spread sufficiently on the stump. Spreading was carried out by hand, squirting inoculum to freshly cut stumps, using back-pack type sprayer equipment. This group of saplings was named as the *fungal treatment*.

Similar to the fungal treatment, about six saplings per tree species per area (altogether 163 saplings) were marked to be cut a brush saw (Husqvarna) and treated with Bio-max. Biomax is a herbicide including glyphosate with a concentration of 41%. It was diluted with tap water (1:10) and sprayed to the sapling stumps individually, using a similar back-pack type sprayer equipment. This group was labelled *herbicide treatment* group.

The fungus and herbicide treatments were compared to the traditional way of inhibiting sprouting, i.e., mechanical cutting only (a brush saw Husqvarna), therefore a third treatment group was established and named as *control treatment*, which included about six saplings per tree species per area (altogether 146 saplings). But unlike the other treatments, saplings belonging to the control treatment were cut only, without any further treatment applied. However, some tree species were few in some areas, and therefore more than six saplings per species were treated in some other areas.

The locations of the saplings were marked in the field with tape. The location of each sapling was also noted on hand-drawn maps of the areas.

After three months, all treated saplings were investigated from 28 to 29 July 2014. The following values were recorded (see Table 3):

- Stump diameter [mm] (variable name: Diameter)
- The number of living sprouts per stump
- Highest living sprout per stump [cm]
- Number of stumps of the same species within a 0.5 m radius (SameSpecies)
- Number of all stumps within a 0.5 m radius (AllSpecies)
- Damage caused to the sprouts by animals [0=no damage, 1=visible damage]
- The number of fruiting bodies on a stump [0=no fruiting bodies, 1=1-3 fruiting bodies on a stump, 2=4-10 fruiting bodies on a stump, 3=more than 10 fruiting bodies on a stump]

Table 3: Description of the collected data: means, standard deviations and numbers of observations (n) have been presented.

Species	Treatment	n	AllSpecies	SameSpecies	Diameter (mm)
Birch	Fungus	47	4.2 ± 3.6	3.9 ± 0.7	14.2 ± 2.8
	Herbicide	45	6.0 ± 4.1	4.4 ± 1	15.1 ± 3.1
	Control	42	4.5 ± 3.437	3.2 ± 0.4	15.9 ± 3.4
Aspen	Fungus	37	2.8 ± 3.3	0.5 ± 0.1	13.2 ± 2.5
	Herbicide	38	3.3 ± 2.6	1 ± NA	14.5 ± 3.1
	Control	33	2.9 ± 2.8	0.9 ± 0.2	14.2 ± 4.3
Rowan	Fungus	47	3.4 ± 2.8	2.4 ± 0.3	13.4 ± 3.9
	Herbicide	37	3.3 ± 2.7	2 ± 1	13.4 ± 3.9
	Control	36	4.3 ± 2.2	3.6 ± 0.4	11.5 ± 2.8
Willow	Fungus	43	4.3 ± 3.3	0.9 ± 0.2	13.3 ± 2.9
	Herbicide	43	4.5 ± 2.9	3 ± 1.5	14.5 ± 3.4
	Control	35	4.4 ± 2.3	1.5 ± 0.2	14.6 ± 3.9

2.2 Root penetration experiment

Regarding the fungal treatment, two stumps with roots per tree species per area were excavated for further analyses (see Table 4).

Table 4: Number of stumps excavated from the field per area per treatment per species. Forest areas in which the experiments were established are coded as [1-8] in the area column.

Treatment	Area	Aspen	Birch	Rowan	Willow
Fungus Total: 60	1	2	2	2	2
	2	1	2	1	2
	3	-	2	1	2
	4	3	2	1	3
	5	2	2	2	2
	6	1	2	2	2
	7	2	2	2	3
	8	2	2	2	2
	Total:	**13**	**16**	**13**	**18**
Control Total: 34	1	-	2	1	
	2	3	1	-	2
	3	-	1	-	1
	4	2	1	2	1
	5	2	1	1	1
	6	1	1	1	1
	7	1	-	-	-
	8	1	2	2	2
	Total:	**10**	**9**	**7**	**8**
Herbicide Total: 30	1	1	1	-	1
	2	-	1	-	-
	3	-	-	1	2
	4	2	1	2	1
	5	-	1	1	1
	6	2	1	1	1
	7	2	1	1	1
	8	1	1	1	1
	Total:	**8**	**7**	**7**	**8**

Regarding the control and herbicide treatments, one stump per tree species per area was excavated in order to analyse whether *C. purpureum* will be found. Stumps were randomly chosen for excavation using the “sample()” function of R statistical programming language. Including all treatments, altogether 124 stumps were excavated from the investigated regeneration areas.

Three small wood chips were cut from each stump from six consecutive point; these sampling points were denoted as *top, middle, bottom, roots 1, roots 2, roots 3*, so altogether 18 wood chips were cut per stump. Before the stumps were investigated in detail, all dirt and bark was removed from the sampling points to prevent contamination of the samples. The first batch of three wood chips, denoted as *top*, was cut right below the cut surface of each stump. The second batch of three wood chips was cut from the location in the middle of each stump, between the top and the bottom, which was denoted as *middle*. The next batch of three wood chips was cut from the base of each stump, and this location was denoted as *bottom*. The first root sampling point, from where three wood chips were cut, was located one cm from the base of each stump towards root tips, which was denoted as *roots 1*. The second sampling point was in the middle, between the furthest tip of the roots and *roots 1*, this location was denoted as *roots 2*. The last sampling point was cut from the furthest root tip, i.e., a root tip of more than 5 mm in diameter, this last sampling point was denoted as *roots 3* (see Figure 4). Herbicide and control treatment samples were only taken from the top of the collected stumps.

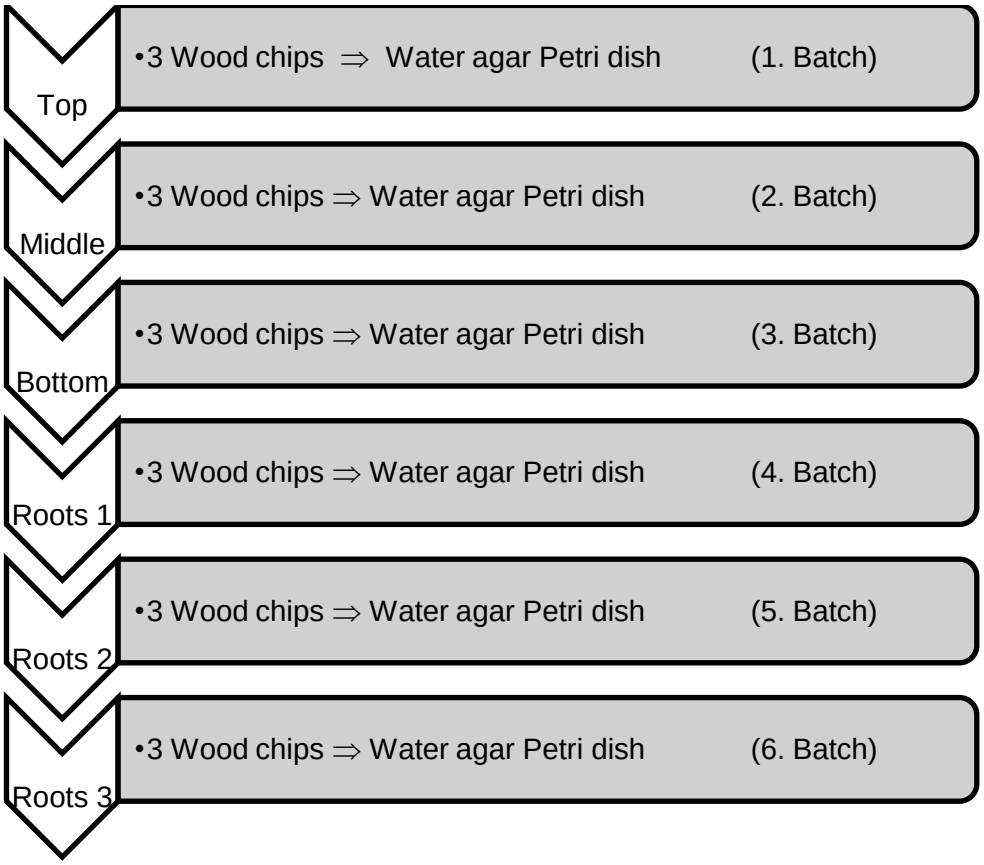

Figure 4: Sampling procedure for each stump belonging to the fungal treatment.

Distances from the top of the stumps to sample points *top, middle and bottom* were measured and recorded for each investigated stump. Considering root samples, distances from the top of the stumps to sample points, *top*, *middle, bottom, roots 1, roots 2* and *roots 3* were measured.Three wood chips from every sampling point were placed to one water agar Petri dish. The water agar plates were kept in room temperature to promote possible growth of *C. purpureum* (see Figure 5).

Figure 5: Petri dishes containing *C. purpureum* samples

2.2.1 Extracting and cultivating *C. purpureum* from wood samples

After about 48 hours of cultivation time, the water agar plates were investigated with a microscope to see whether *C. purpureum* has grown from wooden samples. To verify the fungus species, clump connections and an angle between the branches and the main fungal hyphae of approximately 45 degrees are strong indicators of *C. purpureum* (Uotila, Penttinen and Salingre, 2003). In case it was found, single hyphae was extracted and laid into a potato dextrose agar plate in order to prepare pure *C. purpureum* culture. However, in case of a successful extraction, another single hyphae tip was extracted from the isolated *C. purpureum* culture in order to ensure that pure culture was extracted. To confirm the isolated fungal individual to be the inoculated R5, PCR (polymerase chain reaction) was performed by the Metla lab technicians, to compare the DNA of the isolated fungus to the DNA of R5.

2.3 Statistical analysis

The data collected during field experiments was transformed into data matrix form, in which each column represented one variable and each row represented one observation, i.e., a treated sapling. The following explanatory variables (i.e., fixed effects) from the data matrix were taken into account in the models (see Figure 6):

- Diameter, the basal diameter of the sapling, measured in millimetres.
- Species, the species to which the spalings belong. [birch, aspen, rowan, willow]
- Treatment, a categorical variable consisting of three levels: Fungus, Herbicide, Control.
- AllSpecies, the amount of all saplings within a radius of 0.5 m around an investigated sapling, a non-negative integer.
- SameSpecies, the amount of saplings of the same species within a radius of 0.5 m around an investigated sapling, a non-negative integer.
- Damage, damage visible in sapling, caused by animals, a binary vector consisting of 0's and 1's,
    - 0 = no damage observed
    - 1 = damage observed
- Area, the area in which the sapling grows, a categorical random factor, since many saplings were investigated within the same area (these observations are correlated, i.e., possibly more similar than randomly investigated stumps within regeneration areas in general).

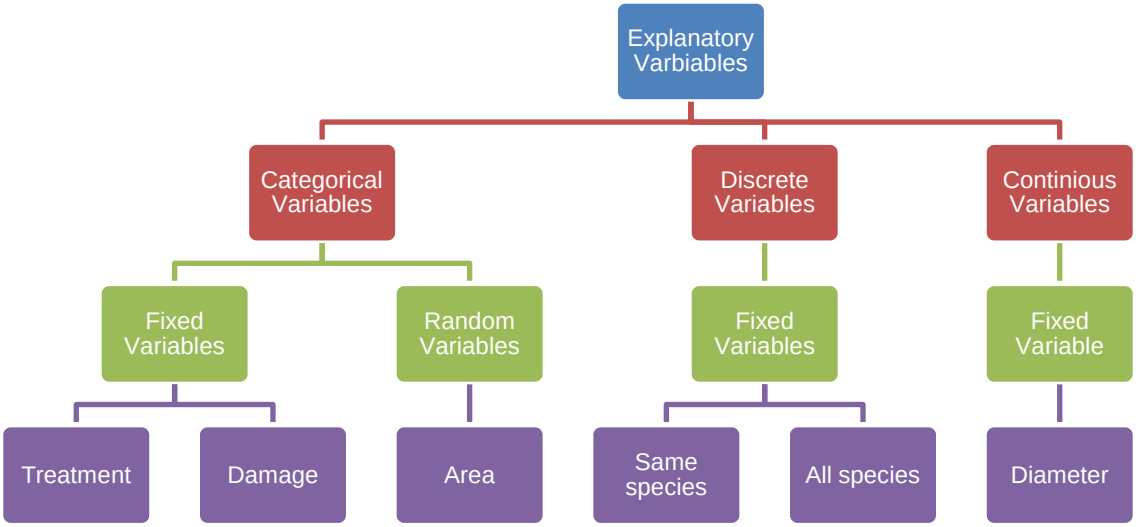

Figure 6: Explanatory variables included in the models. Categorical variables are non-numeric, discrete variables are numeric countable and continuous are numeric non-countable variables

Significances of the explanatory variables were tested against three different response variables (see Figure 7):

- Mortality, a vector consisting of binary data, where 1 indicated the sapling to be dead, meaning there were no sprouts growing, and 0 indicating the sapling to be alive, with at least one sprout growing, binomially distributed:
  - 0 sprouts $\Rightarrow$ the sapling is dead, indicated as 1
  - 1 or more sprouts $\Rightarrow$ the sapling is alive, indicated as 0
- Sprouts, the number of living sprouts per living stump, counted as non-negative integer, Poisson distributed.
- HighestSprout, height of the highest living sprout per living stump, measured in cm, normally distributed.

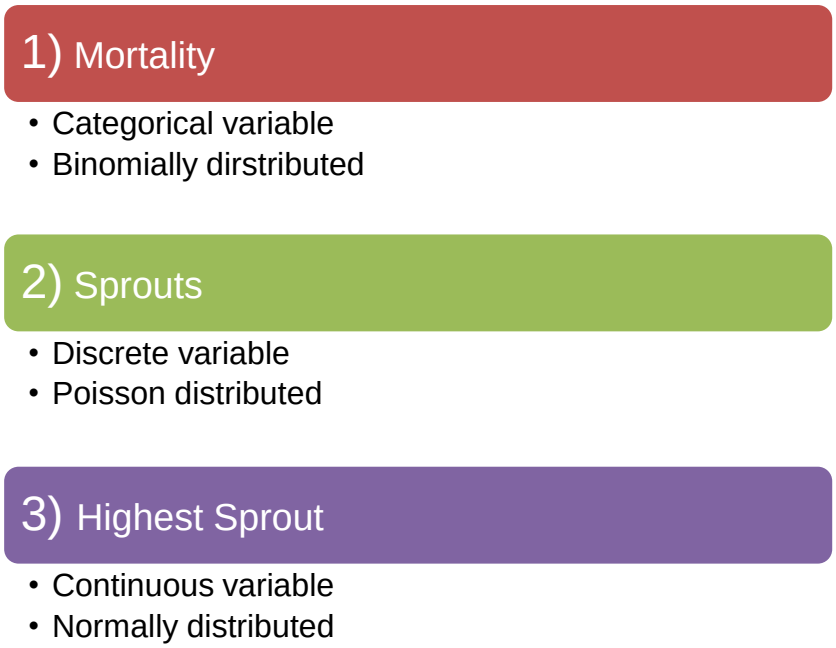

Figure 7: Response variables and their distributions. The significances of the explanatory variables in Figure 2 were tested against these response variables.

2.3.1 Probability distributions and transformations

Ordinary least square regression requires the response variable to be normally distributed, which is the case for HighestSprout but not for Sprouts and Mortality. Since the amount of new sprouts per stump is always a non-negative integer which occurs randomly in a given location, i.e., on the stump of the specific sapling, the response was assumed to be Poisson distributed. However, Mortality consists only of binary digits, meaning the variable can take on only the values 0 or 1, with the probability taking on either of the two digits being constant within each tree species and each treatment. Thus, Mortality was assumed to be binomially distributed.

In order to use models based on regression, the distribution of the response variables Mortality and Sprouts were normalized at the same time when the models were estimated, using a logistic link function for Mortality and a logarithmic for Sprouts, respectively (Weisberg, 1985):

- $g_1()$ indicates the logit transformation functions applied on the response variable Mortality (3) .
- $p_{Mortality}$ indicates the probability of success, i.e. the value is 1.

$$g_1(Mortality) = \log\left(\frac{p_{Mortality}}{1 - p_{Mortality}}\right) \quad (3)$$

- $g_2()$ indicates the logarithmic transformation functions applied for response variable Sprouts (4).
- $\mu_{Sprouts}$ indicates the expected value of living stumps for each tree species

$$g_2(\mu_{Sprouts}) = \log(\mu_{Sprouts}) \quad (4)$$

2.3.2 Correlation tests for the explanatory variables

The continuous explanatory variables were tested for correlations against each other using the Pearson product-moment correlation coefficient method (PPMCC), which is the default procedure in R statistical programming language. The Pearson product-moment correlation coefficient method calculates the correlation coefficient, abbreviated "r". It indicates the strength of a correlation between variables. The correlation coefficients range from -1, describing a perfectly negative correlation, to 1, describing a perfectly positive correlation; 0 describes that there is no correlation; therefore, the closer the coefficient to zero, the less correlation between the explanatory variables (Pearson, 1885).

Strongly correlated continuous variables (r>0.50) were excluded from the models because the variation of the first correlated variable explains the same variation in the corresponding response variable as the variation in another explanatory variable. Because of strong correlations between the variables AllSpecies and SameSpecies, with Pearson correlation coefficients of 0.85, 0.83 and 0.57 for birch, rowan and willow respectively, the variable SameSpecies was excluded from all models. Only aspen showed a lower Pearson correlation coefficient of 0.17 between these two variables. However, the variable SameSpecies was excluded from the aspen models too, in order to make the results more comparable to other tree species.

2.3.3 Type of models

The data were analyzed using generalized linear mixed models, which can both normalize non-normally distributed response variables and take random effects into account (McCulloch and Neuhaus, 2005). Every model was calculated for each tree species individually. To calculate suitable generalized linear mixed models, the lme4 package was installed to R statistical programming software. The notation (1|Area) indicates

that the explanatory variable Area was included to the model as random effect. Even though ANOVA (analysis of variance) is the more traditional approach to statistical modelling in forestry, generalized linear mixed models were used, due to the non-normal nature of the response variables.

2.3.4 Mortality models

Calculating models for Mortality (response variable), the following assumptions were made, since Mortality was expected to be binomially distributed:

- Mortality consists of n trials, each classified as success or failure, every treated sapling was considered a trial.
    - 1 = success, the sapling is dead
    - 0 = failure, the sapling is alive
    - N = amount of observed saplings
- Response and explanatory variables are of *n* length
- i is an element of the sequence 1 to *n* (5)

$$i \in 1:n \tag{5}$$

- The response vector Mortality consisting of binary numbers is indicated as y_i (6)
- The model coefficients i.e., intercept and slopes of each variable, are indicated as β
- The i'th element of the explanatory variable x is indicated as x_i
- The experimental error, indicated as ε, is the sum of all errors occurred during the experiment and is considered to be random

$$logit(y_i) = \beta_0 + \beta_i x_i + \varepsilon \tag{6}$$

Following the above stated assumptions, the mortality models contained the following terms (7):

$$logit(Mortality) \sim \beta_0 + \beta_1 Treatment + \beta_2 AllSpecies + \beta_3 Diameter + \beta_4 (1|Area) + \varepsilon \tag{7}$$

The differences in the number of observations between living and dead saplings, depending on the treatment and tree species, were rather large. Mortality rates were too low for other treatments than herbicide to acquire reliable results from the models for aspen, rowan and willow. Therefore, a model with respect to mortality could only be calculated for birch, because the number of dead stumps among the treatments were

similar enough. However, the treatment level control had to be excluded from the mortality models even for birch, since no mortality was observed in this treatment; therefore, fungus was chosen as base level of this variable and integrated to the Intercept.

2.3.5 Stump sprouts number models

Sprouts as response variable was expected to be Poisson distributed thus the following assumptions were made:

- The i'th element of the response variable y is indicated as y_i (8)
- The response variable Sprouts is Poisson distributed and λ_i is the average of the elements of the response variable (8):
- Number of sprouts of the i'th sapling is indicated as y_i

$$y_i \sim Poisson(\lambda_i) \quad (8)$$

- The logarithm of the response variable depends linearly on the explanatory variables and random error (9):

$$Log(y_i) \sim \beta_0 + \beta_i x_i + \varepsilon \quad (9)$$

Following the above stated assumptions, the models with Sprouts as response variable were calculated in the following way (10):

$$\log(Sprouts) \sim \beta_0 + \beta_1 Diameter + \beta_2 Treatment + \beta_4 AllSpecies + \beta_5 (1|Area) + \varepsilon \quad (10)$$

The treatment level Control was chosen to be the base of the explanatory variable Treatment, which means that the significances of the other two levels, i.e., Fungus and Herbicide, were compared against the base level. Since only one herbicide treated aspen sapling was observed to be alive after the treatment, the herbicide treatment was excluded from the sprout model for aspen.

2.3.6 Stump sprout height models

Since the response variable HighestSprout was expected to be normally distributed, the following assumptions were made:

- The sprout heights were measured randomly
- The average height of HighestSprout is indicated as $\bar{x}$ (11)
- The height of the highest sprout of the i'th sapling is indicated as y_i (11)

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n}(y_i) \tag{11}$$

- Standard deviation of HighestSprout is indicated as s (12)

$$s = sd(HighestSprout) \tag{12}$$

- HighestSprout is normally distributed (13)

$$\gamma_i \sim N(\bar{x}_i, s) \tag{13}$$

- HighestSprout depends linearly on the explanatory variables and random error (14)

$$\gamma_i \sim \beta_0 + \beta_i x_i + \varepsilon \tag{14}$$

Following the above stated assumptions, HighestSprout models were calculated in the following way (18):

$$HighestSprout \sim \beta_0 + \beta_1 Diameter + \beta_2 Treatment + \beta_3 AllSpecies + \beta_4 Damage + \beta_5(1|Area) + \varepsilon \tag{15}$$

Similar as in sprout models, the herbicide treatment was excluded from aspen model since it contained only one observation. The treatment level control was chosen to be the base level, thus not visible. Furthermore, damage was included as an explanatory variable to the model as it may have an effect on the height of a sprout.

2.3.7 Root penetration

Average penetration distances of *C. purpureum* were calculated and compared for each investigated species, and the averages were plotted as bars. The number of sampling points from where an infection caused by *C. purpureum* could be observed, was calculated in percentage of all sampling points of the same location on the stumps within each tree species and presented as a table.

3 Results

3.1 Mortality

Mortality in the fungus treatment for birch saplings was 42.6%, in the herbicide treatment 48.9% and in the control treatment 0% (see Table 5). The fungus treatment seemed to cause higher mortality rate than control treatment. However, no significant difference between the herbicide and the fungus treatment could be found (Table 6, Figure 8). Herbicide treatment tended to cause the highest mortality percentage for all other tree species. Mortality in aspen saplings in the fungus treatment was 13.5%, in the herbicide treatment 97.4% and in the control treatment 9.1% (see Table 5). Mortality of rowan saplings in the fungus treatment was 0.0%, in the herbicide treatment 86.5% and in the control treatment 0.0%. Mortality of willow saplings in the fungus treatment was 0.0%, in the herbicide treatment 88.4% and in the control treatment 0.0% (see Figure 8).

Table 5: Mortality of birch, aspen, rowan and willow stumps in different treatments.

Species	Treatment	n alive	n dead	Mortality [%]
Birch	**Fungus**	27	20	42.6
	Herbicide	23	24	53.3
	Control	42	0	0
Aspen	**Fungus**	32	5	13.5
	Herbicide	1	37	97.4
	Control	30	4	12.1
Rowan	**Fungus**	47	1	2.1
	Herbicide	5	33	89.2
	Control	36	1	2.8
Willow	**Fungus**	43	0	0
	Herbicide	35	1	90.7
	Control	5	39	2.9

Table 6: The effect of different treatments (fungus and herbicide) on the mortality of birch (generalized linear mixed model). Coefficients with standard errors (SE) and *p*-values have been presented. See Figure 4 for mortality percentages.

Response Variable: Mortality			
Species	**Explanatory Variables**	**Coefficients ± SE**	***p*-values**
Birch	Intercept (n=47)	-0.831 ± 1.285	0.518
	Herbicide (n=45)	0.296 ± 0.470	0.529
	AllSpecies	-0.025 ± 0.066	0.707
	Diameter	0.048 ± 0.084	0.571

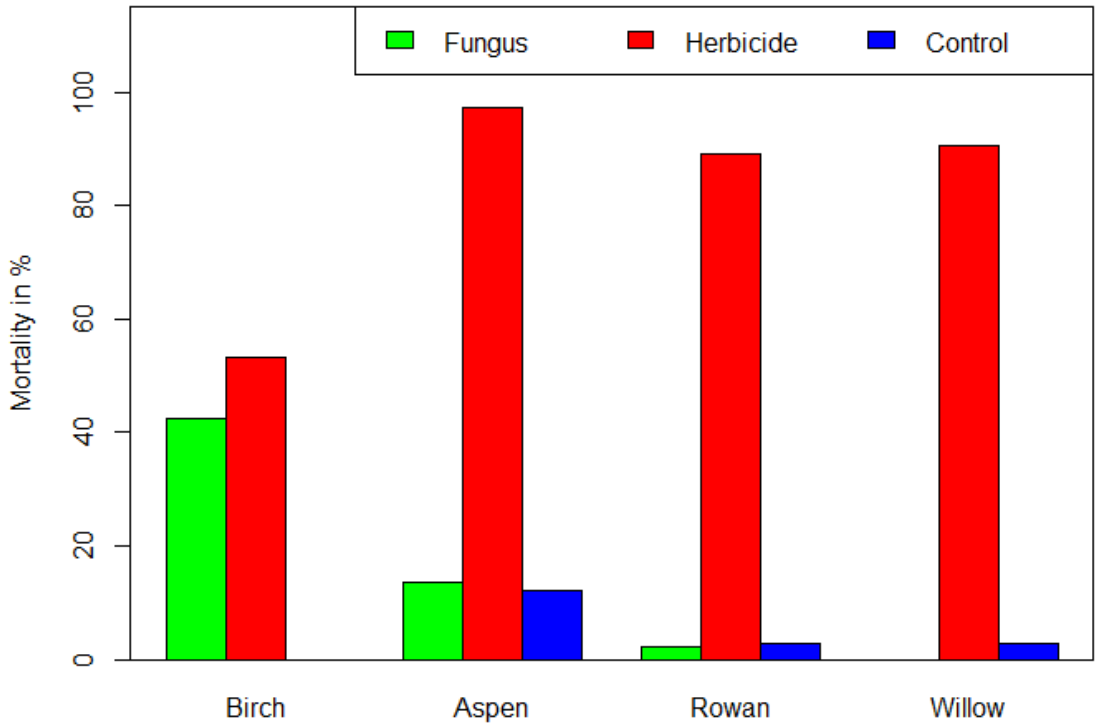

Figure 8: Mortality of birch, aspen, rowan and willow stumps in percentage in different treatments. See Table 6.

3.2 Number of stump sprouts

Concerning living birch stumps, the average number of new stump sprouts per stump in the fungus treatment was 7.3 in the herbicide treatment 21.7 and in the control treatment 7.8 (Table 7, Figure 9). However, no statistically significant difference in terms of the number of sprouts was found between the fungus and the control treatments whereas the number of stump sprouts was significantly higher in the herbicide treatment than in the control treatment (Table 7).

Table 7: Number of sprouts per tree species and treatment (mean ± standard deviation).

Species	Treatment	n	Sprouts
Birch	Fungus	27	7.3 ± 4.9
	Herbicide	23	21.7 ± 20.1
	Control	42	7.8 ± 4.6
Aspen	Fungus	32	2.8 ± 1.5
	Herbicide	1	5.0 ± NA
	Control	30	2.9 ± 2.0
Rowan	Fungus	47	5.5 ± 2.7
	Herbicide	5	23.2 ± 25.3
	Control	36	4.5 ± 2.2
Willow	Fungus	43	8.5 ± 3.2
	Herbicide	5	11.6 ± 11.6
	Control	35	9.3 ± 3.7

Figure 9: The effect of different treatments on the number of sprouts per living stump in birch, aspen, rowan and willow. See Table 8.

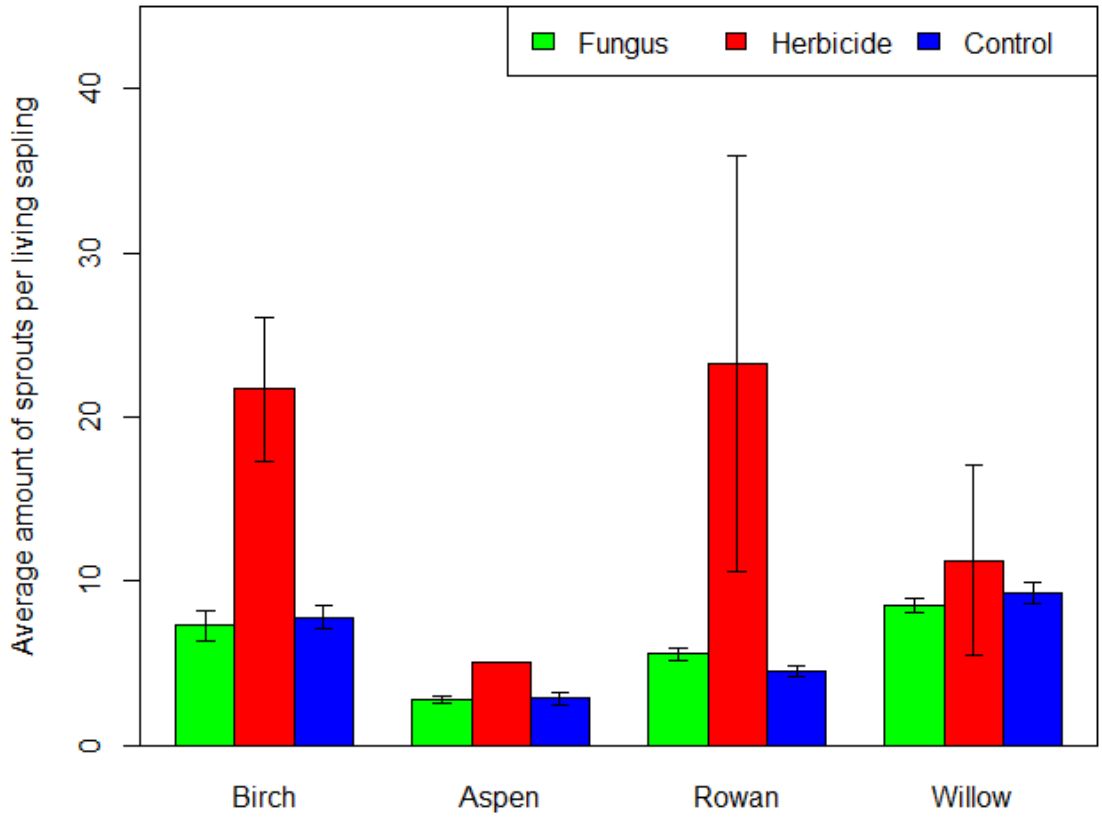

Table 8: The effect of different treatments on the number of stump sprouts per living stump. Coefficients, standard errors and significances of the explanatory variables have been presented (generalized linear mixed models). See Figure 9.

Response Variable: Sprouts			
Species	**Explanatory Variable**	**Coefficients ± SE**	***p*-Value**
Birch	Intercept	3.2.1.1 2.151 ± 0.267	**<0.001**
	Fungus	-0.048 ± 0.102	0.636
	Herbicide	1.138 ± 0.080	**<0.001**
	Diameter	0.004 ± 0.013	0.725
	AllSpecies	-0.064 ± 0.010	**<0.001**
Aspen	Intercept	-0.21 ± 0.426	0.622
	Fungus	0.093 ± 0.160	0.561
	Diameter	0.073 ± 0.023	**0.002**
	AllSpecies	0.055 ± 0.027	**0.045**
Rowan	Intercept	1.275 ± 0.231	**<0.001**
	Fungus	0.137 ± 0.107	0.197
	Herbicide	1.336 ± 0.157	**<0.001**
	Diameter	0.031 ± 0.014	**0.026**
	AllSpecies	-0.04 ± 0.020	**0.042**
Willow	Intercept	1.715 ± 0.204	**<0.001**
	Fungus	-0.032 ± 0.080	0.688
	Herbicide	0.294 ± 0.170	0.083
	Diameter	0.034 ± 0.011	**0.002**
	AllSpecies	-0.003 ± 0.013	0.824

Concerning living aspen saplings, the average number of new sprouts per sapling in fungus treatment was 2.8 saplings, in herbicide treatment 5.0 saplings and in control treatment 2.9 saplings (Table 7). However, no statistically significant difference between the fungus and control treatment was found (Table 8). The herbicide treatment was excluded from this analysis because only one stump was living.

Concerning living rowan saplings, the average number of new sprouts per sapling in the fungus treatment was 5.5 saplings, in the herbicide treatment 23.2 saplings and in the control treatment 4.5 saplings (Table 7, Figure 9). No statistically significant difference between the fungus and the control treatments was found (Table 8). However, in the herbicide treatment the number of stump sprouts was significantly higher than in the control treatment.

Concerning living willow saplings, the average number of new sprouts per sapling in the fungus treatment was 8.5 saplings, in the herbicide treatment 11.6 saplings and in the control treatment 9.3 saplings (Table 7).No statistically significant difference between the fungus and the control treatments was found (Table 8). . However, in the herbicide treatment the number of stump sprouts was indicatively higher than in the control treatment.

An increase in stump diameter increased the number of sprouts in aspen, rowan and willow (see Tables 7 and Table 8). An increase in the number of other saplings around an investigated sapling decreased the number of stump sprouts in birch and rowan, whereas for aspen a significant increase in the number of stump sprouts was observed with increasing number of other saplings around.

3.3 Sprout heights of living saplings

The average height of the highest sprouts in birch in the fungus treatment was 46.7 cm, in the herbicide treatment 11.2 cm and in the control treatment 61.8 cm (Table 1, Figure 10). The sprout height of birch was significantly lower in the fungus and the herbicide treatments than in the control treatment (Table 10).

The average height of the highest sprouts of aspen saplings in fungus treatment was 31.5 cm, in herbicide treatment 11.0 cm and in control treatment 36.7 cm (Table 9, Figure 10). No significant differences between the fungus and the control treatment was found (Table 10). Herbicide treatment was not included in this analysis because only one stump was alive after the herbicide treatment.

Table 9: Stump sprout height per tree species and treatment (mean ± standard deviation).

Species	Treatment	n alive	Height [cm]
Birch	Fungus	27	46.7± 19.2
	Herbicide	23	11.2 ± 6.9
	Control	42	61.8 ± 24.3
Aspen	Fungus	32	31.5 ± 21.2
	Herbicide	1	11.0 ± NA
	Control	30	36.7 ± 19.1
Rowan	Fungus	47	48.8 ± 16.8
	Herbicide	5	8.8 ± 4.6
	Control	36	47.1 ± 19.9
Willow	Fungus	43	91.4 ± 33.8
	Herbicide	5	5.5 ± 2.4
	Control	35	84.0 ± 32.8

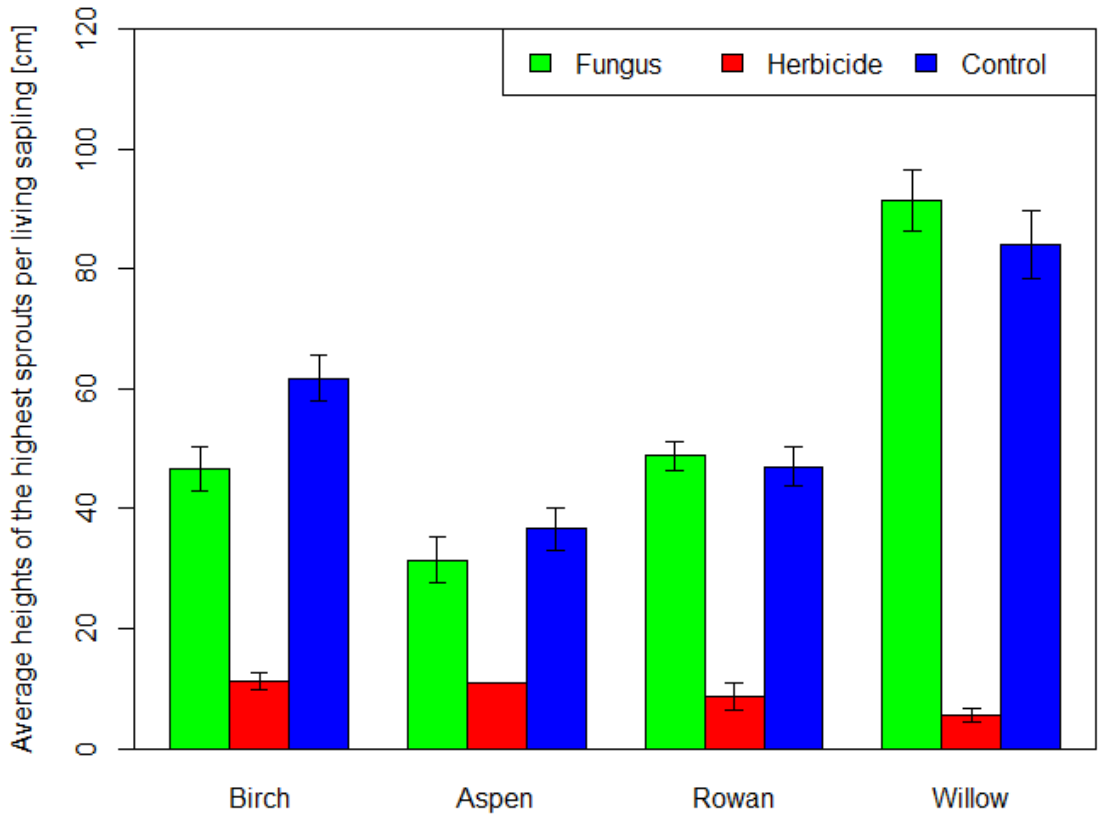

Figure 10: Maximum height of living stump sprouts of birch, aspen, rowan and willow in the fungus, herbicide and control treatments (see Table 10).

Table 10: The effect of different treatments (control, fungus and herbicide) on the maximum height of living birch, aspen, rowan and willow stumps. See Figure 10.

Response Variable: HighestSprout			
Species	**Explanatory Variables**	**Coefficients ± SE**	***p*-value**
Birch	Intercept	39.891 ± 11.178	**0.001**
	Fungus	-9.65 ± 4.513	**0.036**
	Herbicide	-51.776 ± 4.853	**<0.001**
	AllSpecies	0.944 ± 0.543	0.087
	Damage	-7.794 ± 5.607	0.169
	Diameter	1.311 ± 0.700	0.065
Aspen	Intercept	14.62 ± 14.659	0.324
	Fungus	-2.367 ± 5.240	0.654
	AllSpecies	0.746 ± 1.210	0.540
	Damage	2.629 ± 6.738	0.698
	Diameter	1.46 ± 0.822	0.082
Rowan	Intercept	34.622 ± 8.318	**<0.001**
	Fungus	-2.863 ± 3.800	0.454
	Herbicide	-53.257 ± 8.906	**<0.001**
	AllSpecies	-0.671 ± 0.717	0.352
	Damage	-6.381 ± 6.514	0.331
	Diameter	1.465 ± 0.543	**0.009**
Willow	Intercept	45.083 ± 18.268	**0.016**
	Fungus	10.424 ± 6.753	0.127
	Herbicide	-94.85 ± 15.630	**<0.001**
	AllSpecies	0.824 ± 1.145	0.474
	Damage	0.95 ± 7.516	0.900
	Diameter	2.393 ± 0.947	**0.014**

The average height of the highest rowan sprouts in the fungus treatment was 48.8 cm, in the herbicide treatment 8.8 cm and in the control treatment 47.1 cm (Table 9, Figure 10). No significant difference between the fungus and the control treatment could be found (Table 10). However, in the herbicide treatment the height of sprouts was significantly lower than in the control treatment.

The average height of the highest sprouts within willow saplings in the fungus treatment was 91.4 cm, in herbicide treatment 5.5 cm and in control treatment 84.0 cm (Table 9, Figure 10). No significant differences between the fungus and the control treatment could be found (Table 10). However, the height of stump sprouts was significantly lower in the herbicide treatment than in the control treatment. An increase in the basal diameter of rowan and willow increased the height of the highest sprout (Table 7**)**.

3.4 Root penetration

Within birch stumps, *C. purpureum* had penetrated the furthest with an average penetration distance of approximately 20 cm, which is an average distance from top to the bottom of the stumps. However, no clear differences in average penetration distances between aspen, rowan and willow saplings could be found. Fungal infection has been found from root samples of birch sapling stumps (see Table 11 and Figure 11).

Table 11: The fungus, *C. purpureum*, found in each sampling point in percentage (%). See Figure 11.

	Top	***Middle***	***Bottom***	***Roots 1***	***Roots 2***	***Roots 3***
Aspen	76.9	15.4	0	0	0	0
Birch	93.3	86.7	80	73.3	20	13.3
Rowan	46.2	7.7	0	0	0	0
Willow	83.3	5.6	0	0	0	0

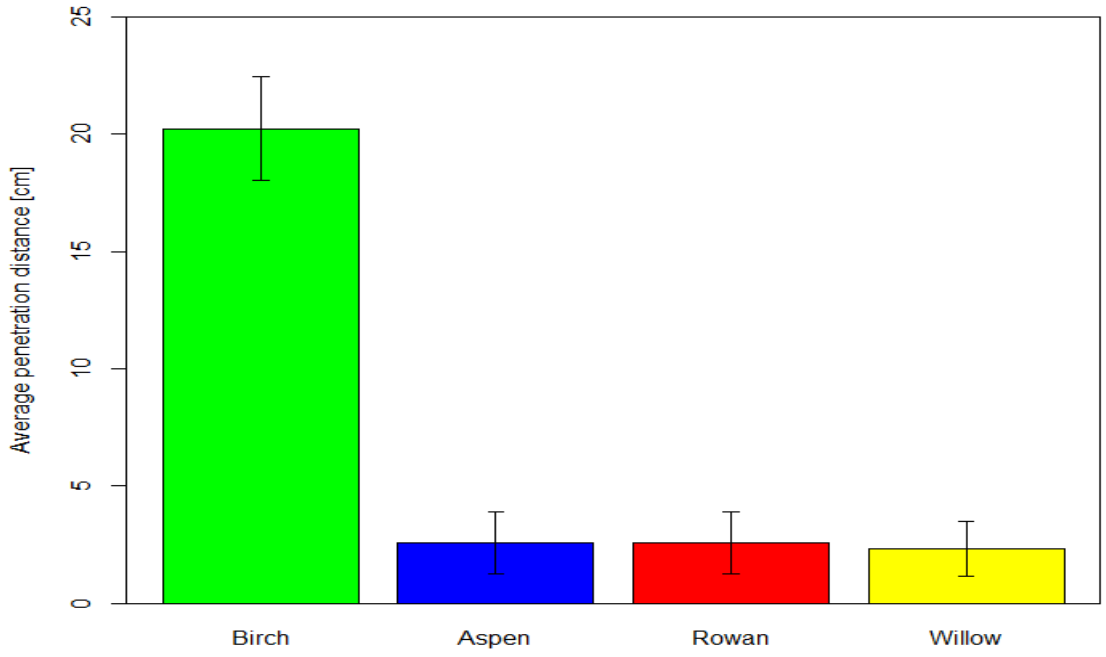

Figure 11: The average fungal penetration distance per tree species in centimetres. See Table 11.

5 Discussion

5.1 Birch

In birch stumps, the fungus treatment seemed to cause higher mortality rate (42.6%) than in the control treatment (0%). This result is in accordance with the results acquired by Vartiamäki et al. (2009), which showed *C. purpureum* to be a suitable biological control agent for birch, causing 70% mortality of inoculated birch saplings after one year. However, observed mortality in my study was also promising as after three months the mortality was already 42.6% although the treated birch stumps were smaller than in the study by Vartiamäki et al. In general, the fungus treatment seemed to perform best in birch stumps. Herbicide treatment seemed to have a positive effect on the number of sprouts per living stump. Possibly those saplings that survived in the herbicide treatment experienced a stress reaction, causing them to mobilize resources to grow more sprouts. The significantly negative effect of the number of saplings growing in proximity (AllSpecies) of birch saplings might be explained by the competition for nutrition, light, and growing space they caused to treated saplings (Coomes and Allen, 2007). Based upon the results, herbicide and fungus treatment seem to delay or even completely stop vertical growth of stump sprouts of birch saplings.

5.2 Aspen

In aspen, no significant difference in mortality between the fungus (13.5%) and the control treatment (9.1%) was found. As it seemed, the exposure time was not long enough for the fungus to decay aspen saplings and cause mortality. It is important to remind that saplings were only exposed to their treatments for about three month, while it has been investigated that the fungus treatment shows its full efficacy after at least two years (Vartiamäki, Hantula and Uotila, 2009). However, Hamberg et al., 2011, have found 57 % mortality in aspen after one year exposure time, which tended to be significantly higher than mortality caused by mechanical cutting. However, high mortality rate caused by the herbicide treatment, shows that Biomax can kill aspen sprouts faster than fungus treatment. Differences between the number and height of stumps sprouts were not found. However, only one aspen stumps was living after the herbicide treatment and therefore this treatment couls not be included in the statistical analyses. In aspen, the number of stump sprouts per living stump was higher when the number of saplings growing in proximity increased, possibly since these saplings tended to be

connected by their belowground stems to other conspecific saplings, which may support each other (DesRochers and Lieffers, 2001). Diameter seemed to have a significantly positive effect on the number of sprouts in aspen; bigger saplings in terms of stump diameter tended to produce more sprouts.

5.3 Rowan

In rowan, no differences in mortality between the fungus and the control treatments was found, as in both treatment mortality was low. However, Hamberg et al., 2014, have found 27% of all fungus treated rowan stumps to be dead after one year exposure time, which was significantly higher than in the mechanical cutting. Herbicide treatment seemed already after ca. three month of exposure time to cause high mortality rates to rowan saplings and is therefore a fast and reliable solution to prevent sprouting in rowan saplings. However, saplings surviving the herbicide treatment tended to have more but lower sprouts compared to the control treatment. This might have been the case since saplings surviving the herbicide treatment experienced a stress reaction, causing them to mobilize resources to grow more sprouts. The significantly negative effect caused by the number of saplings growing in proximity on rowan saplings might be explained by the competition for nutrition, light and growing space they caused. Diameter seemed to have a significantly positive effect on the number of sprouts in rowan stumps; bigger saplings in terms of stump diameter tended to produce more and higher sprouts. Possibly these saplings had a larger pool of resources in belowground stems to allocate to vertical growth.

5.4 Willow

In willow, no significant differences between the fungus and the control treatments were found (mortality was low in both of the treatments). However, herbicide treatment tended to efficiently prevent sprouting in willow already after about three month of exposure time. However, the results indicated that the number of stumps sprouts in the living stumps was higher but the height lower than in the other treatment due to possible willow resource allocation to stumps sprout growth when the growth was efficiently limited by the herbicide. Diameter seemed to have a significantly positive effect on the maximum height of stump sprouts in willow stumps. This might have been the case

because saplings with larger basal diameter also tend to allocate more resources from belowground stems to grow higher sprouts.

5.5 Root penetration

The fungus has penetrated furthest within birch stumps. This result was expected since the fungus was expected to affect birch saplings the fastest because in earlier research the efficacy in the fungus treatment has been high in birch (Vartiamäki et al. 2009). No significant differences among other tree species in terms of average penetration distance were observed. *C. purpureum* was clearly isolated from root samples of birch saplings, which has not been observed before.

6 Conclusion

After an exposure time of three month, *C. purpureum* seems be a valid alternative to chemical herbicides to prevent sprouting in birch. Concerning aspen, rowan and willow, exposure time to the fungus treatment might not have been long enough to distinguish it clearly from the control treatment. However, the herbicide treatment was efficient in aspen, rowan and willow after three months exposure time. Furthermore, this study has shown that *C. purpureum* is able to penetrate into the roots of birch saplings. More research should be conducted to investigate the treatment efficacies after longer exposure time.

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Appendices

Appendix 1: Diagnosing the models

Diagnostic analyses relating to the models were performed in order to ensure that all requirements were met (Figures 12-14). The residuals vs. fits plots depict the expected value of the response variable predicted by the model on the x-axis, against the deviation of each measurement value within the response variable, from the calculated regression line on the y axis.

Figure 12: Residuals vs. fits plot with respect to Mortality

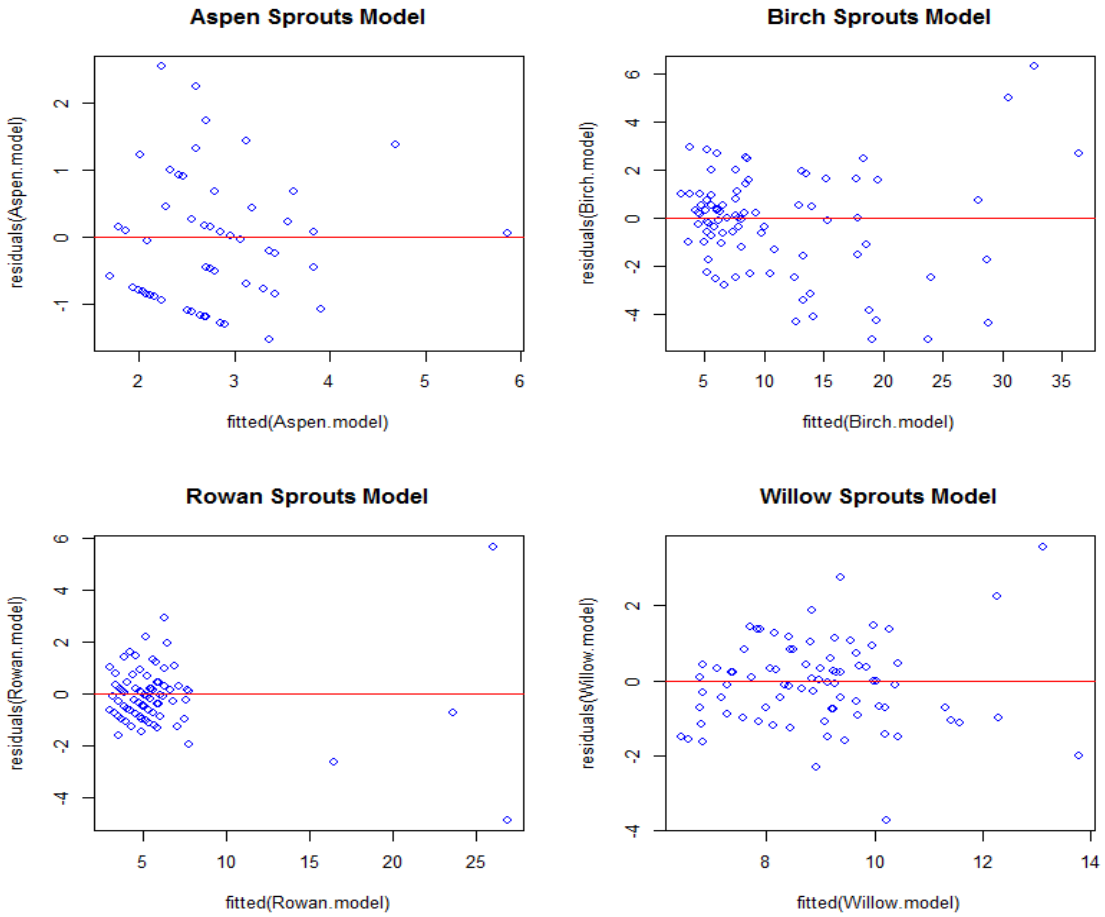

Figure 13: Residuals vs. fits plot within each tree species with respect to Sprouts.

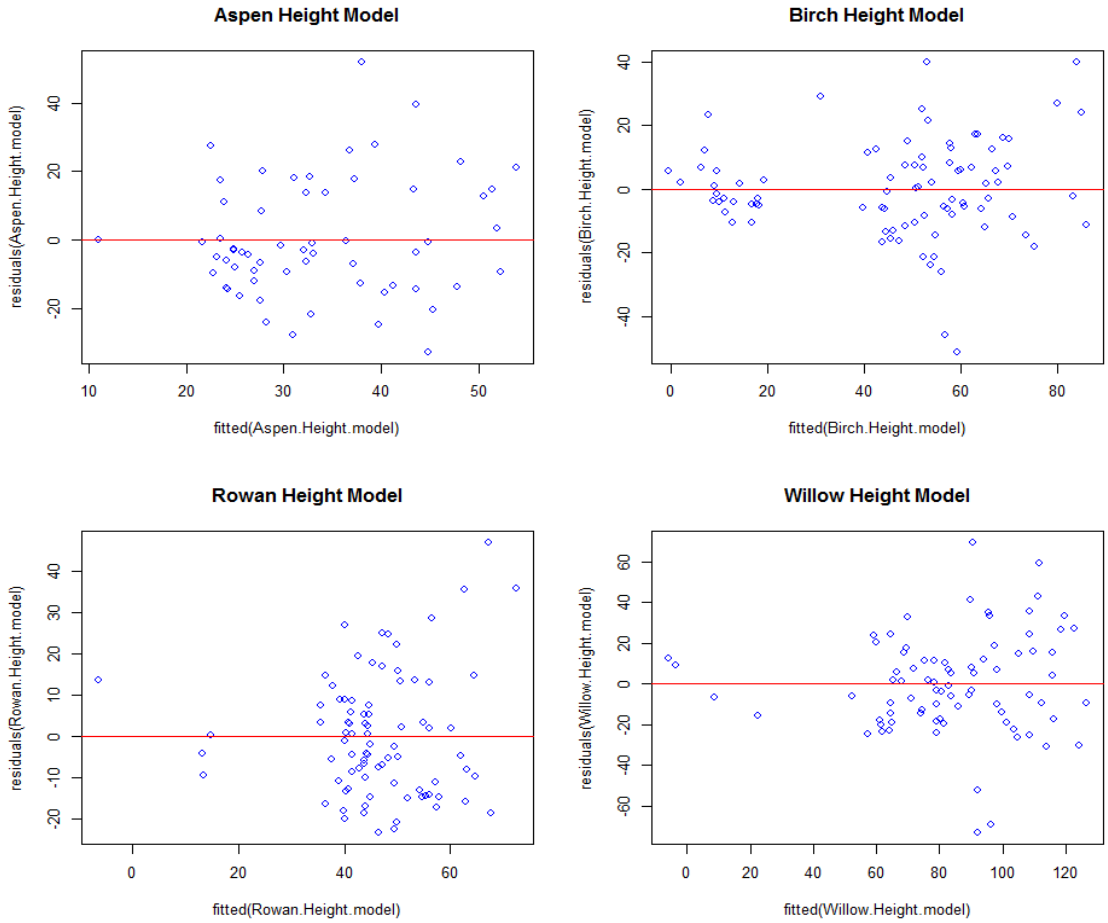

Figure 14: Residuals vs. fits plot of each tree species with respect to HighestSprout.