Annual Report
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Improvement of forest reproductive material for ash: characterizing the resistance against ash dieback (Askskottsjuka)

Project: 2016-015

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Popular Scientific Summary:

Over the last two decades common ash (*Fraxinus excelsior*) has been threatened by an alien invasive fungal pathogen (*Hymenoscyphus fraxineus*) introduced from Asia. The rapid spread and intensification of the disease has resulted in a serious population decline; since 2010 ash is a Red-listed species in Sweden. This is concerning not only for the loss of this important noble broadleaved tree species, but also for the biodiversity of species dependent on ash. Genetic resistance is an important tool for disease management to conserve the species from further population decline. Large variation in susceptibility to the pathogen has been observed within natural populations; with less than 5% of trees showing disease resistance. Studies have shown that suceptibility to damage is a heritable trait that is genetically controlled and that considerable gain can be achieved through selection and breeding.

The aim of the work is to support the development of a more resistant ash population for planting in Sweden. To achieve this, we have initiated two projects that use both traditional selection and modern phenotyping techniques to characterize the resistance in ash..

During 2013-2015 we generated a large inventory of putatively resistant genotypes (showing high levels of natural resistance to the pathogen) across the whole range in Sweden, and propagated a first test population. The first project builds on those efforts to now implement the research into practice. During 2016, we established a field trial at Snogeholm using propagated selections of wild-type genotypes to screen for, and characterize, the resistance. First assessments have been conducted on the planted genotypes and will continue in subsequent years. This work is critical to enable targeted genotypes to be selected for further commercial propagation, breeding and future establishment of new seed orchards.

In the second project we explored possibility of using modern phenotyping techniques to identify resistant ash genotypes. This technique, also referred to as marker-assisted selection, involves rapid and accurate phenotyping of trees that can be conducted in the field based on known resistant characteristics of the host. During 2016, we initiated a study using a state-of-the-art chemical fingerprinting technique known as Fourier-transform infrared (FT-IR) spectroscopy and chemometric modelling on a unique collection of material acquired from genetic field experiments across Europe, to *i)* discriminate between resistant and susceptible ash genotypes and *ii)* predict the concentration of putative phenolic biomarkers associated with resistance. Using a soft independent modeling of class analogy model built with infrared spectra of phloem phenolic extracts, it correctly predicts the tree phenotype, and we have validated this model across large populations of ash in Europe. These results suggest that this modern phenotyping technique can provide a promising approach for identifying disease resistance and can drastically advance the efficiency and timing of selecting genetically resistant ash trees, thus potentially, significantly expedite our current selection and screening protocols for breeding efforts.

Collectively, the results of both projects will be extremely important for the restoration and sustainable management of this important noble broadleaved tree species in Swedish forests, cities and other urban and natural landscapes.

Annual Results:

PROJECT 1:

During 2016, a field trial was established at Snogeholm with 2-year-old ash tree clones (grafted plants propagated with scions) of healthy, vital, mature *F. excelsior* trees. The trees were previously selected based on extensive surveys conducted between the years 2013 and 2015 in forests and the natural landscape including key habitat areas for ash (nyckelbiotop) and known seed stands throughout the natural distribution range of ash in the southern half of Sweden. In those surveys, more than 500 vital ash trees were identified and marked for selection and further monitoring. The estimation of tree vitality was done based on the percentage of crown damage in relation to other damaged trees in heavily diseased areas. For a tree to be considered vital and marked for selection, at least 80 percent of the crown needed to be intact but stem quality and growth characteristics were secondary to tree vitality (Fig. 1).

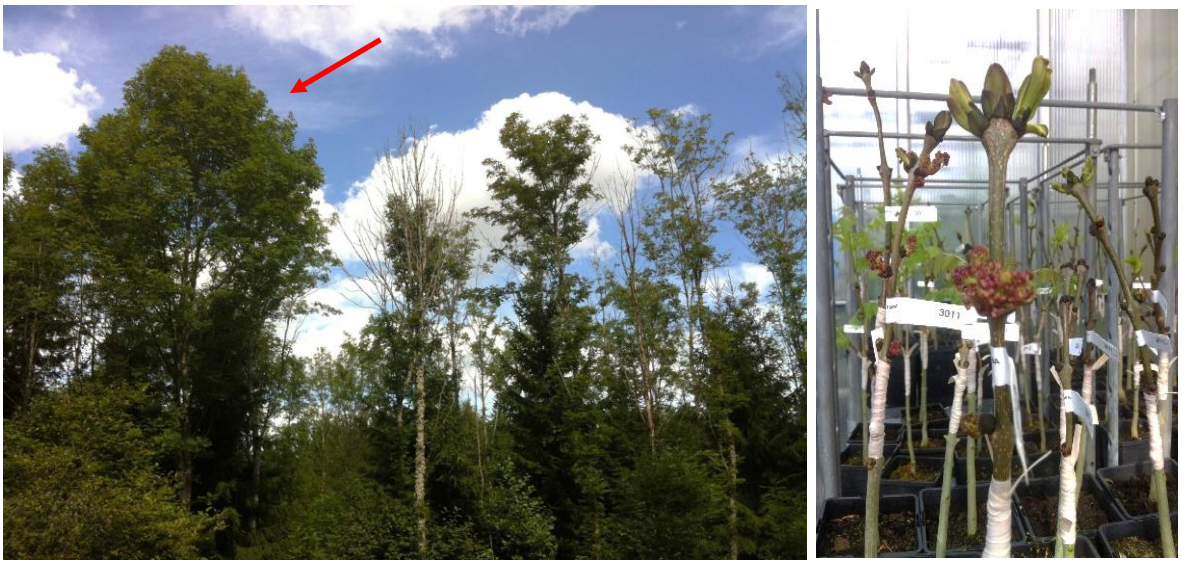

Figure 1. (LEFT) Example of a resistant ash tree showing high vitality with little to no dieback in the crown (red arrow) situated alongside severely diseased trees showing extensive dieback. (RIGHT) Scions collected from those resistant genotypes from the wild population grafted to root stock and grown in a controlled climate chamber for 1 year prior to establishment in the field trial.

Preparatory work for trial establishment included a deep plowing and harrowing of the soil, and herbicide treatment to help control competing vegetation. The trial's perimeter was marked by a fence to prevent browsing of seedlings by rabbits and/or deer (Fig. 2). For this first test population, we selected 56 of those genotypes and up to 13 replicates were propagated for each selected genotype by grafting onto *F. excelsior* rootstock originating from a known resistant genotype from Denmark. We also included in the trial design four known susceptible mature *F. excelsior* tree clones and five Asian *Fraxinus* tree clones, to serve for comparison of genotype performance. The trial at Snogeholm was established using a randomized block design. A total of 65 clones (Table 1) were planted in May 2016 at 1.5 m spacing divided among 12 plots where at least one graft per clone was randomly established in each plot. The Asian *Fraxinus* selections belonging to the species *mandshurica*, *japonica* var. *stenocarpa*, *platypoda*, and *spaethiana*, were selected from known accessions at arboreta and botanical gardens and included to give a unique comparison of what is presumably a known host to *H. fraxineus* in its native origin of Asia. Health assessments were done in late July and September. Disease incidence ratio, survival, and height measurements were determined at the end of the season. At present we are in the process of compiling the project data (from assessments) for further analysis and ranking of genotypes.

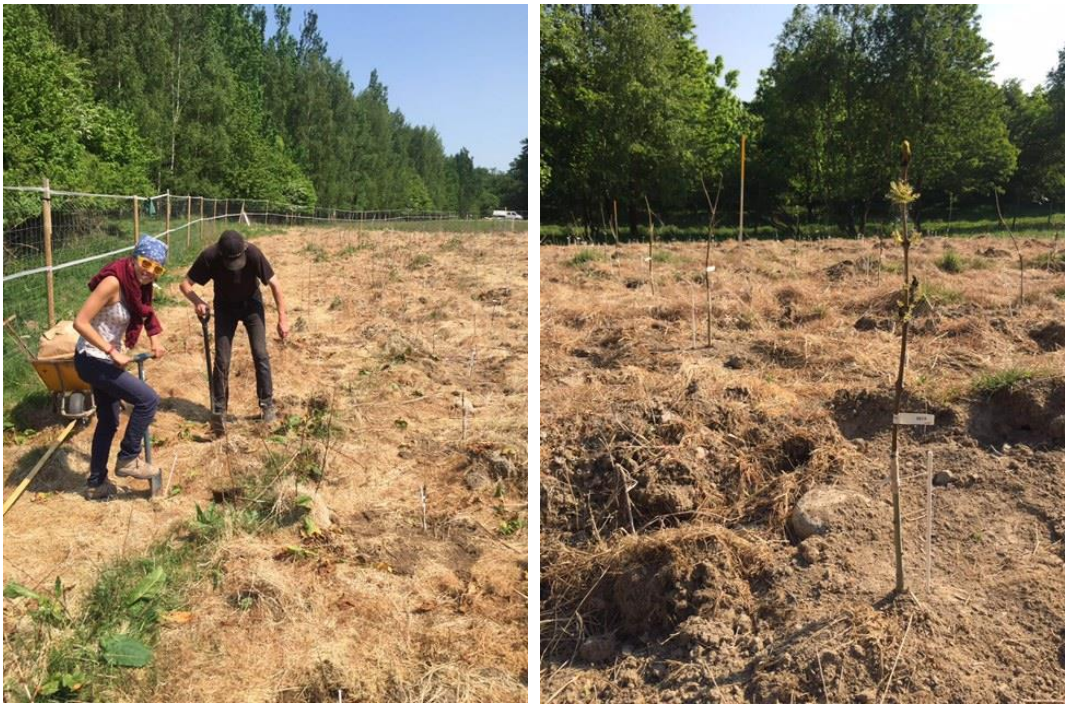

Figure 2. Site preparation and planting of the Snogeholm trial with selected genotypes from wild populations in spring 2016.

Table 1. List of tested clones at Snogeholm trial

Clone number	Origin	Fraxinus species	Susceptibility rating[1]	No. of ramets
8	Trolleholm	*F. excelsior*	Tolerant	13
44	Trolleholm	*F. excelsior*	Tolerant	13
57	Trolleholm	*F. excelsior*	Tolerant	13
62	Trolleholm	*F. excelsior*	Tolerant	13
65	Trolleholm	*F. excelsior*	Tolerant	13
66	Trolleholm	*F. excelsior*	Tolerant	12
89	Trolleholm	*F. excelsior*	Tolerant	9
93	Trolleholm	*F. excelsior*	Tolerant	13
3001	Munkedal	*F. excelsior*	Susceptible	12
3002	Munkedal	*F. excelsior*	Tolerant	11
3003	Munkedal	*F. excelsior*	Tolerant	13
3004	Munkedal	*F. excelsior*	Tolerant	13
3005	Munkedal	*F. excelsior*	Tolerant	8
3006	Öland - Kalkstad	*F. excelsior*	Susceptible	13
3007	Öland - Ismantorp	*F. excelsior*	Tolerant	13
3008	Öland - Ismantorp	*F. excelsior*	Tolerant	12
3009	Öland - Ismantorp	*F. excelsior*	Tolerant	13
3010	Öland - Ismantorp	*F. excelsior*	Tolerant	12
3011	Öland - Ismantorp	*F. excelsior*	Tolerant	11
3012	Öland - Kalkstad	*F. excelsior*	Tolerant	13
3013	Öland - Kalkstad	*F. excelsior*	Tolerant	12
3014	Öland - Kalkstad	*F. excelsior*	Tolerant	13
3015	Öland - Kalkstad	*F. excelsior*	Tolerant	13

3016	Omberg	*F. excelsior*	Tolerant	6
3017	Jönköping	*F. excelsior*	Tolerant	12
3018	Nässjö	*F. excelsior*	Tolerant	13
3019	Nässjö	*F. excelsior*	Tolerant	7
3020	Nässjö	*F. excelsior*	Tolerant	13
3021	Omberg	*F. excelsior*	Tolerant	11
3022	Omberg	*F. excelsior*	Tolerant	13
3023	Omberg	*F. excelsior*	Tolerant	13
3024	Omberg	*F. excelsior*	Tolerant	13
3025	Öland - Löttorp	*F. excelsior*	Susceptible	12
3026	Öland - Löttorp	*F. excelsior*	Tolerant	13
3027	Öland - Löttorp	*F. excelsior*	Tolerant	13
3028	Öland - Löttorp	*F. excelsior*	Tolerant	13
3029	Öland - Löttorp	*F. excelsior*	Tolerant	12
3031	Alnarp	*F. excelsior*	Tolerant	13
3032	Alnarp	*F. excelsior*	Tolerant	9
3033	Alnarp	*F. excelsior*	Tolerant	13
3034	Alnarp	*F. excelsior*	Tolerant	13
3035	Alnarp	*F. excelsior*	Tolerant	12
3036	Sturup	*F. excelsior*	Tolerant	13
3037	Sturup	*F. excelsior*	Tolerant	13
3038	Sturup	*F. excelsior*	Tolerant	13
3039	Sturup	*F. excelsior*	Tolerant	13
3040	Sturup	*F. excelsior*	Tolerant	13
3041	Sturup	*F. excelsior*	Tolerant	13
3042	Sturup	*F. excelsior*	Tolerant	13
3043	Sturup	*F. excelsior*	Tolerant	13
3044	Sturup	*F. excelsior*	Tolerant	13
3045	Sturup	*F. excelsior*	Tolerant	13
3046	Karlsborg	*F. excelsior*	Tolerant	13
3047	Karlsborg	*F. excelsior*	Tolerant	12
3048	Karlsborg	*F. excelsior*	Tolerant	13
3049	Lysekil	*F. excelsior*	Susceptible	13
3050	Lysekil	*F. excelsior*	Tolerant	13
3051	Lysekil	*F. excelsior*	Tolerant	12
3052	Gothenburg	*F. japonica var.stenocarpa*	Tolerant	8
3053	Gothenburg	*F. mandschurica*	Tolerant	11
3054	Gothenburg	*F. mandschurica*	Tolerant	11
3055	Gothenburg	*F. platypoda*	Tolerant	5
3056	Alnarp	*F. spaethiana*	Tolerant	11
3057	Lysekil	*F. excelsior*	Tolerant	9
3058	Lysekil	*F. excelsior*	Tolerant	9

[1] based on field surveys conducted by M. Cleary and L-G. Stener during the years 2013 and 2015.

For Project 1, all research-oriented activities are progressing according to the timeline specified in the original proposal. In addition to this first trial establishment we have collected and grafted scions from an additional 56 selections and propagated those as clones in a controlled climate chambers during 2016. These selections are currently in cold storage and will be planted out as a

second test population at a nearby site located also at Snogeholm in the spring 2017. We plan to proceed with the remaining research activities related to the field trials (genotype assessments) during 2017 as well as information dissemination and extension activities planned for this project.

PROJECT 2:

Vibrational infrared (IR) spectroscopy is a highly sensitive, rapid and high-throughput chemical fingerprinting technique which can separate biological samples into functional groups on the basis of how samples absorb infrared radiation. IR spectroscopy shows great potential for non-invasive measurement of quality parameters that are important in disease resistance (Martin et al. 2005; Conrad et al. 2014; Conrad and Bonello 2015). In order to evaluate the usefulness of this technique for future use in practice, we conducted large scale testing on populations of European ash where the genetic, inheritable resistance of individuals against damage by *Hymenoscyphus fraxineus*, is known based on field evaluations conducted over several years.

For this study, we collected phloem and leaf samples from *F. excelsior* trees with known susceptibility to *H. fraxineus* in six European countries: Austria, Denmark France, Germany, Lithuania, and Sweden, in collaboration with several colleagues (geneticists) in each of those countries. Source material originated from genetic trials established as either clonal seed orchards (Kirisits and Freinschlag 2012; Stener 2013; Enderle et al. 2015) or for testing ash provenance (McKinney et al. 2011) or progeny (Pliūra and Baliuckas 2007, Pliūra et al. 2011, 2014; Muñoz et al. 2016) (See also Table 2). Sample collection was performed between May and June. The timing for sample collection was critical to ensure that samples were collected prior to the normal sporulation period for *H. fraxineus* which typically occurs between June and September, with peak sporulation between mid-July and mid-August.

At each site, a minimum of three and up to eight genotypes, were selected per susceptibility class (low, intermediate and high susceptibility) based on a relative measurement of dieback intensity as determined in previous assessments. In the case of clonal trials, between two and three ramets per clone were sampled. From each individual, the current year's shoots were harvested. Some shoots that were not reachable by ground were taken at a higher height (up to 5 m) with a pole scissor. On susceptible genotypes, most if not all sampled shoots were from epicormic shoots. Leaves collected in the field were labelled according to country, trial, family, ramet and susceptibility status, placed in a plastic bag and immediately stored on dry ice. At the base of each leaf, sections of phloem consisting of outer bark, cortex and some cambium were dissected from the stem with a sterile razor blade, and similarly labelled and stored on dry ice in the field. All samples were then transported cold to SLU laboratory at Alnarp for further processing.

In the lab, phloem and leaf tissues were finely ground in liquid nitrogen and stored at -80˚C. 200 ±1 mg aliquots of either tissue type were placed in individual 2 mL microcentrifuge tubes. Samples were kept cold during this process with liquid nitrogen so as to avoid any warming and oxidation of tissue, and then stored at -20˚C until chemical extraction. Chemical extracts of samples were obtained by adding 700 µl of 70% acetone, 30% water to each tube. Samples were subjected to sonication for 30 min under room temperature, followed by centrifuging at 1600 rcf for 8 min. The supernatant was transferred to a new 2 mL tube and twice the volume of chloroform added. Samples were then centrifuged at 10000 rcf for 2 minutes at 10˚C and the supernatant collected and transferred to a new 2 mL screw-cap tube with O-ring seal. Samples were then lyophilized and stored at room temperature. Crude sample extracts purified on a C18 column using HPLC-grade methanol and collected in new microcentrifuge tubes. Samples were stored in -20˚C until further analysis.

Table 2. Source material of resistant and susceptible genotypes used in the FT-IR study.

			Trial details							No. of samples collected per susceptibility class			
Country	**Location / Trial name**	**Type of genetic trial**	**Coordinates**	**Elevation (m asl.)**	**Est. year**	**Size (ha)**	**No. of clones**	**No. of ramets per clone**	**Spacing of trees (m)**	**Suceptible**	**Inter-mediate**	**Resistant**	**No. ramets per clone**
Austria	Feldkirchen an der Donau, Upper Austria	Seed orchard	48°19'12.5" N, 14°04'15.9" E	264	1993	1.36	51	2 to 4	7.5 x 8.6	7		7	2
Denmark	Tuse næs, Northern Sealand	Seed orchard	55° 45' 57.99" N, 11° 42' 47.48" E	22	1998	2	39	25	3 x 6	3	2	3	3
France	Devecey, East	Provenance + family comparison trial	47°19'31.5" N, 06°01'54.1" E	250	1995	1.36	788	1	4 x 4	7	7	7	1
Germany	Weisweil, Baden-Württemberg	Provenance trial	48°11'29.7" N, 7°42'02.5" E	173	2005	0.22	577	1	2.0 x 2.0	5	0	5	1
Lithuania	Sasnava, Marijampolė	Clonal archive	54°37'32.1" N, 23°33'55.5" E	100	2012	2.5	228	3 to 7	6.0 x 5.4	4	3	5	2-3
Sweden	Snogeholm	Seed orchard	55°32'33.8" N, 13°42'22.7" E	50	1992	4.4	100	40-60	3.5 x 3.5	4	8	7	1-3

Samples representing the extreme susceptibility groupings of *F. excelsior* genotypes, resistant and susceptible, were analyzed on a Cary 630 FT-IR spectrometer. Spectra were collected over a range of 4000-7000 cm-1 at 4 cm-1 resolution and an interferogram of 64 scans was co-added for each sample. Spectral data were displayed in terms of absorbance and viewed using Win-IR Pro Software (Aglient Tehcnologics Inc. Santa Clara, CA, USA), and then analyzed using a multivariate classification software for the selective differentiation and identification of the target classes (susceptible vs resistant). Soft independent modeling of class analogy (SIMCA), a well-developed and accepted pattern recognition method in IR spectroscopic analysis (De Maesschalck et al. 1999) was be used to identify variables important to discriminate between susceptible or resistant individuals. The results of the IR analysis show clear differentitaion between resistant and susceptible genotypes for stem tissue, but not for leaf tissue. The model was validated using random sampling which correctly (100%) identified resistant and susceptible genotypes. The results of this work demonstrate that modern phenotyping techniques are a promising approach for identifying disease resistance and drastically advance the efficiency and timing of selecting genetically resistant trees. This finding is significant as it open the possibility for the development/refinement of advanced instrumentation for chemical metabolite profiling to be used in practice.

For Project 2, all research-oriented activities are progressive according to the timeline specific in the original proposal. At the moment we are preparing a manuscript and will proceed with the information dissemination and extension activities planned for this project.

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