Dynamics of Genetic Resistance to *Hymenoscyphus pseudoalbidus* in Juvenile *Fraxinus excelsior* Clones

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Abstract

The study was aimed to evaluate a temporal change in genetic resistance to *Hymenoscyphus pseudoalbidus* and in level of its genetic variation and heritability in juvenile *Fraxinus excelsior* clones initially tested and selected for resistance in progeny trials. Data on tree growth and ash dieback symptoms of 47 (26 Lithuanian and 21 foreign) clones representing trees of improved resistance against *H. pseudoalbidus* and 7 control clones from random Lithuanian populations were collected during the three growing seasons in seven clonal trials in 2012 and compared with previously reported data of these trials. Evaluations revealed a considerable increase in disease occurrence and damage severity thus showing the continuous spread of infection. Incidence of disease increased from 10.7% in spring 2012 to 72.2% by spring 2013 and remained almost constant till the autumn 2013. In spring 2012, dieback symptoms occurred on 45.9% of the tested clones on 3–50% of their ramets while in 2013 disease occurred on 100% of the tested clones on 2–100% of their ramets. On 23.4% of tested clones disease incidence was equal or below 50%, while mortality increased very little and reached only 1.2%. There were evident differences in ash dieback severity between the investigated populations. The lowest disease mean incidence remained in clones originating from three Lithuanian populations – Ignalina, Žeimelis and Kėdainiai (52.2, 54.3 and 67.4% respectively), while the highest was found in clones of German – Farchau (86.0%) and Lithuanian – Šakiai (88.7%) populations. Most clones shoved significant growth recovery by the end of 2013 vegetation season regaining and slightly increasing the height that was lost after diseased leader shoots had dried-up.

Variance analysis of tree damage traits such as health condition and disease incidence in 5th and 6th growing seasons revealed constantly increasing clonal variance component, coefficients of genetic variation and heritability which indicated permanent substantial genetic control of the disease resistance and therefore good prospects for further tree breeding for resistance at a clonal level. Clonal variance component (vc_c) of disease incidence over all trials increased from 8.15% (19.0–70.5% in individual trials) in spring 2012 to 14.76% (23.9–63.2% in individual trials) in autumn 2013 and broad sense heritability (H^2) increased from 0.33 to 0.40 (means from all 7 trials) thus indicating increasing genetic variation in susceptibility to disease. The vc_c for tree health conditions increased from 13.60% to 16.35% and H^2 decreased from 0.40 to 0.38. Meanwhile vc_c for length of dry/necrotic part of leader shoot decreased from 29.86% to 8.12% and H^2 decreased from 0.74 to 0.51 thus indicating decreasing genetic variation in disease spread rate within trees. Genotype-environment interaction (G × E) vc_c in most of tree damage traits decreased although remained significant thus suggesting presence of genetic variation in plasticity and reaction norms of clones across a range of infection load/pressure environments.

Keywords: *Hymenoscyphus pseudoalbidus, Chalara fraxinea*, common ash, dynamics of dieback, disease resistance, dynamics of genotypic variation, heritability

Introduction

Currently, severe dieback of common ash (*Fraxinus excelsior* L.) is occurring in many European countries, causing massive tree mortality (Timmermann et al. 2011, Anon. 2012a, Gross et al. 2013). The disease is caused by an ascomycete fungus *Hymenoscyphus pseudoalbidus* V. Queloz, C.R. Grünig, R. Berndt, T. Kowalski, T.N. Sieber & O. Holdenrieder (Queloz et al. 2010), which in Europe is considered an invasive species (Husson et al. 2011, McKinney et al. 2012). Symptoms of the disease typically include discolouration and subsequent wilting of leaves and petioles, necrotic, eye-shaped spots on petioles and the bark of young shoots, elongated necrotic lesions, cankers on stem and wood discolouration, followed by gradual dieback of the crowns (Lygis et al. 2005, Bakys et al. 2009a, Kowalski and Holdenrieder 2009a,b, Kirisits et al. 2009, Skovsgaard et al. 2010). The main infection pathway is assumed to be via ascospores that infect leaves and petioles and then spreads to the ligneous tissues (Kirisits et al. 2009, Kräutler and Kirisits 2012, Kirisits and Freinschlag 2012, Cleary et al. 2013). Another infection pathway though lenticels has been proposed by Husson et al. (2012).

In Lithuania, the dieback of *F. excelsior* caused by *H. pseudoalbidus* started around 1995–1996 in forests of the north-central part of the country (Juodvalkis and Vasiliauskas 2002). Subsequently, due to continuous sanitary fellings, the area of ash stands has decreased from 50,800 ha in 1995 (Anon. 2001) to 35,700 ha in 2012 (Anon. 2012b), or from 2.7% to 1.7% respectively of the total forest area in Lithuania. Currently the epidemic is in a chronic phase; all ash stands are damaged and their health conditions continue to deteriorate (Riepšas 2009, Gustienė 2010, Pliūra et al. 2011). No effective means to control the spread of the disease have been identified so far. Artificial re-establishment of ash stands is not recommended due to a high probability of dieback in newly established plantations (Lithuanian State Forest Service, personal communication, 2011). The future of common ash, an important ecosystem-forming tree species, is unclear and adequate gene conservation and management strategies need to be elaborated.

In damaged ash stands, the density of trees producing seeds presently is reduced to few individuals per hectare, meaning that the effective population size (N_e) has decreased substantially thus compromising the genetic diversity of *F. excelsior* in mature stands and in regenerating offspring (Pliūra et al. 2011). Current genetic diversity might be insufficient for further physiological and genetic adaptation and sustainability of ash populations thus predisposing damaged populations to collapse. The infection and severity of disease is under constant changes within – and among – years due to seasonal cycles of disease development and mortality of trees. Therefore it may result in continuous and cyclic changes of genetic variation of disease damage or resistance, adaptedness and growth traits in *F. excelsior* populations and forest plantations. Information on temporal dynamics of both disease development and genetic characteristics of affected populations, is of primary importance for evaluation of genetic variability, sustainability and adaptation potential of *F. excelsior*.

A substantial variation in the degree of damage of individual trees can be observed in natural populations of *F. excelsior*, however, as detected in various progeny trials, only 2–5% of trees remain symptom-free (McKinney et al. 2011, Pliūra et al. 2011). It is unclear whether these healthy-looking trees are truly resistant to infection or whether they tolerate the invaded pathogen. By definition, resistance to pathogens is the ability of the host to hinder the growth of pathogen; it is considered a genetic mechanism enabling a plant to suppress a disease or to retard invasion by a certain pathogen (Tapiero 1999). Investigations in natural stands, clonal orchards and progeny trials in a number of European countries provide evidence that susceptibility of *F. excelsior* to *H. pseudoalbidus* varies considerably between individuals, populations and families, and that there is a significant genetic heritability in disease resistance/tolerance (Pliūra and Baliuckas 2007, McKinney et al. 2011, Pliūra et al. 2011, Kjær et al. 2011, Husson et al. 2012, Kirisits and Freinschlag 2012, Stener 2013). Pliūra et al. (2011) and Kjær et al. (2011) have reported a significant genetic variation in the health conditions among tested half-sib ash progenies pointing to a quantitative resistance system based on multiple genes. Existence of genetically-based resistance at a clonal level has been demonstrated by McKinney et al. (2011) and Stener (2013). Due to the complex life cycle of *H. pseudoalbidus*, resistance or tolerance may be expressed in leaf or/and wood, and their importance may vary between clones (Kirisits and Freinschlag 2012). Relationships between phenology and degree of damage of *F. excelsior* have been documented (McKinney et al. 2011, Stener 2013, Bakys et al. 2013), yet these relationships were not consistent in all studies (Kirisits and Freinschlag 2012). Only a small fraction of the *F. excelsior* populations is likely to survive due to inheritable resistance mechanisms (Pliūra et al. 2011) or beneficial phenological traits such as early leaf senescence that prevents spreading of *H. pseudoalbidus* from infected leaves to wood (McKinney et al. 2011). The genetically inherited resistance may provide a basis for a natural adaptation of *F. excelsior* to this new selection factor. Widely used in plant breeding programs, the development of resistant cultivars is one of the most successful means of controlling plant diseases. Given the low proportion of resistant ash individuals in nature, extensive breeding programmes are undoubtedly needed to save the species (McKinney et al. 2011, Pliūra et al. 2011, Husson et al. 2012, Kjær et al. 2011, Stener 2013). A deeper knowledge on the genetic background of resistance/tolerance of *F. excelsior* to *H. pseudoalbidus* is needed to elaborate efficient breeding programmes. The success of a breeding program is, to a great extent, determined by the presence of sufficient genetic variation in the breeding population. Another important issue in breeding for resistance is precision of evaluations of resistance, identification and selection of truly resistant genotypes. Here the evaluation was based on estimates of heritability of damage traits. Constant changes in occurrence and severity of disease, due to cycles of disease spread, development and tree mortality in breeding populations (progeny or clonal trials) will most likely result in changes of genetic parameters such as coefficient of genetic variation, heritability and genetic correlations among traits. The highest

genetic gain can be obtained when genetic variation and heritability reaches its maximum. Therefore, to maximize genetic gain the most suitable age and time of the year has to be chosen for the most precise evaluation of the breeding values and for selection.

The aim of the present study was to estimate the temporal changes in damage due to *H. pseudoalbidus* and the changes in genetic variability and heritability in juvenile *F. excelsior* clones from Lithuanian and foreign populations, which were initially tested and selected for resistance to *H. pseudoalbidus* in progeny trials (Pliūra et al. 2011).

Materials and Methods

Study sites and clonal material

In the present study, 50 *F. excelsior* clones, preselected for resistance against *H. pseudoalbidus* from eight-years-old *F. excelsior* progeny trials established in 2005 (see Tables 2 and 6 in Pliūra et al. (2011)), were assessed for resistance against *H. pseudoalbidus* in new clonal trials. Of all tested clones, 26 represented four Lithuanian populations (Ignalina, Kėdainiai, Šakiai and Žeimelis) and 21 represented four foreign (Czech, French, German and Irish) provenances, while the Lithuanian mixture-control group (the remaining seven clones) consisted of three preselected clones from progenies of other tested Lithuanian populations, and four clones originating from healthy-looking trees selected in damaged forest stands. Forty-one clones were previously evaluated as very resistant (health condition score of parent ortet was 5), 11 – as resistant (score was 4), and two – of average resistance (score was 3) to *H. pseudoalbidus* (Pliura et al. 2011). Each clone was represented by 10–40 grafted ramets (1300 trees in total). After grafting (in 2010), ash clones were potted in peat containers and grown for two years under greenhouse conditions. All two-year-old root stocks was obtained from a forest nursery in Kaunas SFE (seed lot originated from local forests of Kaunas SFE). In spring 2012, all grafted ash juveniles were replanted in seven clonal trials established in seven geographical locations (Figure 1): Marijampolė, Šakiai, Kuršėnai, Biržai, Radviliškis, Kėdainiai and Dubrava SFE's. The trials were distributed over three provenance regions of *F. excelsior* (Figure 1, Table 1). Six of the trials (Marijampolė, Šakiai, Kuršėnai, Biržai, Radviliškis and Kėdainiai) were planted in the forest land, while Dubrava trial was established in the greenhouse (Table 1). Prior to replanting in the trials, most of grafted ash juveniles were in good health condition (only 2% of grafts had symptoms of disease in autumn 2011) and received identical light, temperature and moisture treatment. The trials were established in a randomized incomplete block design, with 2 blocks, each consisting of three to seven single-tree plots of each clone. Due to large number of clonal trials (7) the sample size for each clone in each clonal trial was small and varied for each block. Trees were planted in rows with 6 m × 5 m spacing.

In all seven clonal trials, measurements and scoring of tree growth and disease damage parameters were performed in May 2012 and repeated in May and August 2013 after fourth, fifth and sixth growing seasons (including four growing seasons in greenhouse), respectively. Tree heights were measured in May 2012

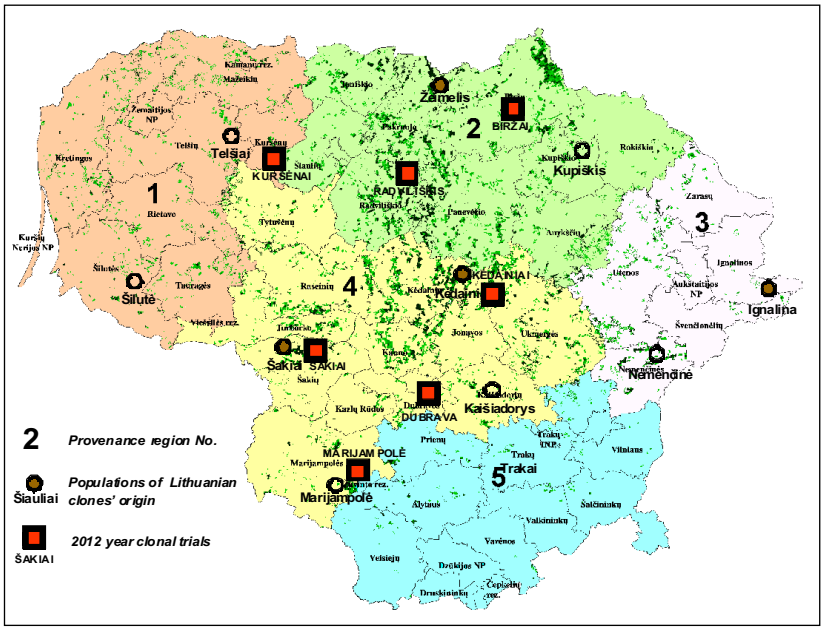

Figure 1. Locations of investigated *F. excelsior* clonal trials – seed orchards of 2012-year series and tested Lithuanian populations. Filled in circles indicate populations selected for resistance, empty circles indicate less resistant populations

Table 1. Site conditions of investigated *F. excelsior* clonal trials of 2012-year series in Lithuania

Name (State Forest Enterprise)	Location (Forest district)	Area, ha	Provenance region[a]	Soil type[b]	Site index[c]	Continentality index[d]
Dubrava	Vaišvydava (greenhouse)	-	4	Peat substrate in pots	Nd	30
Marijampolė	Sasnava	2.50	4	Albic Luvisols	Nd	30
Šakiai	Gelgaudiškis	3.30	4	Gleyic Luvisols	Ld	28
Kėdainiai	Lančiūnava	1.64	2	Gleyic Luvisols	Ld	29
Biržai	Joniškėlis	1.75	2	Hapli-Calc(ar)ic Luvisols	Nd	29
Radviliškis	Liepynė	2.10	2	Hapli-Calc(ar)ic Luvisols	Nd	28
Kuršėnai	Gedinčiai	1.90	1	Gleyic Luvisols	Ld	26

[a] as indicated on Figure 1
[b] according to IUSS Working Group WRB, FAO (2006)
[c] Nd – eutrophic (fertile) mineral soils of normal moisture, Ld – eutrophic (fertile) temporarily over-moisture mineral soils (according to Vaičys 1999)
[d] according to Chromov (1968)

and in May and August 2013 and height increments in 2012 and 2013 year growing seasons were calculated. Three types of disease damage parameters were estimated for each graft of *F. excelsior*: 1) disease incidence rate among ramets in clones, 2) tree health condition score, 3) length of necroses on the bark of leader shoot, lateral shoots and stem. Disease incidence within a certain clone was expressed as a ratio between the number of symptomatic ramets and the total number of ramets in a clone per site. Health condition of trees was scored on a five-point system: 5 – symptomless (no visible symptoms of disease); 4 – slightly damaged (sporadic disease symptoms on separate shoots or leaves (according to Bakys et al. 2009a, Kowalski and Holdenrieder 2009a): including brown wilted or dry leaves and/or single necrotic lesions on shoots), leader shoot is alive; 3 – moderately damaged (necrotic leader shoot and two or three lateral shoots; resprouting from below the damaged shoots); 2 – severely damaged (top shoot and half of the main stem or/and most of the shoots are necrotic, trees are not resprouting from below damaged shoots or is resprouting from the root collar; and 1 – dead (the main stem and all emerged sprouts are dead). For each tree with necrotic leader shoot, length of a necrotic part of leader shoot was measured. Also the length of necroses on lateral shoots was measured as well as total length of necrotic lesions on the plant to estimate the extent of tree damage. Bud flushing phenology was assessed in May 14 to 16 using a categorical scale of five degrees whereby 5 – very early; 4 – early; 3 – of moderate earliness; 2 – late; and 1 – very late (scoring adopted after Douglas et al. 2013). Relative bud flushing was expressed as a ratio between a number of flushed buds and the total number of buds on apical and lateral shoots of a given tree. Data obtained after growing seasons of 2012 and 2013 was analysed in conjunction with results of previous study (Pliūra et al., submitted).

Detection of *H. pseudoalbidus* in *F. excelsior* was done by PCR using a pair of species-specific ITS primers. A total of 48 wood and bark samples were collected in June 2012 from non-symptomatic ash trees growing in Marijampolė, Biržai and Kuršėnai plantations. Three samples were collected from each tree at the edge of necrotic lesions and pooled together to make one bulk sample of ca. 1 g fresh weight. Of each mixed bulk sample, ca. 0.2 g was placed into 2-ml screw cap tubes containing a metal screw and a nut. Before the homogenization procedure, the surface of the samples was washed in running tap water for 30 s to remove fungal spores. Extraction of total genomic DNA form the woody tissues followed procedure described by Cleary et al. (2013). DNA was purified using, JETquick DNA Clean Up Spin Kit' (Genomed, Lohne, Germany) following manufacturer's instructions. Presence of *H. pseudoalbidus* DNA in each sample was checked by PCR using the primers developed specifically for *H. pseudoalbidus* by Johansson et al. (2010) in combination with a CAPs marker (Sma I), following a procedure described by McKinney et al. (2012). PCR products, obtained using the primers by Johansson et al. (2010) were digested with a Sma I restriction enzyme (Thermo Scientific, Vilnius, Lithuania) according to manufacturer's instruction. In PCR, DNA solutions of *H. pseudoalbidus* and *H. albidus* were used as positive controls. The two polymorphisms gave rise to a Sma I digestion site in *H. albidus* (two bands of size 132 bp and 361 bp). This digestion site was not present in *H. pseudoalbidus*; the undigested PCR product yielded a size of 494 bp. Digests were visualised on 1.5% agarose gels.

Variance analysis

The variance analysis of data was done with the MIXED procedure in SAS Software (SAS® Analytics Pro 12.1 2012) which uses Mixed model equations (MME) and the restricted maximum likelihood (REML)

method. The significance of fixed effects (of block and clonal trial) was tested with *F*-tests and the significance of the random effects was tested with the *Z*-test within the MIXED procedure in SAS Software. The combined linear statistic model was used for joint analysis of data from all seven clonal trials together:

$$y_{ijklm} = \mu + z_i + b_j + p_k + c_l + c_l * z_i + \varepsilon_{ijklm} \quad (1)$$

where y_{ijklm} is an observation of the m^{th} tree from the l^{th} clone in the k^{th} provenance/population in the j^{th} block of the i^{th} clonal trial, m is the overall mean, z_i is the fixed effect of the i^{th} clonal trial, b_j is the j^{th} block effect, p_k is the k^{th} provenance/population effect, c_l is the effect of l^{th} clone, $c_l * z_i$ is the interaction effect of l^{th} clone and i^{th} clonal trial, e_{ijklm} is the random residual. The model assumes that random effects are normally distributed with expectation zero and corresponding variances: $\sigma_p^2, \sigma_c^2, \sigma_{c*z}^2$ and σ_e^2. The normality of residuals' distribution and homogeneity of variances were tested with SAS GLM and UNIVARIATE procedures (SAS® Analytics Pro 12.1 2012).

The variance components of random effects of provenances, clones and clone by site interaction (G × E) were computed from corresponding variances obtained in joint ANOVA and expressed in percentage of the total random variation:

$$vc_p^2 = \sigma_p^2 / (\sigma_p^2 + \sigma_c^2 + \sigma_{c*z}^2 + \sigma_e^2),$$

$$vc_c^2 = \sigma_c^2 / (\sigma_p^2 + \sigma_c^2 + \sigma_{c*z}^2 + \sigma_e^2),$$

$$vc_{cz}^2 = \sigma_{c*z}^2 / (\sigma_p^2 + \sigma_c^2 + \sigma_{c*z}^2 + \sigma_e^2),$$

where vc_p^2, vc_c^2 and vc_{c*z}^2 are the provenance, clone, and clone by site (G × E) interaction variance components, σ_p^2 is the provenance/population variance, σ_c^2 is the clonal variance, σ_{c*z}^2 is the variance of clone by site (G × E) interaction, and σ_e^2 is the variance of random residuals.

The simplified linear model was used for variance analysis of data from each individual clonal trial:

$$y_{jlm} = \mu + b_j + c_l + \varepsilon_{jlm} \quad (2)$$

where y_{jlm} is an observation of the m^{th} tree from the l^{th} clone in the j^{th} block, μ is the overall mean, b_j is the j^{th} block effect, c_l is the effect of l^{th} clone, and ε_{jlm} is the random residual.

The model assumes that random effects are normally distributed with expectation zero and corresponding variances σ_c^2 and σ_e^2.

Means of trials, provenances/populations and clones were computed using SAS MEANS procedure (SAS® Analytics Pro 12.1 2012).

Genetic parameters, clonal variance components (vc_c), coefficients of genotypic variation (CV_g), heritability coefficients (H_c^2) and their standard errors (SE) of each trait were assessed using variances and covariances obtained in analysis of variances, SAS MIXED procedure. The variance components of random effects of clones in each clonal trial were derived from corresponding variances and expressed in percentage of the total random variation:

$$vc_c = \sigma_c^2 / (\sigma_c^2 + \sigma_e^2) \cdot 100,$$

where vc_c is the clonal variance component, σ_c^2 is the clonal variance, and σ_e^2 is the variance of random residuals.

The coefficient of genotypic variation of a trait was calculated for each individual clonal trial using a formula:

$$CV_g = \sigma_c^2 \cdot 100 / \overline{X},$$

where $\overline{X}$ is the phenotypic mean of the trial (Falconer 1989, Falconer and Mackay 1996).

The broad sense individual heritability coefficients were calculated using a formula:

$$H_c^2 = \sigma_c^2 / (\sigma_c^2 + \sigma_e^2),$$

where H_c^2 is the individual heritability coefficient.

The broad sense clonal means heritability (clonal means repeatability) coefficients were calculated using a formula:

$$H_{cm}^2 = \sigma_c^2 / (\sigma_c^2 + \sigma_e^2 / n),$$

where H_{cm}^2 is the clonal means heritability coefficient and n is the harmonic mean number of ramets per clone.

Direct conventional genotypic gains were estimated, in relative value, according to Nanson (1988) for a selection differential i equal to 1 (selection of 38% of the clones tested):

$$\Delta G(\%) = i \cdot H_{cm}^2 \cdot CV_P,$$

where CV_p is the phenotypic coefficient of variation in relative value.

Genotypic correlation coefficients (r_G^2) between traits measured at the same time and among the same trait measured at the beginning and the end of vegetation seasons of 2012 and 2013 were estimated as (Becker 1984):

$$r_G = \sigma_{c(xy)} / \sqrt{\sigma_{c(x)}^2 \times \sigma_{c(y)}^2},$$

where $\sigma_{c(xy)}$ is the clone covariance component, $\sigma_{c(x)}^2$ is the clone variance component for the trait x and

$\sigma^2_{c(y)}$ is the clone variance component for the trait y. To calculate genotypic correlation coeficients the data were standardized to mean = 0 and $\sigma = 1$. Because of sampling errors and mathematical approximation, some genotypic correlations exceeded ±1. In these cases they were assumed to be equal to ±1 considering the asymptotic nature of distribution of correlation coefficients. The standard errors of genotypic correlations were estimated using the following equation (Falconer, 1989):

$$\sigma = \frac{1 - r_G^2}{\sqrt{2}} \sqrt{\frac{\sigma_{(H_x^2)} \sigma_{(H_y^2)}}{H_x^2 H_y^2}},$$

where H_x^2 is the heritability estimate of the character x, H_y^2 is the heritability estimate of the character y, $\sigma_{(H_x^2)}$ is the standard error of H_x^2 and $\sigma_{(H_y^2)}$ is the standard error of H_y^2.

Phenotypic correlations among traits were assessed using SAS CORR procedure (SAS® Analytics Pro 12.1 2012)

Results

The incidence rate of disease among all 1300 tested *F. excelsior* juveniles in field trials increased from 10.73% in spring 2012 to 72.16% by spring 2013 and slightly decreased to 71.38% till the autumn 2013 (Table 2, Figures 2, 3). Health condition score of tested trees deteriorated substantially by spring 2013 while

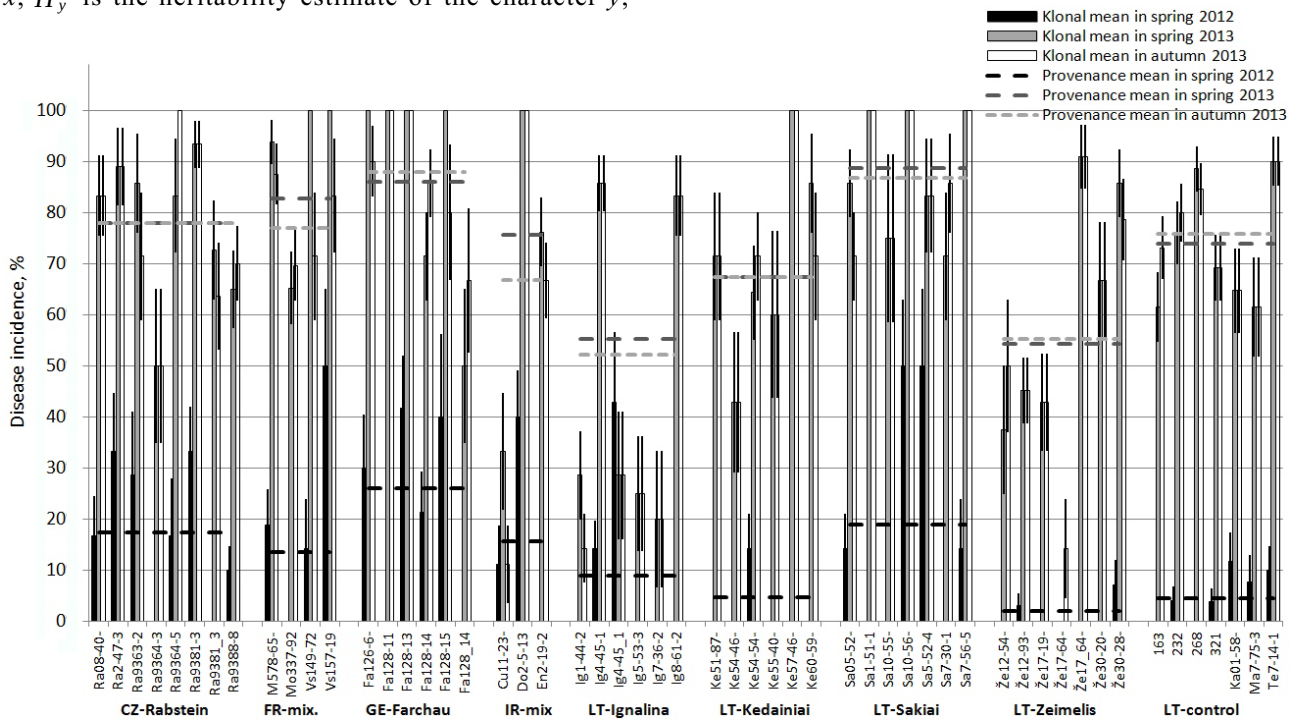

Figure 2. Incidence of ash dieback disease (a proportion of symptomatic ramets in a clone, %) in grafted clones of *F. excelsior* from Lithuanian (LT) and foreign (CZ – Czech Republic, FR – France, GE – Germany and IR – Ireland) populations in spring 2012, spring and autumn 2013 over seven clonal trials. First letters of the clone's code indicate an abbreviated name of a certain population (for population names and their origin please refer to Pliūra et al. 2011). Pins at top of the bars indicate standard errors

Table 2. Mean characteristics of disease damage and growth traits in course of three growing seasons in clonal trials of *F. excelsior*

Tree trait	Mean in spring 2012 ± se	Mean in spring 2013 ± se	Mean in autumn 2013 ± se
Disease incidence, %	10.73±0.86	72.16±1.25	71.38±1.26
Length of necrotic part of leader shoot, cm	14.15±1.24	39.60±0.76	44.82±0.89
Total length of necrotic lateral shoots, cm	14.43±0.68	31.74±0.93	32.27±0.94
Total length of necrotic lesions on stem, cm	7.17±0.85	15.89±1.05	9.84±0.53
Tree health condition score, points	4.54±0.02	3.46±0.04	3.49±0.03
Survival, %	100.00±0.00	98.60±0.33	98.13±0.38
Tree height, cm	69.93±0.71	86.96±0.81	94.47±0.97

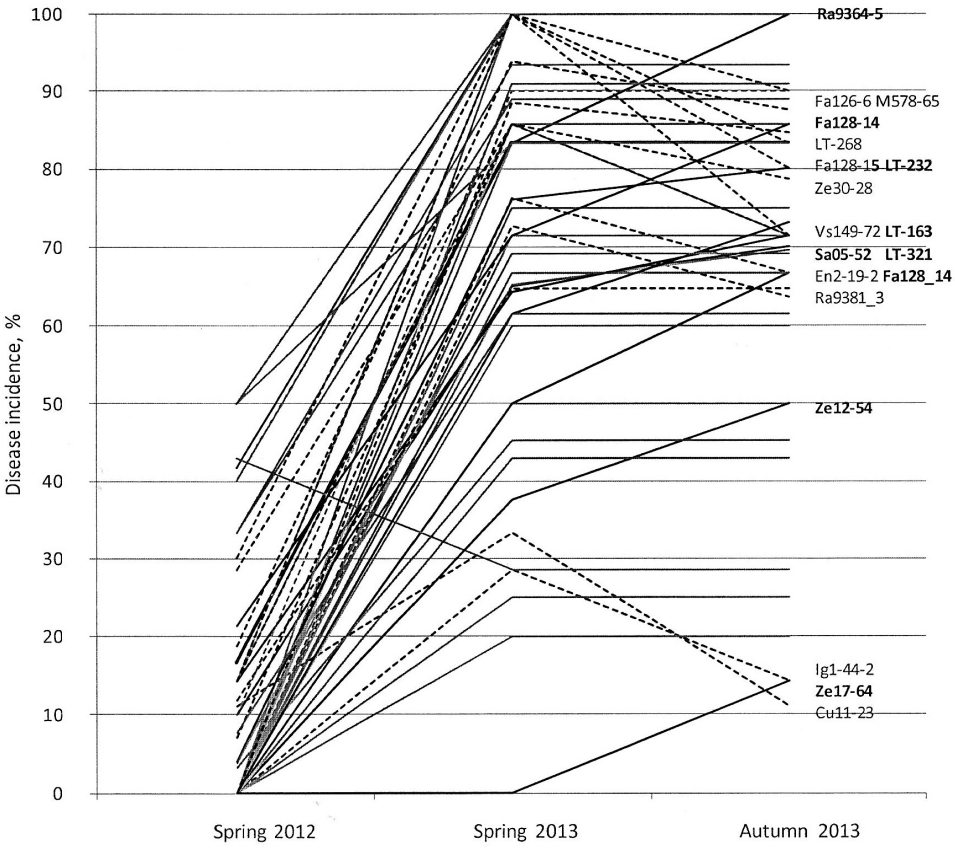

Figure 3. Changes in incidence of ash dieback disease (a proportion of symptomatic ramets in a clone, %) in grafted clones of *F. excelsior* populations during period from in spring 2012 to autumn 2013 over seven clonal trials. Solid bold lines and bold clone number indicate clones of increased disease incidence. Dashed lines indicate clones of decreasing disease incidence while gray lines indicate stabile disease incidence during 2013 vegetation season

it remained almost constant during 2013 vegetation season. DNA of *H. pseudoalbidus* was detected in 19 out of 48 (39.6%) sampled asymptomatic *F. excelsior* trees. Tree height increased constantly during both 2012 and 2013-yr growth seasons from 69.93±0.71 cm to 94.47±0.97 cm even after temporal height loss when diseased leader shoots had died (Table 2). The smallest clone in spring 2012 was Czech clone Ra9364-3, tree height reaching only 34.7±4.7 cm, while the largest was German clone Fa128-11 reaching 111.7±6.9 cm; the smallest clone in autumn 2013 was clone Že12-54 with height of 65.4±6.2 cm while the largest was clone Že12-93 from the same Lithuanian Žeimelis population with height reaching 129.1±5.1 cm (data not shown).

In spring 2012, dieback symptoms occurred on 45.9% of all 54 tested clones, and on 3–50% of their ramets, while in 2013, 100% of the tested clones and 2–100% of their ramets were symptomatic (Figure 2). On 23.4% of tested clones disease incidence rate was equal or below 50%. Mortality of *F. excelsior* juveniles increased very little in vegetation seasons of 2012 and 2013 and reached only 1.2% (Table 2). The average length of necroses in the leader shoots and stems of *F. excelsior* clones increased from 14.15±1.24 cm in spring 2012 to 39.60±0.76 cm by the spring 2013 and to 44.84±0.89 cm in autumn 2013 (Table 2). Total length of necrotic lesions doubled during spring 2012 to spring 2013 period while it decreased by the autumn 2013. Length of necrotic lesions decreased as many of lesions have caused whole leader or lateral shoots to dry completely and thus were registered as length of necrotic leader or lateral shoots.

Among the different clonal trials, the highest mean disease incidence in spring 2012 was observed in Šakiai and Marijampolė clonal trials (17.8±3.2 and 16.4±2.1%, respectively), while the highest mean incidence rate in autumn 2013 was in Kuršėnai and Biržai (84.6±2.9 and 83.5±3.0%, respectively, Table 4). The lowest disease incidence rate was revealed in Dubrava (greenhouse trial) (0 and 52.2±3.7% in spring 2012 and autumn 2013, respectively, Table 4). The length of dry leader shoot in spring 2012 was largest in Kėdainiai and Radviliškis clonal trials (25.87±3.52 cm and 23.15±5.18 cm respectively) while in autumn 2013 – in Dubrava and Šakiai trials (84.77±7.35 and 51.75±2.02 cm respectively, Table 4). The tree health condition scores varied from 3.02±0.1 pt. in Kuršėnai to 4.13±0.08 pt. in Dubrava (Table 5). Severity of assessed disease damage parameters did not correlated with site humidity or continentality index (data not shown).

Joint ANOVA has not revealed significant population effect for the traits studied except bud flushing phenology (Table 3), although some differences among populations in disease incidence in clones represent-

Table 3. Results of joint ANOVA of various tree traits in different years evaluated in seven clonal trials of *F. excelsior*: population, clone, and clone by site (G × E) interaction variance components and its standard errors, significance of site and block effects (*F*–criteria and level of significance)

Tree trait	Season, year	Population variance component, % ±se	Clonal variance component, % ± se	G × E variance component, %	Site effect (*F*-criteria)	Block effect (*F*-criteria)
Disease incidence rate	Spring 2012	2.64±3.04 n.s.	8.15±4.71*[a]	30.74±5.13***	2.56*	2.43*
	Spring 2013	7.69±5.90 n.s.	15.26±5.28**	22.20±3.97***	7.86***	3.20***
	Autumn 2013	2.64±3.04 n.s.	14.76±5.02**	20.58±3.84***	5.85***	5.20***
Length of necrotic part of leader shoot	Spring 2012	n.s.	29.86±14.89*	57.12±14.54***	2.22 n.s.	4.64***
	Spring 2013	0.33±3.92 n.s.	16.21±7.48*	25.37±6.30***	5.71***	1.68 n.s.
	Autumn 2013	n.s.	8.27±5.75 n.s.	42.29±8.47***	9.01***	2.29**
Total length of necrotic lateral shoots	Spring 2012	4.07±5.04 n.s.	n.s	55.73±10.87***	0.46 n.s.	4.80***
	Spring 2013	7.23±5.66 n.s	4.34±5.09 n.s.	34.90±6.85***	3.86**	2.28**
	Autumn 2013	2.76±3.17 n.s	3.08±4.77 n.s.	30.49±6.70***	2.10 n.s.	3.01***
Total length of necrotic lesions	Spring 2012	n.s.	n.s.	48.59± n.e. ***	0.88 n.s.	3.14***
	Spring 2013	21.54±16.09 n.s.	n.s.	41.29±13.14***	0.91 n.s.	1.65 n.s.
	Autumn 2013	17.63±21.02 n.s.	16.91± 30.05 n.s	54.89±24.76***	0.49 n.s.	4.77***
Tree health condition score	Spring 2012	5.17±4.74 n.s.	13.60±5.51*	25.74±4.48***	1.90 n.s.	1.25 n.s.
	Spring 2013	13.23±9.05 n.s.	21.04±6.07***	18.50±3.31***	22.77***	4.02***
	Autumn 2013	4.97±4.58 n.s.	16.35±5.25***	18.07±3.58***	10.72***	5.60***
Tree height	Spring 2012	n.s.	33.54±8.92***	23.70±3.89***	15.71***	2.49**
	Spring 2013	0.44±3.36 n.s.	25.99±7.95***	23.99±4.10***	17.30***	1.26 n.s.
	Autumn 2013	n.s.	20.40±6.17***	24.84±4.34***	49.17***	1.29 n.s.
Bud flushing phenology	Spring 2012	20.88±12.51*	9.76±3.91**	21.38±3.54***	37.83***	1.21 n.s.

[a] levels of significance: * – $0.05 > P > 0.01$, ** – $0.001 > P < 0.01$, ***– $P < 0.001$; n.s. – not significant at $P \leq 0.05$, n.e. – not estimable

ing different populations were evident (Figure 2). The lowest disease mean incidence was recorded in clones originating from three Lithuanian populations – Ignalina, Žeimelis and Kėdainiai (52.2%, 54.3% and 67.4%, respectively), while the highest was in clones of Lithuanian Šakiai (88.7%) and German Farchau (86.0%) populations/provenances. All Lithuanian populations (except Šakiai) demonstrated lower disease incidence as compared to foreign (Czech, French, German and Irish) provenances (Figure 2). There were substantial differences in ash dieback severity between the investigated populations, which revealed that the Lithuanian Šakiai population and Irish populations are most severely damaged (Figure 5, 6). One clone of Lithuanian Žeimelis population (Ze17-64) was the healthiest clone (disease incidence rate was 2.1%). Disease incidence rate of three other healthiest clones from this population did not exceeded 50%. Three clones out of the 9 clones from Lithuanian Ignalina population (Ig7-36-2, Ig5-53-3 and Ig1-44-2) were among the healthiest, with disease incidence below 30%. The disease incidence in most clones was stable since spring 2013 while disease incidence slightly increased in 8 clones and decreased in 13 clones (Figure 3).

Joint ANOVA showed that clonal variance component was significant ($P < 0.05$ and $P < 0.01$) and increased substantially since spring 2012 to spring 2013 and remained almost constant later on (Table 3). Meanwhile clonal variance component of length of necrotic part of leader shoot decreased from 29.86±14.89 to 8.27±5.75%. Clonal effect was non-significant for total length of necrotic lateral shoots, for total length of necrotic lesions on stem and for survival. The significance of clonal effect for tree health condition increased from spring 2012 to autumn 2013 with clonal variance component varying from 13.60±5.51% to 21.04±6.07%. Clonal effect was also significant for growth and phenology traits ($P < 0.001$ and $P < 0.01$, respectively).

Significance of site and block effects for disease incidence increased from weakly significant in spring 2012 ($P < 0.05$) to highly significant in autumn 2013 ($P < 0.001$, Table 3). Site effect for length of necrotic part of leader shoot and tree health condition was non-significant in spring 2012 and became highly significant ($P < 0.001$) since spring 2013, while site effect was not significant for total length of necrotic lesions on stem (Table 3). Site effect for tree growth and bud

Table 4. Genotypic parameters of disease incidence, evaluated in different years in seven clonal trials of *F. excelsior*: trait mean, clonal variance component (vc_c), coefficient of genotypic variation (CV_G), coefficient of broad sense individual heritability (H_c^2) and genotypic gain ΔG

Clonal trial	Season, year	Mean ± se, %	vc_c ± se, %	CV_G, %	H_c^2	Δ G, %
Biržai	Spring 2012	13.9±2.8	70.49±23.91**	228.86	0.70	235.77
	Spring 2013	88.6±2.5	10.01±7.62 n.s.	11.41	0.10	16.03
	Autumn 2013	83.5±3.0	24.16±12.87*	22.67	0.24	31.04
Kuršėnai	Spring 2012	7.7±2.1	37.81±14.94**	221.00	0.38	276.43
	Spring 2013	83.3±3.0	59.50±21.18**	39.24	0.59	40.55
	Autumn 2013	84.6±2.9	54.49±19.59**	34.84	0.54	37.84
Kėdainiai	Spring 2012	9.6±2.3	34.21±13.44**	184.59	0.34	231.51
	Spring 2013	86.6±2.7	21.14±10.41*	18.37	0.21	24.54
	Autumn 2013	81.7±3.0	30.06±13.28*	26.94	0.30	34.40
Marijampolė	Spring 2012	16.4±2.1	24.44±7.37***	112.28	0.24	148.53
	Spring 2013	63.5±2.7	47.26±10.93***	52.27	0.47	63.80
	Autumn 2013	63.5±2.7	36.75±9.28***	46.16	0.37	58.73
Radviliškis	Spring 2012	6.1±1.9	19.03±10.32*	175.11	0.19	224.32
	Spring 2013	75.6±3.4	48.16±16.20**	41.25	0.48	47.84
	Autumn 2013	70.7±3.6	50.66±16.77**	48.48	0.51	55.02
Dubrava	Spring 2012	0	n.t.	n.t.	n.t.	n.t.
	Spring 2013	47.8±3.7	29.37±14.06*	59.01	0.29	84.04
	Autumn 2013	52.2±3.7	23.86±12.50*	48.22	0.24	72.21
Šakiai	Spring 2012	17.8±3.2	45.89±15.21**	148.88	0.46	172.35
	Spring 2013	71.2±3.8	61.27±18.26***	51.70	0.61	56.21
	Autumn 2013	74.0±3.6	63.19±18.57***	48.66	0.63	52.96

[a] levels of significance: * – $0.05 > P > 0.01$, ** – $0.001 > P < 0.01$, ***– $P < 0.001$; n.s. – not significant at $P \leq 0.05$

Table 5. Genotypic parameters of length of necrotic part of leader shoot, evaluated in different years in seven clonal trials of *F. excelsior*: trait mean, clonal variance component (vc_c), coefficient of genotypic variation (CV_G), coefficient of broad sense individual heritability (H^2) and genotypic gain ΔG

Clonal trial	Season, year	Mean ± se, cm	vc_c ± se, %	CV_G, %	H^2	Δ G, %
Biržai	Spring 2012	9.20±1.53	24.17±20.89	44.10	0.24	26.89
	Spring 2013	37.90±1.77	31.32±16.87*	29.03	0.31	35.31
	Autumn 2013	40.49±2.06	21.76±12.99*	25.59	0.22	32.35
Kuršėnai	Spring 2012	14.31±4.26	98.68±49.64*	169.95	0.99	149.7
	Spring 2013	43.93±1.56	13.60±9.62 n.s.	13.81	0.14	15.72
	Autumn 2013	46.05±1.95	37.33±17.78*	29.25	0.38	33.86
Kėdainiai	Spring 2012	25.87±3.52	89.50±42.36*	81.02	0.90	67.24
	Spring 2013	40.96±2.23	49.92±19.10**	40.14	0.50	44.83
	Autumn 2013	47.70±2.23	58.50±23.20**	39.49	0.59	39.21
Marijampolė	Spring 2012	8.97±1.86	97.71±37.36**	135.96	0.98	121.7
	Spring 2013	34.25±1.15	29.48±13.58*	20.05	0.29	17.29
	Autumn 2013	39.10±1.66	49.11±16.02**	36.28	0.49	35.29
Radviliškis	Spring 2012	23.15±5.18	91.35±47.73*	126.39	0.91	104.2
	Spring 2013	33.02±1.82	60.75±21.87**	44.77	0.61	46.98
	Autumn 2013	36.52±1.80	36.17±17.41*	29.68	0.36	31.19
Dubrava	Spring 2012	5.67±1.63	95.49±68.96 n.s.	161.03	0.95	115.73
	Spring 2013	42.00±0.0	n.t.	n.t.	n.t.	n.t.
	Autumn 2013	84.77±7.35	80.77±46.63*	40.37	0.81	37.36
Šakiai	Spring 2012	11.71±2.41	25.52±18.36 n.s.	66.75	0.26	40.83
	Spring 2013	49.63±2.29	48.48±19.65**	30.55	0.48	30.85
	Autumn 2013	51.75±2.02	69.75±23.66**	32.03	0.70	31.68

[a] levels of significance: * – $0.05 > P > 0.01$, ** – $0.001 > P < 0.01$, ***– $P < 0.001$; n.s. – not significant at $P \leq 0.05$

flushing were highly significant ($P < 0.001$). Genotype by environment interaction effect was highly significant for all damage traits, tree height and bud flushing phenology ($P < 0.001$). Analysis of data within individual clonal trials revealed significant clonal effect in disease incidence rate in all trials except Biržai in spring 2013 and Dubrava (in the greenhouse) in spring 2012 (Table 4). Clonal variance components varied from 21.14±10.41 to 70.49±23.91% and it increased since spring 2012 to autumn 2013 in Kuršėnai, Marijampolė, Radviliškis and Šakiai clonal trials while it decreased or was variable in Kėdainiai, Biržai and Dubrava clonal trials. Very high CV_G values were obtained for disease incidence in spring 2012 (112.28–224.72%), while it decreased and remained within range 18.37 and 39.24% in spring 2013 and autumn 2013 in Biržai, Kėdainiai and Kuršėnai clonal trials and within 41.25 and 59.01% in the rest of the trials (Table 4). Broad sense individual heritability (H^2) followed the similar pattern of changes that was observed for clonal variance components as it was derived from the same variances. Very high expected genetic gain (ΔG) estimates were obtained for disease incidence in spring 2012 (55.1–173.6%) then they decreased to more realistic although very variable among trials estimates within range of 16.03 and 84.04 (Table 4).

Clonal variance components of necrotic part of leader shoot were very large in spring 2012 in all clonal trials except Biržai and Šakiai (89.50±42.36 to 97.71±37.36%, Table 5), then it decreased in spring 2013 and autumn 2013. While in Šakiai clonal trial clonal variance components increased or remained rather low in Biržai clonal trial. CV_G was very large in spring 2012 in Kuršėnai, Dubrava, Marijampolė and Radviliškis (126.39–169.95%) and large in Kėdainiai, Šakiai and Biržai (44.10–81.02%). CV_G decreased substantially in 2013 and varied from 13.81 to 44.77% (Table 5). Changes in values of H^2 followed the pattern of changes of clonal variance components with high values in spring 2012 (except Biržai and Šakiai) and substantial decrease in 2013. Expected ΔG estimates were large in spring 2012 in all clonal trials except Biržai and Šakiai reaching 67.24 to 149.7%. By the autumn 2013 expected genetic gain dropped in all trials to 31.19–39.19% (Table 5).

Clonal effect of tree health condition score was highly significant in all clonal trials except Dubrava ($P < 0.001$ or $P < 0.01$, Table 6). Clonal variance components were not very variable over years and were rather low in Dubrava and Marijampolė and moderate in all the rest of clonal trials. CV_G of tree health condition was low in spring 2012 varying from 3.83 to 18.02% and increased in spring and autumn 2013 reaching from 8.95 to 33.64% (Table 6). H^2 was rather low in

Table 6. Genotypic parameters of tree health condition score evaluated in different years in seven clonal trials of *F. excelsior*: trait mean, clonal variance component (vc_c), coefficient of genotypic variation (CV_G), coefficient of broad sense individual heritability (H^2) and genotypic gain $\ddot{A}G$

Clonal trial	Season, year	Mean ± se, pints	VC_c ± se, %	CV_G, %	H^2	Δ G, %
Biržai	Spring 2012	4.52±0.06	47.54±18.05**	12.51	0.48	14.75
	Spring 2013	2.98±0.09	38.37±15.64**	23.14	0.38	30.47
	Autumn 2013	3.10±0.10	37.74±15.67**	25.52	0.38	32.70
Kuršėnai	Spring 2012	4.62±0.06	48.32±17.41**	12.16	0.48	14.64
	Spring 2013	2.86±0.09	54.48±19.81**	33.64	0.54	36.34
	Autumn 2013	3.02±0.10	45.93±17.55**	28.94	0.45	33.45
Kėdainiai	Spring 2012	4.43±0.07	40.68±14.87**	13.87	0.41	17.04
	Spring 2013	2.89±0.10	50.83±17.46**	32.81	0.51	37.81
	Autumn 2013	3.77±0.07	22.53±10.90*	21.56	0.23	28.51
Marijampolė	Spring 2012	4.58±0.05	30.08±8.23***	10.00	0.30	13.03
	Spring 2013	3.85±0.06	39.88±9.75***	18.56	0.40	23.37
	Autumn 2013	3.77±0.07	32.17±8.57***	17.37	0.32	22.49
Radviliškis	Spring 2012	4.47±0.06	35.02±13.68**	11.35	0.35	13.84
	Spring 2013	3.31±0.09	54.60±17.47***	28.15	0.55	31.65
	Autumn 2013	3.60±0.09	38.01±13.95**	20.64	0.38	25.41
Dubrava	Spring 2012	4.75±0.03	20.86±11.41*	3.83	0.21	5.86
	Spring 2013	4.50±0.05	32.46±14.82*	8.95	0.32	12.46
	Autumn 2013	4.13±0.08	28.10±14.11*	14.52	0.28	20.68
Šakiai	Spring 2012	4.34±0.08	59.02±17.69***	18.02	0.59	20.06
	Spring 2013	3.34±0.11	65.08±18.93***	32.97	0.65	35.67
	Autumn 2013	3.45±0.10	59.15±17.71***	26.30	0.59	29.24

[a] levels of significance: * – $0.05 > P > 0.01$, ** – $0.001 > P < 0.01$, ***– $P < 0.001$; n.s. – not significant at $P \leq 0.05$

Dubrava and Marijampolė and moderate in Kuršėnai, Kėdainiai, and Šakiai clonal trials. ΔG estimates were low in spring 2012 varying from 5.86 to 20.06% and increased in spring and autumn 2013 reaching from 20.68% in Dubrava to 37.81% in Kėdainiai clonal trials (Table 6).

Clonal effect of tree survival rate was significant ($P < 0.01$ or $P < 0.05$, data not shown) only in Biržai, Kėdainiai, Marijampolė and Šakiai clonal trials in spring 2013 and in Kuršėnai, Marijampolė and Šakiai clonal trials in autumn 2013. Clonal variance component was high in Kėdainiai and Kuršėnai trials (49.58±21.14 and 53.17±19.65%, respectively, data not shown) and low in the rest of the trials (from 16.22±8.04 to 25.85±12.21%).

Clonal effect of tree height was significant in all clonal trials ($P < 0.001$ or $P < 0.01$, data not shown). Clonal variance component was high in spring 2012 (varied from 44.26±16.76% to 67.20±21.03%) and showed tendency of slight decrease by autumn 2013 (varied from 28.34±12.75% to 55.17±18.81%, data not shown). Rather modest CV_G values obtained in spring 2012 (16.94–33.09%) also slightly decreased by the autumn 2013 (15.22–26.95%, data not shown). Changes in values of H^2 followed the pattern of changes of clonal variance components with moderate to high values in spring 2012 (0.44–0.67) and low to moderate by the autumn 2013 (0.28–0.55). ΔG of tree height varied within range 22.46 to 39.96% in spring 2012 and slightly decreased by the autumn 2013 to 18.56–32.47% (data not shown).

Genotypic correlations between disease incidence in spring 2012 and spring 2013 and autumn 2013 were moderate (r_G = 0.435±0.031 and r_G = 0.454±0.034, Table 7). Disease incidence in spring 2013 positively correlated with length of necrotic part of leader shoot in spring 2012, spring 2013 (r_G = 0.718±0.035 and 0.252±0.048) while correlations in autumn 2013 were weak (Table7). Length of dry/necrotic part of leader shoot in spring 2012 and 2013 moderately to strongly correlated with tree health condition in 2012 and 2013 (r_G ranged from -0.316±0.062 to -0.810±0.017). A weak although significant genotypic correlation was obtained between disease incidence rate in spring 2012 and tree health condition in 2012 and 2013 (r_G = -0.950±0.004 – -0.588±0.027, $P < 0.001$, Table7). Genotypic correlations between health condition in spring 2012, spring and autumn 2013 were strong (r_G = 0.624±0.028 – 0.926±0.007, $P < 0.001$).

Discussion

The study revealed a considerable increase in disease occurrence and damage severity in the first

Table 7. Genotypic (above diagonal) and phenotypic (below diagonal) correlations between traits of tree health condition evaluated in seven clonal trials of *F. excelsior*. Standard errors of genotypic correlations and significance of phenotypic correlations are indicated in *italics*

	Disease incidence			Length of dry/necrotic part of leaner shoot			Tree health condition		
	Spring 2012	Spring 2013	Autumn 2013	Spring 2012	Spring 2013	Autumn 2013	Spring 2012	Spring 2013	Autumn 2013
Disease incidence in spring 2012		0.534	0.454	1.000	0.392	0.231	-0.950	-0.598	-0.588
		±0.031	*±0.034*	*±-0,304*	*±0.041*	*±0.042*	*±0.004*	*±0.029*	*±0.027*
Disease incidence in spring 2013	0.114		1.000	0.718	0.252	-0.477	-0.614	-0.899	-0.898
	<0.001		*±0.000*	*±0.035*	*±0.048*	*±0.037*	*±0.030*	*±0.009*	*±0.008*
Disease incidence in autumn 2013	0.097	0.674		0.054	-0.199	-0.236	-0.540	-0.890	-0.933
	0.001	*<0.001*		*0.071*	*±0.048*	*±0.043*	*±0.033*	*±0.010*	*±0.006*
Length of dry leader shoot in spring 2012	0.425	0.205	0.101		1.000	1.000	-0.716	-0.407	-0.316
	<0.001	*0.003*	*0.147*		*±-0.061*	*±-0.165*	*±0.037*	*±0.064*	*±0.062*
Length of dry leader shoot in spring 2013	0.096	0.258	0.085	0.327		0.726	-0.477	-0.763	-0.590
	0.015	*0.002*	*0.031*	*<0.001*		*±0.025*	*±0.041*	*±0.022*	*±0.031*
Length of dry leader shoot in autumn 2013	0.032	-0.020	0.197	0.165	0.742		-0.509	-0.810	-0.635
	0.404	*0.612*	*<0.001*	*0.065*	*<0.001*		*±0.036*	*±0.017*	*±0.027*
Tree health condition in spring 2012	-0.811	-0.195	-0.178	-0.663	-0.106	-0.021		0.662	0.624
	<0.001	*<0.001*	*<0.001*	*<0.001*	*0.007*	*0.588*		*±0.028*	*±0.028*
Tree health condition in spring 2013	-0.154	-0.749	-0.602	-0.301	-0.442	-0.152	0.245		0.926
	<0.001	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*		*±0.007*
Tree health condition in autumn 2013	-0.114	-0.574	-0.769	-0.050	-0.250	-0.428	0.166	0.676	
	<0.001	*<0.001*	*<0.001*	*0.476*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	

and second growing season after transplanting grafted *F. excelsior* to field clonal trials thus revealing the continuous breakage of disease resistance of majority of clones, selected for resistance in progeny trials of 2005-year (see Pliūra et al. 2011). Similar disease spread rate was registered in provenance trial established in 2005 on four sites in Germany where disease incidence increased from 13% in 2007 up to 94% in 2012 (Enderle et al 2013). In a clonal study performed in Denmark the repeated assessments showed a development of disease symptoms at two sites over the 3 years of observations with percent damage score increasing from 32 to 55% (McKinney et al. 2011). Meanwhile the study performed in Sweden on one site showed a substantial decrease in ash damage between years 2006 and 2007 but no strong 5 year temporal trend was apparent while on second site there was a weak improvement between years 2010 and 2011 (Stener 2013). In recent clonal study in Austria, mean ash dieback intensity reached only 18.1% in 2009 and 17.6% in 2010 (Kirisits and Freinschlag 2012). It was explained that diseased trees often respond intensively with the formation of auxiliary and epicormic shoots in order to compensate the loss of killed shoots and that results in decreased estimates of disease severity. Also it was supposed that climatic factors might have contributed to the slight decrease in disease intensity (Kirisits and Freinschlag 2012). The relatively low disease rate can be explained by low infection rate in neighboring stands due to short history of ash dieback in Austria in comparison to chronic ash dieback in Lithuania. The difference in age of studied trees of tested clones in these studies may also influenced the disease rate and severity.

In spring 2012, dieback symptoms occurred on almost half of the tested clones while in 2013 disease occurred in all clones but with differing disease incidence rate. This corresponds to results of similar studies on resistance of *F. excelsior* genotypes, where none of the tested clones showed total resistance to ash dieback, and only few exhibited reduced susceptibility (McKinney et al. 2011, Stener 2013). In a study performed in Denmark, the average degree of damage varied among genotypes from 1% to 69% (McKinney et al. 2011). In a seed plantation of ash in Austria disease intensity varied greatly between clones ranging from almost no dieback to 80% (Kirisits and Freinschlag 2012). A natural genetic variation in pathogenicity of *H. pseudoalbidus* has been demonstrated in pathogenicity tests with *H. pseudoalbidus* (Bakys et al. 2009b, Bakys et al. 2013). Based on evidence, that intra-population genetic variability of isolates obtained from ascospores can be greater than genetic variability of isolates obtained from necrotic tissue on ash shoots it was assumed that *H. pseudoalbidus* isolated from necrotic ash tissue were of greater pathogenicity (Kraj et al. 2012, Kraj and Kowalski 2013). It also may be assumed that only a part of the fungal genotypes most likely those which are of greater pathogenicity are able to brake the resistance of host trees and that more resistant ash genotypes may be infected only by most pathogenic *H. pseudoalbidus* genotypes. Thus it can be hypothesized that more resistant clones, that were healthy in 2012, were infected in 2013 by more aggressive strains of *H. pseudoalbidus.* Similar regularities have been indicated by Eisenhauer (1991) in studies on oak decline. The fact that DNA of *H. pseudoalbidus* was detected in 39.6% of sampled asymptomatic *F. excelsior* trees can be considered as indication that some genotypes/strains of *H. pseudoalbidus* does not cause the disease or that the symptoms have not appeared yet.

In spite of high disease incidence rate, a mortality of trees increased very little and reached only 1.2%. while clonal study in Sweden have shown 33% mortality during the 5 year period (at age 15 to 19 years) on one site and 7% mortality during last 2 years on another site (Stener 2013). In the Lithuanian progeny trials of 2005 year series, almost 90% of the trees died between the establishment of the experiment in 2005 and the assessment year in 2010 (Pliūra et al. 2011). Moreover, numerous diseased trees of most clones in present clonal trials demonstrated significant growth recovery by the end of 2013 vegetation season. This indicates that population of preselected for resistance clones has potential to cope with the disease. On 23.4% of tested clones disease incidence rate was equal or below 50%. These clones are potential candidates for further breeding for resistance against *H. pseudoalbidus*.

Evidence that population effect in all assessed traits (except phenology) was weak and non-significant in currently investigated clonal trials, is in good agreement with results of other studies (Olrik et al. 2007, Stener 2007, McKinney et al. 2011) that found little differences in resistance to *H. pseudoalbidus* among ash populations. In contrast, in our older studies on resistance of half-sib *F. excelsior* progenies the population effect proved to be significant (Pliūra et al. 2011). The absence of significant population effect in the present study could be due to high inter-clonal variation within populations because of the rather low number of clones representing populations (3–8) and number of tested populations (8). Nevertheless, the differences in presence and severity of symptoms, caused *by H. pseudoalbidus* between the investigated populations are evident (Figure 2, 4, 5, 6).

By the end of 2013 the lowest disease mean inci-

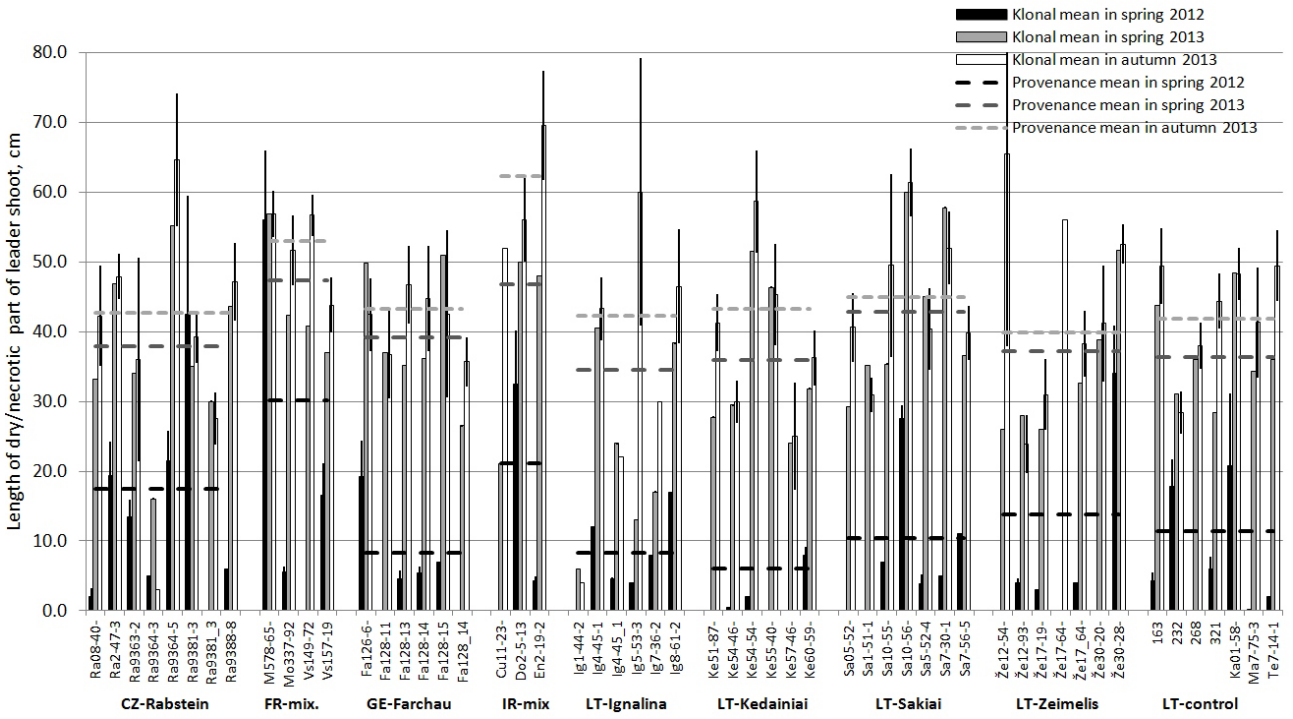

Figure 4. Length of necrotic part of leader shoot in grafted clones of *F. excelsior* from Lithuanian and foreign populations (for population names see legend of Figure 2) in spring 2012, spring and autumn 2013 over seven clonal trials. Bars are clonal means ± standard error

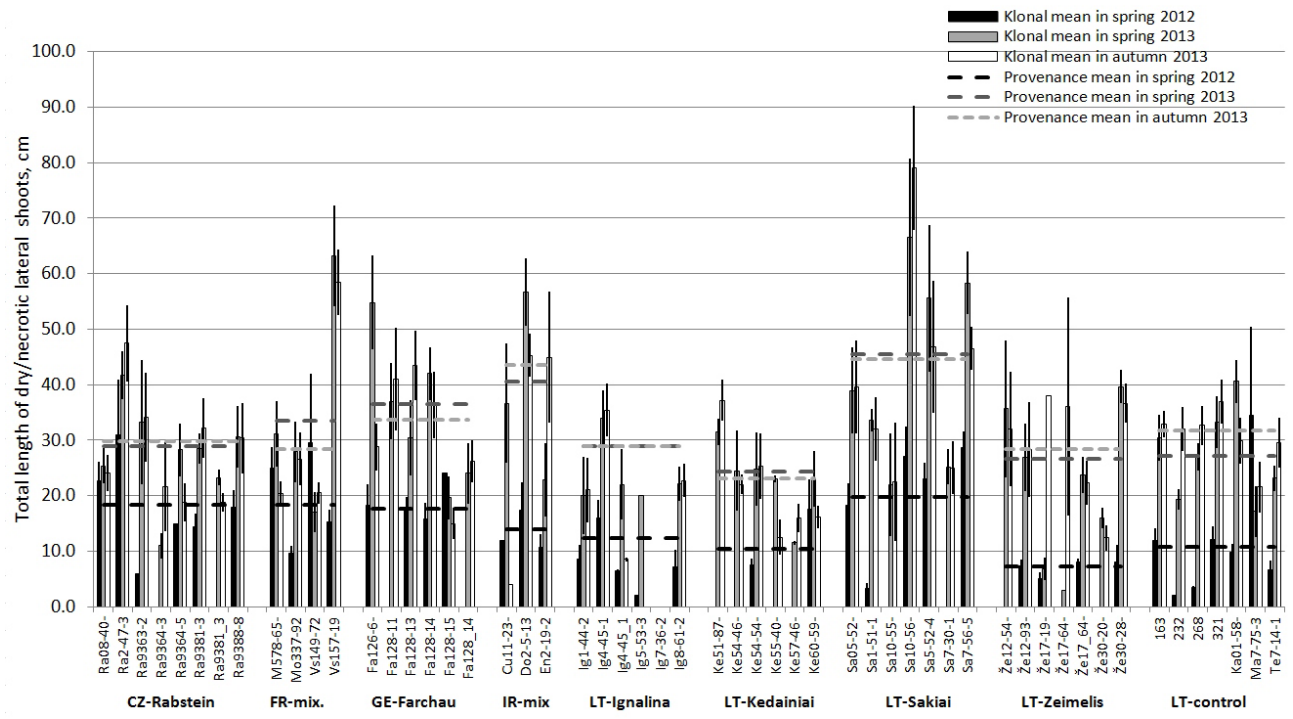

Figure 5. Total length of necrotic lateral shoots in grafted clones of *F. excelsior* from Lithuanian and foreign provenances (for provenance names see legend of Figure 2) in spring 2012, spring and autumn 2013 over seven clonal trials. Bars are clonal means ± standard error

dence rate remained in clones originating from three Lithuanian populations – Ignalina, Žeimelis and Kėdainiai, while the highest – in clones of German Farchau and Lithuanian Šakiai populations. The lower disease incidence observed Lithuanian populations could be explained by the fact that the tested clones represented an outcome from a second selection cycle. The material for their grafting has been selected from the most healthy and vigorous parent trees within the most healthy families and populations in the progeny trials established in 2005, where general disease incidence at the age of 9 years was as high as 99% (Pliūra and Baliuckas 2007). The ranking of Lithuanian populations by lowest disease incidence rate in spring 2012 was as follows: 1 – Žeimelis, 2 – LT control, 3 – Kėdainiai, and 4 – Ignalina. By autumn 2013 is has changed to: 1 – Ignalina, 2 – Žeimelis, 3 – Kėdainiai and 4 – LT control (Figure 2). Similar changes of ranking was also obtained in tree health condition score (Figure 6).

In addition, generally better health conditions of clones of Lithuanian origin (including the Lithuanian control group) as compared to those from the foreign provenances could also be a consequence of ongoing intense natural selection process in Lithuanian ash stands in favor of trees with a better resistance against *H. pseudoalbidus* (seeds for establishment of progeny trials of 2005 (Pliūra et al. 2011) were collected from healthy seed trees in already diseased ash stands). Whereas seeds from foreign populations were collected from healthy stands, before the outbreak of ash dieback disease in those respective countries where susceptibility-driven natural selection had not yet started.

Significant genetic control (characterised by H^2 and coefficient of genotypic variation) of damage traits revealed during the present and former study in general corresponded well to results of recent studies, performed in Denmark and Sweden at the clonal level (Stener 2007, Olrik et al. 2007, McKinney et al. 2011, Stener 2013). The heritability coefficients obtained for health condition score in our clonal trials (H^2 = 0.21–0.65) were similar to these calculated by Danish (H^2 = 0.25–0.54, McKinney et al. 2011) and Swedish (H^2 = 0.07–0.57, Stener 2013) researchers in *F. excelsior* trials. In our clonal trials the highest H^2 was obtained in spring 2013, which means that the most precise detection of resistant tree genotypes by phenotype can be done in spring when the winter period of disease development ends with maximum appearance of symptoms. Genotypic variation of health condition was also highest in spring 2013, indicating that the differences among clones in health condition is also most expressed in spring time. This is related to consequenc-

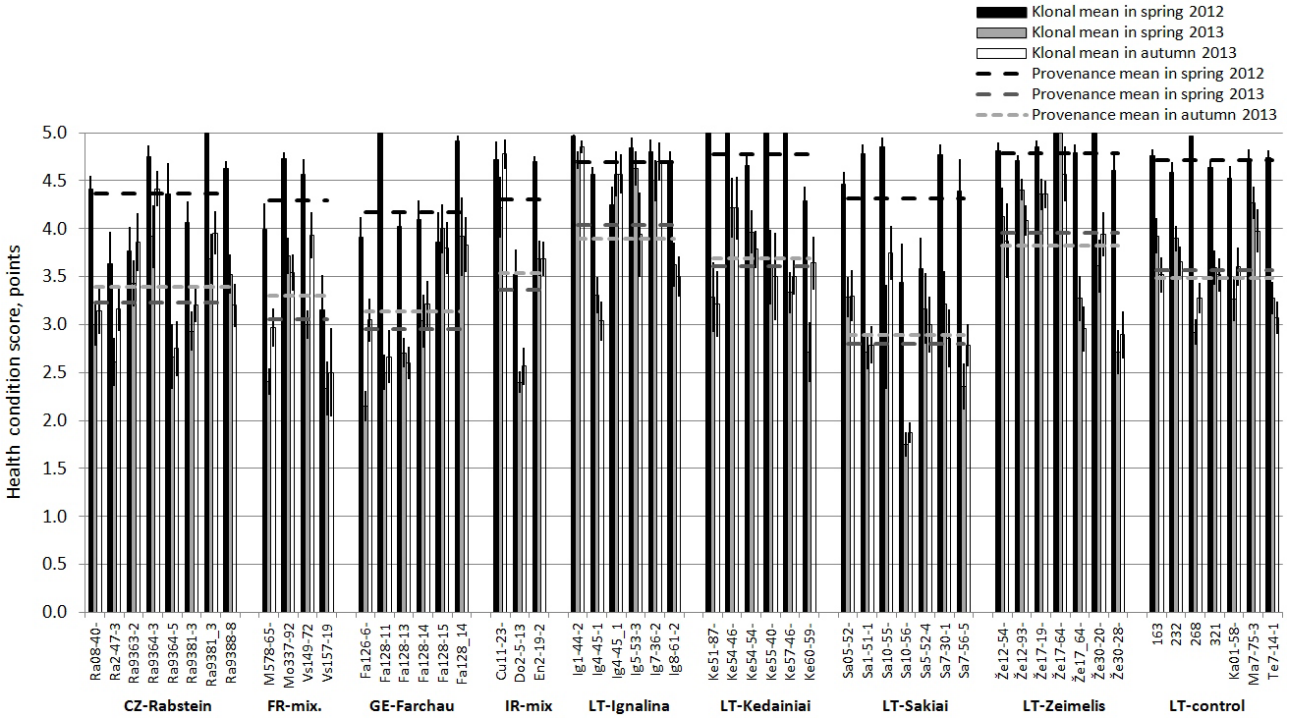

Figure 6. Mean health condition scores in grafted clones of *F. excelsior* from Lithuanian and foreign populations (for populations names see legend of Figure 2) in spring 2012, spring and autumn 2013 over seven clonal trials. Bars are clonal means ± standard error

es of disease development during a winter period (Bengtsson 2013). In all evaluations dates in year 2013 CV_gs in our clonal trials remained lower than ones observed in Denmark (CV_g = 0.38–0.87%, McKinney et al. 2011) and Sweden (CV_g = 19.0–56.9%, Stener 2013), and lower than additive coefficient of genetic variation obtained in progeny studies in Denmark (CV_A = 37–61%, Kjær et al. 2011) and Lithuania (CV_A = 30–39%, Pliūra et al. 2011). This could be explained by lower clonal differences due to putative resistance of the clones used in present study which were obtained from selected healthiest individuals in open pollination progeny trials of 2005-year series.

Extremely high coefficients of genotypic variation of disease incidence in spring 2012 were likely exaggerated by application of rather low means (due to low disease incidence rate) used as denominators in computing CV_gs for this trait (Pliūra et al., submitted). In spring and autumn 2013 disease incidence CV_g have decreased substantially although it remained higher than that obtained for health condition.

Durable long-term resistance of *F. excelsior* against ash dieback needs combination of different plant defence mechanisms to diminish probability of breaking the resistance due to mutation and/or adaptation of a pathogen. Thus, the strategy to build the long-term resistance should be based on application of so-called 'Pyramid principle' that integrates two types of resistance inheritance: qualitative and quantitative (horizontal), i.e. resistance determined both by individual major genes and by interaction of many minor genes, additive effects, epistasis, pleiotropy, etc. (Tapiero 1999, McDonald and Linde 2002a,b, Baniulis et al. 2008). The fact that broad-sense heritability coefficients of resistance/health conditions traits revealed during the present study were of similar magnitude as narrow-sense heritability coefficients (which accounts the additive component), obtained in Denmark (H^2 = 0.37–0.52, Kjaer et al. 2011) and earlier – in Lithuania (H^2 = 0.40–0.49, Pliūra et al. 2011), suggests that resistance of *F. excelsior* is largely inherited additively. This would allow development of durable quantitative resistance highly desirable in ash breeding programs. In breeding of ash for resistance to *C. fraxinea*, a controlled crossing should be planned instead of open pollinated mating model as the latter may compromise the achieved genetic gain because of possible significant uncontrolled gene flow from non-resistant pollen sources (Douglas et al. 2013). It is also emphasized that vegetative propagation could be a very efficient option in breeding for resistance as resistance/tolerance of ash is generally observed more at the individual genotype level in comparison to population or family levels (Douglas et al. 2013).

In spring 2012, environmental conditions of the clonal trial sites likely had too little time to influence the disease incidence rate of the outplanted ash juveniles and therefore the site (clonal trial) effect in damage traits was low. Although, based on increased *F*-criteria of site effect (Table 3), the influence of site increased by spring 2013. Disease incidence rate was lowest in Dubrava greenhouse trial (52.2±3.7%) likely due to their partial isolation from outdoor sources of infection by a greenhouse cover and due to the absence of diseased trees of ash in the vicinity to the greenhouse. As disease damage traits did not correlated with site humidity or climate continentality index, the most probable factor contributing to site effect might be different infection pressure (amount of ascospores) from the neighbouring diseased stands located at different distances to the clonal trials.

As revealed by joint ANOVA (Table 3), G × E interaction for disease incidence and health condition had similar or even higher variance component values as for tree height and bud flushing phenology. This indicates a presence of genetic variation in plasticity and reaction norms of clones across sites providing that resistance to disease of the most susceptible clones unequally depends on site conditions (infection pressure at given site). Similar studies performed with *F. excelsior* clones in Denmark and Sweden showed weak G × E interaction (McKinney et al. 2011, Stener 2013), although each Scandinavian study has covered only two clonal trials (seed orchards), while the present study was performed in seven clonal trials established across three provenance regions at different infection pressure from neighbouring diseased stands of *F. excelsior*. Moreover, clonal trials of present study were established using material from Lithuanian and foreign populations, and with likely inclusion of clones with specific reaction norms. All these factors contributed to increased G × E interaction effect. The high B-type site-by-site genotypic correlation obtained in study in Denmark indicated that the genotype-by-environment interaction in the degree of susceptibility was mainly an effect of scale rather than rank differences among clones across sites (McKinney et al. 2011). In agreement with our results, significant G × E interactions have been found for height growth of 22 British and continental European *F. excelsior* provenances tested at six sites in Wales and England (Cundall et al. 2003) and of 52 provenances tested at 26 sites in continental Europe (Kleinschmit et al. 1996).

Molecular detection of *H. pseudoalbidus* in 39.6% of symptomless *F. excelsior* trees indicates either a higher resistance of those trees which are able to defeat the disease or to slow down its development (delayed appearance of symptoms), or just an initial

stage of infection (here more time is needed for symptoms to develop). The real rate of infection was most likely underestimated, but in the future it will be interesting to observe how these infected but symptomless trees perform: the ability to suppress the pathogen is currently one of the most desirable traits of *F. excelsior*.

Moderate genotypic correlation between disease incidence rate in spring 2012 and in spring and autumn 2013 probably can be explained by numerous disease-free clones in spring 2012 (just after establishment of the trials), which showed *H. pseudoalbidus* infection symptoms in 2013, which resulted in changes in ranking of clones in disease incidence rate. The same explanations can be made for moderate genotypic correlation between health condition score in spring 2012 and in spring and autumn 2013.

Low positive and low to moderate negative genotypic correlation between disease incidence in spring and autumn 2013 and length of dry/necrotic part of leader shoot in spring and autumn 2013 indicates that these two traits are genetically regulated separately and reflect two different and little related features of resistance: the disease incidence rate reflects resistance to infection while length of dry/necrotic part of leader shoot reflects the rate of damage or spread of disease within the tree.

Lower mean disease incidence in most Lithuanian populations among tested populations and some of the clones from these populations so far provides possibilities for breeding of common ash for resistance against *H. pseudoalbidus*. Further testing of *F. excelsior* resistance in the clonal trials and a new cycle of selection could provide a potential for obtaining a set of genetically most resistant clones for crossings in tree breeding, for establishment of resistant seed orchards and for vegetative mass propagation.

Conclusions

Studies of temporal dynamics of *F. excelsior* resistance to *H. pseudoalbidus* in clonal trials during two growth seasons after transplanting revealed a considerable increase in disease occurrence and damage severity thus demonstrating the continuous increasing infection of trees from tested and selected for resistance clones with only few clones remaining with infrequent disease incidences. It shows that material initially considered as resistant becomes susceptible and this raises serious concern in forestry community as regards existence of a *stricto sensu* genetic resistance in common ash. Nevertheless, disease occurrence in the clones remained lower than in base population of half-sib families in progeny trials thus showing the certain success of first cycle of selection aimed for resistance to disease. Most clones shoved significant growth recovery by the end of 2013 vegetation season regaining the height that was lost after diseased leader shoots had died.

Population effect for most of traits studied was not significant, although, the tested clones from Lithuanian provenances showed better health conditions as compared to the foreign (Czech, French, German and Irish) ones. Lower mean disease incidence among the tested Lithuanian clones, so far, provides promising a future prospect for breeding of common ash for resistance against *H. pseudoalbidus*.

Even at increased disease incidence rate in 2012 and 2013 growth seasons, genetic effect of a clone remained significant with a constantly increasing clonal variance component, coefficients of genetic variation and heritability, which indicates permanent and substantial genetic control of the disease incidence and therefore good prospects for obtaining high genetic gain in tree breeding, for resistance at a clonal level.

Low positive and low to moderate negative genotypic correlation between disease incidence rate and length of dry/necrotic part of leader shoot in spring and autumn 2013 indicates that these two traits are genetically regulated separately and reflect two different and little interrelated features of resistance: the disease incidence reflects resistance to infection while length of dry/necrotic part of leader shoot reflects the rate of damage and spread of disease within infected tree.

Constant and significant genotype-environment interaction (G × E) in all disease damage traits points to possible existence of certain genetic variation in plasticity and reaction norms of clones across a range of infection load environments.

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