Susceptibility of *Fraxinus angustifolia* Clones to *Hymenoscyphus fraxineus* in Lowland Croatia

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Abstract

In this study on the narrow-leaved ash's resistance to *Hymenoscyphus fraxineus,* significant differences were obtained between the nine tested clones, among which the clones BJ25, BJ38, NG03 and NG31 turned out to be the least susceptible to the pathogen. A significant difference was obtained between genotypes in clonal seed orchards for the leaf unfolding parameter as well. Earlier leaf unfolding was found in clone NG03, which revealed smaller necrosis development in the inoculation experiment, while the clone NG55 revealed later leaf unfolding and longer necrosis lengths, leading to the conclusion that phenology could play an important role in narrow-leaved ash clones' resistance to *H. fraxineus.* Preliminary results on three types of agar medium with leaf extracts from native Croatian *Fraxinus* species revealed that the nutrition status of the ash host could also play a role in resistance to the pathogen. The experiment revealed the fastest growth of the pathogen isolates on the agar enriched with *F. excelsior* leaf extract, followed by the agar with *F. ornus* leaf extract, while the slowest growth was obtained on the agar with *F. angustifolia* leaf extracts.

Keywords: narrow-leaved ash, clones, fungal pathogen, resistance

Introduction

Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz, Hosoya (basionym: *Chalara fraxinea*) a new invasive pathogen, has shown its presence to be the cause of severe dieback of ash (*Fraxinus* spp.) throughout Europe since the 1990s. In Croatia, it was first recorded on the common ash (*Fraxinus excelsior* L.) in 2009 in Gorski Kotar, a mountainous area close to Slovenia (Barić and Diminić 2010). Typical symptoms of the disease: discolouration and wilting of leaves and petioles, formation of necrotic tissue progressing to elongated necrotic lesions and subsequently forming open cankers on the stem, and inner wood discolouration have been observed and described by many authors (Lygis et al. 2006, R. Bakys et al. 2009a, Kowalski and Holdenrieder 2009, Kirisits 2012, Gross et al. 2014). Two main infection pathways of the pathogenic fungus have been proposed, one via ascospores that infect leaves and petioles and then progress to the ligneous tissue (Kirisits et al. 2009, Kräutler and Kirisits 2012), and the other through lenticels (Husson et al. 2012).

Since 2009, *H. fraxineus* in Croatia has been found and confirmed in natural forest stands of the two native ash species, the common and the narrow-leaved ash (*Fraxinus angustifolia* Vahl) (Barić et al. 2012). Attempts to isolate the pathogen from the third native Croatian ash species, *Fraxinus ornus* L., have not been successful in natural conditions to date.

The narrow-leaved ash in central Europe and the Pannonian Basin in the Balkans occurs mainly in riparian and floodplain forests (Fraxigen 2005). In Croatia, the narrow-leaved ash is distributed in the Pannonian lowland area in mixed stands with the pedunculate oak (*Quercus robur* L.). The largest complexes of *F. angustifolia* in Croatia

(80%) and the majority of its genetic variabilities are located in the area of 30 000 ha along the Sava river, hence two clonal seed orchards of the narrow-leaved ash were established. The first established clonal seed orchard of *F. angustifolia* (Nova Gradiška) represents the eastern Sava river seed provenance, and the second clonal seed orchard (Čazma) represents the western Sava river seed provenance. The implementation of feasible conservation measures in order to preserve the ash in its natural environment is of great importance as a part of sustainable forest management policies in Croatia.

In affected ash stands, the number of trees which are capable of producing seeds is reduced to a 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 (Pliûra et al. 2011). The current genetic diversity might be insufficient for further physiological and genetic adaptation and sustainability of ash populations, thus predisposing damaged populations to collapse (Pliûra et al. 2014). Several European authors have implied that among the limited disease control measures, the most promising potential option would be to take advantage of the naturally occurring individuals' resistance to the fungus (Bakys et al. 2009b, Kirisits et al. 2009, McKinney et al. 2011, McKinney et al. 2012b, Kirisits and Freinschlag 2012). So far, the work carried out on *F. excelsior* has revealed that there is a significant difference among individuals, populations and families regarding their tolerance to *H. fraxineus* (Kirisits et al. 2009, Pliūra et al. 2011, McKinney et al. 2011, McKinney et al. 2012b, Stener 2013). However, there is very little work published regarding the individual resistance of *F. angustifolia* to ash dieback (Hauptman et al. 2016), Schwanda & Kirisits 2016). Temporal dynamics of the disease and genetic characteristics of the affected populations of ash stands are also crucial for the evaluation of genetic variability, sustainability and adaptation potential of ash species.

A substantial variation has been observed in the degree of damage and symptoms occurrence in natural populations of ash stands in Croatia. Similar reports have been published all across Europe on *F. excelsior* natural stands, but as detected from the various progeny trials, only 2-5 % of trees remained symptom-free (McKinney et al. 2011, Pliûra et al. 2011).

A large number of European countries, through research on natural stands, progeny trials and clonal seed orchards, provide evidence that susceptibility of *F. excelsior* to *H. fraxineus* varies considerably between individuals, populations and families, and that there is a significant genetic heritability in disease resistance/tolerance (Pliūra and Baliuckas 2007, Pliûra et al. 2011, McKinney et al. 2011, Kjær et al. 2012, Husson et al. 2012, Kirisits and Freinschlag 2012, Stener 2013).

The aim of this study was to provide initial results about possible differences in susceptibility to *H. fraxineus* among the studied clones of *F. angustifolia* from two clonal seed orchards in Croatia. As part of the research, an analysis was conducted of the leaf unfolding phase in the clonal seed orchard of the narrow-leaved ash to determine intrapopulation and interpopulation variability and the existence of ecotypic forms in relation to the beginning of leaf unfolding. A correlation among the phenology and the degree of damage on *F. excelsior* has been reported (McKinney et al. 2011, Stener 2013, Bakys et al. 2013), but the data on *F. angustifolia* is still insufficient. An additional preliminary test was done on *H. fraxineus* isolates growing on agar with leaf extracts of the three native Croatian *Fraxinus* species to complete the data on possible differences in resistance or tolerance in ash.

Materials and methods

According to the aim of the research of investigating possible differences in susceptibility of *F. angustifolia* to *H. fraxineus*, an inoculation experiment was performed on seedlings selected from the two clonal seed orchards of Nova Gradiška and Čazma. Isolates of *H. fraxineus* used in the experiment were obtained from *F. excelsior* from the site where the disease was recorded for the first time in Croatia in 2009. Phenology of the narrow-leaved ash clones was included in the study and focused on the phase of the beginning of leaf unfolding as one of the possible factors which could influence the host's susceptibility to the pathogen. The host-pathogen relationship was tested in a growth experiment on the *H. fraxineus* isolates growing on different media containing leaf extracts of the three native *Fraxinus* species of Croatia. The obtained data were statistically analysed.

Clonal material

In the study, nine narrow-leaved ash clones originating from two clonal seed orchards were preselected for susceptibility trials against the invasive pathogenic fungus *H. fraxineus*. Narrow-leaved ash clones originated from the two clonal seed orchards of Nova Gradiška and Čazma, each representing one of the two most important provenance seed regions of *F. angustifolia* in Croatia. The first clonal seed orchard, Nova Gradiška, was established in 2005 on an area of 3.5 ha with a total of 56 clones from the eastern Sava river seed provenance. The grafts were planted with a 4 × 4 m spacing. The second clonal seed orchard, Čazma, was established in 2007 on an area of 7.3 ha. The grafts were planted with a 5 × 5 m spacing, and the orchard contains a total of 50 clones representing the western Sava river seed provenance. Nine clones in total were used in the research, four of which represented the eastern Sava river seed provenance clones from the orchard in Nova Gradiška:

clones NG03, NG31, NG41 and NG55; and five of which represented the western Sava river seed provenance clones from the orchard in Čazma: clones BJ25, BJ28, BJ32, BJ35 and BJ38.

Fungal isolates

The two isolates of *H. fraxineus* used for inoculations were obtained in spring 2014 from the Educational and Research Centre Zalesina of the Faculty of Forestry, University of Zagreb, located in the Gorski Kotar region, where the disease was first detected in Croatia (Barić et al. 2010). Isolate no. 1 was obtained from the micro-location with the coordinates: 45.383858°N, 14.873822°E; from a symptomatic solitaire tree of *F. excelsior*, 20 cm in diameter and 18 m in height, from the wood tissue sample taken as a cross-section of the trunk, approximately 1 m from the ground level. Isolate no. 2 was obtained from the micro-location with the coordinates: 45.385710°N, 14.872510°E; from a symptomatic young tree of *F. excelsior,* 4 cm in diameter and 3 m in height, from the shoots of the crown with visible necrosis, and the tissue sample for isolation was taken from the wood tissue just beneath the bark. Shoots were collected at approximately 2 m from the ground level.

Samples were processed according to the EPPO diagnostic standard PM 7/117 (1) (EPPO 2013) for *H. fraxineus*. Pieces of plant tissues (woody material without bark) were disinfected in 10 times diluted commercial bleach (20 s), rinsed three times in sterile water, cut into pieces of approximately 5 mm^2 in size, and placed on 2% malt extract agar (MEA Oxoid™ CM0059) supplemented with 100 mg/l streptomycin sulphate (Sigma-Aldrich GmbH). Petri dishes were incubated at room temperature in the dark for 4–5 weeks. Isolates after purification were subcultured on 2% MEA.

Inoculation of grafts of Fraxinus angustifolia

Narrow-leaved ash clones were produced by grafting one-year-old scions onto one-year-old rootstocks in the nursery. One year before the beginning of the experiment, clone seedlings were replanted into plastic containers of 7.5 l to ensure their proper establishment and adaptation. Seedlings were placed in the nursery of the Faculty of Forestry in order to be exposed to natural climatic conditions. Selected clones were inoculated by two *H. fraxineus* isolates in order to monitor the development of induced necroses in plant stems during the specified period of time. The five clones from the clonal seed orchard of Čazma were represented with 15 ramets each, while the four clones from the clonal seed orchard of Nova Gradiška were represented with nine ramets each, resulting in a total of 111 inoculated plants. Each *H. fraxineus* isolate was used for inoculation of five ramets of every clone originating from the Čazma orchard, leaving the remaining five ramets of each clone as a control group. The procedure was repeated for the clones originating from the Nova Gradiška orchard, with the difference of inoculating three ramets of every clone with each isolate and leaving three ramets per clone as a control group.

Prior to the wound formation (inoculation spot), each plant stem was sterilized with 96% ethanol in the area 10 cm above and below the planned inoculation spot, approximately 20 cm above the root collar. Inoculation wounds were made with a special circular sharp tool of 0.5 cm in diameter, and potential bark remains were removed with a sterile scalpel. Mycelium plugs (0.5 cm in diameter) were cut from the margin of 4-week-old *H. fraxineus* cultures grown on the PDA (Difco™ No. 213400) at 20 °C and placed into the wound. Inoculation spots were covered with a Parafilm™ sealing tape and additionally covered with aluminium foil. All the tools and equipment were sterilized with ethanol (96%) and flame prior to each use. Control plants were inoculated with sterile PDA (Difco™ No. 213400) plugs (0.5 cm in diameter), following the same procedure as for inoculations with isolates.

Seedlings were inoculated on 30th September 2014, and stem diameter at the inoculation spot of each ramet was measured. Plants were monitored and necroses measured every two weeks in the period from 14th October 2014 to 17th February 2015. Plants were inspected once more one year after the inoculation, on 22nd September 2015, to observe the one-year progression of the disease and to establish the survival rate of the experimental plants.

Phenology observation in clonal seed orchard

The Nova Gradiška clonal seed orchard of the narrow-leaved ash contains 56 clones (plus trees) selected in natural stands. The clonal seed orchard has been regularly maintained by pruning and with other agrotechnical and pomotechnical treatments from the moment of establishment (Kajba et al. 2008).

Through a period of three years (2012, 2014, 2015), phenological clonal differences of flushing phases to determine interclonal and intraclonal variability were observed and studied. Analysis was focused exclusively on the phase of the beginning of leaf unfolding. The average number of days from 1st January and the start date of the leaf unfolding stage were defined as the point at which the entire leaf blade and leaf stalk were visible. Each year, observation began before any buds began to break (10th March), and was performed every seven days until 1st June. Monitoring included 42 clones originating from three populations (Jasenovac, Novska and Stara Gradiška), with the ramets randomly selected across the entire area of the orchard. Each clone was represented with four ramets (168 plants in total).

Fungal growth on leaf extracts

Due to a complex life cycle of *H. fraxineus* and susceptibility of the plant which may be expressed in the leaf or/and wood (Kirisits and Freinschlag 2012), the conducted experiment was based on the growth rate of the selected two isolates on four different growth media for two weeks at 20 °C. The experiment was designed as a preliminary test which could reveal possible differences in the growth of the same isolate on a different agar medium with added leaf extracts of the three native *Fraxinus* species in Croatia.

Three growth media referred to potato dextrose agar (PDA Oxoid™ CM0139) enriched with ash leaf extracts from *F. excelsior, F. angustifolia* and *F. ornus* respectively, and the fourth one used as control contained only PDA, all without antibiotics. Leaves for the experiment were collected from individual trees of *F. excelsior* and *F. ornus.* Leaves of *F. angustifolia* were collected from the individuals of five clones used in the inoculation experiment belonging to the west seed provenance of Čazma, and mixed together to form a unified sample.

Exactly 50 g of fresh leaf fragments of each species of ash was added to one litre of prepared PDA respectively, prior to autoclaving (similar as described by Kirisits et al. 2013, Carrari et al. 2015). Before fragmentation with a sterile scalpel, the leaves were superficially scrubbed and the surface sterilized with 70 % ethanol on cotton pads and rinsed with sterile water three times. PDA containing leaf fragments was then autoclaved at 121 °C for 15 min. Liquid medium was poured through a sterile sieve, so the leaf fragments were separated and removed before it was poured into the Petri dishes. Finally, 20 ml of each media was poured into 90 mm Petri dishes in a laminar flow chamber. For each isolate, a total of 40 replicates (cultures) were made (10 replicates of each of the medium leaf extract contained and 10 replicates for control without the leaf extract). Growth of the cultures was measured every two days in four opposite directions (cross) previously marked on the Petri dish. Average growth, calculated as the average of differences between growths measured every two days, was taken into account for the statistical analysis.

Statistical analysis

Regarding the final measured lengths of necroses, coefficient of variation (CV) was calculated for each clone as an indicator of the intraclonal variability in order to determine whether susceptibility is on the clonal level or an individual feature. Assumptions of normality were checked using the Shapiro-Wilk W test, and the assumption of homogeneity of variance using Levene's and Brown-Forsythe tests. Due to non-normal variable distribution, unequal variances among observed groups and small sample size in each group (n=3 or 5), non-parametric statistical tests were used, Mann-Whitney U Test for the comparison of two independent groups (difference in final necroses length between the two isolates used), and Kruskal-Wallis ANOVA by Ranks for the comparison of multiple independent groups (difference in final necroses length among the observed clones). Post-hoc comparisons of mean ranks of all pairs of clones analysed with Kruskal-Wallis ANOVA by Ranks were also computed to determine which clones differ significantly.

The data on leaf unfolding were analysed and shown by standard descriptive statistical parameters (arithmetic mean, standard error of the mean, standard deviation, and coefficient of variation). The significance of difference for the given property among the studied genotypes and populations was tested using the analysis of variance. The affiliation of clones to ecotypic forms regarding the beginning of leaf unfolding (early and late flushing) was established using the algorithm for the classification of objects into clusters, i.e. k-means cluster analysis.

The calculated average growth of isolates on different growth media was shown by standard descriptive statistical parameters: arithmetic mean, median, maximum, minimum, standard deviation, and coefficient of variation. The significance of difference in the growth rate between the two *H. fraxineus* isolates, regardless of the growth media used, was tested with non-parametric Mann-Whitney U Test for two independent samples, and the significance of difference in the growth rate among the four used media for each of the two isolates was tested with non-parametric Kruskal-Wallis ANOVA by Ranks for multiple independent samples. Non-parametric tests were used because of the non-normal distribution of the analysed variable (growth rate of isolates) and inhomogeneous variances among observed groups (growth media) for the given variable. These were tested with the Shapiro-Wilk W test for normality, and Levene's and Brown-Forsythe tests for homogeneity of variances.

All data were statistically analysed in the StaSoft. Inc. (2011) STATISTICA version 10 software package.

Results

Inoculation of grafts of Fraxinus angustifolia

The inoculation experiment revealed a high capability of *H. fraxineus* isolates to cause and develop tissue necroses in the tested clones of *F. angustifolia*. In four months, necroses developed in 72 ramets (97.3%), and only 2 ramets (2.7%) of clones BJ38 and BJ25 (inoculated with isolate no. 1) did not develop necroses. All the control plants remained symptomless during the whole monitoring period.

The coefficient of variation (CV) was calculated in order to analyse intraclonal variability regarding the final measured lengths of necroses in plant stems (Figure 1) for each *H. fraxineus* isolate used. The results revealed a very high intraclonal variability, except within the clone NG55,

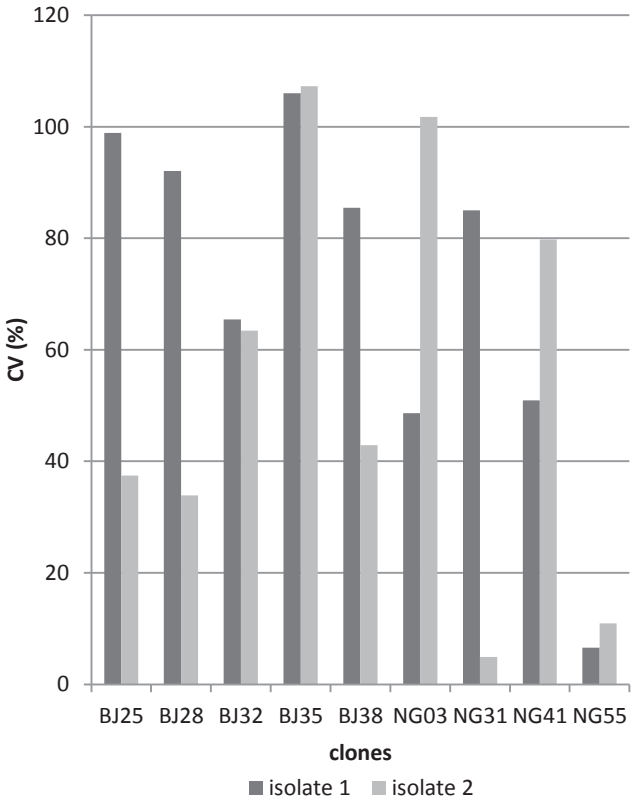

Figure 1. Coefficient of variation (CV) within clones (%) regarding the final measured lengths of necroses for *H. fraxineus* isolate

where intraclonal variability was the lowest for both isolates. With isolate no. 1, the rate of intraclonal variability in the clones from the Čazma orchard remained very high (above 60%), but lowest intraclonal variability was shown in three of the four clones from the Nova Gradiška orchard (clones NG03, NG41, and NG55). With isolate no. 2, the lowest intraclonal variability was shown in three of the five clones from the Čazma orchard (clones BJ25, BJ28 and BJ38), and also in two of the four clones from the Nova Gradiška orchard (clones NG31 and NG55).

Analysis of the data on developed necroses demonstrates that isolate no. 1 caused the longest necroses in clones BJ32, BJ28 and BJ25, NG41 and NG55, and the shortest necroses in clones NG03 and NG31. Isolate no. 2 caused the longest necroses in clones BJ35, BJ28, NG41 and NG55, and the shortest in clones NG03 and NG31. This correlates with the results of inoculation with isolate no. 1, revealing clones NG03 and NG31 as the least susceptible or with the most pronounced tolerance to *H. fraxineus* among the tested clones (Figure 2).

Kruskal-Wallis ANOVA by Ranks was used to determine if there was a significant difference between clones in the final length of the developed necroses (Figure 3). Analyses revealed a highly significant difference ($p = 0.0249$) between the clones regardless of their origin and the *H. fraxineus* isolates used in the test. Post-hoc comparisons of mean ranks of all pairs of clones revealed a significant difference between clones NG03 and BJ28 (p value with Bonferroni adjustment = 0.032272) (Figure 3).

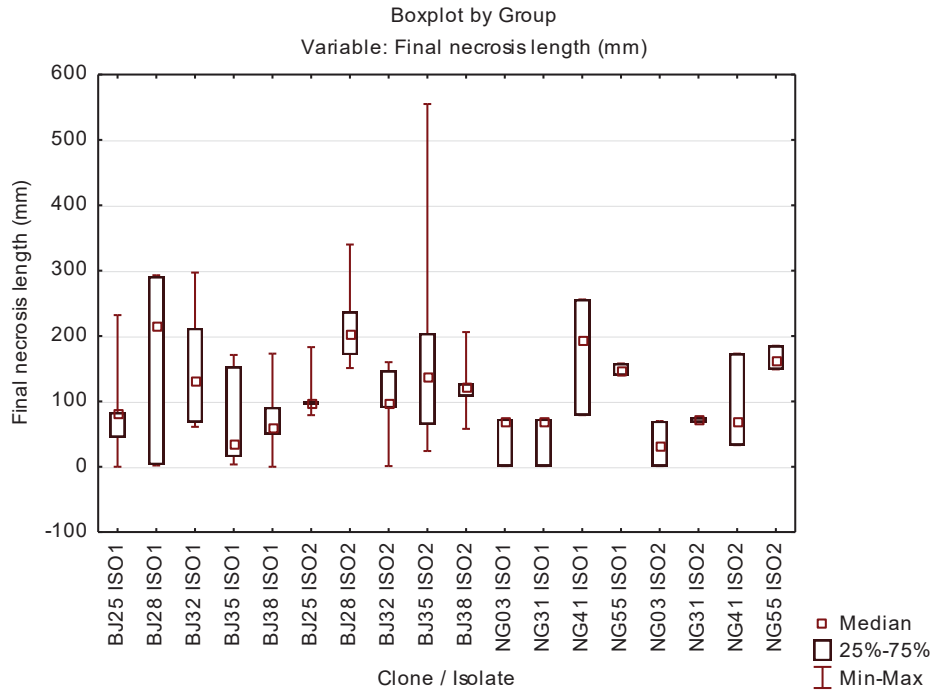

Figure 2. Final necrosis lengths for the clones inoculated with isolate no. 1 (ISO1) and no. 2 (ISO2)

According to the performed phenology study in Nova Gradiška (Figure 4), Kruskal-Wallis ANOVA by Ranks was repeated to determine if there was a significant difference in the final length of necroses between the tested clones NG03 and NG55 originating from the same clonal seed orchard. A significant difference was obtained (p=0.0063) when both fungal isolates were taken into consideration. Post-hoc comparisons of mean ranks of all pairs of clones for the Nova Gradiška clonal group revealed a significant difference between clones NG03 and NG55 as well (p value with Bonferroni adjustment = 0.013198) (Figure 3).

Average necrosis growth rate was calculated as the mean value of differences between necrosis lengths measured every two weeks for a period of the first four weeks of monitoring. Mann-Whitney U Test showed no statistically significant difference between the growth rates of the two isolates used in the experiment (p = 0.321938). Kruskal-Wallis ANOVA by Ranks revealed a significant difference between clones (p = 0.0094) in the development rate of the necroses.

One year after the inoculation, when the plants were examined, it was determined that 36 (48.6%) of a total of 74 inoculated seedlings were completely dead. Additional 4 seedlings (5.4%) revealed no live tissues above the inoculation point (including the crown), although resprouting beneath the inoculation point was observed.

In total, 54% of the inoculated plants had dieback or were in critical condition and expected to die in a very short period of time. From the remaining 46% of the inoculated plants, the majority were heavily affected, revealing severe symptoms of the disease and degradation of vitality.

Phenology observation in clonal seed orchard

The collected data on phenology - leaf unfolding and the performed analysis of variance revealed a statistically significant difference between the studied genotypes in the clonal seed orchard for each year of investigation (F = 5.95, F = 7.57, F = 5.66, Pr <.0001). No statistically significant differences were found between the three studied populations (Jasenovac F=0.43, Novska F=2.04, Stara Gradiška F=0.27). The average number of days from 1st January to the beginning of leaf unfolding was 98 days in 2012, 93 days in 2014, and 103 days in 2015. The results of k-means clustering of the clones according to their leaf unfolding clearly classified genotypes into two ecotypic forms: early and late flushing. Statistically significant differences were found for intrapopulation variability for the beginning of leaf unfolding; however, no statistically significant differences were found between the studied populations (Figure 4).

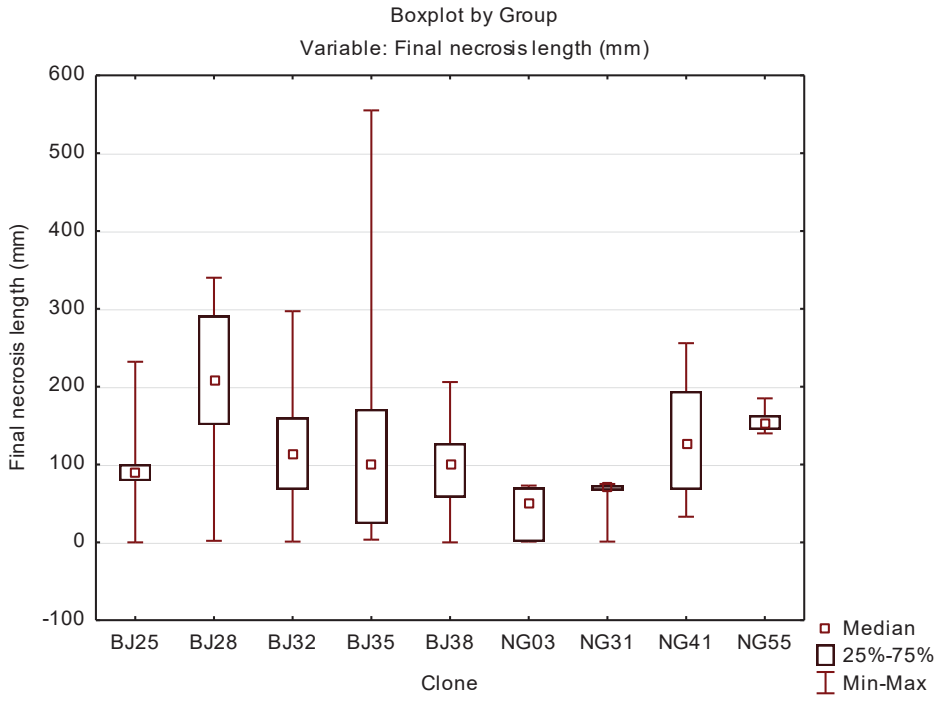

Figure 3. Final necrosis lengths for the clones regardless of the isolate

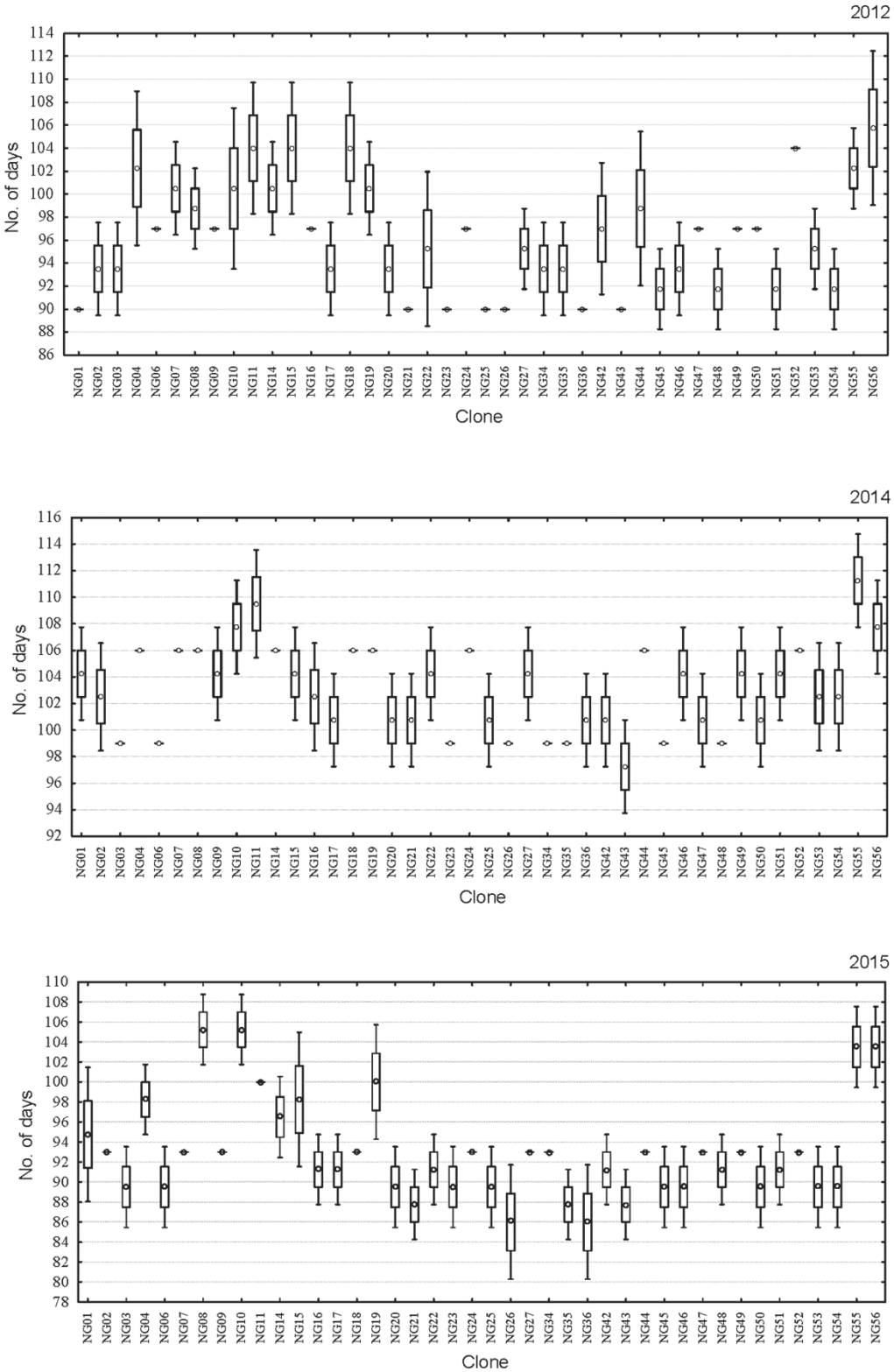

Figure 4. Average number of days per clone required for leaf unfolding for all three investigation years (2012, 2014, 2015) of the Nova Gradiška clonal seed orchard. The horizontal hyphen represents the aritmethic mean of the clone in a certain year, the box represents the standard error of the mean, and whiskers represent the standard deviation

The affiliation of the clones to ecotypic forms did not coincide with their geographic origin, which additionally confirmed important intrapopulation variability of the narrow-leaved ash. Intraclonal values of the coefficient of variability (C.V. %) for the property of leaf unfolding decreased with the age of the plantation and on average amounted to 15.22% at age 2 + 8 years, 13.46% at age 2 + 10 years, and 7.8% at age of 2 + 11 years, indicating higher stability and uniformity of phenological characteristics among the ramets as their age increased. In this period, the clonal seed orchard was also continuously monitored and checked for the presence of *H. fraxineus*. The presence of the pathogen was not confirmed, and clones did not reveal symptoms of the disease.

Fungal growth on leaf extracts

H. fraxineus isolates achieved growth on all tested media with and without the leaf extracts. Morphology of the mycelium varied according to different media (Kirisits et al. 2013), but corresponded well with that described by Kowalski (2006). Leaf extracts influenced growth variations between each medium. Variability of isolates growth within each media was expressed with the coefficient of variation (CV), which was very or relatively weak for both of the isolates and all of the growth media used, except for isolate no. 2 replicates grown on agar enriched with *F. angustifolia* leaf extract (Table 1).

Regarding the growth rates of both isolates, the fastest growth was observed on agar enriched with *F. excelsior* leaf extract, followed by agar with *F. ornus* leaf extract, and the slowest growth was observed on agar with *F. angustifolia* leaf extract as shown in Table 1. Mann-Whitney U Test revealed statistically significant differences between growth rates of the two isolates ($p < 0.0000001$). Isolate no. 2 grew faster on all four media when comparing mean and maximum average growth rates. In order to determine if differences among growth rates on the different media used were statistically significant, Kruskal-Wallis ANOVA by Ranks test was conducted and showed high statistical significance (Table 2, Figure 5). As shown in Figure 5, the growth of both isolates revealed the highest range (minimum and maximum values) on PDA with *F. angustifolia* leaf extracts when compared to other media used in the experiment.

Discussion and conclusions

In order to supplement the body of knowledge for the future forestry management policy on preserving the narrow-leaved ash in lowland forests of Croatia, in view of the new disease threatening its existence, the susceptibility of *F. angustifolia* clones to *H. fraxineus* has been studied.

The inoculation experiment revealed a considerable increase in the disease progression already in the first four months after the inoculation. Development of symptoms, damage severity, and development of necroses were observed in 97.3% of all the ramets tested in the study. High susceptibility of *F. angustifolia* clones to the pathogen was observed, thus confirming similar reported studies on natural susceptibility and resistance to *H. fraxineus*, especially in the first progeny trials with resistance abilities showing great variation (McKinney et al. 2011, Pliûra et al. 2011, Enderle et al. 2013). 54% of the plants had dieback or were in critical condition after the first year of disease progression monitoring, revealing continuous breakage of resistance in the majority of the tested clones. Many authors have shown similar results even after the first selection of resistant clones and progeny trials. Lithuanian clones were selected for resistance in progeny trials in 2005 and revealed inconsistency in resistance abilities in subsequent trials (Pliûra et al. 2014). In Germany, the rate of disease spread in a provenance trial established in 2005, increased from 13% in 2007 up to 94% in 2012 (Enderle et al. 2013).

Table 1. Descriptive statistics for all the growth media and isolates used. Mean, median, minimum and maximum correspond to the values (mm) for a two-day period

Average growth at 20 °C	Mean	Median	Minimum	Maximum	St. Dev.	Coeff. of variation (%)
Isolate 1 Medium 1	1.89	1.87	1.69	2.15	0.18	9.37
Isolate 1 Medium 2	1.57	1.55	0.67	2.27	0.42	27.02
Isolate 1 Medium 3	1.84	1.83	1.71	2.02	0.10	5.65
Isolate 1 Medium 4	1.07	1.02	0.77	1.56	0.27	25.29
Isolate 2 Medium 1	4.68	4.81	3.29	6.06	0.94	20.11
Isolate 2 Medium 2	2.50	1.85	0.42	5.58	1.73	69.53
Isolate 2 Medium 3	3.16	3.15	2.79	3.88	0.35	11.04
Isolate 2 Medium 4	3.37	3.31	2.85	4.27	0.40	11.84

Medium 1 – PDA (Oxoid™ CM0139) with *F. excelsior* leaf extracts
Medium 2 – PDA (Oxoid™ CM0139) with *F. angustifolia* leaf extracts
Medium 3 – PDA (Oxoid™ CM0139) with *F. ornus* leaf extracts
Medium 4 – PDA (Oxoid™ CM0139) without leaf extracts

Table 2. Kruskal-Wallis ANOVA by Ranks p values

	Both isolates	Isolate 1	Isolate 2
p value according to the Kruskal Wallis ANOVA by Ranks	0.0069	< 0.00001	p = 0.0014

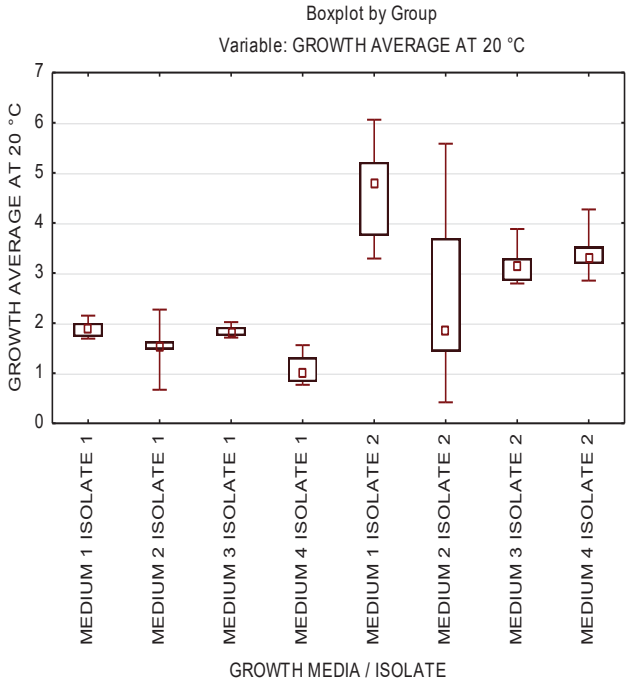

Figure 5. Growth rates of isolates no. 1 and no. 2 on four media. Medium 1 – PDA (Oxoid™ CM0139) with *F. excelsior* leaf extracts, Medium 2 – PDA (Oxoid™ CM0139) with *F. angustifolia* leaf extracts, Medium 3 – PDA (Oxoid™ CM0139) with *F. ornus* leaf extracts, Medium 4 – PDA (Oxoid™ CM0139) without leaf extracts

In a clonal study in Denmark, on two sites disease symptoms increased over three years, from 32 to 55% (McKinney et al. 2011). Only a few studies reported a decrease in ash damage, such as the study performed in Sweden, but the study revealed only a one-year decrease in damage, while in the five-year temporal trend there were some periods of increased damage (Stener 2013).

In a clonal study in Austria, mean ash dieback intensity reached only 18.1% in 2009, and 17.6% in 2010 (Kirisits and Freinschlag 2012). The explanation for the results obtained was that affected trees often responded intensively with the formation of epicormic shoots to compensate for the loss of dieback ones, which resulted in a temporary appearance of decreased dieback symptoms. The authors also assumed that climatic factors might contribute to the phenomenon. One-year monitoring of the disease rate in clones may vary substantially, and every progeny trial revealed a very high disease incidence rate and more uniform results after three or more years of monitoring.

In our study, regardless of the high disease incidence rate, mortality of trees reached almost 49% in one year, while in Lithuanian clone resistance trials mortality increased very little and reached only 1.2% (Pliûra et al. 2014). This could be expected and explained by the long temporal exposure to the pathogen in Lithuania, and as a consequence of the intense natural selection process in Lithuanian *F. excelsior* stands (Pliûra et al. 2014). In Croatia, plus trees in clonal seed orchards were collected from healthy stands of *F. angustifolia* before the outbreak of the disease, where susceptibility-driven natural selection according to the pathogen had not yet started. Also, the age of the seedlings used in our trials may have been the reason for the high mortality rate, as young trees are very susceptible to the disease.

On the other hand, a clonal study in Sweden revealed a 33% mortality range during a five-year period (Stener 2013). If we consider the results from the first Lithuanian progeny trials which are far more similar to our first susceptibility experiment regarding the first trials of selecting possibly resistant clones, there was an almost 90% mortality rate in the five-year period (Pliûra et al. 2011). Second-generation progeny trials always show a significantly lower mortality rate because of greater resistance and/or tolerance inside the group of clones that has previously been selected for exhibiting resistant genetic attributes.

In our study, none of the tested clones revealed total resistance to ash dieback, but a few exhibited reduced susceptibility, which is also in accordance with similar studies performed on resistance of *F. excelsior* genotypes (McKinney et al. 2011, Stener 2013, Pliûra et al. 2014). The study showed a great variety, with an average degree of damage among genotypes consistent with research conducted in Denmark, ranging from 1 to 69% (McKinney et al. 2011), and in Austria, where the degree of damage between clones varied from 0 to 80 % (Kirisits and Freinschlag 2012).

The results of the experiment revealed clones less susceptible or more tolerant to *H. fraxineus,* BJ25, BJ38, NG03 and NG31. These were the clones that exhibited some sort of reduced susceptibility to the pathogen, which should be tested in future experiments. If the resistance remains solid, vegetative propagation could be a very efficient option for breeding, as resistance/tolerance of ash is generally observed more at the individual genotype level than on the population or family levels (Douglas et al. 2013). The coefficient of variation on necrosis lengths revealed that only a small percentage of the tested clones have a small coefficient of variation which correlates to a uniform result in necrosis length. Only the result with small necrotic lengths and a small coefficient of variation could be considered for second-generation progeny trials.

Based on the results of phenological monitoring, we were able to classify narrow-leaved ash clones into early and late flushing groups. Some authors reported a correlation between early leaf shedding and susceptibility to *H. fraxineus* (McKinney et al. 2011, Bakys et al. 2013, Stener 2013, Hauptman et al. 2016). According to our preliminary results, they correlate with the results of McKinney et al. (2011) and Bakys et al. (2013), who claim that clones with earlier leaf unfolding and leaf shedding are less susceptible to the disease. Significantly smaller necrosis lengths were obtained in clone NG03, and longer necrosis lengths in NG55 (Figure 3). Clone NG03 revealed earlier leaf unfolding, approximately 8 to 14 days before clone NG55 in study years 2012, 2014, and 2015 (Figure 4). Earlier leaf unfolding could be one of the important characteristics in the narrow-leaved ash clones' resistance to *H. fraxineus*, as stated in the research done on *F. excelsior* (McKinney et al. 2011, Bakys et al. 2013, Stener 2013). However, according to the study by Hauptman et al. (2016), this correlation was not confirmed in *F. angustifolia*. Due to the data obtained only on two clones, NG03 and NG55, for the length of necroses and leaf unfolding parameter, a conclusion cannot be made. To test the hypothesis on the role of the leaf unfolding parameter as one of the possible phenological characteristics important in *F. angustifolia* resistance or tolerance to the disease, future experiments should be performed and include a larger number of clones and *H. fraxineus* isolates.

Intraclonal variability during the investigated years indicated a higher stability and uniformity of phenological characteristics among the ramets as their age increased. The proportion of symptomatic ramets in a clone for ash dieback disease was highly variable during the studied years (Pliūra et al. 2014). The results of investigation by Stener (2013) suggest that resistant trees in natural ash stands are quite rare, implying that it will be necessary to select a large number of candidate individuals. In the context of global climate change, the composition and structure of genetic variability of the narrow-leaved ash, particularly in terms of adaptive potential such as growth, survival and leaf phenology, should be considered.

Statistically significant differences in our study were obtained between genotypes in clonal seed orchards for the leaf unfolding parameter, but no statistically significant differences were found between the studied populations. Intraclonal values of the coefficient of variability (C.V. %) for the property of leaf unfolding parameter decreased with the age of the plantation, indicating a higher stability and uniformity of phenological characteristics among the ramets as their age increased.

A preliminary experiment carried out on three types of agar with leaf extracts from native *Fraxinus* species in Croatia revealed that the fastest growth of *H. fraxineus* isolates was present on agar enriched with *F. excelsior* leaf extract, followed by agar with *F. ornus* leaf extract, and the slowest growth was obtained on agar with *F. angustifolia* leaf extracts. According to Carrari et al. (2015), growth rates on the media containing extracts of susceptible hosts are higher than those on the media containing extracts of resistant hosts. The results obtained from our experiment cannot be explained at this stage, but could serve as a good basis for further research on *H. fraxineus* isolates. In new tests, isolates should be obtained from different ash host species and host tissues, and the tests should be more focused on the nutrition status of the hosts as a possible susceptibility / resistance characteristic.

The presented preliminary study on the narrow-leaved ash revealed clones less susceptible or more tolerant to *H. fraxineus*: BJ25, BJ38, NG03 and NG31. These were the clones that exhibited some sort of reduced susceptibility to the pathogen, which should be tested in future experiments. If the resistance remains solid, vegetative propagation could be a very efficient option for breeding. The data obtained on clones less susceptible to *H. fraxineus* can be a good basis for future research targeting host genotypes more resistant to the disease, and these studies should provide better insight for the future forestry management policy on preserving the narrow-leaved ash in lowland forest ecosystems of Croatia.

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