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# *Pinus sylvestris* Breeding for Resistance against Natural Infection of the Fungus *Heterobasidion annosum*

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**Abstract:** Increasing resistance against biotic and abiotic factors is an important goal of forest tree breeding. The aim of the present study was to develop a root rot resistance index for Scots pine breeding and evaluate its effectiveness. The productivity, branch diameter, branchiness, stem straightness, spike knots, and damage from natural infection of root rot in 154 Scots pine open-pollinated families from Latvia were evaluated through a progeny field trial at the age of 38 years. Trees with decline symptoms were sampled for fungal isolations. Based on this information and kriging estimates of root rot, 35 affected areas (average size: 108 m<sup>2</sup>; total 28% from the 1.5 ha trial) were delineated. Resistance index of a single tree was formed based on family adjusted proportion of live to infected trees and distance to the center of affected area. Heritability for resistance to root rot based on the value of this index, was high (0.37) and comparable to indices of growth traits. Correlations of family breeding estimates between resistance to root rot and the other traits were not significant, except for a weak, yet significant, positive correlation with diameter at breast height and branch diameter. Selection index including only growth traits (height and stem volume) had a negligible effect on damage by root rot. We detected a maximum genetic gain in resistance index of 33.7% when incorporating it into the selection index with positive gains for growth traits (6.5–11.0%). Two-stage selection with prior selection of the most resistant families was not superior to the use of selection index with only rot resistance included. Overall; rot resistance index appeared to be an effective tool in tree breeding for the selection of more resistant families, using the existing trials with natural (uncontrolled) infection

**Keywords:** selection index; root rot; growth; genetic gain; two-stage selection; heritability

## 1. Introduction

Scots pine (*Pinus sylvestris* L.) is one of the most economically important tree species in the Baltic States and Scandinavia. In this region, it is regenerated primarily by planting, and most of all the plant material is a result of tree breeding; seeds for plant production are collected from seed orchards. Thus, it is both relatively straightforward as well as important to improve a particular trait in significant portion of Scots pine stands. Scots pine breeding primarily had been focused on achieving gain in traits related to productivity (height, diameter, volume production), and frost hardiness in the northern part of the region. In these traits, considerable gain has been achieved [1]. However, little has been done in resistance breeding at practically applicable scale; the most prominent activities include selection

of resistance against needle cast (*Lophodermium* spp.) across the region and against pine blister rust (*Cronartium flaccidum* and *Peridermium pini*) in northern Sweden [2–4].

Two species of the fungus genus *Heterobasidion* are found in Northern Europe: *H. annosum* (Fr.) Bref. and *H. parviporum* Niemelä and Korhonen. Scots pine is highly susceptible to root rot caused by *H. annosum* [5]. The biology of the pathogen has been studied intensively [6], nevertheless host genotypes with total resistance to root rot have not been observed for Scots pine or Norway spruce [7]. Considerable effort has been made to study genetic control of root-rot resistance in Norway spruce [8,9]. However, in context of changing climate, Scots pine must also be given a high priority. Genetic control of resistance has been previously detected in *P. sylvestris* [10], and clones with apparently varying resistance have been detected in seed orchards [11] and among seedlings [12]. At the genetic level, a recent study about terpenoids associated with *P. sylvestris* defensive mechanisms against *H. annosum* has indicated higher expression of genes encoding certain terpenoid compounds, thus showing potential to identify susceptibility of trees based on inherent genetic and chemical properties [13]. Moreover, copy number variation polymorphism had been detected for a gene encoding a thaumatin-like protein, involved in antimicrobial activity against 12 fungal species. As increased gene copy number can lead to increased gene product amounts in cells, candidate of more resistant genotypes can be detected [14].

Previous studies have recommended planting the most resistant genotypes, though it remains difficult to perform the mass testing of these genotypes for their selection and use in practical breeding. One of the methods—genetic screening—can be biased, while controlled inoculation is expensive at large scale. Besides reported results from inoculation experiments allow to evaluate resistance against pathogen spread from already infected trees [15–17], but not the ability of trees to avoid infection. It would be beneficial (saving time and resources) to use existing trials with naturally occurring infection. *Heterobasidion* spp. has a mixed infection biology: (i) Primary infections via airborne spores can originate from freshly exposed sapwood, (ii) secondary infection via vegetative growth of mycelium to neighboring trees. Other ways—e.g., via browsing damage—are very unlikely for Scots pine [5].

Regarding substantial economic losses due to dieback caused by the pathogens, inclusion of resistance to *H. annosum* in Scots pine breeding strategies should be considered. Resistance to disease commonly exhibits strong gains and lacks undesirable correlations with other priority traits, hence the common integration of this strategy in tree breeding programs [18]. Incorporation of root rot resistance into the selection criteria—e.g., index selection [19]—alongside genetic gains of economically important traits, such as tree height and stem volume, may represent a viable strategy to increase net genetic improvement [20]. For Norway spruce, early selection for resistance to *H. parviporum* observed not to have negative effect on growth and wood quality later [9]. However, an additional trait in the selection criteria reduces the gains on already included traits [18]. Thus, a reasonable measurement of resistance against root rot—one that preferably possesses substantial genetic variance and would provide practically important genetic gains—is required. As such, the aim of the present study is to construct a root rot resistance index for Scots pine and evaluate its effectiveness.

## 2. Materials and Methods

### 2.1. Studied Trial and Measurements

The assessed Scots pine progeny trial consisted of 154 open-pollinated families from 13 Latvian populations. The trial site was located in eastern Latvia (56°40' N, 25°57' E), in flat terrain, ca. 110 m above sea level, and was characterized by a rather continental climate [21]. Mean annual temperature was +6.0 °C, and mean monthly temperature ranged from −6.4 °C to +17.1 °C in February and July, respectively. Mean annual precipitation was ca. 700 mm [22]. The trial was established in 1979 using one-year-old bare-rooted seedlings with an initial spacing of 2 × 1 m. The site corresponded to the *Cladinoso-callunosa* forest type according to Latvian forest typology [23]. A randomized block design was used with eight blocks and eight trees row-plot. In total, 4695 trees were available for analysis.

No thinning or other silvicultural measures had been carried out during the trial, thus the only source of natural infection of *H. annosum* had been old Scots pine stumps remaining from previous stand (harvested at mature age).

Evaluations were performed after 38 years of growth, and height (H), diameter at breast height (DBH), and diameter of the thickest branch until the stem height of 2 m (BrD) was measured for each tree. Stem volume (StVol) was calculated according to Liepa [24], and the occurrence of spike knots (SpKn) was recorded as a binary variable (1 = present, 0 = absent). Arbitrary scores using a three-point scale of stem straightness (StStr) and branchiness (Branch) were assessed (1 = straight stem/relatively thin branches, 3 = crooked stem/relatively thick branches).

Regarding their potential resistance to *H. annosum*, trees were allocated in three categories: (1) Asymptomatic trees; (2) symptomatic trees based on crown conditions and fruit bodies on root collar; and (3) dead trees. To evaluate the proportion of actually infected trees, all trees from the Category 2 and 3 (altogether 421) were cut. Wood samples (cross sections) were collected from those stumps. Asexual sporulation (conidiophores) was used to confirm the presence of the pathogen. Isolation and identification of *Heterobasidion* from all samples was attempted according to previous work [17].

Provided data were analyzed using the procedures VARIOGRAM and KRIGE2D in SAS 9.3 software (SAS Institute, Cary, NC, USA) [25] to define affected areas of root rot. Kriging was used as a geostatistical interpolation method to estimate unknown value for trees considering both the distance and the degree of variation between known data points when estimating values in unknown areas. A kriged estimate of root rot infection for a tree was a weighted linear combination of the known sample values (infected trees with registered *H. annosum*) around the point to be estimated as an optimal and unbiased estimate. A semivariogram of the data was constructed to weight nearby sample points when interpolating [26]. Based on kriged estimates larger than zero, presumably infected plots were delineated.

## 2.2. Data Analysis

Within the established affected area, root rot resistance index (*RotResist*) was formed:

$$RotResist = IndDist + IndRot + IndRotFam \quad (1)$$

where *IndDist* is inverted distance from the tree to the plot center (average plot radius minus tree distance to the plot center), *IndRot* is the ratio of number of trees in the plot to number of killed by *H. annosum* trees in the plot, and *IndRotFam* is the ratio of number of certain families' trees in the parcel to number of trees in the certain parcel that are included into a delineated root-rot plot. The index *IndDist* is inverted distance, because the larger value should indicate higher resistance for a living tree closer to the plot center. Similarly, *IndRot* and *IndRotFam* are inverted proportions of killed trees by root rot in the plot and trees per family parcel that is included in the plot, respectively, by this means securing larger resistance index values for alive trees under higher risk conditions.

Variance components for variables were estimated using the SAS Mixed (PROC MIX) and Generalized Linear Mixed (PROC GLIMMIX) procedures with the restricted maximum likelihood approach [27,28]; and standard error values were calculated using Dickerson's approximation [29]. Root rot variance components for heritability calculations were estimated using data only from affected areas. The following model was used:

$$y_{ijk} = \mu + B_i + F_j + BF_{ij} + \varepsilon_{ijk}, \quad (2)$$

where  $y_{ijk}$  is the observation on the  $k$ th tree from the  $j$ th family in the  $i$ th block;  $\mu$  is the overall mean;  $B_i$  is the fixed effect of the  $i$ th block;  $F_j$  and  $BF_{ij}$  are the random effects of the  $j$ th family and interaction of the  $i$ th block and the  $j$ th family, respectively, and  $\varepsilon_{ijk}$  is the random residual effect. For estimating the variance components of the root rot resistance index, block effect was substituted in the model

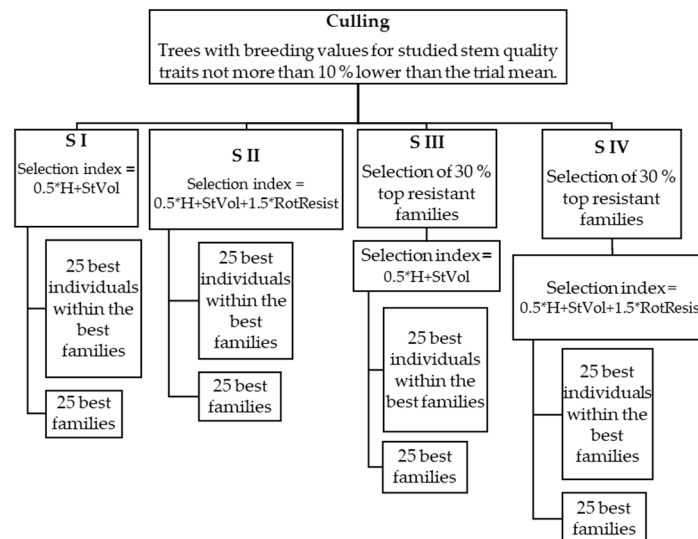
by plot effect since resistance index was calculated only in established affected areas that represented more homogenous growing conditions than blocks.

Estimates of narrow-sense heritability ( $h^2$ ) were calculated as follows:

$$h^2 = \frac{4 \times \hat{\sigma}_f^2}{\hat{\sigma}_f^2 + \hat{\sigma}_{fb}^2 + \hat{\sigma}_\varepsilon^2} \quad (3)$$

where  $h^2$  is narrow-sense heritability and  $\hat{\sigma}_f^2$ ,  $\hat{\sigma}_{fb}^2$ , and  $\hat{\sigma}_\varepsilon^2$  are the estimated variance components of the family, family  $\times$  block interaction, and the residual, respectively.

Family breeding values were estimated as double general combining ability values [19] using a best linear unbiased predictors (BLUP) procedure in SAS [25]. For binary traits, predicted probabilities were estimated by applying the inverse of the link function [27]. We applied different alternative criteria for the selection. However, all of the alternative strategies had independent culling levels [18] set as a first step. Namely, trees with breeding values for studied stem quality traits no more than 10% lower than the trial mean were selected. In combination with the culling, further criteria were applied (Figure 1).



**Figure 1.** Scheme of the different selection criteria applied. Abbreviations: H—tree height; StVol—stem volume; RotResist—root rot resistance index.

First, standard Scots pine breeding methodology in Latvia was applied for a selection of the best performing families and trees [30]. Tree height and volume were used (selection index =  $0.5 \times$  (height breeding estimate) +  $1.0 \times$  (stem volume breeding estimate)). We selected the 25 best trees within the 25 top-ranked families (one from each selected family) and weighted each tree by the number (proportion) of ramets for a seed orchard. None of the families was represented by less than 10–15 progeny nor were any of the best families' clones allowed to comprise more than 10% of the total number of ramets. Second, root rot resistance index was included separately to the selection index formula by weighting it equally to the productivity traits (Selection index =  $0.5 \times$  (height breeding estimate) +  $1.0 \times$  (stem volume breeding estimate) +  $1.5 \times$  (root rot resistance index breeding estimate)). The third and the fourth approaches were similar to the aforementioned approaches but involved applying two-stage selection (tandem selection in the same breeding cycle) [18] with an initial selection of the 30% most resistant families. Thereafter, the four selection criteria were labelled as S I, S II, S III, and S IV (Figure 1).

### 3. Results

For 149 trees, 28 different *H. annosum* genotypes were registered over a total area of 1.55 ha, and 35 affected areas of root rot obtained. The mean affected area was 108 m<sup>2</sup>. Distance between areas ranged from 2 to 74 m, and in total they covered 27.5% of the trial area.

Narrow-sense heritability for *RotResist* was high (0.37, cf. Falconer and Mackay [19]) and comparable to the heritability of productivity traits, which were moderate to high (0.25–0.45). Stem quality traits, such as stem straightness, branchiness, branch diameter, and the occurrence of spike knots, had low (0.02–0.13) heritability estimates (Table 1). Correlations of family breeding values of *RotResist* and the remaining analyzed variables were not significant, with the exception of a weak (cf. Evans [31]), positive correlation with DBH ( $r = 0.16$ ,  $p = 0.05$ ), StVol ( $r = 0.19$ ,  $p = 0.02$ ), and BrD ( $r = 0.28$ ,  $p < 0.01$ ). Productivity traits (H, DBH and StVol) showed very high correlation ( $r \geq 0.88$ ,  $p < 0.01$ ) among themselves and moderate to high ( $0.51 < r < 0.65$ ,  $p < 0.01$ ) correlation with BrD (Table 2).

**Table 1.** Mean, minimum (Min), and maximum (Max) values and narrow-sense heritability indices of the studied traits.

Trait	Mean $\pm$ Standard Deviation	Min	Max	Narrow-Sense Heritability $h^2 \pm$ standard Error
Height (m)	9.7 $\pm$ 2.42	1.9	16.1	0.45 $\pm$ 0.094
Diameter at breast height (cm)	10.3 $\pm$ 4.06	1.0	23.0	0.25 $\pm$ 0.069
Stem volume (dm <sup>3</sup> )	55.6 $\pm$ 43.39	0.3	274.9	0.28 $\pm$ 0.072
Branch diameter (cm)	1.5 $\pm$ 0.43	0.3	4.5	0.13 $\pm$ 0.053
Branchiness (score)	1.4	1.0	3.0	0.10 $\pm$ 0.048
Spike knots (% of trees)	21.8	-	-	0.03 $\pm$ 0.077
Stem straightness (score)	1.2	1.0	3.0	0.02 $\pm$ 0.042
Root rot resistance index	0.78 $\pm$ 1.34	-1.9	8.6	0.37 $\pm$ 0.220

**Table 2.** Family mean breeding value correlations (significant correlations with  $p \leq 0.05$  in bold) in the upper diagonal part and their  $p$ -values in the lower diagonal part. Abbreviations: H—height; DBH—diameter at breast height; StVol—stem volume; BrD—branch diameter; Branch—branchiness; StStr—stem straightness; SpKn—spike knots; *RotResist*—root rot resistance index.

	H	DBH	StVol	BrD	Branch	StStr	SpKn	<i>RotResist</i>
H	1	<b>0.89</b>	<b>0.88</b>	<b>0.51</b>	-0.10	<b>0.39</b>	<b>-0.27</b>	0.15
DBH	<0.01	1	<b>0.98</b>	<b>0.65</b>	<b>-0.34</b>	<b>0.31</b>	<b>-0.25</b>	<b>0.16</b>
Vol	<0.01	<0.01	1	<b>0.62</b>	<b>-0.34</b>	<b>0.26</b>	<b>-0.25</b>	<b>0.19</b>
BrD	<0.01	<0.01	<0.01	1	<b>-0.58</b>	0.04	-0.13	<b>0.28</b>
Branch	0.19	<0.01	<0.01	<0.01	1	0.15	0.02	-0.15
StStr	<0.01	<0.01	<0.01	0.59	0.06	1	<b>-0.24</b>	-0.03
SpKn	<0.01	<0.01	<0.01	0.11	0.76	<0.01	1	0.07
<i>RotResist</i>	0.06	0.05	0.02	<0.01	0.06	0.71	0.38	1

The applied different selection criteria highlighted a varying response to selection (Table 3). Selection index considering only H and StVol (S I) would not notably alter damage by root rot via selection of the 25 best individuals from each family (genetic gain 2.3%) or from the 25 top families (genetic gain 4.9%). If the tree root rot resistance index was included to the selection index formula with equal weighting-to-productivity traits (S II), then the observed increase in root rot resistance comprised 30.0% of cases where individuals were selected and 33.7% when top families were selected. Genetic gain estimates for H, DBH and StVol were reduced considerably, but the other traits were quite robust with the inclusion of *RotResist* to the selection index. The two-stage (tandem) selection process allowed increased genetic gain for root resistance index when applying both S III and S IV. Initial selection of the top 30% of resistant families resulted in an improved resistance index among 15.4% (the best individuals) and 27.0% (the best families) when applying S III. The inclusion of resistance in the selection index in S IV increased the genetic gain for rot resistance to 27.7 and 35.2% for the individuals and the families, respectively. Generally, when selecting the best 25 families instead of the best 25 individuals from each family, the estimated genetic gains for H, DBH, and StVol were less sensitive to the inclusion of resistance in the selection index (Table 3).

**Table 3.** Estimated genetic gain for the studied variables using different selection criteria: S I—selection index with only height (H) and stem volume (StVol) included; S II—selection index with root rot resistance index (*RotResist*) included; S III—two-stage selection with the selection of 30% of individuals from families with the greatest resistance prior to the application of conventional selection index; S IV—two-stage selection with the selection of 30% of families with the greatest resistance prior to the application of selection index with *RotResist* included. For all criteria, the selection of the 25 best individuals within the best families or selection of the 25 best families regarding the selection index are applied.

Selection Criterion		Genetic Gain (%)							
		Height	Diameter at Breast Height	Branch Diameter	Branchiness	Stem Straightness	Spike Knots	Stem Volume	Root Rot Resistance Index
S I ( $0.5 \times H + \text{StVol}$ )	25 best individuals	16.5	13.2	3.0	−0.6	0.1	−1.1	44.3	2.3
	25 best families	14.5	17.8	6.3	−2.4	0.2	−15.4	50.6	4.9
S II ( $0.5 \times H + \text{StVol} + 1.5 \times \text{RotResist}$ )	25 best individuals	6.5	4.7	1.6	0.3	0.1	−1.6	10.9	30.0
	25 best families	10.4	13.5	6.0	−2.4	0.0	−0.5	38.1	33.7
S III (Two-stage selection/ $0.5 \times H + \text{StVol}$ )	25 best individuals	16.4	12.5	2.8	−0.2	0.0	−1.0	41.0	15.4
	25 best families	11.5	14.3	4.8	−2.4	0.1	−2.3	39.7	27.0
S IV (Two-stage selection/ $0.5 \times H + \text{StVol} + 1.5 \times \text{RotResist}$ )	25 best individuals	6.5	4.6	1.7	0.2	0.1	−1.4	10.7	27.7
	25 best families	9.5	12.8	5.5	−2.3	0.0	0.6	35.7	35.2

## 4. Discussion

### 4.1. Genetic Parameters

Although there are no conifer species with genetically determined total resistance against the pathogen *Heterobasidion* spp. [6,7], estimated high heritability of resistance index (Table 1) suggests existing variation in the susceptibility of Scots pine to this fungus being strongly genetically controlled. Genetic control has been commonly reported in the resistance of Norway spruce *P. abies* to *Heterobasidion* spp. [8,15,32–37], moreover the genetic component in the resistance of Scots pine has also been previously detected [10]. The narrow-sense heritability ( $h^2 = 0.37$ ) for *RotResist* was similar or higher to the indices estimated for *H. parviporum* lesion length in the phloem ( $0.17 < h^2 < 0.33$ ) and fungal growth in sapwood ( $h^2 = 0.42$ ) in Norway spruce open-pollinated progenies [9,37]. Also, for Norway spruce clones, moderate broad-sense heritability ( $H^2 = 0.21$ ) has been estimated for lesion length [15]. Swedjemark and Karlsson [33] reported moderate broad-sense heritability ( $H^2 = 0.28$ ) for the frequency of stems infected with *H. annosum* in a progeny trial of 44-year-old Norway spruce full-sib families. For Scots pine, the estimated  $h^2$  in open-pollinated families for *RotResist* was similar to the observed resistance in full-sib families to *Dothistroma* needle blight ( $h^2 = 0.38$ ) [38].

Notably, we detected only very weak undesirable relationships between root rot resistance index and other valuable traits (Table 2), implying potential simultaneous improvement. Besides, the highest estimated correlation (0.28) with BrD might be somewhat exaggerated because of the *RotResist* construction characteristics. Namely, trees with higher *RotResist* in the root-rot affected areas tended to face higher mortality around them, thus promoting development of thicker branches because of improved light conditions.

Overall, fungal growth and lesion length in the phloem were not found to correlate with growth and wood quality traits in a study evaluating the incorporation of resistance to *H. parviporum* in the Norway Spruce Breeding Program [9]. The rather weak, yet significant, positive correlations between family breeding values of *RotResist* and StVol (Table 2) might be explained by reduced growth among more infected families. In Southern Sweden, assessing relative small sample size, significantly reduced the growth nine years after the first thinning had been detected for trees with even a small proportion of roots being infected; general decline in volume increment was detected as early as three years after the first thinning [39].

### 4.2. Selection Methods

Root rot resistance can be considered a high priority trait alongside H and StVol for incorporation into the selection index since it shows notable genetic variance, has high economic impact on timber production value, and does not show an undesirable relationship with growth [18], as also observed for Norway spruce [37]. Unsurprisingly, the reduction of gains in H and StVol were observed (Table 3) with the inclusion of *RotResist* as an additional trait into the selection index [18]. Gains in productivity remained positive (6.5–10.4%), while genetic gains for *RotResist* increased from ca. 2%–5% to 30%–33.7% (Table 3). Considerable heritability for *RotResist* (Table 1) might have determined high genetic gain in our case, when assigning equal weight to productivity traits.

Still, higher gains for *RotResist* and smaller reductions in gains in growth could be achieved by selecting the best (based on selection index) families (backward selection) instead of the best individuals within the best families (Table 3), indicating a higher effectiveness in family selection likely due to sufficiently large environmental deviations affecting the phenotypic variance of the traits [19].

The two-stage selection (S III and S IV) appears to be as effective as single-stage selection (S I and S II), having a small impact on growth and quality traits (Table 3). Under two-stage selection, the aforementioned effectiveness of family selection seems to be retained, providing higher gains for growth traits. However, regarding similar improvement in *RotResist* in the index and two-stage selection processes, S II should be preferred because little yet improved gains in growth traits were observed for S II compared to S IV (Table 3).

Overall, the monetary value of forest products must reflect genetic gains for resistance index, and evaluation is necessary regarding whether the added value of *RotResist* outweighs selection for pure productivity [37]. In our study, we applied equal weights for growth traits and *RotResist*. Therefore, potential further research should address the optimal economic weight for each trait in the selection index to achieve the greatest economic gain; however, uncertain future economic conditions makes this a difficult task [18]. The loss of merchantable timber because of pathogens is hardly predictable because of numerous site factors affecting the spread of infections [6,40] and reduced resistance to wind damage [6].

Although inclusion of *RotResist* into the selection criteria indicates substantial improvements in genetic gain, some practical issues may arise regarding the application of the index. In the present study, *RotResist* was calculated for trees in the sample plots derived from the kriging estimates, which were based on detected rot in cross-sectional wood samples. Such a destructive method involves laborious extra work to include in breeding activities, thereby increasing breeding costs. However, *H. annosum* may infect Scots pine root systems without any obvious crown symptoms; thus, visual assessment alone may result in the underestimation of disease distribution [7,39,40]. As only Category 2 and 3 trees have been sampled, calculations are based on the minimum size of the pathogen genets. To estimate the exact borders of *Heterobasidion* genets more accurate sampling would be required (excavated root systems). Still, the observed effectiveness of *RotResist* may be successfully applied in combination with other, less resource-consuming root rot detection methods at an earlier age, such as non-destructive resistography [41] or assessment during inoculation experiments [15].

## 5. Conclusions

Root rot resistance index appeared to be an effective measure for inclusion in the selection criteria because of its notable genetic variance and lack of undesirable relationship to other growth and quality traits. We suggest the incorporation of the root rot resistance into the conventional selection index of growth traits for family selection, which would considerably improve resistance to root rot causing dieback while maintaining reasonable gains in growth traits. Notwithstanding, further analysis is necessary to adjust economic weights for the traits in the selection index to obtain optimal index performance.

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