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Greenhouse Screening for pH Stress in *Rhododendron* Genotypes

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Abstract: The genus *Rhododendron* is known for its preference for acidic soils, although some genotypes can tolerate a more neutral or alkaline pH. In this study, a greenhouse experiment was set up for 140 days to examine different parameters to assess pH stress in the progeny of *R. fortunei* and the cross combination *R.* 'Pink Purple Dream' x 'Belami'. Additional cultivars 'Gomer Waterer' and 'Cunningham's White' were included in the greenhouse test. The plants were divided into two groups. One group was planted in a substrate with a neutral pH (treatment, pH 6.3) and the other group of plants was planted in an acidic pH substrate (control, pH 4.5). Tolerance to pH stress was evaluated for the individual genotypes on both substrates 140 days after the start of the experiment. The following parameters were analyzed: shoot length, root development, chlorophyll fluorescence (Fv/Fm), leaf color and weight (fresh and dry). In intolerant genotypes, all parameters except for number of shoots were negatively affected by pH stress; especially, the development of roots was negatively impacted by the neutral pH, resulting in above-ground symptoms of pH stress, including decreased height and lower fresh and dry weight. The results show variation in pH tolerance within the genotypes tested and point to the potential for the selection of *Rhododendron* genotypes with improved tolerance to neutral pH.

Keywords: abiotic stress; alkalic soil; calcium; chlorophyll fluorescence; pH stress; *Rhododendron fortunei*

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1. Introduction

With nearly 1000 species, *Rhododendron* L. is the largest genus in the family of Ericaceae. Despite the popularity of rhododendrons for use as ornamental garden plants, the economic potential of rhododendron is limited as this genus only thrives in acidic soils (pH 4.5–6.0). *Rhododendron* grown in pH-neutral or alkaline soils shows chlorosis symptoms, reduction in growth of shoots and roots, leaf wilting, defoliation and, finally, plant death [1]. In a study that combined databases on soil characteristics with the natural occurrence of *Rhododendron* species in China, 76 rhododendron taxa out of 525 taxa were predicted to have potential lime tolerance [2]. The abundant genetic variation and the variation in pH tolerance allow for breeding by cross-hybridization and selection of rhododendron cultivars that may be better suited for gardens with neutral or high soil pH. The selection of genotypes adapted to higher soil pH requires appropriate bioassays.

Many authors show that, in soils with elevated pH, it is not the calcium level that causes stress in rhododendron but rather the elevated levels of bicarbonate (HCO_3^-) that lead to reactions between these soil carbonates, water and carbon dioxide [3–5]. For this reason, rhododendron plants treated with high levels of gypsum ($CaSO_4$) grow better than in substrates with elevated levels of $CaCO_3$ [5,6]. This response might be attributed to bicarbonate (HCO_3^-) toxicity [4,5]. In soil with an elevated pH, bicarbonates also create

an environment where insoluble forms of iron, manganese, phosphorus and other plant nutrients become unavailable to plant roots [7]. Bicarbonate is a major anion component of calcareous soils; at the concentrations likely to be found in those soils, it can inhibit root growth (cell elongation) in calcifuge plants and disrupt iron uptake, resulting in chlorosis [8].

The breeding and selection of *Rhododendron* genotypes that can grow in more alkaline soils is important to penetrate the market for gardens with neutral or high soil pH. It is known that some *Rhododendron* species, subspecies or botanical varieties have a tolerance to an elevated soil pH as they are found in natural habitats with a soil pH above pH 7. For example, *R. fortunei* (used in the current study) grows in habitats with a higher soil pH [2]. Chaanin [5] studied what pH level should be used to test lime tolerance in rhododendron. A study was performed with 200 lime-treated rhododendron species and hybrids at different pH levels adjusted with HCO_3^-. All grew well at a low pH of 4.2, but stunted growth and iron chlorosis were already noticed at a moderate pH of 6.4. At the highest pH of 7.1, all plants died except for a few seedlings of *R. micranthum*, *R. occidentale* and *R. schlippenbachii*.

In a previous study, we tested seedlings of *Rhododendron* genotypes for tolerance to higher soil pH. Using germination experiments in tissue culture, the effects of alkaline pH and extra Ca^{2+} on the germination and growth of seedlings of *R. fortunei*, *R. vernicosum* and *R. chihsinianum* were investigated [9]. Four seedling populations obtained from crossings between commercial rhododendron cultivars were also tested in a similar way [10]. The results indicated no significant effect of alkaline pH on germination, while a dramatic increase in the number of abnormal seedlings and seedling mortality was observed. Wang et al. [9] also described an alkaline pH shock experiment in well plates where seedlings of various rhododendron species were treated with $NaHCO_3$. Already on the second day after treatment, the negative influence of alkaline pH on seedlings could be detected by chlorophyll fluorescence imaging. Those experiments revealed a genotype-dependent response to higher pH, enabling efficient selection of seedlings for tolerance to a higher soil pH.

In the current study, a greenhouse experiment was set up and plants were submitted to two pH levels in a pot experiment with substrate. The aim of the greenhouse experiment was twofold: (1) to validate the differences in pH stress between the different genotypes and (2) to evaluate different parameters to estimate the pH stress in the greenhouse plants.

2. Materials and Methods

2.1. Plant Material

Two groups of plant material were used for the greenhouse experiment: *Rhododendron* plantlets that had survived the tissue culture selection process in a neutral pH medium and a control group grown in a low pH medium in tissue culture without selection [9]. The genotypes in both groups were seedlings of *R. fortunei* and genotypes of a manual pollination between *R.* 'Pink Purple Dream' × 'Belami' (Table 1). These seedlings were obtained after sowing in tissue culture as described by [9] and clonal propagation in tissue culture, followed by three months of acclimatization in the greenhouse. All *R. fortunei* seedlings were coded as RF and subnumbered per seedling genotype. The *R.* 'Pink Purple Dream' × 'Belami' hybrids were coded as "PB". The subnumbered PB genotypes refer to individual genotypes clonally propagated in tissue culture. Only the PB group without subnumbers refers to a group of individual seedlings not clonally propagated but grown as a group of individual genotypes.

Two additional cultivars were included in the experiment, 'Gomer Waterer' and 'Cunningham's White'. Both are commonly grown and well-known cultivars. 'Cunningham's White' and *R. fortunei* are known to be the parents of the higher-pH tolerant rootstock Inkarho® [5]. Both cultivars were obtained from a commercial grower, Raf Goossens BV (Moerbeke-Waas, Belgium). 'Gomer Waterer' was obtained as a tissue culture propagated plant. 'Cunningham's White' was not propagated in tissue culture and was the only cultivar propagated through cuttings. Before the experiment, all plants were grown in propagation trays with 150 cells of approx. 20 mm^3. All plants were between 2 to 6 cm high before they

were potted at the start of the experiments. Information on preceding experiments and plant numbers is given in Table 1.

Table 1. Genotypes used in the experiments were obtained as tissue culture grown seedlings from *R. fortunei* (RF) and from the cross *R.* 'Pink Purple Dream' × 'Belami' (PB), tissue-cultured plants of 'Gomer Waterer' (GW) and cuttings of 'Cunningham's White' (CW). For genotypes screened during tissue culture in preceding experiments, pH of the medium is mentioned.

Genotypes	Not Selected (NS)/Selected (S) in Previous Seedling Selection in Tissue Culture	pH Used for Selection	Number of Plants per Treatment
PB-T3-1	S	7.7	4
PB-T3-4	S	7.7	4
PB-T4-2	S	7.5	4
PB	NS	5.8	12
RF-T-2	S	8.9	3
RF-T-3	S	8.9	3
RF-T-5	S	8.9	3
RF-T-6	S	8.9	3
RF-C-1	NS	6.2	3
GW	NA	NA	12
CW	NA	NA	12

NA: not applicable.

2.2. Substrate and Experimental Setup

An extra fine-sieved substrate for seedlings and cuttings, Lp307z, was obtained from Greenyard Horticulture (Ghent, Belgium) and comprised sod peat, perlite, white peat and Irish peat and calcium at 0.5 kg m^{-3} (pure calcium) supplemented with 0.1 kg m^{-3} Micromax® premium (ICL, Waardenburg, The Netherlands). The manufacturer's estimate of the pH of the substrate was 4.4 and an EC of 49 µS cm^{-1}. In-house analysis resulted in a pH of 4.5 and an EC of 85 µS cm^{-1}.

The substrate without addition of extra calcium was used as the control. For the treatment with a neutral soil pH, $CaCO_3$ in a concentration of 2 g powder per L of potting soil was mixed into the substrate for a final pH of 6.3. All 9 RF and PB genotypes as well as 'Cunningham's White' (CW) and 'Gomer Waterer' (GW) (Table 1) were randomly divided into two groups; one group was transplanted in neutral pH and the other in control substrate.

All plants were watered with the ebb and flow system in the greenhouse at a frequency of 3 times per week and were fertigated with NPK(Mg) 20-5-10 (+2) adjusted to an EC of 1 mS cm^{-1}. pH levels remained stable throughout the experiment.

2.3. Measurements

All measurements were taken 140 days from the start of the treatment. Only root development was scored after 70 and 140 days. Parameters analyzed were fresh and dry weight, chlorophyll a fluorescence, CIELab color analysis and growth of shoots and roots. For the analysis of the fresh weight, the above-ground parts of the plants were cut and analyzed using a balance (Mettler Toledo XS104, Zaventem, Belgium). The individual plants were dried at 70 °C for 3 days before analysis of the dry weight on the same balance.

Chlorophyll fluorescence was analyzed after the plants had undergone at least 1 h of dark adaptation with a pulse amplitude-modulated fluorometer (PAM 2100, Heinz Walz GmbH, Effeltrich, Germany). For every plant, the youngest fully developed leaf was exposed to a low intensity (<0.1 µmol m^{-1} s^{-1}) measuring light to estimate the initial (F0) fluorescence value. Consecutively, the leaves were flashed with a saturating light pulse (±8000 µmol m^{-2} s^{-1}) for 0.8 s to determine the maximum (Fm) fluorescence. By subtracting F0 from Fm, the variable fluorescence, Fv, was calculated (Fm − F0 = Fv). Then,

the parameter Fv/Fm was determined as a measure for the efficiency of excitation energy capture by open photosystem II reaction centers, as Fv/Fm is an indicator for plant stress.

For the analysis of L*a*b* values in the CIELab color space, a portable spectrophotometer (CM-700D, Konica Minolta, Inc., Erfurt, Germany) was used. Color can be quantified using L* as a value for brightness ranging from 0 (black) to +100 (white), a* as a value ranging from −128 (green) to +128 (red) and b* as a value ranging from −128 (blue) to +128 (yellow) [11].

Root development was scored on a scale from 0 to 5. After plants were lifted out of the pot, root development was scored based on the appearance of the root ball. Score 0 was assigned when no roots were visible, score 1 for a small number of roots visible at one side or when a single root appeared on more than one side, score 2 for more than 1 root visible on at least 2 sides, score 3 for some roots visible on all sides, score 4 for roots visible around the entire root ball and score 5 for roots visible from top to bottom and all around the entire root ball (Figure S2). Plant height and length of the youngest shoot flush were measured using a ruler (Figure S3). The number of shoots and flushes was counted per individual plant.

2.4. Statistical Analysis

During the experiment, the plant pots were randomly placed in trays on one table in the greenhouse. Prior to measuring and scoring, they were rearranged to a new random placement on the table.

All statistical analyses in this study were performed using R 4.1.3 and R-studio [12]. In the R package "ggboxplot", global *p*-values of variations were calculated by the Kruskal–Wallis test, and pairwise comparisons between each group and the overall group mean (grand mean) were analyzed by a Wilcoxon test. The principal component analysis (PCA) was performed with the package "prcomp", and Pearson's correlation analyses were performed with "ggcorrplot".

3. Results

3.1. Comparison of pH Stress between Acidic and Neutral Substrate pH

First, an overall analysis of the effect of pH on the plant traits of the 11 genotypes was performed. The overall effect of the higher pH of the neutral substrate showed significant differences for most parameters (Figure 1). The root score (day 70 and 140), plant height, fresh and dry weight, number of flushes, length of the new flush, Fv/Fm and CIELab (a) of plants in the neutral pH substrate significantly decreased when compared with the plants in the acidic (control) substrate ($p < 0.0001$). The CIELab values L* and b* of the plants on neutral pH were significantly higher than in the control ($p < 0.0001$). No significant difference was found in the number of shoots.

3.2. Comparison of pH Stress between Genotypes

To evaluate differences in the performance of different genotypes, the individual genotypes were compared with the grand mean of all genotypes for all parameters. The grand mean is defined as the sum of all data for all genotypes divided by the total number of samples.

Differences in root development were found between genotypes. After 70 days in the control substrate, significantly better root development was found in PB-T3-4 ($p < 0.01$), while CW showed a significantly lower development of roots ($p < 0.01$) when compared with the grand mean of all genotypes (Figure 2A,B). The root scores of the different genotypes in the neutral substrate showed no significant differences on day 70 (Figure 2B). On day 140, however, the root score of PB-T3-4 was significantly ($p < 0.001$) higher than the grand mean both in the control and in the neutral pH. Only PB-T2-4 showed a significantly lower value ($p < 0.001$) compared with the grand mean in the neutral pH (Figure 2D).

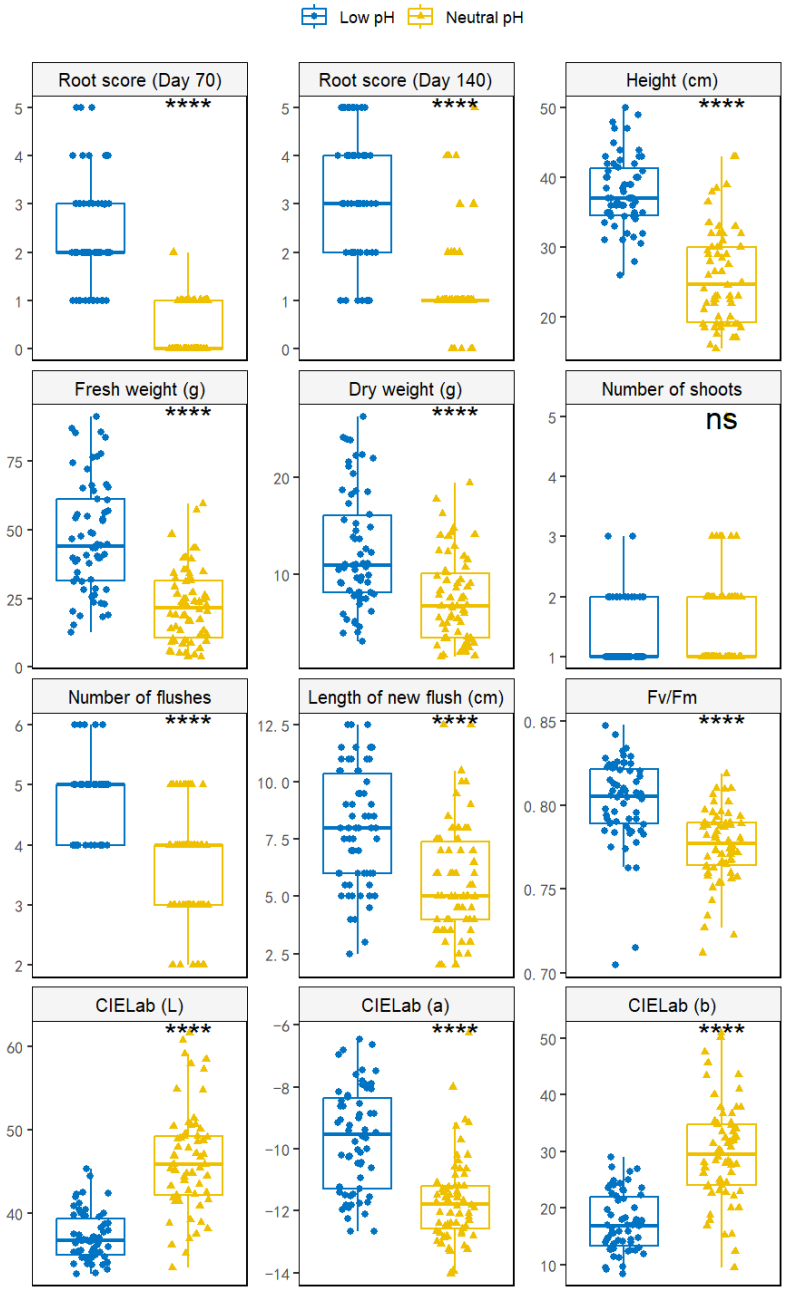

Figure 1. Overall effects of low pH (control) and neutral pH ($CaCO_3$ supplemented) substrate on root score, plant height, fresh weight, dry weight, number of shoots, number of flushes, length of the new flush, Fv/Fm and CIELab (L, a, b) of all genotypes. Pairwise comparisons against the control were calculated by a Wilcoxon test and indicated by **** ($p < 0.0001$) and ns (not significant), respectively.

In the randomly selected progeny plants of R. 'Pink Purple Dream' × 'Belami' (PB), the fresh weight was significantly ($p < 0.05$) higher than the grand mean (Figure 2A) in the control treatment and showed the largest variation when compared with the other genotypes. In the neutral pH treatment, fresh weight was approximately equal to the grand mean. The dry weight of PB genotypes (Figure 2C,D) showed similar results. Both genotypes GW ('Gomer Waterer') and PB-T3-4 performed significantly better than the grand mean in the control and neutral pH (both $p < 0.01$) treatment for fresh weight and dry weight. The genotype CW ('Cunningham's White') performed significantly worse ($p < 0.0001$) when compared with the grand mean.

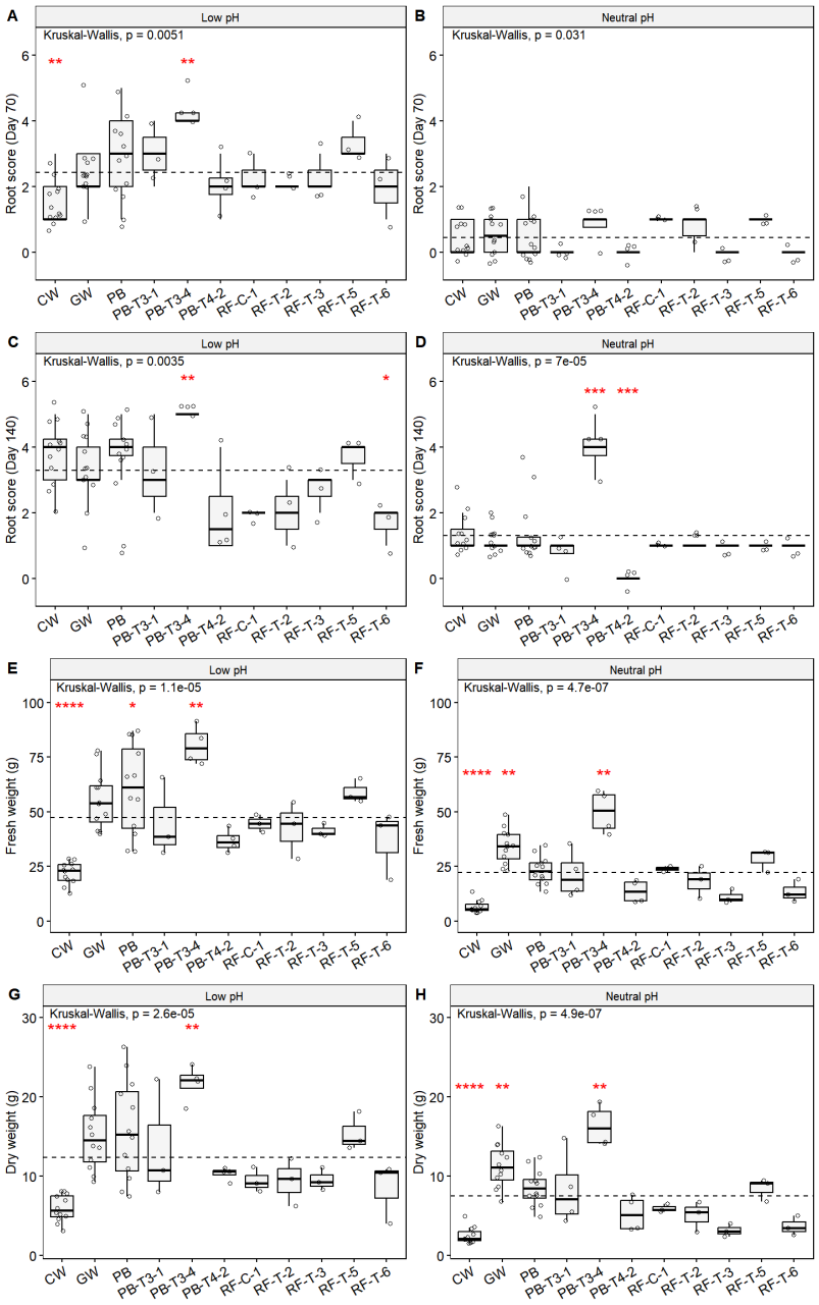

Figure 2. Performance of different genotypes evaluated by (**A**,**B**) root score on day 70 and (**C**,**D**) root score on day 140, (**E**,**F**) fresh and (**G**,**H**) dry weight on day 140 under low and neutral ($CaCO_3$) pH treatments. Global *p*-values of variations among genotypes were calculated by the Kruskal–Wallis test. Pairwise comparisons against all (grand mean, illustrated by the dotted line) were calculated by the Wilcoxon test indicated by **** ($p < 0.0001$), *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$) and no marks (not significant), respectively.

Above-ground growth was measured after 140 days. Also for growth parameters differences in performance were found among genotypes. Genotype PB-T3-4 grew tallest in both the control and neutral pH substrates and was significantly ($p < 0.01$) taller than the grand mean (Figure 3A,B). Genotype PB-T4-2 grew significantly less tall in the control and neutral pH substrate compared with the grand mean ($p < 0.05$). Only in the neutral pH substrate were genotypes CW and GW significantly less tall and taller than the grand mean, respectively ($p < 0.05$) (Figure 3A,B). GW developed significantly ($p < 0.05$) fewer shoots than the grand mean in the low and neutral pH. PB developed significantly more shoots

in the neutral pH ($p < 0.05$) (Figure 3C,D). The number of shoot flushes was significantly higher in RF-T-2 in the control ($p < 0.05$) but not in the neutral pH. In the neutral pH, significantly fewer flushes developed in CW ($p < 0.001$) and significantly more flushes developed in RF-C-1 ($p < 0.05$) (Figure 3E,F). The length of the new flush of genotype PB-T3-4 was only significantly higher than the grand mean in the neutral substrate but not in the control ($p < 0.01$). In two other genotypes, RF-C-1 ($p < 0.01$) and RF-T-3 ($p < 0.05$), the flushes were significantly less long than the grand mean compared in the neutral pH, and, for RF-C-1 and RF-T-2, the flush length was also significantly less long in low pH ($p < 0.05$) (Figure 3G,H).

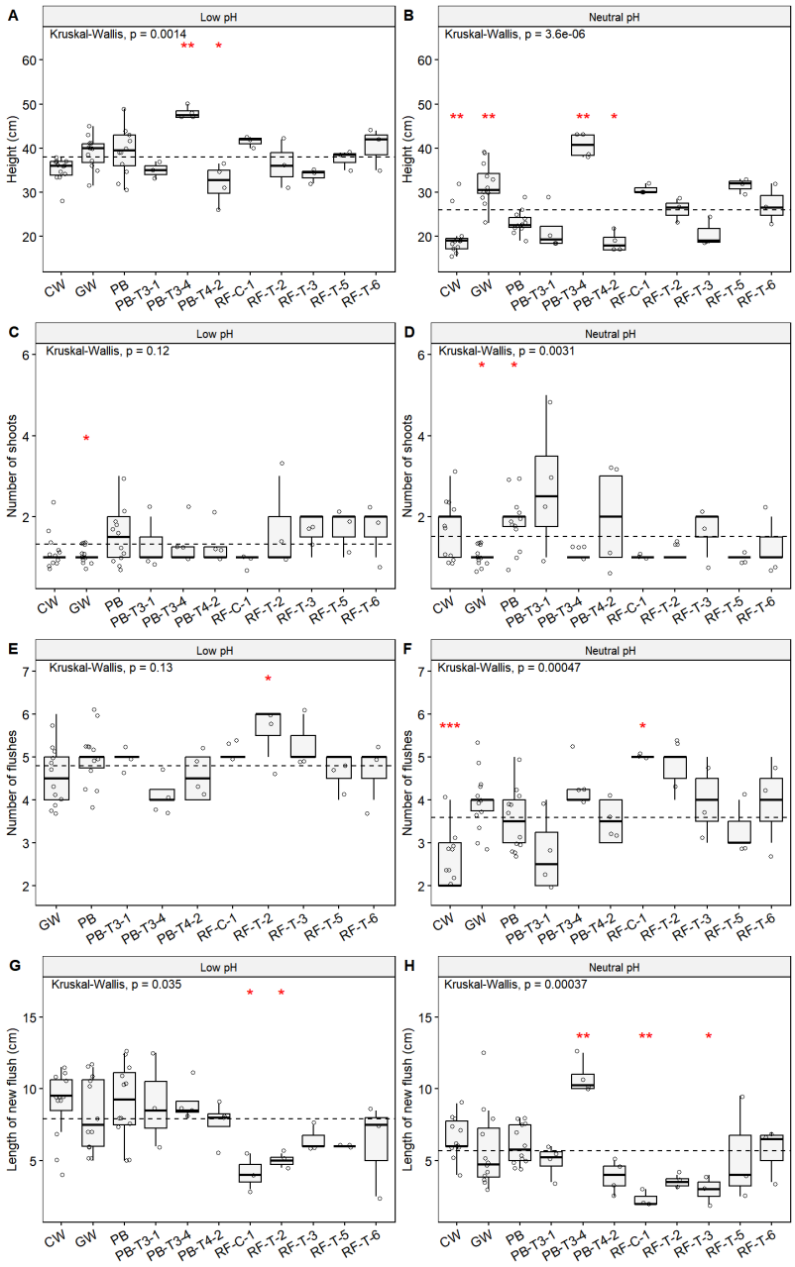

Figure 3. Performance of different genotypes evaluated by (**A**,**B**) height, (**C**,**D**) number of shoots, (**E**,**F**) number of flushes (CW not included) and (**G**,**H**) length of the new flush on day 140 under control and neutral (CaCO3) pH treatments. Global *p*-values of variations among genotypes were calculated by the Kruskal–Wallis test. Pairwise comparisons against all (grand mean, illustrated by the dotted line) were calculated by Wilcoxon's test, and indicated by *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$) and no marks (not significant), respectively.

Chlorophyll fluorescence was measured as Fv/Fm in this experiment. In the control (low pH), the Fv/Fm values were all around 0.8. Values were significantly higher than the grand mean in two genotypes, CW and RF-T-3 (both $p < 0.05$), and lower in PB ($p < 0.01$) than the grand mean in the control (Figure 4C). In the neutral pH, the grand mean value dropped to around 0.75 and was significantly lower for CW ($p < 0.01$) than the grand mean and higher in GW ($p < 0.01$) and PB-T3-4 ($p < 0.05$) (Figure 4A,B). The value for PB-T3-4 was the highest among all genotypes in the neutral pH; this was higher than the Fv/Fm value this genotype had in the low pH substrate.

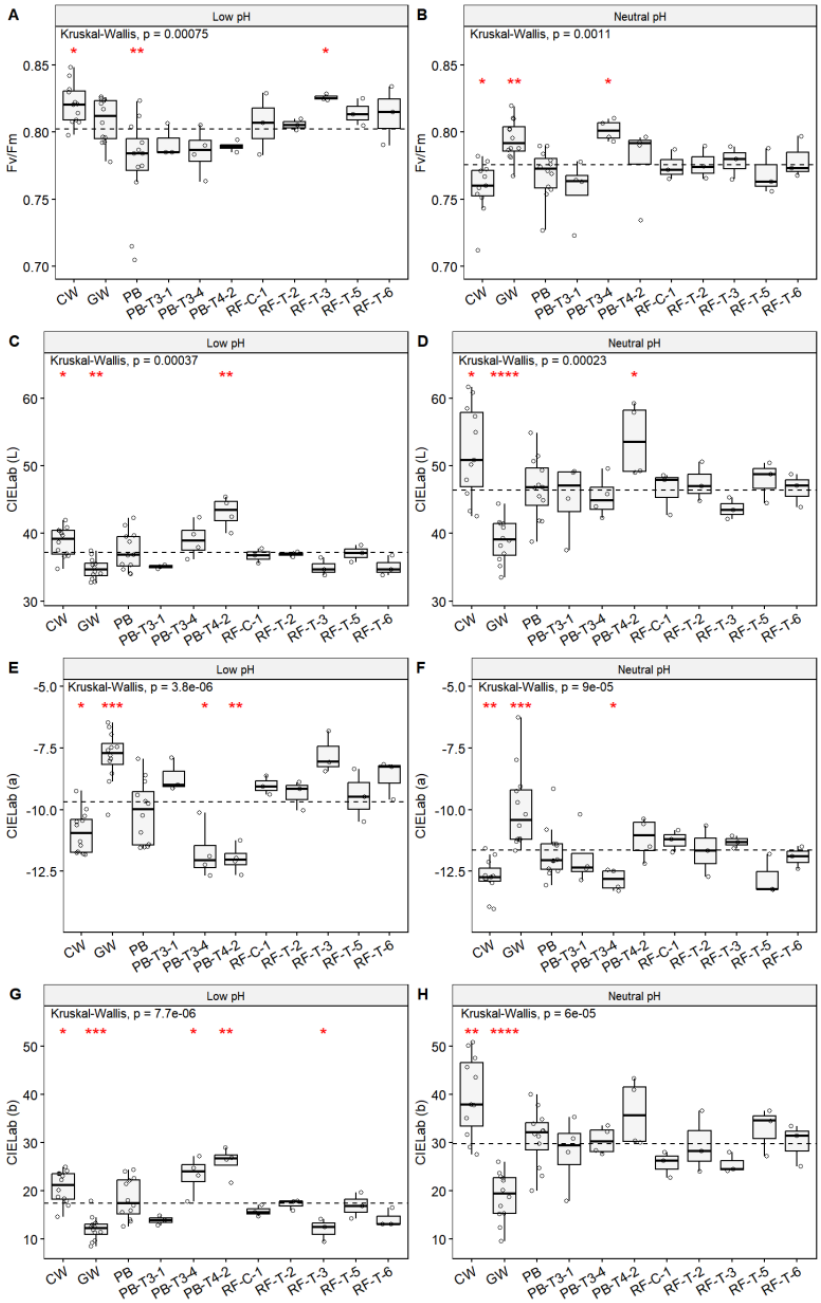

Figure 4. Performance of different genotypes evaluated by (**A**,**B**) Fv/Fm and (**C**–**H**) CIELab (L*, a*, b*) on day 140 under control and neutral ($CaCO_3$) pH treatments. Global *p*-values of variations among genotypes were calculated by the Kruskal–Wallis test. Pairwise comparisons against all (grand mean, illustrated by the dotted line) were calculated by the Wilcoxon test, and indicated by **** ($p < 0.0001$), *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$) and no marks (not significant), respectively. Performance of different genotypes evaluated by height, number of shoots and number of flushes.

L*a*b* values in the CIELab color space measured by the portable spectrophotometer CM-700D were important non-destructive parameters for plant phenotyping. A higher L* value indicated a whiter color, a lower a* value indicated more green and a higher b* value indicated more yellow in the leaves. Differences in CIELab values were found among different genotypes. L* and b* of CW were significantly higher than the grand mean in both the control and neutral substrate, while L* and b* of GW showed the opposite. The darker colored leaves of GW can be seen in Figure S4. In both the control and neutral pH, significant differences from the grand mean were found for L* in CW, GW and PB-T4-2 (Figure 4C,D). These variations only showed a genetic difference in leaf color without the influence of pH. For a* CW, GW and PB-T3-4 differed from the grand mean in both pH levels, while only PB-T4-2 showed a significantly lower a* value when compared with the grand mean in the low pH ($p < 0.05$). In the neutral pH, the value was higher than but not significantly different from the base mean (Figure 4E,F). For b*, significantly different values in the low pH were found for CW, GW, PB-T3-4, PB-T4-2 and RF-T-3. In the neutral pH, there was no longer a significant difference for PB-T3-4, PB-T4-2 and RF-T-3 when compared with the grand mean (Figure 4G,H).

A PCA analysis of root score day 70 and 140, fresh and dry weight, height, number of shoots, length of new flush, Fv/Fm and CIELab of selected (T) and non-selected (C) genotypes in neutral and control substrates indicated that the two PCs (PC1 and PC2) accounted for 61.7% and 17.0% variance, respectively (Figure 5).

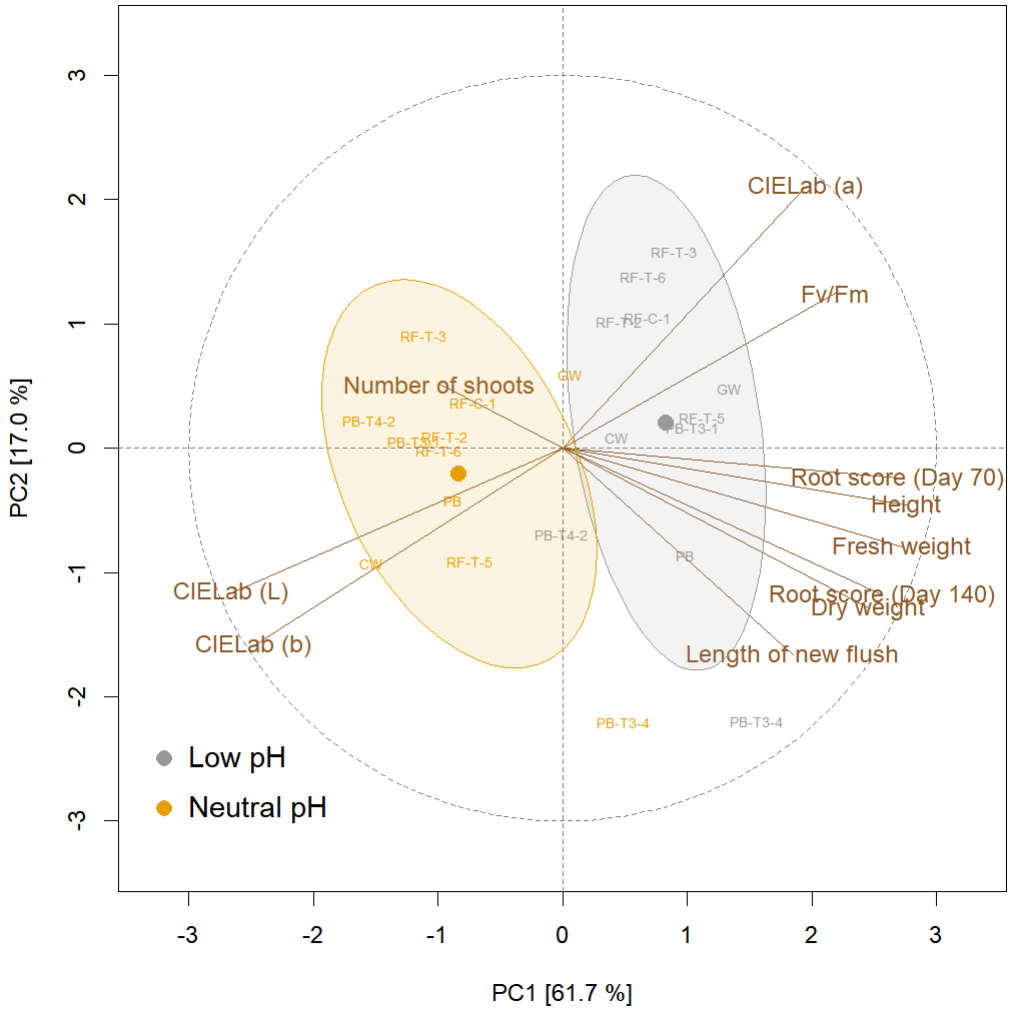

Figure 5. PCA biplots of PC1 and PC2 of root and shoot growth parameters, Fv/Fm and CIELab of the different genotypes in neutral pH and acidic control substrates.

The growth parameters of root score, height, length of new flush, fresh and dry weight had the same direction and were close to each other, so they were highly positively correlated, while the number of shoots was in the opposite direction and was thus negatively correlated. The chlorophyll fluorescence parameter Fv/Fm had the same direction and was highly positively correlated with CIELab (a*), while it was negatively correlated with CIELab (L* and b*). The genotypes in the control (indicated in blue and green) and neutral (in grey and yellow) substrate had a different group/ellipse structure that could be distinguished by PC1, indicating that the genotypes growing in the neutral substrate had different characteristics to the genotypes in the control substrate. Differences in performance between genotypes could be shown. The genotype PB-T3-4 was best performing and far from the other genotypes both in the control and neutral substrates (Figures 5 and S4). For PB-T3-4, the root score after growing the seedlings for 70 days in the neutral pH was significantly ($p < 0.05$) worse than the control (Figure S1). However, after 140 days, the root score of PB-T3-4 in the neutral pH was not significantly different from the control. In contrast, for other genotypes such as CW, GW, PB, PB-T3-1 and PB-T4-2, the root score after 140 days in the neutral pH was still significantly lower than the control (Figure S1). In addition, after 140 days in PB-T3-4, significant ($p < 0.05$) differences for height, fresh weight and b* were observed, but no significant differences were noted for dry weight, number of shoots, number of flushes, length of new flush, Fv/Fm, L* and a* (Figure S1).

Further, the correlation analysis indicated that most parameters were significantly correlated, except for the number of shoots, which only negatively correlated with height (Figure 6). CIELab (L* and b*) had the highest correlation (0.98), followed by fresh and dry weight (0.96). The chlorophyll fluorescence parameter Fv/Fm had a significant negative correlation with b* (r = −0.76) and L* (r = −0.75) and a significant positive correlation with height (r = 0.63), root score (r = 0.52 both on day 70 and 140) and fresh weight (r = 0.46). CIELab (L* and b*) had a significant negative correlation with most root and shoot growth parameters except for the number of shoots and the length of the new flush. CIELab (a*) showed only a significant positive correlation with the root score (day 70) and height. The root score (day 140) had a significant positive correlation with all shoot parameters except for the number of shoots; for example, a good correlation was found with the length of the new shoot (r = 0.81).

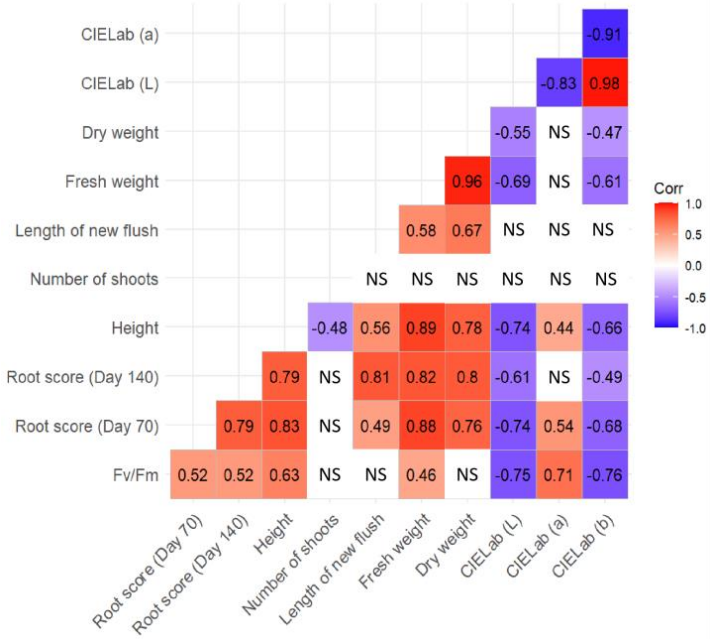

Figure 6. Correlation plot of the root and shoot growth parameters, Fv/Fm and CIELab of all tested genotypes. The values in the squares with different colors show the correlation coefficients (Pearson's r) between two parameters, and "NS" indicates no significant correlation ($p > 0.05$).

4. Discussion

In calcifuge plants, the higher pH of neutral or alkaline soils can damage plant root structure, reduce root activity and affect plant nutrient utilization, which leads to yellowing of leaves and, in severe cases, death [13]. The damage caused in plants by alkaline stress is a complex physiological and biochemical process that affects the entire growth and development of the plant from seed germination onward [14]. *Rhododendron* is a textbook example of a calcifuge plant.

Previously, we selected germinating seedlings in media with a higher pH [9,10]. As *R. fortunei* again showed tolerance for a higher pH, we included seedlings of this species (RF) in the current study [9]. Seedlings from crosses between commercial rhododendrons 'Pink Purple Dream' × 'Belami' (PB) were also screened in tissue culture and showed genotypic variation in lime tolerance [10]. The results of the aforementioned studies show that a high pH stress has no significant effect on the germination of rhododendron seeds but does inhibit the growth of seedlings. Moreover, the differences in alkalinity tolerance during the germination period are dependent upon the genotype [9]. Genotypes used in the current study were the progeny of RF and PB selected on tissue culture media with a high pH and seedlings that had not undergone selection. All seedlings were propagated in tissue culture. Additionally, plantlets of two cultivars ('Cunningham's White' (CW) and 'Gomer Waterer' (GW)) were used to test pH stress in a greenhouse pot experiment. 'Gomer Waterer' and 'Cunningham's White' were used as tissue cultured plantlets and cuttings, respectively. In total, seven of the tested genotypes (RF and PB) were preselected on an alkaline medium; four genotypes, one PB, one RF, CW and GW were not preselected in the seedling stage. The number of not-selected plants was too small to make a good comparison between tissue culture selection in the seedling stage and greenhouse performance. When the individual genotypes selected on a high pH tissue culture were compared with not-selected genotypes, in general no better performance was found in the selected genotypes, the RF progeny, or the hybrids of PB. This was in contrast with what was expected. Nevertheless, the best performing genotype, PB-T3-4, was obtained after selection in the seedling stage in a tissue culture medium with an elevated pH level.

In the acidic control substrate, a large variation in the parameters tested was found between the genotypes. These were considered as genotypical differences, while, in the neutral pH, differences reflected the response between genotype (G) and environment (E) (or a so called genotype–environment interaction (GxE)). The effect of a pH of 6.3 on plant growth could be shown visually by changes in biomass indicators. In the present experiment, fresh and dry weights of all plants were significantly reduced after 140 days in the neutral pH substrate. Other discriminative growth parameters involved were root score, plant height, number of flushes and the length of the new flush. The number of shoots was not dependent upon the substrate pH, probably because not many plants developed lateral shoots. The plants were not pruned during the experiment and no other action was taken to induce the formation of lateral shoots. The PCA plot shows very well how the different parameters, except for the number of shoots, were commonly influenced by the neutral pH. The correlation matrix shows good correlations, especially between plant height, fresh and dry weight and root scores after 70 or 140 days. The PCA shows how the genotypes clustered together per treatment. The results therefore confirm that alkaline stress inhibits the normal growth of rhododendrons, which is in agreement with previous studies on rhododendron [6,15–17] and is observed in other pH susceptible plants [18–20]. The growth and development of the roots and the inter-root environment directly affected the growth and development of the whole plant. In our experiment, it was shown that in rhododendron roots subjected to long-term (140 d) alkaline soil stress, plant height, number of shoots, number of flushes and the length of the new flush were significantly inhibited in all genotypes of *R.* 'Pink Purple Dream' × 'Belami' and *R. fortunei*. Especially, the lack of development of roots was remarkable; in the stressed plants, roots did not develop in the neutral pH substrate and they did not grow beyond the original transplanted root plug volume. Chaanin and Preil [5] noticed a complete inhibition of root formation of

rhododendron in tissue culture supplemented with $NaHCO_3$. Also, the results of Turner et al. [21] suggest that root morphology and function might be the limiting factor under alkaline conditions. They tested rhododendrons grown 49 days in a pH nutrient solution with pH 6.5 and observed smaller root systems than plants grown in an optimal pH. In nutrient solutions with pH 6.5, the roots developed as clusters of short highly branched roots, which was not the case for plants grown in nutrient solutions with a pH of 5.5. Demasi et al. [22], who also used a hydroponic system, observed no changes in root system development in higher pH after 21 days. In an experiment with potted plants and a longer experimental timeline, Demasi et al. [23] found that growth and biomass of different evergreen azalea species and cultivars decreased at a high pH in varying degrees, showing that certain genotypes are more tolerant of a high pH than others. Roots were not evaluated in that study.

Interestingly, after 70 and 140 days in the control, the root score of genotype PB-T3-4 showed a significant growth advantage above the mean baseline. This significantly better root growth was also found in the acidic substrate after 140 days, while this was not reflected after 70 days of pH stress. The root system is the main organ for nutrient uptake in plants and the first to sense changes in inter-root environmental conditions, requiring a certain adaptation period to adapt to changes in the environment [24]. In addition, the analysis of above-ground phenotype and growth indicators like plant height and the length of the new flush confirmed this superior pH tolerance in PB-T3-4.

Chlorophyll fluorescence was used as a parameter to assess plant stress. Fv/Fm reflects the maximum light energy conversion efficiency of PSII, which is usually significantly affected by biotic or abiotic stress and can be used to determine the damage to plant photosynthetic organs. This makes it an effective indicator of the photosynthetic physiology of plants under stress [25]. For the detection of abiotic stresses, chlorophyll fluorescence measurements have been applied and examples can be found for stress related to heat [26], cold [27,28], drought [29,30], salt [31] and nutrient deficiency [32] and (high) light stress [33,34]. Also, for biotic stresses, chlorophyl fluorescence has been used to examine stress caused by nematodes [34], pathogens [35] and insects [36]. Chlorophyll fluorescence imaging is also an efficient way to perform high-throughput screening of rhododendron seedlings for their tolerance to alkaline growing media [9]. Most genotypes of the species *R. fortunei* and the cross *R.* 'Pink Purple Dream' × 'Belami' and also CW showed a significant down-regulation of Fv/Fm after 140 days in a neutral pH when compared with plants in a low pH. In the genotype PB-T3-4, the values for Fv/Fm were slightly higher in a neutral pH when compared with a low pH. Also, GW had a good Fv/Fm value of around 0.8 in the neutral substrate, although this was significantly lower than the control.

The CIELab (L*) and CIELab (b*) indicators of each genotype were significantly up-regulated after alkaline stress as photosynthetic efficiency was reduced, which indicated that alkaline stress caused chlorosis of the leaves. Our results indicated that, other than Fv/Fm, CIELab (especially L* and b*) was also a good predictor for the biomass of PB and RF. However, unlike Fv/Fm, the correlations between CIELab and biomass were genotype-specific. Recorded chlorosis under lime stress increased significantly after 4 to 7 weeks in evergreen potted azalea depending on the genotype. In our greenhouse test, GW responded differently from the other genotypes as its L* value remained lower, meaning the leaves were still dark-colored. A typical symptom of pH stress is interveinal chlorosis caused by iron deficiency [37]. The ability to take up iron depends on plant enzymes like ferric chelate reductase (FCR) that reduce Fe^{3+} to Fe^{2+}. Genotypic differences in levels of ferric chelate reductase activity under iron deficiency were found by Demasi et al. [1]. Lower foliar Fe^{2+} concentrations were measured by Demasi et al. [23], but the authors concluded that the pH of the substrate hampered azalea ornamental performance more than Fe deficiency. No relationship between iron levels and chlorophyll content was observed. In a study on the content of elements in the leaves of rhododendron plants in their natural habitat, it was found that deficiencies of manganese, and to a lesser extent iron, related to the growth of rhododendron on alkaline soils [38,39]. Also, Chaanin and Preil [4] found a reduction in

iron and manganese levels in young leaves of rhododendron artificially subjected to high levels of $CaCO_3$ in the substrate.

Although 'Cunningham's White' is known to be one of the parents of the high pH-tolerant rootstock Inkarho® [40], its performance in our greenhouse test was poorer when compared with 'Gomer Waterer' and many PB and RF genotypes. Preil and Ebbinghaus [15] mention a reduced shoot and root development for 'Cunningham's White' grown on a substrate with $CaCO_3$, but chlorosis was less profound in comparison with other genotypes. They found that shoot and root development did not always respond in the same way to pH stress. The lime tolerance screens from Preil and Ebbinghaus [15] have led to the development of the Inkarho® (Interessengemeinschaft Kalktolerante Rhododendron) rootstock tolerant of soil pH 6.5–7.0, derived from a cross between *R. fortunei* and 'Cunningham's White' [5]. Other authors describe 'Cunningham's White' as a nineteenth-century cultivar with moderate lime tolerance used extensively as rootstock [6,40]. Another breeding product is Bloombux®, released in 2014 as a lime-tolerant hybrid from *R. micranthum* and *R. hirsutum* advertised as tolerant of soils up to pH 7.5 [40].

In the present study, the *Rhododendron* genotype PB-T3-4, well performing in both the control and neutral pH, has the potential to grow in soils with an elevated pH. The identification of this genotype is important for the development of new cultivars that can thrive in alkaline soils, which may be useful for landscape gardening and for the development of new ornamental plants. Further studies are needed to investigate the underlying mechanisms that enable *Rhododendron* genotypes to grow in alkaline soils and to identify other candidate genotypes and select for other ornamental characteristics to obtain a marketable cultivar with enhanced adaptability to alkaline soils.

5. Conclusions

Various evaluation methods were used to assess pH stress in rhododendron. After 140 days of growth in substrate with a pH of 6.3, root development was especially negatively affected. This observation had a good correlation with observed plant height, flush length and fresh and dry plant weight. The genotype PB-T3-4, progeny of 'Pink Purple Dream' × 'Belami', resulted from this selection process and outperformed the other genotypes tested, including seedlings of *R. fortunei* and the commercial rhododendrons 'Cunningham's White' and 'Gomer Waterer'.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9121302/s1, Figure S1: Effects of neutral ($CaCO_3$) treatment on root score (Day 70 and 140), height, fresh weight, dry weight, number of shoots, number of flushes, length of new flush, Fv/Fm and CIELab (L*, a*, b*) of different genotypes. Pairwise comparisons against the control treatment were calculated by Wilcoxon test, and indicated by **** ($p < 0.0001$), *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$) and "ns" (not significant) respectively; Figure S2: Root scores 0 to 5, with score 0: no roots are visible; score 1: a little bit of roots visible at one side or just a single root on a few sides of the root ball; score 2: roots at two sides appear; score 3: roots are visible around the pot; score 4: roots are present around the root ball; score 5: roots from top to bottom are all around the potting soil; Figure S3: Measurements of above ground growth (height) and number of flushes; Figure S4: Pictures taken at the end of the experiment 140 days after potting in acidic or neutral pH.

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