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Evaluation of the genetic diversity and root architecture under osmotic stress of common grapevine rootstocks and clones

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Running title of the work: Variability assessment in rootstocks

Abstract

Grapevine is grown as a grafted plant, mainly using phylloxera-resistant rootstocks obtained when this aphid destroyed European vineyards, and the use of a reduced number of rootstocks in each production area is common. This indicates that the genetic variability that is being used could be insufficient to tackle new stress constraints. Changes that will be produced as a consequence of climate change are promoting the development of new rootstocks and the study, in a deeper manner, of those already in use, mainly in relation to drought stress. In this work, we have studied 40 rootstock accessions, including clones of common rootstocks, others developed later, some recovered from old abandoned fields and other, resprouted rootstocks. From these accessions, 19 unique SSR profiles were obtained and chlorotypes were assigned, as no information was available for them in the *V*IVC database, thus generating new knowledge. Genetic variability was analysed in the 110 Ritcher, 140 Ruggieri and 1103 Paulsen rootstocks (derived from *Vitis berlandieri* and *Vitis rupestris*), commonly used in the countries of greater wine production (Spain, France and Italy), and in the 19 rootstocks with unique profiles. As expected, higher variability was found in the latter. Fortunately, variability was also found in the small sample of which reflects there is variability among the three more-commonly-used rootstocks despite they are half and/or full sibs. Considering all the germplasm analysed, the relationships found agree with a recent report stating that some genotypes had been erroneously assigned, previously, and show that another genotype may not be correct. Variability was also found in clones of several rootstocks, with considerable variability in some of them, including two rootstocks rescued from old abandoned vineyards. This result suggests the possibility of evaluating these materials for other traits. Finally, evaluation of osmotic-stress tolerance was carried out in *in vitro* culture, using media containing PEG. Micropropagated plants of one rootstock classified as drought-resistant, another reported as sensitive and two others whose classification in the field is variable were used. The results indicate that this methodology can

be useful in breeding programmes, to screen the variability in osmotic-stress tolerance among clones and to study root architecture and plasticity.

Keywords: AFLPs/M-AFLPs; chlorotypes; genetic variability; *in vitro* culture; PEG; SSR; *Vitis*; water deficit

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most economically-important crops cultivated worldwide. Global grape production currently amounts to close to 78 million metric tonnes per year, with a wine production of 292 million hectolitres in 2018. Today, Spain, China, France, Italy and Turkey account for more than 50 % of the area dedicated to grapes. Italy, France and Spain are the top wine-producing countries (OIV, 2019).

Since the grape root aphid phylloxera (*Daktulosphaira vitifoliae* Fitch) arrived in Europe in the mid-1800s and devastated the vineyards, grapevine has been grown as a grafted plant using phylloxera-resistant rootstocks (Pouget, 1990). Firstly, accessions of the American species *Vitis riparia* Michaux and *Vitis rupestris* Sheele, selected for their phylloxera resistance and rooting ability, were used as rootstocks. Subsequently, crosses with *Vitis berlandieri* Planchon, another American phylloxera-resistant species, were made to produce rootstocks able to grow on chalky soils (Imazio et al., 2002). Among them, 110 Richter (110 R) was obtained in France in 1902 and 140 Ruggeri (140 Ru) and 1103 Paulsen (1103 P) were obtained in Italy from *V. berlandieri* x *V. rupestris* crosses (Bavaresco et al., 2015). These three rootstocks are produced in high quantities in Spain, France and Italy, the main rootstock-producing countries (Zavaglia et al., 2016). Among the *V. berlandieri* x *V. riparia* rootstocks, one of the most popular is Selektion Oppenheim 4 (SO4). *Vitis vinifera* was also used in other breeding programmes to increase the compatibility between the rootstock and the scion (e.g. 41 B

Millardet et de Grasset (41 B) and 333 École de Montpellier (333 EM)) (de Andrés et al., 2007). Subsequently, other rootstocks have been developed to deal with new constraints like nematodes, which have become a problem since the use of methyl bromide for soil disinfection was banned. For example, the Harmony and Freedom rootstocks, which have resistance to root-knot nematodes, were derived from complex crosses which involved *V. champinii* Planchon (Weinberger and Harmon, 1966). Also, this species and others - like *V. acerifolia* Rafinesque, *V. aestivalis* Michaux, *V. labrusca* Linneo, *V. longii* Prince*, V. rotundifolia* Michaux and *V. vulpina* Linneo - have been evaluated to develop rootstocks with tolerance of lime, low pH, salinity, drought or high/low temperatures (Burger et al., 2009). *Vitis champinii* itself (e.g. Salt Creek rootstock) has also been used to confer salt tolerance (Walker et al., 2007). Nowadays, several programmes are in progress to derive or select rootstocks more adapted to climate changes (Fraga et al., 2013; Berdeja et al., 2015). It is predicted that there will be increased evapotranspiration and crop water needs, which will greatly affect vineyards. Under drought stress, the root architecture plays an important role: the overall root system size is related to the acquisition of water and nutrients, as are the density and length of hairy roots, which enlarge the surface area over which soil-root contact occurs and enhance water uptake (Comas et al., 2013).

Recently, Riaz et al. (2019) studied the pedigree of 47 rootstocks and 98 wild or cultivated species - including *V. berlandieri*, *V. riparia*, *V. rupestris*, *V. vinifera*, *V. champinii* and *V. acerifolia* - and found mistakes in the historical pedigrees assigned to some common rootstocks. In addition, their results revealed that grape rootstocks have a narrow genetic base: three accessions belonging to three *Vitis* species (*V. rupestris, V. riparia* and *V. berlandieri*) contributed 39 % of the genetic content in the evaluated rootstocks. Besides, it is common to find that a single or few rootstocks predominate in a vineyard area, which indicates that a tiny variability is being used. For instance, Renouf et al. (2010) mapped 400 ha in Bordeaux and

found that Riparia Gloire de Montpellier was, by far, the most-used rootstock, covering around 45 % of the extension. In Montpellier, the largest grape-production area in France, the main rootstocks used include: 110 R (39 %), 140 Ru (18 %), SO4 (17 %), 3309 Couderc (3309 C; 7.2 %) and 1103 P (4.8 %) (Dry, 2005). In Spain, 110 R is also the most-employed rootstock, followed by 140 Ru and 1103 P (Pongrácz 1983; Hidalgo, 1999; Gambetta et al., 2012). Data from Valencian nurseries, which produce around 70 % of the grafted plants in Spain, corroborate this information: around 1100 ha of 110 R rootstock mother plants were grown and around 25,000,000 grafted plants were produced in 2018. In second place, in terms of rootstock production, were 140 Ru and 1103 P (personal communication). Based on these data, our hypothesis is that the variability used nowadays is probably low, which can be a problem in the face of new constraints.

The aim of the present work is to analyse the genetic variability in, and deepen our knowledge of, the most-common rootstocks and others in use. Concretely, the objectives are: 1) To identify or confirm the rootstocks using SSRs, assign chlorotypes and study their relationships, 2) To assess the genetic diversity in the most-commonly-used rootstocks (110 R, 140 Ru and 1103 P) *vs.* that of the total rootstock collection analysed (40 accessions, including the most-used clones and rootstocks recovered from old vineyards), 3) To evaluate the variability among the clones of the rootstocks by the study of AFLPs and M-AFLPs and 4) To assess the usefulness of *in vitro* culture for studying the variability in root architecture and osmotic-stress tolerance in a reduced sample of rootstocks.

2. Material and methods

2.1. Identification, variability analysis and chlorotype determination

2.1.1. Plant material

A total of 40 rootstock accessions - including six unidentified rootstocks (BR, FON-1, LOSI-1, SA-1, SA-2 and XA-1), several clones for the six commonly-used rootstocks (110 R, 140 Ru, 1103 P, 41 B, SO4 and 161-49 C) and other, more-recent rootstocks (like Fercal, Gravesac, Harmony, Freedom and Salt Creek) supplied by several nurseries - were used (Table S1 [supplementary]).

2.1.2. DNA extraction

Fully-expanded leaves were used for DNA extraction with the DNeasy Plant Mini Kit (Qiagen). The DNA quality and quantity were assessed using gel electrophoresis and spectrophotometry.

2.1.3. Identification by SSRs

The nuclear SSRs VVS2 (Thomas and Scott, 1993), VVMD5, VVMD6, VVMD7, VVMD21, VMD24, VVMD25, VVVMD27, VVMD28, VVMD32 (Bowers et al., 1996; 1999), VrZAG62, VrZAG79 (Sefc et al., 1999) and VMC1b11 (Zyprian and Töpfer, 2005) were analysed. The SSRs VVS2, VVMD5, VVMD7, VVMD27, VrZAG62 and VrZAG79 SSRs were proposed by OIV (http://www.oiv.int/) to identify varieties and rootstocks. Two sets of multiplex PCR reactions were performed as described by Peiró et al. (2018). The system of reference alleles used as genetic descriptors (OIV801-OIV806) was used for microsatellites VVS2, VVMD5, VVMD7, VVMD27, VrZAG62 and VrZAG79 and for the rest of the SSRs the *V*IVC database (*V*IVC, 2019) was used.

2.1.4. Chlorotypes

The chlorotypes were determined by analysing the SSRs: cpSSR3, cpSSR5 and cpSSR10 (Arroyo-García et al., 2003), and ccSSR9 and ccSSR14 (Arroyo-García et al., 2006). In addition to the rootstock accessions, four grapevine varieties showing the chlorotypes A (Monastrell), B (Muscat of Alexandria), C (Botó de Gall) and D (Planta Mula) (Jiménez et al., 2019) were used as controls. Each amplification was performed in a total volume of 11.5 µl containing PCR buffer (Multiplex Hot Short PCR, Takara), 20-30 ng of DNA, 30 µM of each dNTP, 0.5 U of Taq DNA polymerase (Takara) and labelled multiplexed SSR primers (10

pmol). The PCR amplification was performed in an ABI9700 thermocycler using the following thermal cycles: 15 min at 95 ºC followed by 35 cycles of denaturation (30 s at 95 ºC), annealing (1 min at 57 ºC) and extension (1 min at 72 ºC), with a final step of 10 min at 72 ºC. The multiplex PCR product was previsualised using gel electrophoresis; later, electrophoresis was carried out on an ABI 3100 platform using 0.13 µl of an internal size standard (GeneScan™ 500 LIZ, Applied Biosystems), 1.00 µl of PCR product and 10.87 µl of formamide. The mixture was heated at 94 °C for 3 min and then cooled in icy water. The lengths of the alleles were sized with the software package GeneScan 3.7 (Applied Biosystems).

2.1.5. Genetic variability assessment

The allelic richness (A), the effective number of alleles (Ne) and the number of genotypes (Ge) were determined for each SSR locus using PowerMaker v.3.0. The observed heterozygosity (Ho), the expected heterozygosity (He) and the Shannon diversity index were computed for each SSR locus using GenAlEx v.6.501. The major allele frequency (MaAF), which refers to the frequency of the most-common allele occurring in the population, was also estimated. To evaluate the discriminatory power of the microsatellite loci, the Polymorphic Information Content (PIC) value for each locus was determined (Botstein et al., 1980). Genetic similarities were calculated and an Unweighted Pair Group Method with Arithmetic mean (UPGMA) phenogram was produced using PowerMaker software and plotted using TreeView v.1.6.6. Correlations between several genetic variability parameters, like the number of alleles and estimated heterozygosity, were estimated using Statgraphics Centurion XVII software.

For AFLPs and M-AFLPs analysis, the restriction, ligation and pre-amplification conditions were similar. The restriction-ligation of genomic DNA (150 ng) was performed using 5 U of each restriction enzyme (*EcoR*I and *Mse*I), 1 U of ligation enzyme (T4 ligase), 10 mM ATP (adenosine triphosphate), 50 µM of *MseI* adapter and 5 µM of *EcoRI* adapter, in 1× restriction-ligation buffer (20 mM Tris acetate, 20 mM magnesium acetate, 100 mM potassium

acetate, 5 mM dithiothreitol, 2.5 mg of bovine serum albumin). Then, the pre-amplification was performed using 5.0 µl of DNA (ten-fold diluted, digested and ligated) in 15.0 µl of reaction mixture containing 5.5 µM of *EcoRI*+N and *MseI*+N primers, 1× PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl), 40 µM dNTPs and 1.25 U of Taq DNA Polymerase (Takara Clontech). The pre-amplification conditions were an initial step of 2 min at 72 °C, 20 cycles of 30 s at 94 °C, 1 min at 56 °C and 1 min at 72 °C, a cycle of 2 min at 72 °C and a final extension cycle of 5 min at 60 °C. The AFLP analysis was performed using a labelled *EcoR*I+3 primer and an unlabelled *Mse*I+3 primer (three selective nucleotides; Table S2 (Supplementary)). Each 20 µl PCR reaction contained 0.20 µl of the pre-amplified DNA, 0.92 pmol of labelled *EcoR*I+3 primer, 5.50 pmol of unlabelled *Mse*I+3 primer, 2.00 µl of 10× PCR buffer, 40 µM dNTPs and 1.25 U of Taq DNA Polymerase (Takara Clontech). The cycling conditions for the labelled PCR were 1 cycle of 2 min of denaturation at 94 °C, 1 cycle of 20 s at 94 °C, 30 s at 66 °C and 25 s at 72 °C and a touch-down profile (10 cycles with -0.7 °C/cycle, annealing temperature) for the annealing step, followed by 20 cycles at a constant annealing temperature of 56 °C and, finally, an extension cycle of 30 min at 60 °C. Similarly, the M-AFLP analysis was performed using, in the second amplification, a labelled SSR primer in combination with an *Mse*I+3 primer. Fragment size was determined using an ABI3100 Platform. Amplified fragments derived from the AFLP and M-AFLP analyses were evaluated using Genographer (v.2.1.4) and only well-resolved fragments were used for the analysis. These fragments were scored according to the presence (1) or absence (0) of homologous bands and then they were transformed into a binary matrix. The similarity index was estimated using the Dice coefficient of similarity of Nei and Li (1979). Subsequently, cluster analyses were carried out using a UPGMA procedure with the PHYLIP software package (v.3.69). To verify the robustness of the nodes, resampling of the matrix with 1,000 samples and a replacement of 30 % of the data was performed. The dendrogram was visualised with the program TreeView. Besides, Principal

Components Analysis (PCA) was performed, removing all non-informative alleles (alleles present in all individuals). The PCA was performed on centred, non-scaled data and the hierarchical cluster analysis used Ward's minimum variance method with pairwise Manhattan distances between individuals. This analysis was carried out using Statgraphics Centurion XVII software.

2.1.6. *Root architecture and osmotic-stress tolerance evaluation*

Shoots of the rootstock accessions 41 B St, 110R 163, 161-49 C 176 and SO4 E3 were firstly introduced into *in vitro* culture after previous disinfection using the same conditions previously described for seeds by Peiró et al. (2015). From each established plant, eight clones were obtained (eight nodes, each bearing a single axillary dormant bud) and cultured in MW medium supplemented with 0.2 mg L^{-1} indole-butyric acid (IBA) (San Pedro et al., 2017). Several cycles of multiplication were carried out to obtain around 70 plants per rootstock. Clones of these four rootstocks were evaluated for osmotic-stress tolerance in modified MW medium (solidified by adding Gelrite^{TM} at 6 mg L^{-1}), which was the clearest medium. PEG-6000 (DUCHEFA, The Netherlands) was added at 0, 1, 2, 4, 8 and 16 % to this modified MW culture medium. For each rootstock, the apical shoot (1 cm) was isolated and sown in a tube with the modified MW medium, with or without PEG. Ten replicates per rootstock accession and for each set of conditions (stressing or control) were set up. The pH was adjusted to 5.8 before autoclaving for 20 min at 121 ºC and a pressure of 1 atmosphere; 0.2 mg L^{-1} of IBA was added after autoclaving, when the culture medium had cooled. The conditions in the *in vitro* chambers were: 70 % humidity, a temperature of 25 ± 1 ºC and a photoperiod of 16 h (achieved using Sylvania Agro-Lux F36W/CRO tubes).

The height (cm), number of leaves and rooting ability, measured as a visual index on a scale from 0 to 3 (Figure S1), were noted after 15 and 45 d of culture. On day 45, the plants were recovered from the tubes. The roots were carefully rinsed with water to remove the Gelrite

™ and the fresh weight (FW) of the roots and aerial parts (shoots) was noted. The dry weight (DW) of the roots and shoots of each plant was measured after incubation for 72 h at 65 ºC. Previous to the determination of the DW, scanned images of the roots were analysed using WinRHIZO software and the following parameters of the root system were estimated: total length (cm), average diameter (mm), total surface area (cm^2), total volume (cm^3) and number of tips and forks. The percentage growth inhibition was calculated with respect to control data (0 % PEG) and data from the least-stressing PEG treatment (1 %). The YSI index described by Bouslama and Schapaugh (1984) for drought tolerance, based on biomass reduction (FW or DW in stressing conditions/FW or DW in non-stressing conditions), was complementary to the growth reduction measured for both traits (YSI = 100-% reduction).

Multifactor ANOVA was used to test differences among treatments (three levels of PEG concentration: 0, 1 and 2 %) and rootstocks (four levels: 110 R 164, SO4 E3, 41 B St and 61-49 C 176) for the plant growth traits and root architecture data. Differences between combinations were estimated by the LSD (Least Significant Difference) at P = 0.05 of Fisher´s test using Statgraphics Centurion XVII software.

3. Results

3.1. Identification, chlorotypes and relationships among rootstock accessions

The SSR profiles of the 40 rootstock accessions analysed using 13 SSR markers, shown in Table S3 [supplementary], confirmed the identities of the rootstocks supplied by nurseries and identified AGU as 161-49 C, FON-1 as 196-17 C, BR as 333 EM, XA-1 as 41 B, LOSI-1 as Rupestris and SA-2 as Ritcher 31 (31 R), whereas SA-1 remained unknown. A total of 19 unique rootstocks were found in the set of rootstocks analysed.

For chlorotype assignment, five chloroplast SSRs described by Arroyo-García et al. (2003; 2006) were used: fragments of two sizes differing in 1 bp were amplified for cpSSR5 (101 and 102 bp) and ccSSR9 (166 and 167 bp), whereas three alleles were obtained for

ccSSR14 (203, 204 and 205 bp). However, for cpSSR3 the amplified fragments differed by less than 1 bp (124.4 and 124.8 bp), and by 18 bp from the values reported previously. For cpSSR10, two peaks of difficult assignment, in the range from 116 to 118 bp, were obtained. Considering the first three polymorphisms and following the classification of Arroyo-García et al. (2006), chlorotype D could be assigned to rootstocks 41 B, 333 EM, Fercal and SA-2 (identified as 31 R) since a band of 101 bp in cpSSR5 was found (Table 1). Similarly, the small fragment in ccSSR14 (204 bp) was observed in rootstocks 161-49 C, 420 A, Gravesac and 196-17 C. However, the latter rootstock differed in the ccSSR9 locus (167 *vs.* 166 bp). Therefore, chlorotype B was assigned to rootstocks 161-49 C, 420 A and Gravesac whereas rootstock 196-17 C was classified as A. Chlorotype C was assigned to the rest of the rootstocks (5 BB, 110 R, 140 Ru, 1103 P, 3309 C, Freedom, Harmony, Rupestris, SA-1, Salt Creek and SO4). As expected, no genetic variability among clones was obtained - neither for nuclear nor for chloroplast SSRs.

Dendrograms showing the relationships among the rootstocks are displayed in Figure S2 [supplementary] and Figure 1. In the former, as expected, all the clones for a unique rootstock are in the same cluster and thus possessed the same SSR alleles. Therefore, in Figure 1, only one clone per rootstock was analysed to determine the relationships among the rootstocks. The unique genotypes are clustered into two groups in Figure 1. Rootstocks including *V. champinii* in their pedigrees (Salt Creek, Freedom and Harmony) are clustered in group A. The remaining rootstocks are clustered in group B, with the rootstocks 333 EM, 420 A and SA-1 subclustered in B1 and rootstocks with *V. riparia* in their pedigree (196-17 C, 5BB, SO4, 3309 C, 161-49 C and Gravesac) in B2, while B3 comprises one subgroup (B3.1) containing *V. berlandieri* x *V. rupestris* hybrids (110 R, 140 Ru and 1103 P) and *V. rupestris*, and another subgroup (B3.2) including rootstocks 41 B, Fercal and 31 R (SA-2).

3.2. Analysis of genetic variability using SSRs: the three most-commonly-used rootstocks vs. the whole set of analysed rootstocks

In order to study the variability in the three rootstocks used most in the main wine-producing countries (110 R, 140 Ru and 1103 P - all derived from *V. berlandieri* x *V. rupestris*), the molecular diversity was analysed and compared to the variability present in a bigger population that included these rootstocks and other available rootstocks. Greater variability implies a greater possibility of overcoming constraints. In the first population (three rootstocks), the highest heterozygosity and PIC values were obtained for VVMD5, VVMD24, VVMD27, VVMD28 and VrZAG79, with four different alleles (Table 2). Despite their similar origins, only VrZAG62 had the same genotype in all three rootstocks (Table S3). In the bigger population, high polymorphism was found for all the loci, with a mean number of alleles per locus close to 12 and an average number of genotypes close to 15 (Table 3). The least-polymorphic SSR was VVMD6, with five alleles. By contrast, VVMD27, VVS2 and VMC1b11 showed the highest number of genotypes (18) and 15, 14 and 12 alleles per locus, respectively. The highest number of alleles was obtained for VVMD27. The observed heterozygosity ranged from 0.32 (VVMD32) to 0.95 (VVMD27 and VrZAG79), with a mean value close to 0.85. Overall, the expected heterozygosity was close to the observed heterozygosity. However, differences were observed for some SSRs, especially for VVMD32 (0.32 *vs.* 0.90). All the loci were highly informative since all the PIC values were higher than 0.50; VVMD27, VVMD32 and VMC1b11 were the most-informative SSRs (PIC 0.89) whereas VVMD6 was the least informative (PIC 0.67). Most of the SSRs had a Shannon index higher than 2.00 and a MaAF lower than 0.40. Besides, a positive correlation was observed between the number of alleles and the level of polymorphism (r = 0.88 between A and He, r = 0.89 between A and PIC and r = 0.94 between A and Shannon's index). Comparing the two populations (Table 3 *vs.* Table 2), the mean PIC value was reduced by approximately one-third (0.833 *vs.* 0.576) and Shannon´s

index by one-half (2.149 *vs.* 1.109) in the three most-used rootstocks. The total number of alleles in the three most-used rootstocks was 43 whereas in the whole population it was 150.

3.3. Analysis of genetic variability using AFLPs and M-AFLPs

The variability among the clones from rootstocks 110 R (163, 180, E32, E35), 140 Ru (265, E30), 1103 P (113, 166, E37), 41 B (153, E15, Vd3), Rupestris (E7, LOSI-1), 196-17 C (99, FON-1), 161-49 C (176, AGU, E25), SO4 (102, 157, E2, E3) and 333 EM (27-72, BR) was also assessed using AFLPs and M-AFLPs. A total of 10,007 reproducible amplifications were obtained: 737 fragments in the range of 50 to 300 bp were analysed, and 666 of these were polymorphic (90.4 %). Variability among clones was found for all the evaluated rootstocks. Thirty-two polymorphic fragments corresponded specifically to nine genotypes: 41 B Vd3 (2), 110 R 163 (2), 161-49 C 176 (5), 196-17 C 99 (2), 333 EM 27-72 (1), Rupestris E7 (2), SO4 102 (4), BR identified as 333 EM (6) and FON-1 identified as 196-17 C (8). Other fragments were rootstock-specific: 1103 P clones shared four fragments; 140 Ru clones shared one; 333 EM clones shared three and 161-49 C clones shared 16. The clones SO4 102 and 110 R E32 had the lowest (296) and highest (440) number of total fragments, respectively. In the clones AGU and BR, both recovered from old abandoned fields, a higher number of specific amplified bands (present in these clones but absent from the other clones of the rootstocks) were obtained (Figure S3). The dendrogram based on the AFLPs/M-AFLPs analysis (Figure 2) clustered the 161-49 C clones (cluster A) separately from the rest of the rootstocks (cluster B), for which three subclusters were observed: in B1, 196-17 C clones; in B2, Rupestris and the hybrids of *V. berlandieri* x *V. rupestris* (110 R clones, 140 Ru and 1103 P) in one subgroup and SO4 clones, SA-1, Fercal and SA-2 in another; and in B3, the rootstocks with *V. vinifera* in their pedigree (41 B and 333 EM clones). The dendrogram results are in accordance with those of the PCA, in which the first two principal components accounted for around 30 and 15 % of the total

variation, respectively (data not shown). The M-AFLPs markers were more polymorphic than the AFLPs (93.1 % *vs.* 88.5 %).

3.4. Evaluation of variability in root architecture and osmotic-stress tolerance

The data for root and shoot development on MW medium in the presence or absence of PEG, a compound of high molecular weight which cannot pass through the cell wall and is commonly used to impose osmotic stress, are shown in Table 4 (for 1 and 2 % PEG). At 4 % PEG, several plants initiated rooting although good development was not achieved in any rootstock (Figure 3). When the medium contained 8 or 16 % PEG, the plants suffered complete damage, finally undergoing necrosis (data not shown).

Under control conditions, the highest vigour was shown by the rootstock 110 R 163, the plants of which had taller shoots, larger root systems and higher FWs and DWs. The rootstock SO4 E3 also had a greater root biomass than 161-49 C 176 and 41 B St, and a lower number of leaves were noted in 161-49 C (Table 4). In the presence of PEG, inhibition of growth and rooting was produced in all rootstocks (Tables 4). In addition, collapsed shoots (unable to grow or root) were occasionally found at the lower PEG concentrations. At 1 % PEG, the 110 R plants showed the highest root index (2.5), in agreement with the fact that they also exhibited the greatest root length (8.6 cm) and root FW and DW (125 mg and 5.1 mg, respectively). In this medium, the rootstock SO4 also had taller shoots (6.1 cm), higher shoot FW and DW (210 mg and 25 mg, respectively) and higher root DW (4.6 mg) than both 161-49 C and 41 B (which showed similar values for all analysed traits, with the exception of shoot length that was greater in 41 B). At 2 % PEG, greater inhibition of growth was noted although, in general, it was lower in 110 R that also showed greater growth (number of leaves, root index and length, shoot length, shoot and root FW and DW) than the rest of the rootstocks. The SO4 rootstock had a greater shoot length than 161-49 C and 41 B, while rootstocks SO4 and 41 B had higher values of root

length than 161-49 C (4.2 *vs.* 3.2 cm). The lowest inhibition at 2 % PEG, relative to 1 % PEG, for all the studied parameters was produced in 110 R (Table 4).

The values of the root architecture parameters obtained from the WinRHIZO software also differed among rootstocks and were lower in plants growing under stressing conditions, with the exception of the average root diameter of 110 R plants, which was similar in the three culture media (Table 5). Under stressing and non-stressing conditions, the root length, area and volume and the number of tips and forks were greatest in the 110 R rootstock. Therefore, the growth and rooting ability were superior for this rootstock, which clearly differed in its root system architecture (RSA; Figure 4). Comparison of the data at 1 % PEG with those of 0 % PEG also indicates some tolerance in SO4, with a greater total root length relative to 161-49 C and 41 B (30.6 *vs.* 15.1 and 16.1 cm, respectively). The growth of the plants of this rootstock was also less inhibited than that of the others at 1 % PEG regarding total root length, projected area and number of tips and forks. With respect to root volume, the smallest reduction was noted in 110 R. Besides, the reductions for all root parameters at 2 % PEG, *vs.* 1 % PEG, were lower in this rootstock.

4. Discussion

Nuclear SSRs - the markers employed most frequently to identify germplasm in different species, including grapevine and rootstocks (Imazio et al. 2002; Jahnke et al., 2011) - confirmed the identity of all the rootstocks provided by nurseries and identified five of the six unknown rootstocks. One of the unknown rootstocks turned out to be *V. rupestris*, which was the rootstock used most commonly in the first plantations set up after the phylloxera attack and which was later replaced in the area of the survey by others like 41 B or 161-49 C, more adequate for chalky soils (Hidalgo, 1999) and also found among the recovered rootstocks. Also, it is of interest to determine the chlorotypes of these rootstocks because there is only such information for some accessions of *V. rupestris* and *V. riparia* in the *V*IVC database. Of the

five chloroplast SSRs described by Arroyo-García et al. (2003; 2006), the three with better performance (ccpSSR5, ccSSR9 and ccSSR14) were sufficient to assign chlorotypes. Considering the sizes of the amplified fragments separated by capillary electrophoresis and those in the grapevine cultivars used as controls, chlorotype A was assigned only to rootstock 196-17 C. This result fits its pedigree (Couderc 1203 x *V. riparia*), as Couderc 1203 resulted from the cross of *V. vinifera* Monastrell, which had chlorotype A, and *V. rupestris* Ganzin. Chlorotype C was noted for Freedom, Harmony, SA-1, Salt Creek (Ramsey) and 3309 C, all the rootstocks in which *V. berlandieri* Rességuier nº 2 or *V. berlandieri* Boutin B was involved (110 R, 140 Ru, 1103 P and SO4) and 5 BB (*V. berlandieri* x *V. riparia*). This chlorotype was also assigned to Rupestris. However, the last does not concord with the chlorotype obtained by Arroyo-García et al. (2003). Chlorotype B was found for 161-49 C and, as expected, for Gravesac because the former is the mother of the latter. Although 161-49 C is thought to be derived from *V. berlandieri* x *V. riparia* crosses, an unknown mother has been proposed recently (Riaz et al., 2019). Chlorotype B was also found for 420 A, whose pedigree is also described as *V. berlandieri* x *V. riparia*. Considering this pedigree and previous results, chlorotype C could be expected. Another possibility was the use of an accession of *V. riparia* with chlorotype B as mother in the cross to produce 420 A; some *V. riparia* accessions have female and hermaphrodite flowers and chlorotype B was reported in some *V. riparia* accessions like GrandGlabre (*V*IVC, 2019). Among the assessed rootstocks, chlorotype D was also found in 333 EM and 41 B, which agrees with the chlorotypes of their mothers (*V. vinifera* cv. Cabernet Sauvignon and cv. Chasselas, respectively). This chlorotype was also found in other rootstocks like 31 R and Fercal. The pedigree of Fercal is Blanchard 1 B (*V. berlandieri* Lafont 9 x Trebbiano Toscano) x Ritcher 31. As Trebbiano Toscano has chlorotype D, it was probably the female parent of Blanchard 1 B. Ritcher 31 can be ruled out as the mother in the pedigree

of Fercal as it has male flowers. The pedigree of 31 Ritcher (*V. berlandieri* Rességuier 2 x Novo Mexicana) is probably wrong.

Figures 1 and 2 show the clusters produced when comparing the genetic distances obtained after SSR and AFLP/M-AFLPs analysis, respectively. Accessions assessed in both analyses are generally grouped in the same way although there are some exceptions. In both analyses, as expected, 110 R, 140 Ru and 1103 P clustered together and grouped with Rupestris. Accessions 110 R and 140 Ru were closer to 1103 P, in accordance with Riaz et al. (2019), who proposed that *V. berlandieri* cv. Boutin is the mother of both 110 R and 140 Ru, whereas Rességuier 2 was confirmed as mother for 1103 P. By contrast, not all the *V. berlandieri x V. riparia* rootstocks (5 BB, 420 A and SO4) clustered together (Figure 1). In concordance with the chlorotypes data, rootstock 420 A clustered in another subgroup. Probably, the historical pedigree for this rootstock needs to be confirmed. More distant from these three rootstocks was 161-49 C (Table S1, Figure 1), also described historically as resulting from a *V. berlandieri* x *V. riparia* cross but suggested by Riaz et al. (2019) as the result of an unknown x *V. riparia* Gloire de Montpellier cross. As expected, the rootstock Gravesac clustered with its parents (161-49 C and 3309 C), with higher similarity to the former - that was used as the mother in the original cross (*V*IVC, 2019). Another group in the dendrogram of Figure 1 is that which clusters Freedom and Harmony, which share alleles from Fresno and Dog Ridge no. 5. Besides, the rootstock Salt Creek (Ramsey), an accession of *V. champinii*, clustered with them. This result agrees with the origin of Dog Ridge no. 5, derived from *V. champinii* (Weinberger and Harmon, 1966). The rootstocks 41 B and 333 EM, obtained by crossing *V. vinifera* and *V. berlandieri*, did not cluster together in Figure 1, but when AFLPs were used both were grouped in the subcluster B3. Great differences in SSR alleles exist between the cultivars used to yield these rootstocks: Chasselas and Cabernet Sauvignon (*V*IVC, 2019). The rootstocks 31 R and Fercal were also found in the same group in both analyses, which agrees with the fact that 31 R was

involved in the development of Fercal (they shared at least one allele in 11 of the 13 SSRs analysed; Table S3). In the AFLP analysis, both rootstocks also grouped with the unknown SA-1; these three genotypes showed the same profile for chloroplastic SSRs.

The pedigrees of 110 R and 140 Ru (*V. riparia* Boutin B x *Rupestris* du Lot) and that of 1103 P (*V. berlandieri* Rességuier 2 x *V. rupestris* du Lot) indicate that the 110 R rootstock is a half-sib of 1103 P and that 140 Ru is a full-sib (Riaz et al., 2019). Considering this, the variability found in our analysis of these rootstocks is higher than expected; high heterozygosity was observed in all SSRs (PIC > 0.5) (Table 2). This is a good result, from a genetic point of view, considering the high percentage of plants which are grafted onto these three rootstocks (Zavaglia et al., 2016). As expected, the genetic variability was clearly lower in this population with respect to that observed in the sample of unique rootstocks (Table 3): the number of alleles and the PIC value were around one-third lower and Shannon´s index was decreased by one-half. The heterozygosity found in our population of 19 rootstocks with unique profiles is similar to that reported by Emmanuelli et al. (2013) for 127 unique rootstocks, which indicates high variability in the former. This variability is convenient because the higher the variability the greater the possibilities of withstanding constraints and reducing the chances of pathogens overcoming resistance.

Variability among clones of the same rootstock was not found using SSRs, as occurred in previous works (Silvestroni et al., 1997; Imazio et al., 2002; Peiró et al., 2018), but was found when AFLP/M-AFLPs were used, as in grapevine (Sensi et al., 1996; Upadhhyay et al. 2007; Peiró et al., 2018). To the best of our knowledge, these markers have not been used previously to analyse clone variability in grapevine rootstocks. In our work, about 90 % of the amplified fragments were polymorphic and reflected a higher variability in the SO4 and 161-49 C clones as well as in rootstocks rescued from old vineyards, in comparison to clones of 110 R, 140 Ru or 1103 P (Figure 2; Figure S3). Rootstock-specific or even clone-specific bands were also

amplified and could be of interest to initiate future studies. Among these, high variability was found in two rootstocks from abandoned vineyards: 114 amplifications were found in the accession 161-49 C AGU, and not in the other two clones of this rootstock (176 and E25), another 174 fragments of the same size were shared by all three, 40 fragments were common between AGU and 176 and five were common between AGU and E7. Similarly, more specific sequences were found in the accession BR than in 27-72 (Figure S3). This is of interest as a source of variability to be used in selection programmes. Due to the rootstocks' heterozygosity, as occurred previously with grapevine varieties, clonal selection programmes are of great interest. In this assay, M-AFLPs markers were more polymorphic than AFLPs, similar to previous results obtained for *V. vinifera* clones (Meneguetti et al., 2012; Peiró et al., 2018).

In the context of climate change, more prolonged drought periods are expected. Among the strategies that could be employed to face up to this constraint, the selection of more-resistant rootstocks is proposed in grapevine (Berdeja et al., 2015; Bianchi, 2018). Among the commonly-used rootstocks, the *V. berlandieri* x *V. rupestris* hybrids (like 1103 P, 140 Ru or 110 R) are among the most resistant (Serra et al., 2014; Ollat et al., 2016). However, great variability in the response to drought stress was reported for rootstocks with *V. riparia* in their pedigree. For instance, tolerance of hydric stress in SO4 is considered high (Carbonnaeau, 1985; Cirami et al., 1994), medium (Whiting, 2005) or low/very low (Galet, 1998; Dry, 2007). Also, the rootstock 41 B (*V. vinifera* cv. Chasselas x unknown) has been classified from drought resistant to sensitive (Peccoux, 2011). This variability may be the result of differences in soil and growth conditions and also the variability present in the clones of the rootstocks. In our assay, higher genetic variability was found among the clones of SO4 or 41 B than among the clones of 110 R. Therefore, the variability in drought tolerance in clones is of interest. In this work, we assessed the adequacy of *in vitro* culture for the evaluation of osmotic-stress tolerance, since in this system the conditions are more controlled than in soil screenings. For this purpose,

a high number of clones were obtained for four rootstocks which differ in their drought tolerance: 110 R, considered highly resistant to hydric stress (Keller, 2012; Peccoux, 2011), 161-19 C, with medium-low water-stress tolerance (Peccoux, 2011; Serra et al., 2014), and 41 B and SO4, with variability in their classification as stated before.

Generally, with *in vitro* systems, water deprivation is achieved by adding non-metabolised compounds like polyethylene glycol (PEG) or sorbitol to the culture medium (Carvalho et al., 2019; Mozafari et al., 2019). However, to discriminate among tolerant or sensitive genotypes, the concentrations of the stressing agent need to be appropriate. In our assay, the stressing agent PEG was added to MW medium. This polymer does not penetrate plant cells and its addition decreases the osmotic potential of the medium and, therefore, water availability. Generally, under osmotic stress different metabolic processes are disturbed, provoking: lower cell enlargement, enhanced leaf senescence, lower photosynthesis rates, a reduction in the rate of respiration, a reduction in the number of leaves and/or changes in leaf shape and size, etc. (Manivannan et al., 2007; Hussain et al., 2019). The evaluation of osmotic-stress tolerance in *in vitro* culture media containing PEG has been proposed as an alternative to field evaluations (Gopal and Iwama, 2007; Marssaro et al., 2017; Tang et al., 2019). However, PEG has not been used in the evaluation of grapevine rootstocks. Some limitations in the use of PEG must also be considered: hypoxia can be produced in PEG solutions because the high viscosity compromises the diffusion of oxygen to the roots (Verslues and Bray, 2006), and the accumulation of PEG in plant roots (or in the cut area) might result in root dysfunction, impacting leaf dehydration in an unpredictable way (Blum et al., 2017). The latter may have occurred in some shoots that collapsed in our assay and which were discarded. In our assessment, PEG concentrations of 4 % or higher resulted in great/total damage to plants and cannot be used for comparisons. However, differences among rootstocks were found in the media with 1 or 2 % PEG. Gopal and Iwama (2007) found that the best concentration of PEG,

in solid media, for the differentiation of potato genotypes according to their osmotic-stress tolerance was 2.4 %.

As expected, the addition of PEG to the MW medium inhibited both shoot and root growth as well as decreasing the number of leaves, as occurs in field conditions under hydric stress. In addition, 110 R was the most-tolerant rootstock whereas no tolerance was found in 161-49 and 41 B. This agrees with previous results observed in field conditions (Peccoux, 2011; Serra et al., 2014; Lovisolo et al., 2016; Romero et al., 2018). Similarly, Zhang et al. (2011) evaluated the behaviour of two artichoke cultivars, differing in drought tolerance in field, in PEG-containing medium. The results of the *in vitro* assays agreed with their drought-tolerance classification. In an assay involving the use of sorbitol in the culture media that we performed to evaluate plant storage conditions for the rootstocks 110 R 163 and 41 B St, promotion of growth was observed with 0.4 % sorbitol whereas a reduction occurred at 1.2 % (Figure S4). In concordance with these results, growth inhibition due to PEG was lower in 110 R than in 41 B. These results indicate that culture media can be used to test water-deficit tolerance in grapevine rootstocks and clones because the results obtained agree with those commonly found in the field. Besides the availability of plants is not a problem because MW medium is adequate for their micropropagation.

Also, RSA and plasticity are associated with the drought tolerance of grapevine rootstocks (Serra et al., 2014; Tsegay et al., 2014). The overall root system (number of roots, length and area) is related to the acquisition of water and nutrients from the soil. Deep rooting is a critical factor influencing the ability to take up water from the deeper layers (Franco et al., 2011). A greater percentage of fine roots, able to penetrate the smaller soil pores, presumably optimises the exploratory capabilities. Scanned roots from plants cultured *in vitro* greatly differed in their RSA: 110 R had the best RSA, followed by SO4, and the least-developed roots were those of 41 B and 161-49 C, which agrees with their relative osmotic-stress tolerances

(Tables 4 and 5, Figure 3). Whereas the roots of 110 R plants in control conditions (0 % PEG) had the highest values for all root traits, under stressing conditions lower values were found, being similar for both degrees of stress. Only the average root diameter was similar in plants from the three culture media, whereas lower values for this trait were observed in the rest of the rootstocks. In a recent report, functional transcripts involved in wax and suberin formation (caffeic acid 3-methyltransferase, ceriferum 3, 3 ketoacyl-CoAsynthase) were significantly upregulated in 110 R under drought stress, whereas no induction of these genes was found in 41 B or 5 BB (Yildirim et al., 2018). In the case of SO4 plants, the root characteristics were similar in the control medium and in the presence of 1 % PEG - with the exception of average root diameter, which was lower. However, great inhibition was found at 2 % PEG. Therefore, root plasticity can also be studied using *in vitro* culture, with the advantages that a higher number of plants can be used for comparison and the extraction of roots is easy. Also, it may facilitate the selection of the most-appropriate time for transcriptomic analysis.

5. Conclusion

These results increase our knowledge about 40 rootstock accessions, specifically for chlorotype SSRs as well as for genetic variability and relationships. Some mistakes recently reported for rootstock pedigrees were confirmed and others were suggested. The variability present in the three main rootstocks produced in Europe has been found to be greater than expected, which is important since they are commonly used. As higher genetic variability is convenient to overcome constraints, the number of rootstocks in use will have to be increased. In the sample of 19 rootstocks with unique SSR profiles, great variability was found. Interestingly, variability was also found among the clones of common rootstocks and in rootstocks recovered from old, abandoned vineyards. These rootstocks represent another source of genetic variation. In addition, the *in vitro* culture system has resulted useful for comparing

osmotic-stress tolerance and root architecture. The results obtained agree with those commonly found in the field and open the way to use this methodology for studying the behaviour of clones under water deficit. This work will guide further exploration of rootstock diversity and facilitate its practical use; for instance, to increase genetic diversity in plantations and/or to develop new rootstocks. Besides, *in vitro* culture facilitates the determination of the optimal development stage for the performance of molecular studies with diverse purposes.

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Table 1. Sizes of the amplification fragments obtained with cpSSR5, ccSSR9 and ccSSR14, and chlorotype assignation according to Arroyo et al. (2003, 2006).

Rootstock[1]	cpSSR5	ccSSR9	ccSSR14	Chlorotype
1103 P	102	166	205	C
110 R	102	166	205	C
140 Ru	102	166	205	C
161-49 C	102	166	204	B
196-17	102	167	203	A
3309 C	102	166	205	C
333 EM	101	166	204	D
41 B	101	166	204	D
420 A	102	166	204	B
5 BB	102	166	205	C
Fercal	101	166	204	D
Freedom	102	166	205	C
Gravesac	102	166	204	B
Harmony	102	166	205	C
Rupestris du Lot	102	166	205	C
SA-1	102	166	205	C
SA-2 (Ritcher 31)	101	166	204	D
Salt Creek (Ramsey)	102	166	205	C
SO4	102	166	205	C

[1] No clonal variability was observed among the evaluated clones.

Table 2. Molecular diversity of the three rootstocks produced most in the Comunitat Valenciana nurseries (110 R, 140 Ru, 1103 P), determined using 13 SSRs.

SSR	A	Ne	Ge	H_0	H_e	PIC	SI
VVMD27	4	3.60	2	1.00	0.72	0.67	1.33
VVMD5	4	3.60	3	0.67	0.72	0.67	1.33
VVS2	3	2.57	2	1.00	0.61	0.54	1.01
VrZAG62	2	2.00	1	1.00	0.50	0.38	0.69
VVMD7	3	2.57	2	1.00	0.61	0.54	1.01
VrZAG79	4	3.60	2	1.00	0.72	0.67	1.33
VVMD24	4	3.00	3	1.00	0.67	0.62	1.24
VVMD32	3	3.00	3	0.00	0.67	0.59	1.10
VVMD25	3	2.57	2	1.00	0.61	0.54	1.01
VMC1b11	3	2.57	2	1.00	0.61	0.54	1.01
VVMD28	4	3.60	3	1.00	0.72	0.67	1.33
VVMD6	3	2.57	2	1.00	0.61	0.54	1.01
VVMD21	3	2.57	2	1.00	0.61	0.54	1.01
Mean	3.3	2.910	2.2	0.897	0.645	0.576	1.109

A: Allelic richness; Ne: Effective number of alleles; Ge: Number of genotypes; H_0: Observed heterozygosity; H_e: Expected heterozygosity; PIC: Polymorphic information content; SI: Shannon's diversity index.

Table 3. Molecular diversity of 19 rootstocks (110 R, 140 Ru, 1103 P, 161-49 C, 41 B, SO4, 196-17 C, 5BB, 333 EM, 420A, 3309 C, Rupestris du Lot, Fercal, Freedom, Gravesac, Salt Creek, Harmony, Ritcher 31 and SA-1), determined using 13 SSR markers.

SSR	A	Ne	Ge	H_0	H_e	PIC	SI	MaAF
VVMD27	15	9.63	18	0.95	0.90	0.89	2.47	0.18
VVMD5	13	7.37	17	0.84	0.86	0.85	2.23	0.24
VVS2	14	7.85	18	0.89	0.87	0.86	2.34	0.26
VrZAG62	12	9.38	15	0.84	0.89	0.88	2.35	0.18
VVMD7	13	9.38	15	0.89	0.89	0.88	2.38	0.18
VrZAG79	11	5.87	14	0.95	0.83	0.81	2.03	0.29
VVMD24	8	4.17	12	0.79	0.76	0.73	1.67	0.37
VVMD32	14	9.89	15	0.32	0.90	0.89	2.44	0.16
VVMD25	11	5.87	15	0.89	0.83	0.81	2.04	0.32
VMC1b11	12	10.31	18	0.84	0.90	0.89	2.39	0.13
VVMD28	11	8.11	16	0.89	0.88	0.87	2.24	0.24
VVMD6	5	3.54	7	0.89	0.72	0.67	1.36	0.37
VVMD21	11	5.51	14	0.74	0.82	0.80	1.99	0.29
Mean	11.5	7.452	14.8	0.826	0.850	0.833	2.149	0.247

A: Allelic richness; Ne: Effective number of alleles; Ge: Number of genotypes; H_0: Observed heterozygosity; H_e: Expected heterozygosity; PIC: Polymorphic information content; SI: Shannon's diversity index; MaAF: Major allele frequency.

Table 4. Number of leaves (and percentage increase or decrease in brackets), root index, shoot and root length, shoot and root FW and shoot and root DW, after 45 days of culture in modified MW medium with different PEG concentrations (0, 1 and 2 %), for the rootstocks 110 R 163, 161-49 C 176, 41 B St and SO4 E3.

PEG	0 %	1 %	2 %
Number of leaves			
110R 163	6.2 ^{B,b} (100 %)	5.7 ^{A,b} (-8 %)	5.7 ^{A,b} (-8 % / 0 %)
161-49 C 176	5.6 ^{C,a} (100 %)	5.0 ^{B,a} (-11 %)	4.3 ^{A,a} (-23 % / -14 %)
41 B St	6.3 ^{C,b} (100 %)	5.3 ^{B,ab} (-16%)	4.2 ^{A,a} (-32 % / -21 %)
SO4 E3	5.8 ^{C,ab} (100 %)	5.7 ^{B,b} (-1 %)	4.3 ^{A,a} (-26 % / -25 %)
Root index			
110R 163	2.7 ^{C,b} (100 %)	2.5 ^{B,b} (-8 %)	2.1 ^{A,b} (-23 % / -14 %)
161-49 C 176	2.2 ^{B,a} (100 %)	2.1 ^{B,a} (-8 %)	1.6 ^{A,a} (-23 % / -14 %)
41 B St	2.4 ^{B,ab} (100 %)	1.9 ^{AB,a} (-8 %)	1.7 ^{A,a} (-23 % / -14 %)
SO4 E3	2.4 ^{B,ab} (100 %)	2.1 ^{AB,a} (-8 %)	1.7 ^{A,a} (-23 % / -14 %)
Shoot length (cm)			
110R 163	11.2 ^{C,b} (100 %)	8.6 ^{B,d} (-23 %)	7.5 ^{A,c} (-33 % / -13 %)
161-49 C 176	8.5 ^{C,a} (100 %)	4.5 ^{B,a} (-48 %)	2.1 ^{A,a} (-75 % / -53 %)
41 B St	8.2 ^{C,a} (100 %)	5.1 ^{B,b} (-38 %)	2.5 ^{A,a} (-69 % / -51 %)
SO4 E3	7.9 ^{C,a} (100 %)	6.1 ^{B,c} (-23 %)	3.2 ^{A,b} (-59 % / -48 %)
Shoot FW (mg)			
110R 163	392 ^{B,b} (100 %)	232 ^{A,b} (-41 %)	197 ^{A,b} (-50 % / -15 %)
161-49 C 176	252 ^{C,a} (100 %)	136 ^{B,a} (-46 %)	51 ^{A,a} (-80 % / -63 %)
41 B St	248 ^{C,a} (100 %)	128 ^{B,a} (-48 %)	59 ^{A,a} (-76 % / -54 %)
SO4 E3	344 ^{C,ab} (100 %)	210 ^{B,b} (-39 %)	55 ^{A,a} (-84 % / -74 %)
Shoot DW (mg)			
110R 163	47.4 ^{B,b} (100 %)	26.1 ^{A,b} (-45 %)	20.0 ^{A,b} (-58 % / -23 %)
161-49 C 176	26.2 ^{B,a}(100 %)	12.1 ^{AB,a} (-54 %)	6.7 ^{A,a} (-74 % / -45 %)
41 B St	28.2 ^{B,a} (100 %)	15.6 ^{AB,a} (-45 %)	9.4 ^{A,a} (-67 % / -40 %)
SO4 E3	34.1 ^{B,ab} (100 %)	25.0 ^{AB,b} (-27 %)	10.7 ^{A,a} (-69 % / -57 %)
Root length (cm)			
110R 163	13.1 ^{B,b} (100 %)	8.1 ^{B,b} (-38 %)	7.6 ^{A,c} (-42 % / -6 %)
161-49 C 176	7.4 ^{C,a} (100 %)	4.5 ^{B,a} (-39 %)	3.2 ^{A,a} (-57 % / -29 %)
41 B St	7.0 ^{C,a} (100 %)	5.2 ^{B,a} (-25 %)	4.2 ^{A,b} (-40 % / -19 %)
SO4 E3	7.9 ^{C,a} (100 %)	5.8 ^{B,a} (-25 %)	4.2 ^{A,b} (-47 % / -28 %)
Root FW (mg)			
110R 163	316 ^{B,c} (100 %)	125 ^{A,b} (-60 %)	87 ^{A,b} (-72 % / -30 %)
161-49 C 176	80 ^{B,a} (100 %)	30 ^{A,a} (-63 %)	17 ^{A,a} (-79 % / -43 %)
41 B St	88 ^{B,a} (100 %)	37 ^{A,a} (-58 %)	17 ^{A,a} (-81 % / -54 %)
SO4 E3	116 ^{B,b} (100 %)	57 ^{A,a} (-51 %)	25 ^{A,a} (-78 % / -56 %)
Root DW (mg)			
110R 163	24.1 ^{B,c} (100 %)	5.1 ^{A,b} (-79 %)	3.9 ^{A,b} (-84 % / -24 %)
161-49 C 176	2.7 ^{B,a} (100 %)	2.0 ^{A,a} (-25 %)	0.9 ^{A,a} (-67 % / -55 %)
41 B St	3.8 ^{B,a} (100 %)	1.9 ^{A,a} (-51 %)	1.6 ^{A,a} (-58 % / -16 %)
SO4 E3	7.4 ^{B,b} (100 %)	4.6 ^{AB,b} (-38 %)	2.8 ^{A,ab} (-62 % / -39 %)

Within columns, means followed by the same lower-case letter are not significantly different according to the LSD (0.05).

Among columns, means followed by the same upper-case letter are not significantly different according to the LSD (0.05).

Table 5. Total root length (and percentage increase or decrease in brackets), root proje area, average root diameter, root volume and number of tips and forks, after 45 d of cultu modified MW medium with different PEG concentrations (0, 1 and 2 %). The values ar the rootstocks 110 R 163, 161-49 C 176, 41 B St and SO4 E3, and were measured u WinRHIZO software.

Total length (cm)	0 %	1 %	2 %
110R 163	76.6 ^{B,b} (100 %)	34.8 ^{A,b} (-55 %)	34.1 ^{A,b} (-56 % / -2 %)
161-49 C 176	24.0 ^{B,a} (100 %)	15.1 ^{AB,a} (-37 %)	7.6 ^{A,a} (-68 % / -49 %)
41 B St	27.9 ^{B,a} (100 %)	16.4 ^{AB,a} (-41 %)	9.7 ^{A,a} (-65 % / -41 %)
SO4 E3	33.9 ^{B,a} (100 %)	30.6 ^{B,b} (-10 %)	8.5 ^{A,ab} (-75 % / -72 %)
Projected area (cm^2)			
110R 163	5.6 ^{B,b} (100 %)	3.1 ^{A,b} (-45 %)	2.4 ^{A,b} (-58 % / -23 %)
161-49 C 176	1.7 ^{B,a} (100 %)	0.9 ^{AB,a} (-50 %)	0.4 ^{A,a} (-77 % / -56 %)
41 B St	1.9 ^{B,a} (100 %)	0.9 ^{AB,a} (-52 %)	0.5 ^{A,a} (-75 % / -44 %)
SO4 E3	2.4 ^{B,a} (100 %)	1.6 ^{B,a} (-33 %)	0.5 ^{A,a} (-79 % / -69 %)
Average diameter (mm)			
110R 163	0.74 ^{A,a} (100 %)	0.73 ^{A,b} (-2 %)	0.72 ^{A,b} (-4 % / -1 %)
161-49 C 176	0.72 ^{B,a} (100 %)	0.55 ^{A,a} (-24 %)	0.51 ^{A,a} (-30 % / -7 %)
41 B St	0.69 ^{B,a} (100 %)	0.54 ^{A,a} (-21 %)	0.50 ^{A,a} (-23 % / -7 %)
SO4 E3	0.68 ^{B,a} (100 %)	0.57 ^{A,a} (-17 %)	0.57 ^{A,a} (-15 % / 0 %)
Volume (cm^3)			
110R 163	0.33 ^{C,b} (100 %)	0.21 ^{B,b} (-38 %)	0.14 ^{A,b} (-59 % / -33 %)
161-49 C 176	0.10 ^{B,a} (100 %)	0.04 ^{AB,a} (-62 %)	0.02 ^{A,a} (-84 % / -50 %)
41 B St	0.11 ^{B,a} (100 %)	0.04 ^{AB,a} (-62 %)	0.02 ^{A,a} (-82 % / -50 %)
SO4 E3	0.14 ^{B,a} (100 %)	0.07 ^{AB,a} (-50 %)	0.03 ^{A,a} (-82 % / -57 %)
Tips			
110R 163	88 ^{C,b} (100 %)	61 ^{B,b} (-31 %)	40 ^{A,b} (-55 % / -34 %)
161-49 C 176	26 ^{B,a} (100 %)	17 ^{AB,a} (-34 %)	8 ^{A,a} (-70 % / -53 %)
41 B St	28 ^{A,a} (100 %)	17 ^{A,a} (-49 %)	13 ^{A,a} (-54 % / -24 %)
SO4 E3	32 ^{B,a} (100 %)	31 ^{B,a} (-3 %)	8 ^{A,a} (-74 % / -74 %)
Forks			
110R 163	90 ^{B,b} (100 %)	46 ^{C,b} (-49 %)	38 ^{A,b} (-57 % / -17 %)
161-49 C 176	22 ^{B,a} (100 %)	10 ^{A,a} (-55 %)	4 ^{A,a} (-80 % / -60 %)
41 B St	23 ^{B,a} (100 %)	13 ^{A,a} (-41 %)	6 ^{A,a} (-75 % / -54 %)
SO4 E3	30 ^{B,a} (100 %)	21 ^{B,a} (-29 %)	4 ^{A,a} (-87 % / -81 %)

Within columns, means followed by the same lower-case letter are not significantly different according to the LSD (0.05).

Among columns, means followed by the same upper-case letter are not significantly different according to the LSD (0.05).

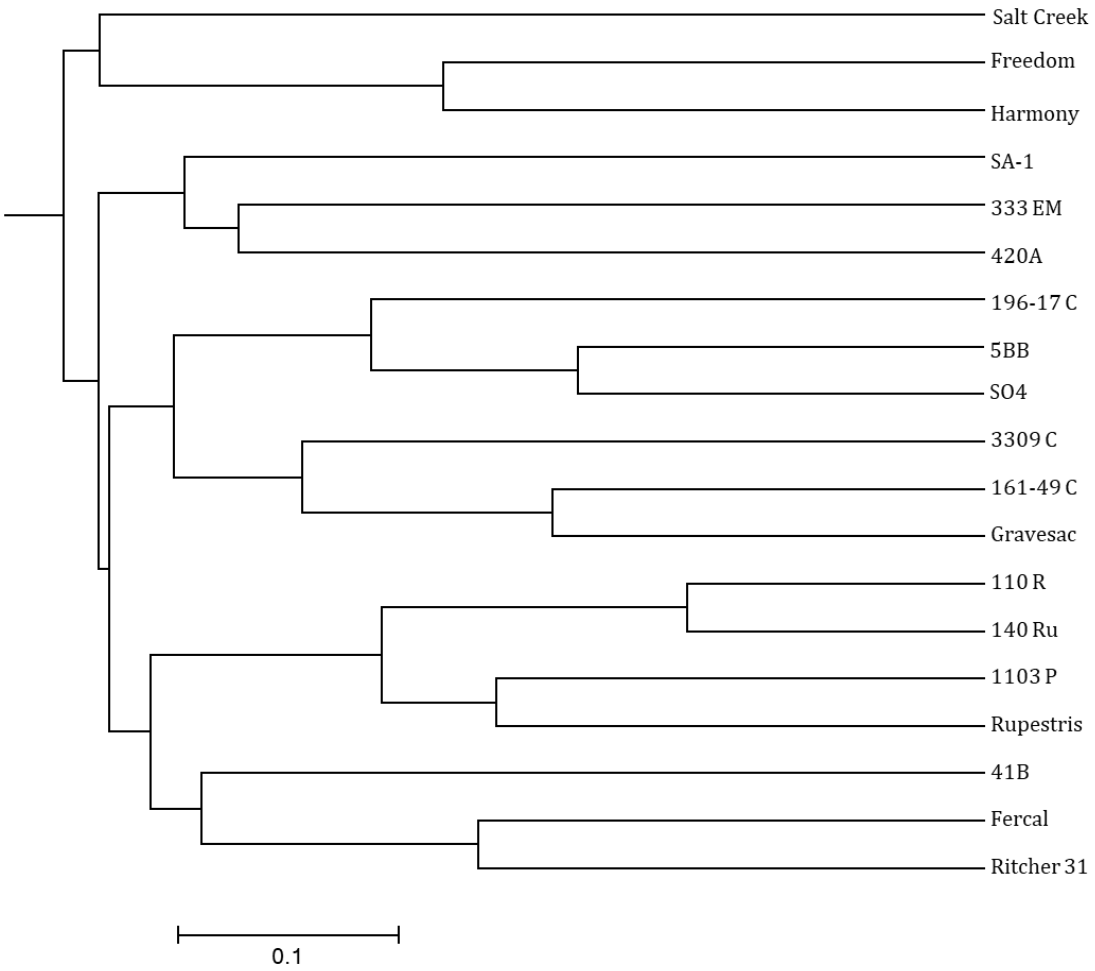

Figure 1. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering resulting from the analysis of 19 unique rootstocks using 13 SSR markers. Bootstrap values were higher than 0.85.

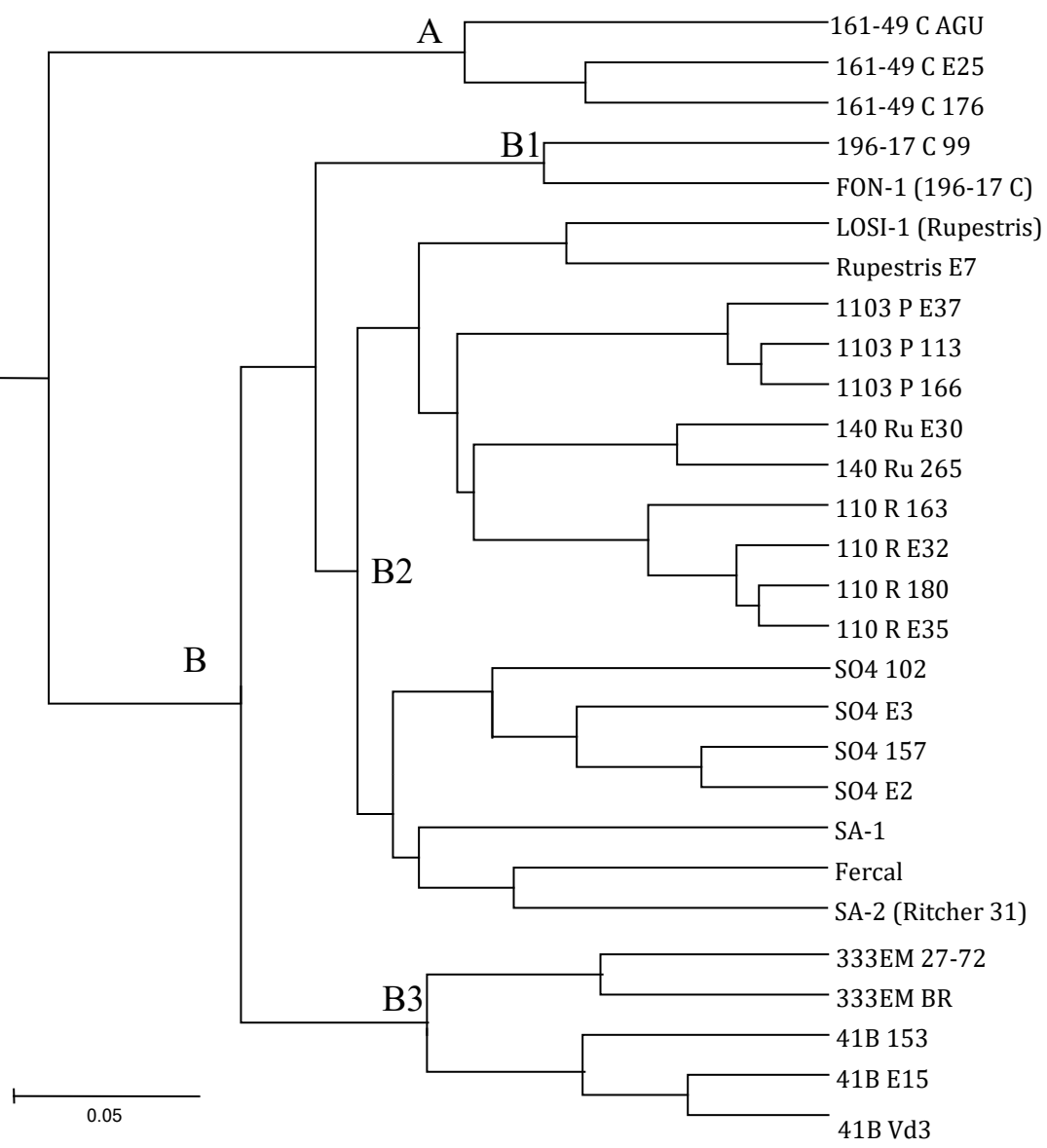

Figure 2. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering resulting from 28 rootstocks analyzed through AFLPs markers. Bootstrap values are higher than 0.70.

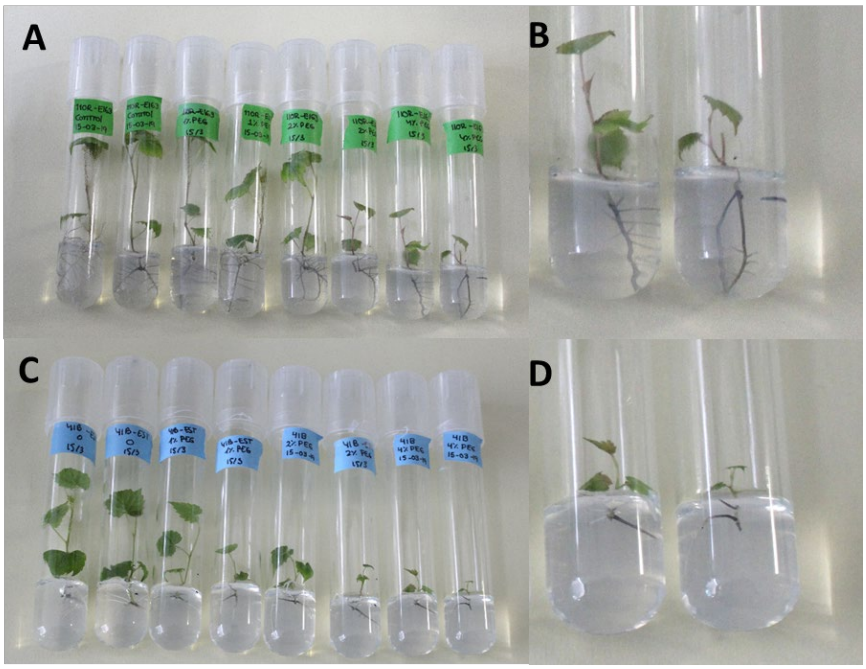

Figure 3. Plants of rootstocks 110 R (A and B) and 41 B (C and D) growing on modified MW containing medium supplemented with 0, 1, 2 or 4 % PEG after 45 d of culture. C and D detail of plants rooted on medium with 4 % PEG.

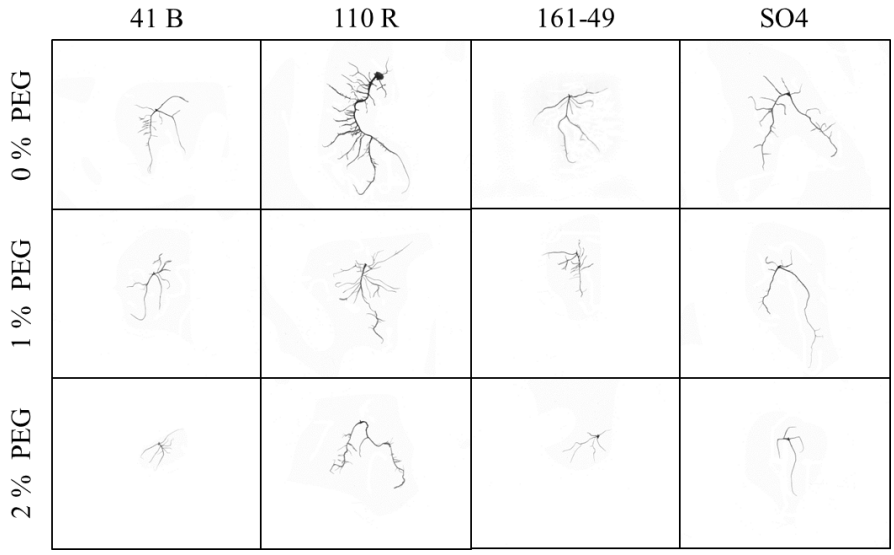

Figure 4. Roots of plants grown *in vitro* in modified MW medium supplemented with 0, 1 or 2 % PEG, after 45 d of culture. Roots were scanned with the WinRHIZO software.