Cultural Conditions and Propagule Type Influence Relative Chloride Exclusion in Grapevine Rootstocks

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Abstract: Breeding for salt resistance in grapevines and other crops has made slow progress despite decades of research. One factor contributing to this problem in grapevines is the weak or nonexistent correlation of field and greenhouse performance observed in some studies when salt resistance is assessed by chloride accumulation in leaf tissue. To develop a rapid chloride exclusion assay for use in rootstock breeding, multiple systems were tested. Results were obtained wherein the well-established field performance of specific genotypes was augmented, equalized, or reversed when in containerized culture. One assay using fritted clay media and herbaceous cuttings yielded a rank order and relative chloride uptake among the tested genotypes that was similar to published values from long-term studies in experimental vineyards. This assay used only 14 days of high salt exposure, was inexpensive, required relatively little space and maintenance, and has continued to provide reliable data in subsequent experiments. The results demonstrate the potential for considerable plasticity in chloride exclusion exhibited by ungrafted grapevines when assayed in containers. This underscores the importance of system design wherein genotypes of known capacity for chloride exclusion are accurately calibrated to their established field performance. This study describes an empirically derived assay that replicates these conditions closely enough to be used in a rootstock breeding program for improved chloride exclusion.

Key words: chloride exclusion, grapevine, rootstock, salinity resistance, salt resistance

Salt-affected soils used in agriculture have diverse chemical composition. Such soils have been broadly classed into four categories: (1) saline, (2) sodic or alkali, (3) gypsiferous, and (4) magnesium- and acid sulfate-based. Plants grown in such soils must tolerate abiotic stress factors that include high osmotic pressure, high or low soil pH, poor physical properties of the soil, and high levels of toxic ions (Pessarakli and Szabolcs 1999). Studies of salt resistance in crop plants could be performed using any of the minerals found in the array of ions that are known to accumulate to problematic levels. In practice, however, sodium has been the focal point for salt resistance research and reviews because many major crops are sodium sensitive (Munns and Tester 2008, Teakle and Tyerman 2010). Sodium uptake has been extensively studied in grains (Munns et al. 2006, Colmer et al. 2006), and genes that regulate sodium transport across cell membranes, such as *SOS1* in Arabidopsis (Shi et al. 2000), have been cloned and characterized.

In viticulture, chloride was identified as early as 1933 to be the most problematic ion for grapevines in salt-affected soils (Hickinbotham 1933), and similar chloride sensitivity has been observed in other crops such as citrus, avocado, and soybean. In early studies, chloride was found to be in high concentration in leaves, which manifested the classic symptom of excess salt uptake in the shoot, namely marginal leaf burn. In the first controlled study establishing this correlation, leaves sampled with burn symptoms contained an order of magnitude higher chloride content compared to sodium and had higher levels of chloride compared to sodium at each stage of expression (Ehlig 1960). Subsequent studies have consistently demonstrated relatively low levels of sodium when grape leaves exhibit salt stress, and sodium analysis is, therefore, often not performed (Downton 1977a, 1977b, Sykes 1987b). Variability in chloride accumulation in the leaves by different genotypes has been demonstrated, at least in some cases, to occur due to differential exclusion of chloride in the root (Tregeagle et al. 2010).

Unlike the clear correlation of chloride concentration and marginal leaf burn, chloride exclusion analyzed at the level of *Vitis* species has shown inconsistencies in rank order and degree of tissue concentration in some published screens. One reliable trend is the relative inability of the European winegrape *Vitis vinifera* to exclude chloride when compared to American *Vitis* species used as rootstocks. For example, the *V. vinifera* cultivar Thompson Seedless accumulated 2 to 4 times the chloride of non-*V. vinifera* rootstocks in a 3-year field trial (Sauer 1968). However, other reports of chloride exclusion among *V. vinifera* cultivars and *Vitis* spp. rootstocks varied widely: a

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Acknowledgments: Funding from the California Grape Rootstock Improvement Commission, the Fruit Tree, Nut Tree and Grapevine Improvement Advisory Board, and California Table Grape Commission is gratefully acknowledged. The authors thank Novavine, Sunridge, and Vintage Nurseries for dormant rootings used in these experiments. The authors also thank Geoffrey Dervishian, Jason Harink, Kurt Kabica, Daniel Ng, Nina Romero, Cindy Yip, and Alicia Zuniga for laboratory, field, and greenhouse assistance. Finally, comments and advice from Jim Richards and André Läuchli are gratefully acknowledged.

Supplemental data is freely available with the online version of this article.

Manuscript submitted May 2012, revised Nov 2012, accepted Jan 2013

doi: 10.5344/ajev.2013.12073

2-fold difference (Alexander and Groot Obbink 1971), an 11-fold difference (Downton 1977a), a 16-fold difference (Bernstein et al. 1969), and a 67-fold difference (Downton 1977b). Within non-*V. vinifera* germplasm, phenotypic reversals can be found. For example, while Antcliff (1983) found *V. rupestris* to accumulate more chloride than *V. berlandieri*, Downton (1977b) reported the opposite. Outside the established difference in chloride uptake between *V. vinifera* and non-*V. vinifera*, screens of diverse *Vitis* species and rootstocks derived from these species show no consistent pattern from which to infer superiority of chloride exclusion by one species over another (Downton 1977b). In addition to these problems, relative capacities for chloride exclusion between grapevine genotypes tested in greenhouse culture often differ from parallel tests conducted in the field (Sykes 1985a, 1987a), and studies using multiple screening methods have shown variability in chloride accumulation in the leaves depending on the method used (Gong et al. 2011, Tregeagle et al. 2010).

To effectively breed grapevine rootstocks for improved chloride exclusion, an efficient and reliable greenhouse-based assay that replicates the field performance of mature vines would be invaluable. This study tested systems that varied by irrigation method, growth media type, media volume and geometry, salt concentration, presence or absence of a high salt ramping period, duration of high salt exposure, grafting status, and cutting type. The factors varied in these experiments represented an array of practical decisions that had an impact on time and resource requirements but with an unknown effect, if any, on chloride uptake. The primary objective was to define conditions that minimized inputs and provided data corresponding to long-term field trials of mature vines. The experiments studied the performance of multiple genotypes within these systems and evaluated the success of each assay using two criteria: (1) replication of genotype rank orders for chloride uptake based on long-term field studies (Tregeagle et al. 2006, Walker et al. 2010) and (2) replication of relative uptake of chloride by *Vitis* plant materials in these same studies as measured by chloride concentration in the leaves.

Materials and Methods

Five experiments designed to span a range of experimental costs and a presumed corresponding level of data quality were conducted. Following these tests, a time-series analysis of the most effective system and a further test of media types were performed (Table 1). All experiments were performed at the University of California, Davis.

Irrigation method and frequency. Drip irrigation in Experiment 1 used 7.8 L/hr emitters. Irrigation was performed for 5 min, three times daily (07:00, 12:00, and 15:00), for a total irrigated volume of 1.95 L/day/plant. Drip irrigation in Experiment 7 used variable rate emitters, and was performed for 3 min, two times daily (06:00 and 17:00). The watering duration in both experiments provided a volume far in excess of field capacity to leach residual salts from the previous irrigation.

Ebb and flow irrigation in Experiments 2, 3, and 4 was performed in black plastic flood trays (Hydrotek, Mirabel, Canada) with internal dimensions of 222 x 102 x 11 cm. For Experiments 2 and 3, trays were filled to capacity every third day using an irrigation timer. For Experiment 4, irrigations were performed every second day, an increased frequency due to the lower total water content capacity of the smaller media volume (Table 1). In all three experiments the entire media volume was saturated for ≥15 min, and trays were drained of solution within 30 to 60 min by adjusting the rate of tray outflow.

Top watering by hand in Experiments 5 and 6 was performed once daily. Irrigations used 500 mL aliquots of solution from a fluted pitcher spread evenly over the media surface or a hose connected to a 5 M NaCl concentrate injected into the irrigation stream with a dosimeter (Dosatron International, Tresses, France). Care was taken to pull back foliage as needed so as not to wet leaf surfaces. When using a pitcher, irrigation solution was maintained at room temperature in 121-L reservoirs made of dark plastic and covered to exclude light and discourage algae growth.

Environment, containers, and media. Experiments 2, 3, 5, 6, and 7 were performed in a greenhouse. Experiments

Table 1 Comparison of experimental variables used in Experiments (Exp) 1 through 7 and costs for each.

Exp	Irrigation[a]	Media[b]	Vol/Diam/Dep[c] (cm^3/cm/cm)	Final NaC1concn (mM)	Ramp[d]	Time[e] (days)	Graft[f]	Propagule[g]	Cost
1	Drip	Sand	766/10/10	50	no	18	no	HC	Low
2	Bottom	Mix	766/10/10	50	yes	28,35,56	yes	HC/BG	Moderate
3	Bottom	Mix	5104/27/10	50	yes	37	no	DR	Moderate/high
4	Bottom	Mix	193/6/7	50	yes	18	no	Herb	Low
5	Hand	Frit	2146/15/15	25	no	14	no	Herb	Moderate
6	Hand	Frit	2146/15/15	25	no	1,4,7,14,35	no	Herb	Moderate
7	Drip	Mix/frit	2146/15/15	25	no	14	no	Herb	Low

[a]Irrigation method: drip, drip irrigation; bottom, bottom watering; hand, top watering by hand.
[b]Potting media: sand, 100% sand; mix, potting media; frit, fritted clay.
[c]Container geometry characterized by media volume, media surface diameter, and media depth. For Exp 1, 3, and 4 that used squared containers, media surface diameter was the shortest possible.
[d]Inclusion of a ramping period for salinity concentration.
[e]Total time of exposure to high salt irrigation treatment, not including ramping time.
[f]Inclusion of some common-scion grafted plant material.
[g]Propagule type: HC, hardwood cuttings; BG, bench-grafted cuttings; DR; dormant rootings, Herb; herbaceous cuttings.

1 and 4 were located outdoors in a shadehouse covered with 70% shade cloth. Air temperature in the greenhouse was maintained between 16 and 35°C. Air temperature in the shadehouse ranged from 13 to 33°C for Experiment 1 and from 5 to 29°C for Experiment 4.

Four container sizes were used and the media volume and dimensions within each are summarized (Table 1). Media dimensions rather than container dimensions were used to more accurately reflect root-zone geometry in bottom-watered experiments, as container capacity was underutilized to reduce media depth and to ensure full media saturation upon irrigation. This principle was most marked in Experiment 3, as large containers were necessary to accommodate the bulky root systems of dormant rootings. Although these containers had a 10,719 cm^3 capacity, media volume was approximately halved. All containers were dark plastic, and media volume varied ~26-fold across experiments.

Three media types were used: 100% coarse sand, a mixed potting media, and fritted clay. The mixed media consisted of 40% washed sand, 20% sphagnum peat moss, 20% redwood compost, and 20% pumice rock with the following amendments: 148 g/m^3 potassium nitrate, 148 g/m^3 potassium sulfate, 1.48 kg/m^3 single superphosphate, 4.45 kg/m^3 dolomite lime, and 1.48 kg/m^3 calcium carbonate lime. Fritted clay (Turface All Sport, Profile Products, Buffalo Grove, IL) was chosen for its high porosity, good CEC (33 meq/100 g), and ease of removal from root systems. This material has been characterized previously (van Bavel et al. 1978).

Fertilization and timing of high salt exposure. Experiment 1 was irrigated with tap water (71.7 mg/L Na, 16.0 mg/L Cl, EC = 0.52 dS/m) and was fertilized with commercial slow release pellets, ~2 g/container (Osmocote 14-14-14 NPK, Scotts Co., Marysville, OH). In all subsequent experiments, reverse osmosis water was used with a modified Hoagland's solution. NaCl was introduced downstream of fertilization using a 5 M concentrate and a Dosatron; final concentration was verified with an EC meter and/or a chloridometer. For hand-watered experiments, NaCl was added manually to the described fertigation water in a reservoir and concentrations verified as in the automated systems.

Final salt concentrations were either increased gradually ("ramped") over a 13-day period or introduced at full concentration (Table 1). When ramped, NaCl concentration was increased in 10 mM NaCl steps every third day beginning with 10 mM NaCl. Total days of plant exposure to full salt concentration is listed in Table 1.

Plant materials, propagation, sample collection, and chloride analysis. Plant materials, parentage, and abbreviations used in the text and figures of this paper are listed in Table 2. Because increased salt resistance in a plant is often associated with reduced salt accumulation in the leaves (reviewed in Munns and Tester 2008), the results of a rootstock survey comparing salt resistance of selected rootstocks is presented as a guideline for expected results. There are currently no published surveys that simultaneously compare the salt resistance of all rootstocks in widespread use, therefore most attempts to rank a new set of rootstocks will include unknowns.

All plant material was propagated with standard methods. Dormant hardwood cuttings or bench-grafted hardwood cuttings were callused in a mixture of equal parts of moistened peat, perlite, and vermiculite at 27°C for 2 weeks. Cuttings were then waxed, planted in a similar media, and placed on a shaded mist bed with 27°C bottom heat. After 6 weeks, root and shoot development were sufficient to allow transplanting

Table 2 Genotypes used in Experiments 1 through 7, including abbreviations, anticipated salinity resistance for selected genotypes, parentage, and specific experiments (Exp) in which each genotype was used.

Genotype	Abbreviation	R/S[a]	Parentage	Exp
V. champinii cv. Ramsey	Ramsey	4	*V. candicans* x *V. rupestris*[b]	1–7
Kober 5BB	5BB		*V. berlandieri* x *V. riparia*	3,5
Malégue 44-53	44-53		*V. riparia* x (*V. cordifolia* x *V. rupestris*)	3,5
Millardet et de Grasset 41B	41B		*V. vinifera* x *V. berlandieri*	1
Millardet et de Grasset 420A	420A	1	*V. berlandieri* x *V. riparia*	3
Millardet et de Grasset 101-14	101-14	4	*V. riparia* x *V. rupestris*	3,5
O39-16	O39-16		*V. vinifera* x *V. rotundifolia*	3,5
Paulsen 1103	1103P	3	*V. berlandieri* x *V. rupestris*	1,3,5
Richter 110	110R	2	*V. berlandieri* x *V. rupestris*	3
V. riparia cv. Riparia Gloire	Riparia		Not an interspecific cross	1–5
Ruggeri 140	140Ru	4	*V. berlandieri* x *V. rupestris*	3,5
Schwarzmann	Schwarzmann		*V. riparia* x *V. rupestris*	3,5
SO4	SO4	1	*V. berlandieri* x *V. riparia*	3,5
V. vinifera cv. Cardinal	Cardinal	1	Not an interspecific cross	1
V. vinifera cv. French Colombard	Colombard	1	Not an interspecific cross	1,2
V. vinifera cv. Pinot noir[c]	Pinot	1	Not an interspecific cross	2
V. vinifera cv. Thompson Seedless	Thompson	1	Not an interspecific cross	2–7

[a]Salinity resistance: 4, resistant; 3, moderately resistant; 2, moderately susceptible; 1, susceptible. From Southey 1992; *V. vinifera* was not directly compared here, but is widely reported as susceptible.
[b]A naturally occurring hybrid of these species.
[c]This genotype was only used as a scion.

into the final container and media. Dormant rootings were waxed and planted directly into the final container and media. Herbaceous cuttings were dipped into rooting hormone powder (Rootone, Bayer Crop Science, Research Triangle Park, NC), inserted into sponge plugs and placed on a shaded mist bed with 27°C bottom heat for 2 to 3 weeks. Rooted plantlets were removed from the mist bed and planted into small containers described in Experiment 4 (see Table 1) in a mixture of 75% sand, 22% crushed pumice, and 3% topsoil for 4 weeks. Young plants were transplanted to their final container and media for 2 weeks, except for Experiment 6 when this period lasted 17 days.

To equalize the quantity of leaf tissue between genotypes, some pruning was performed just before the onset of the salt treatment in some experiments. In Experiment 1, plants were pruned to a single shoot early in development, but no further pruning was performed. In Experiments 2 and 4, all plants were pruned to a height of ~15 cm on the first day of the ramping period. In Experiment 3, all plants were pruned to the single, lowermost fully expanded leaf on the first day of ramping. No pruning was performed in Experiments 5, 6, and 7.

In Experiment 1, leaf tissue was sampled by selecting the two fully expanded and most apical leaves at the onset of the salt treatment. In all subsequent experiments, all leaves were harvested except for leaves growing below the top edge of the container. These leaves were excluded to prevent contamination from chloride introduced externally during irrigations. In all cases, this represented a small fraction of the total leaf tissue and this omission was thought to have a negligible impact on the final measured chloride concentration. Leaf tissue was dried to a constant weight at 67°C, ≥1 week. Dried leaf tissue was ground through a 40-mesh screen with a Wiley Mill (Thomas Scientific, Swedesboro, NJ). Ground tissue was extracted with deionized water according to Jones (2001). Chloride concentration was measured using silver ion titration on a Haake Buchler chloridometer (Labconco, Kansas City, MO) according to manufacturer instructions.

Quantification of leaf necrosis. Necrosis was quantified two days prior to the final harvest in Experiment 2 and the day before harvest in Experiment 4. For Experiment 2, percent necrosis was scored by measuring total shoot length and calculating the fraction of this length that contained leaves with necrotic symptoms, ranging from small amounts of marginal leaf burn in young leaves to completely necrosed older leaves. Experiment 4 used a visual estimation of necrosis and a four-tiered index, as follows: 0 = asymptomatic; 1 = 1 to 50% of all leaves with necrosis; 2 = 51 to 90% of all leaves with necrosis; and 3 = 91 to 100% of all leaves with necrosis.

Experimental design, chronology, and data analyses. Experiments 1, 5, 6, and 7 were completely randomized. Experiments 2, 3, and 4 used a randomized complete block design with flood trays as blocks. Experiments 2 and 3 had four blocks; Experiment 4 had two salt-treated blocks and two unsalinized control blocks. Average replications (reps) per treatment by experiment number were: (1) 7 reps, (2) 17 reps, (3) 4 reps, (4) 6 reps, (5) 4 reps, (6) 7 reps, and (7) 3 reps. Replicates in Experiment 2 were highly variable (standard deviation ±10), primarily due to the limited propagation success of grafted cuttings of Ramsey and Thompson. Replicates in Experiment 4 averaged five subsamples per rep; a single rep was the mean of individuals for a genotype in a 25-well plastic tray. Harvest dates by experiment number were: (1) 6 Sept 2005, (2) 23 July 2008 (harvest 1), (3) 9 Dec 2008, (4) 31 Oct 2008, (5) 29 Sept 2009, (6) 11 Sept to 15 Oct 2010, and (7) 12 Aug 2010.

Simple descriptive statistics were generated in Microsoft Excel. Student's *t* test and analysis of variance were performed using Statistical Analysis Software (ver. 9.1.3; SAS Institute Inc., Cary, NC). Blocks in Experiments 2, 3, and 4 were treated as random effects. Post hoc mean separations used a Tukey multiple comparison test. For the chloride uptake and necrosis data of Experiment 2, complete factorial models were tested for significance prior to the analysis of individual factors. For days 4 and 7 in Experiment 6, Welch's ANOVA was performed to accommodate heteroscedasticity in these two periods.

Experimental costs. Experimental cost (Table 1) is a subjective assessment taking into account set-up time, maintenance time, cost of materials, space requirements, and throughput capacity. For example, Experiment 4 was labeled "low" because it used small containers, an automated irrigation system, easy to propagate plant materials, and inexpensive shadehouse space. Experiment 3 was labeled "moderate/high" because it used large dormant rootings, large containers, a high volume of media, and greater greenhouse space.

Results

Rank order of genotypes and relative uptake. The genotype Ramsey is widely considered a strong chloride excluder, and the comparison of this genotype to cultivars of the weak chloride excluder species *V. vinifera* has been made in multiple publications (Walker et al. 2010, 1997, Sykes 1985a, Downton 1977b). Thus, the Ramsey versus *V. vinifera* comparison serves here as a benchmark.

Experiment 1 resulted in an expected Ramsey to *V. vinifera* rank order, with Cardinal and Colombard accumulating almost 2-fold more chloride than Ramsey (Figure 1; $p = 0.0090$). However, the rootstock 1103P, considered a strong excluder (Table 2), accumulated chloride at a level approaching that of *V. vinifera*.

Experiment 2 was destructively harvested at three time points (Figure 2). Plants were asymptomatic at 28 days, but the unexpected appearance of marginal leaf burn in ungrafted Ramsey within 1 week after this initial harvest initiated an unplanned random sampling of all ungrafted genotypes at day 35. Subsequent rapid shoot necrosis in ungrafted Ramsey and ungrafted Riparia led to an early harvest of the entire experiment at day 56. Subsequent chloride analyses revealed differences in the chloride concentration in leaf tissue over time ($p < 0.0001$) and among genotypes ($p < 0.0001$). Orthogonal contrasts showed differences in uptake between grafted and ungrafted genotypes in both the first and third harvests (day 28: $p < 0.0001$; day 56: $p < 0.0001$); however, there was no genotype effect in a factorial analysis ($p = 0.1274$) or in any

genotype by harvest interaction ($p = 0.7160$). Mean separation of chloride concentrations in ungrafted genotypes corresponded to the observed leaf burn phenotype of Ramsey by day 35 and further showed that this elevated chloride uptake in Ramsey had already commenced by day 28 when all plants were symptom-free. At the third and final harvest, chloride uptake was the most similar among all ungrafted genotypes as compared to the two earlier harvests, as Ramsey and Riparia individuals were reaching full necrosis and hence had accumulated a maximum salt concentration.

Experiment 3 was harvested at day 46 before the onset of any leaf tissue symptoms. Chloride analysis of leaf tissue revealed differences among genotypes ($p < 0.0001$), with Ramsey and Thompson at the extreme ends and in the expected rank order (Figure 3). Chloride uptake in Thompson leaves was 9.7-fold that of Ramsey. Unlike Experiment 1, 1103P took up chloride at very low levels and nearly identical to uptake in Ramsey. The rootstocks Schwarzmann and 140Ru, both with precedent as strong chloride excluders (Walker et al. 2010), took up ~2.6-fold more chloride than Ramsey and 1103P, but ~3.7-fold less than Thompson.

In Experiment 4, Thompson accumulated ~1.5-fold more chloride than Ramsey, with Riparia statistically equivalent to Ramsey (Figure 4; $p = 0.0023$). Variation in this experiment

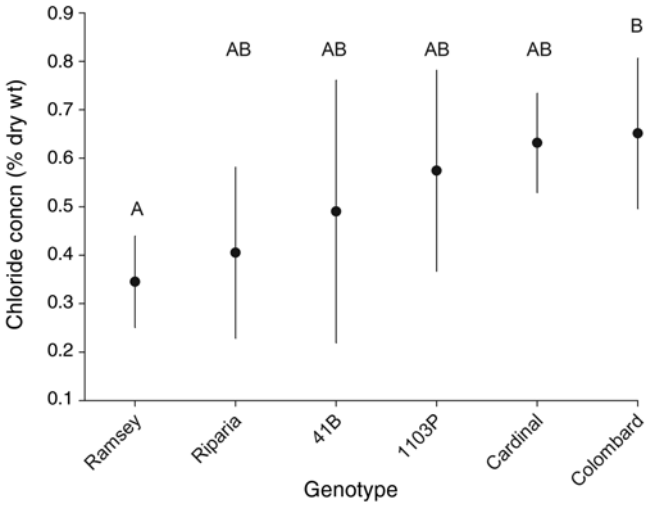

Figure 1 Experiment 1: chloride concentration of young, fully expanded leaves. System components listed in Table 1; abbreviations in Table 2. Data points are means ± 1 SD. Mean values labeled with the same letter were not significantly different. Statistical analysis described in text.

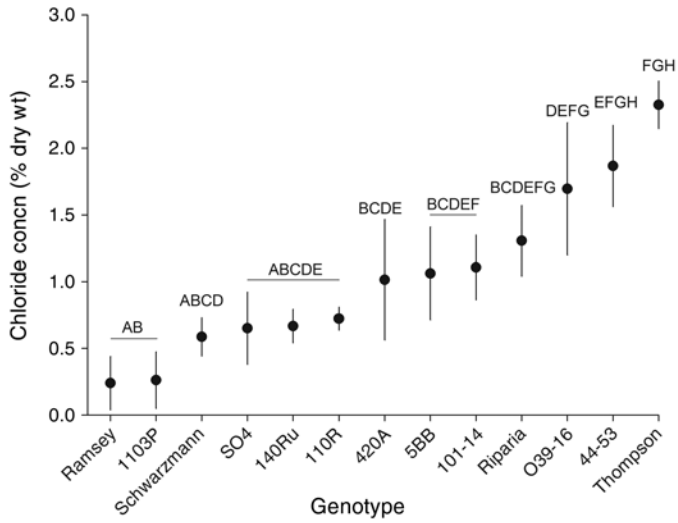

Figure 3 Experiment 3: chloride concentration of pooled total leaves. System components listed in Table 1; abbreviations in Table 2. Data points are means ± 1 SD. Mean values labeled with the same letter were not significantly different. Statistical analysis described in text.

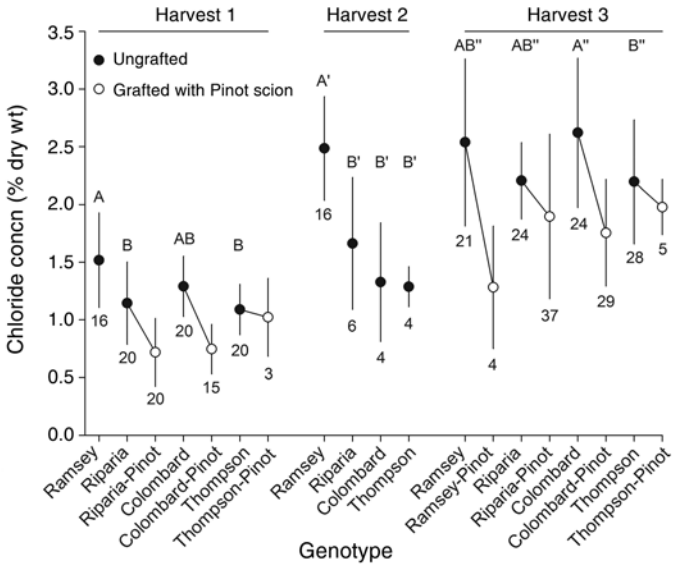

Figure 2 Experiment 2: chloride concentration of pooled total leaves. System components listed in Table 1; abbreviations in Table 2. Data points are means ± 1 SD. Replicate number listed below each data point. Mean values labeled with the same letter were not significantly different. Statistical analysis described in text.

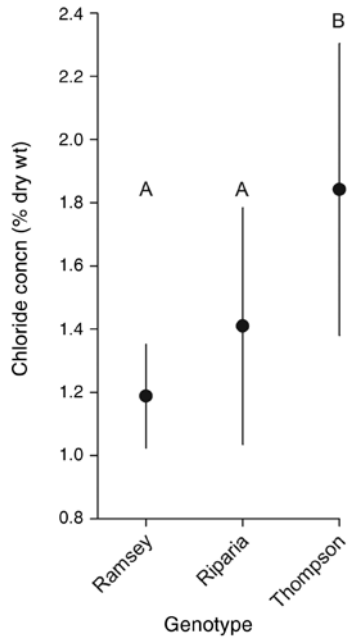

Figure 4 Experiment 4: chloride concentration of pooled total leaves. System components listed in Table 1; abbreviations in Table 2. Data points are means ± 1 SD. Mean values labeled with the same letter were not significantly different.Statistical analysis described in text.

was high relative to other experiments (e.g., the σ^2 for all Ramsey individuals was ~8-fold higher than observed in Experiment 5), but the use of five subsamples per replication provided sufficient statistical power to demonstrate a difference between Thompson and Ramsey.

Experiment 5 resulted in statistical differences in chloride uptake (Figure 5; $p < 0.0001$) and with the lowest variability within genotypes when compared with other experiments. Thompson accumulated ~2.3-fold more chloride than Ramsey. Importantly, the genotypes that were pure *V. vinifera* (Thompson) or partial *V. vinifera* (O39-16, Table 2) had higher mean chloride concentration than in all genotypes with literature precedent as strong chloride excluders (Schwarzmann, 140Ru, 101-14, Ramsey, 5BB, and 1103P). Interestingly, 44-53, a rootstock with a more complex parentage than the other genotypes (Table 2), accumulated 1.8-fold more chloride than Thompson. In a subsequent experiment using 44-53, this genotype again performed as a very weak chloride excluder (data not shown).

Shoot necrosis. The variability in shoot necrosis in Experiment 2 was very high; however, there were statistically significant differences among genotypes ($p = 0.0016$). In the ungrafted state, the two non-*V. vinifera* genotypes had 2.7-fold more necrosis than both *V. vinifera* genotypes, despite their lack of differences in leaf chloride concentration at this time (Supplemental Figure 1). Some Ramsey and Riparia individuals were completely necrosed at the time of measurements; alternatively, some Thompson and Colombard individuals were completely asymptomatic. These same four genotypes grafted with Pinot as a scion showed intermediate necrosis phenotypes despite lower chloride concentration in their leaves compared to all ungrafted genotypes. Shoot necrosis in Experiment 4 was again highly variable, but in this case there were no statistically discernible differences (Supplemental Figure 2; $p = 0.4136$) despite the differences observed in chloride uptake (Figure 4).

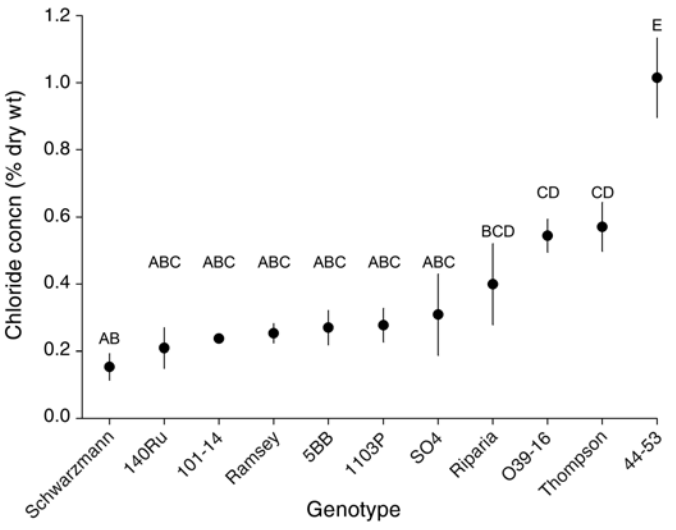

Figure 5 Experiment 5: chloride concentration of pooled total leaves. System components listed in Table 1; abbreviations in Table 2. Data points are means ± 1 SD. Mean values labeled with the same letter were not significantly different. Statistical analysis described in text.

Time series analysis. In Experiment 6, Ramsey and Thompson were assayed in a time series and with the identical protocol as in Experiment 5. No detectable chloride was found in the leaf tissue after 24 hr (Supplemental Figure 3). After 4 and 7 days, chloride could be detected but Ramsey and Thompson were not statistically different. These same genotypes could be distinguished at day 14, with uptake in Thompson 2.2-fold that of Ramsey, but by day 35 they were again not statistically different.

Media testing. Experiment 7 compared the outcome of a screening protocol similar to that in Experiment 5 using mixed media and fritted clay as separate treatments. This experiment differed from Experiment 5 only in the time plants were in their final container before salinization (52 days in Experiment 7 compared to 42 days for Experiment 5; Table 1) and in the use of twice daily automated irrigation rather than once daily hand watering. In a factorial analysis, there were no detectable differences in media type or in genotypes (Supplemental Figure 4, $p = 0.9153$ and $p = 0.4552$, respectively).

Discussion

Container studies of salt exclusion by grapevine rootstocks will have limited use for future breeding efforts if the data generated do not accurately reflect salt uptake in the field by mature vines. Even without the confounding effects that containers can introduce (Campbell et al. 1985), studies of salt resistance generally must contend with the complexity of the trait coupled with environmental interactions (Arzani 2008, Cuartero 2006, Flowers and Flowers 2005). An additional obstacle is the increased sampling error arising from small sample sizes necessary to accommodate greenhouse constraints on space. Assay development therefore seeks to discover cultural conditions that generate accurate phenotypic data while simultaneously minimizing the size of the assay.

There were problematic results in all trials that used plants originating from hardwood cuttings. In Experiment 1, which used rooted hardwood cuttings, Ramsey and *V. vinifera* cvs. Cardinal and Colombard performed as expected with an ~2-fold difference in chloride accumulation. However, 1103P, with precedent as a strong chloride excluder (Fisarakis 2001, Southey 1992), was not statistically distinguishable from either *V. vinifera* cultivar (Figure 1, Figure 6) or 41B, a rootstock reported to be salt sensitive (Arbabzadeh and Dutt 1987). In Experiment 2, which also used rooted hardwood cuttings and hardwood benchgrafts, the usual rank order of chloride accumulation in Ramsey and one cultivar of *V. vinifera* was reversed by day 28 and was reversed relative to both cultivars of *V. vinifera* by day 35 (Figure 2, Figure 6). The ability of Ramsey to exclude chloride relative to *V. vinifera* is widely reported; however, there is one other report of Ramsey accumulating more chloride than *V. vinifera,* but noting these plants may have been affected by tissue culture regeneration (Skene and Barlass 1988). Results with grafted material in Experiment 2 were also anomalous. Although rootstock genotypes grafted with a common scion have been shown to accumulate chloride in the shoot at different rates (Downton 1977b, Sauer 1968), in this experiment they were statistically

indistinguishable in each of two harvest periods (Figure 2), with lower chloride accumulation as a group than the same genotypes in the ungrafted state. In Experiment 3, which used hardwood dormant rootings, a more standard rank order of chloride uptake was obtained, with Thompson accumulating the most chloride and three rootstocks considered to be strong excluders (Ramsey, 1103P, and Schwarzmann) accumulating the least. However, the range of observed differences was far in excess of what would be expected in a field setting (as seen for Thompson, 1103P, and Ramsey in Walker et al. 2004), with a nearly 10-fold difference between Thompson and Ramsey.

Because these hardwood-propagated trials were not intended to isolate the effect of hardwood propagules per se, other factors could be contributing to or responsible for the observed results. For example, anomalous data could have resulted from effects of different irrigation methods, different container volume or geometry, different exposure times to high salt, differences in root development variables (unmeasured in this study), or some combination of these factors. Hardwood propagules are also developmentally older than herbaceous cuttings. In citrus, developmental stage was related to chloride accumulation in the shoot (Sykes 1985b) and may also be important in these results.

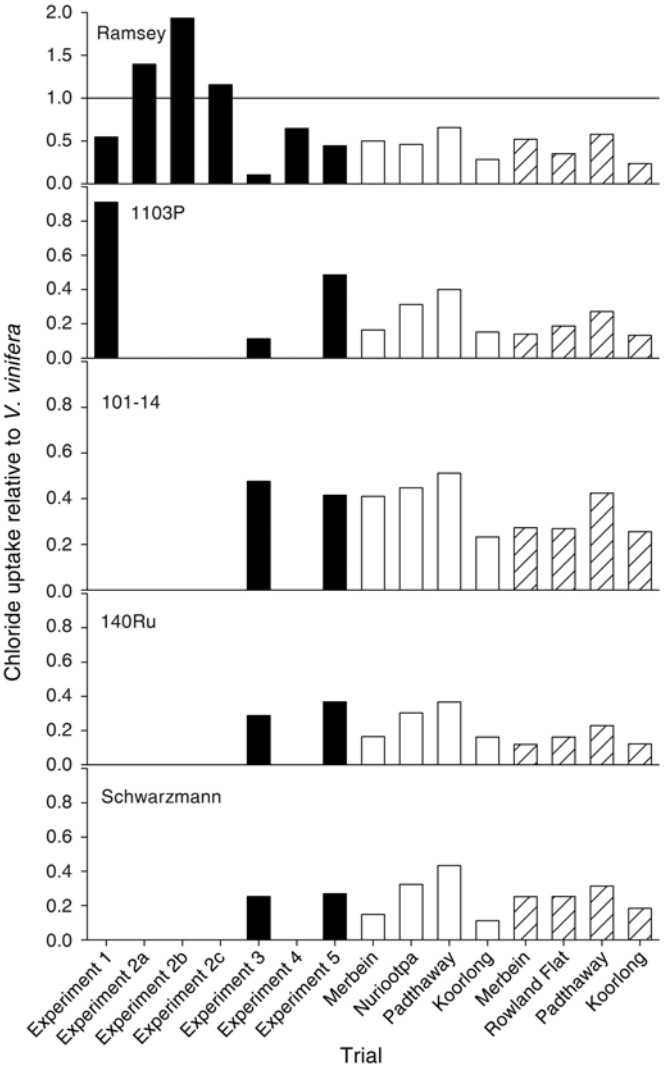

Figure 6 Mean chloride uptake of selected genotypes relative to mean chloride uptake of *V. vinifera* in the same experiment. Black bars are data from Experiments 1 to 5 in this paper. Experiment 2a, 2b, and 2c correspond to harvest 1, 2, and 3 of Experiment 2, respectively, as described in text. White bars derived from field data presented in Walker et al. 2010, and represent *V. vinifera* cv. Chardonnay grafted on the same rootstock as for Experiments 1 to 5 in each graph. Hatched bars derived from field data of *V. vinifera* cv. Shiraz grafted on the same rootstock as for Experiments 1 to 5 in each graph, presented in the same paper. For Walker et al. (2010) data, trials are listed by site.

However, an uneven pace of growth and development with hardwood propagules was noted, which may have played an important role in the observed differences in chloride uptake. In Experiment 1, 1103P accumulated an unexpectedly high level of chloride and also had ~50 to 140% higher final shoot length compared with all other genotypes in this trial (data not presented). In Experiment 2, Ramsey took up relatively high levels of chloride and also had ~30 to 125% more lateral shoot production (data not presented). Experiment 3 had the most striking differences in growth and development, with Ramsey and 1103P pushing buds one to two weeks after Thompson. Although plants were pruned to a single leaf prior to the onset of salt treatment, Thompson still grew more vigorously than all other genotypes, accumulating approximately double the dry leaf biomass when compared to the five best chloride excluders in this trial: Ramsey, 1103P, Schwarzmann, SO4, and 140Ru (Figure 3; biomass data not presented). Dormant hardwood cuttings of *V. vinifera* gathered from the field for propagation were in general heavier and thicker per unit length than rootstock cuttings. In general, it was difficult to equalize the growth and development of rootstocks and *V. vinifera* cvs. taken from hardwood cuttings, and given the correspondence of skewed growth patterns to anomalous chloride uptake, it is possible that inconsistent growth and developmental patterns accounted for at least some of the final variation in chloride uptake. Because of the high variability in timing of budbreak from ungrafted dormant rootings, this form of propagation appears to be especially unsuited for chloride exclusion studies. Taken as a whole, the anomalies discussed here highlight what is unknown regarding how genotype-specific differences in the shoot may influence chloride uptake in the root. Although commercial vineyards generally use one or more common scions, rootstock breeding is simplified when common scion grafting is not needed. Further work in understanding how shoot differences may influence chloride uptake may prove to be important to better align greenhouse and field results.

In contrast to Experiments 1, 2, and 3, the plants generated from herbaceous cuttings in Experiment 5 were visually more uniform in growth and development. Because the period of establishment and salt exposure was minimized (6 and 2 weeks, respectively), there was insufficient time for significant differences in shoot growth among genotypes and no need for any pretreatment pruning. The results of this trial accorded well with expected genotype rank order from previously reported field data and their relative chloride uptake (Figure 6). Thompson accumulated 2.25-fold more chloride in its leaves

than Ramsey, and O39-16, a rootstock with *V. vinifera* parentage (Table 2), was indistinguishable from Thompson. The six genotypes in this study with precedent for strong salt exclusion (Walker et al. 2010) accumulated the least amount of chloride (Schwarzmann, 140Ru, 101-14, Ramsey, 5BB, and 1103P, Figure 5). Although there are no previous reports of chloride exclusion in 44-53, the very weak chloride exclusion observed in this rootstock in Experiment 5 is a feasible trait given that this genotype was also the highest non-*V. vinifera* chloride-accumulating rootstock observed in Experiment 3 (Figure 3). Plants used in Experiment 4 were also propagated from herbaceous cuttings, but these were assayed outdoors and in very small containers. In this experiment, Ramsey and Thompson accumulated chloride in an expected rank order and relative tissue concentration; however, the data were highly variable and may not have separated statistically if not for high subsampling (4 to 6 subsamples per rep). The standard deviation for all Ramsey individuals in Experiment 4 was nearly 8-fold higher than that observed in Experiment 5, approximately doubled for Riparia, and ~4-fold higher for Thompson.

When comparing genotypes that have known differences in their ability to exclude chloride in a container-based assay, as with Ramsey compared with *V. vinifera* cvs., there are four possible outcomes: (1) strong excluders take up less chloride than weak excluders and do so proportionally to values observed in the field; (2) strong excluders take up less chloride than weak excluders, but with differences that exceed field observations; (3) strong excluders take up an equivalent amount of chloride compared to weak excluders; and, least likely, (4) strong excluders take up more chloride than weak excluders. Conditions under which all four possibilities occurred were documented together with literature values generated in the field (Figure 6). The field values presented by Walker et al. (2010) were chosen for comparison based on several factors in the experimental design: the use of five different field sites; randomization at each site; conventional vine training; a long study period (~5 years); and the use of two different *V. vinifera* common scions. This study stands in contrast to many older trials reported in the literature, which were much shorter term or had design flaws such as a lack of randomization. In Walker et al. (2010), own-rooted *V. vinifera* accumulated on average 2.5-fold more chloride than Ramsey and 4.2-fold more chloride than all strong chloride excluders examined.

An analysis of leaf necrosis might serve as an effective substitute for chloride uptake. If true, the assay would need to run longer to allow for symptom development, but length of time could be shortened if the relatively time-consuming step of leaf tissue chloride analysis could be eliminated. There was theoretical support for this possibility in a previous study linking leaf burn symptoms in grape to high tissue chloride (Ehlig 1960) and correlations between sodium and chloride in leaf tissue with leaf necrosis in other crops (Dasgan et al. 2002, Mickelbart and Arpaia 2002, Wahomea et al. 2001). In Experiment 2, the percentage of shoot necrosis was highest for ungrafted Ramsey, in correspondence with the high chloride uptake by this genotype in this trial (Figure 2, Supplemental Figure 1). However, ungrafted Riparia also had significantly higher shoot necrosis compared to both ungrafted genotypes of *V. vinifera,* although there was no difference in leaf tissue chloride among these genotypes during any of the three harvest periods. Grafted genotypes exhibited intermediate levels of shoot necrosis despite lower chloride uptake in the first and third harvests. In Experiment 4, Ramsey and Riparia had significantly lower chloride concentrations in the leaf compared to Thompson, but there were no statistically distinguishable differences in percent shoot necrosis (Figure 5, Supplemental Figure 2). These results from Experiments 2 and 4 suggest differences in leaf tissue tolerance of chloride among genotypes, with highest sensitivity in Ramsey and Riparia, and indicate that leaf necrosis cannot be used as a reliable index for chloride uptake in the shoot. Even without this problem, further work would be needed to ensure the reliability of chloride concentrations derived from leaf tissue with varying levels of necrosis.

To determine if the salinized treatment period could be reduced to less than two weeks, and using methods identical to that in Experiment 5, a time-course analysis using Ramsey and Thompson was performed (Supplemental Figure 3). Of the five time periods sampled, only day 14 provided results comparable to field studies. Some individuals of Thompson began actively accumulating chloride by day 7, but measurable uptake in Ramsey occurred between 7 and 14 days. This developmental delay mirrors that seen in Ramsey and other strong chloride excluders in the dormant rootings of Experiment 3. Interestingly, after further development to day 35, the chloride uptake phenotypes of these two genotypes had equalized. The change in relative uptake of salt over time has been well-documented in many crops, including citrus (Sykes 1985b). It seems that the high chloride accumulation by Ramsey in Experiment 2 may be the final phase of a developmental pattern wherein chloride uptake is initially delayed in Ramsey, but given sufficient time Ramsey equals or exceeds *V. vinifera* cvs. in total integrated chloride accumulation when plants are in the ungrafted state. The plants in Experiment 2 developed in containers (i.e., after removal from the mist bed) for 77 days prior to salt treatment originated from woody cuttings and likely contained more carbohydrate reserve than the plants prepared from herbaceous cuttings used in Experiments 4, 5, 6, and 7. The protocol in Experiment 5 nearly halved this presalt developmental period to 42 days.

The impact of different potting media was tested in Experiment 7 using a soil mix and fritted clay (Supplemental Figure 4) and a similar protocol to Experiment 5, but allowed the plants to develop 52 days prior to salt treatment. This experiment showed no difference in chloride uptake due to media type using the same container size as in Experiment 5, but resulted in the equalization of chloride uptake in Ramsey and Thompson similar to that seen at day 35 of Experiment 6. The effect of increased developmental time in Experiment 7 may have been exacerbated by the warmer greenhouse temperatures during July and August when mean greenhouse highs were ~35°C. The enhancement of salt uptake in the shoot due to warmer temperatures has been shown in

tomato (West and Taylor 1980) and may also occur in grape. The phenotype reversal of Ramsey and *V. vinifera* earlier described for Experiment 2 also occurred during elevated temperatures in July and August. The protocol of Experiment 5 has been used successfully in subsequent screens wherein Ramsey and *V. vinifera* cvs. were used as benchmark genotypes (data not presented) and greenhouse high temperatures were <30°C. However, three of these screens were conducted when greenhouse temperatures were the warmest of the season and also did not distinguish salt uptake between Ramsey and *V. vinifera*.

Although some rootstock genotypes exhibit a robust phenotype of strong chloride exclusion in the field, especially when compared to self-grafted *V. vinifera* cultivars, the experiments here demonstrate the potential for considerable plasticity in chloride exclusion that can occur on ungrafted grapevines when assayed as recently propagated plants in containers. In the field, variability of chloride uptake in grapevines is clearly impacted by spatial (Sykes 1987a) and seasonal (Tregeagle et al. 2006) environmental heterogeneity and can also change over the course of development (Tregeagle et al. 2006). Because environmental conditions and cultural practices in commercial settings are highly variable, additional work that examines how specific variables impact the degree of chloride accumulation in the leaves of both field- and greenhouse-grown plants could improve management practices and accelerate the pace of rootstock breeding for improved chloride exclusion.

Conclusion

The relative ability to exclude chloride in ungrafted recently propagated grapevine rootstocks, as measured by chloride concentration in leaf tissue, exhibited a high degree of phenotypic plasticity. Both rank order of genotypes and relative uptake of chloride can be dramatically altered by simple changes in cultural conditions. For the purposes of rootstock breeding, a simple greenhouse-based assay is presented that mimics the behavior of mature vines grown in the field under conventional cultivation. Optimal results were obtained in Experiment 5, using herbaceous cuttings propagated and tested in a greenhouse, a fritted clay growth medium, no presalinization pruning, 8 weeks of presalinization development, and 2 weeks of 25 mM NaCl exposure.

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