



A rapid method for generating sufficient amounts of uniform genotype-specific material from the woody perennial grapevine for ion transport studies

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Abstract

Experimentation with woody perennials may be difficult due to slow plant growth and a lack of sufficient amounts of uniform plant material. In this study, we sought to determine whether rooted leaves could be used as a substitute for whole plants in ion accumulation studies. Grapevine leaves are particularly amenable for rooting since their petioles are of sufficient length for dipping in rooting hormone and for holding the leaf above the soil surface. To determine whether rooted leaves would be useful for salinity experiments, we investigated the ion uptake characteristics of rooted leaves derived from a backcross population that differed in Cl⁻ accumulation. Long-term ion accumulation studies conducted over several weeks and short-term radioactive uptake studies conducted over several hours were performed. The data showed that the Cl⁻ content of rooted grapevine leaves from different genotypes grown at 25 and 50 mM NaCl was similar to data reported by others. Short-term radioactive uptake assays did not always reveal differences in uptake between the genotypes. Therefore, we suggest that rooted leaves under certain conditions may provide a space-efficient method for generating sufficient amounts of plant material. This material could be used for studying whole plant, molecular and electrophysiological aspects of ion transport and for conducting experiments where root material from specific genotypes is required.

Introduction

The link between salt tolerance and Cl⁻ exclusion has been well documented in citrus (Storey and Walker, 1999) and legumes such as soybean (Abel, 1969; Abel and Mackenzie, 1964; Lauchli, 1984; Lauchli and Wieneke, 1979). While growth experiments with grapevine are difficult to conduct because plant material is not uniform, some data suggest that Cl⁻ exclusion is correlated with salt tolerance (Alexander and Woodham, 1968; Woodham, 1956). To develop *Vitis vinifera* varieties that are better able to exclude Cl⁻, we began working with a backcross population that segregates for a single dominant gene involved in Cl⁻ exclusion (Sykes, 1987). The aim of our work was

to understand the mechanisms of chloride exclusion and the action of a single dominant gene involved in reducing chloride uptake.

We investigated a novel system for studying ion transport and accumulation under saline conditions in grapevines. In general, studies on woody perennial plants may be difficult, because in some cases sufficient plant material of a specific genotype may not be available, and in other cases, certain genotypes are difficult to root. Since whole plant biology experiments require a large amount of replication to overcome individual plant variation, a method that allows for rapid production of large numbers of plants would be valuable. In our specific case, we were limited by the number of canes for clonal propagation that could be taken each year from an individual grapevine backcross genotype, which was represented in the vineyard

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by a single plant, and by a difficulty of rooting the backcross genotypes. We established two plants from each genotype in the greenhouse and tried to root single and multiple node cuttings from greenhouse grown plants. This was not successful, but we found we could root leaves. Similar problems with insufficient plant material may also arise for scientists who are working with plants that may be part of a botanical garden collection where only a few plants are available and clonal propagation would be difficult and time consuming (Williams and Antcliff, 1984).

To test whether roots from rooted leaves had the same ion accumulation characteristics as roots from whole plants, we used a backcross population of grapevines that segregate for Cl^- accumulation. This population segregates for a single dominant gene for Cl^- exclusion (Sykes, 1987). The backcross population was generated 25 years ago when the *Vitis vinifera* variety Biancone was backcrossed to an F1 hybrid. The F1 hybrid is the result of a cross between a single *Vitis berlandieri* plant and *Vitis vinifera* var. Sultana which was the pollen donor (Antcliff et al., 1983; Newman and Antcliff, 1984). *Vitis berlandieri* was used in the cross because of its ability to exclude chloride (Antcliff et al., 1983; Downton, 1977). The outcome of the backcross was a population of approximately 90 plants, of which approximately 20 are still maintained at a CSIRO vineyard in Victoria, Australia. The Cl^- accumulation of these backcross lines was documented previously (Newman and Antcliff, 1984; Sykes, 1987).

Materials and methods

Long-term and short-term experiments were conducted in order to determine whether rooted leaves from grapevines had the same Cl^- accumulation characteristics as the plants from which they were derived.

Leaves were excised from grapevines that were growing in the greenhouse. Only petioles from older leaves that were lignified or were starting to become lignified were suitable for rooting. These leaves were immersed in water to reduce transpirational water loss. The petioles were dipped in 0.1% indolebutyric acid in a 50% ethanol solution for 30–60 s (Williams and Antcliff, 1984) and then placed in a moist medium consisting of 50% vermiculite and 50% perlite in 0.5 L pots. The laminae were above the surface of the pot. Pots were placed on a heating pad to provide bottom heat (approximately 30 °C); a clear plastic dome

Table 1. Backcross and parental genotypes used in the long term experiments

| Genotype | Classification according to Sykes (1987) | Number of rooted leaves used for 25 mM experiment | Number of rooted leaves used for 50 mM experiment |
|-----------|--|---|---|
| MU27-38 | Excluder | 4 | 5 |
| MU27-56 | Excluder | 1 | 4 |
| MU26-91 | Excluder | 6 | 5 |
| MU26-94* | Excluder | 4 | 6 |
| MU27-54* | Excluder | 3 | 5 |
| MU27-42 | Excluder | 3 | 4 |
| MF80-42 | Excluder | 4 | 0 |
| MF77-13 | Excluder | 6 | 5 |
| MU27-31 | Excluder | 0 | 3 |
| MU27-08 | Non-excluder | 5 | 6 |
| MU27-15 | Non-excluder | 4 | 5 |
| MU27-25 | Non-excluder | 6 | 5 |
| MU26-95 | Non-excluder | 4 | 6 |
| MU27-23 | Non-excluder | 3 | 6 |
| MU27-04 | Non-excluder | 1 | 0 |
| Biancone* | Non-excluder | 6 | 6 |

*Also used in short term experiments.

was placed over the pots to keep the leaves in a high humidity environment. Pots were watered periodically with a one-fifth dilution of Hoagland #1 solution (Hoagland and Arnon, 1938). The fungicide Benlate (0.5 g l⁻¹) was sprayed on the leaves periodically to prevent fungal growth. Shade cloth (85%) was initially used to cover the dome on sunny days to reduce temperatures under the dome (30 °C). Callus formation followed by root initiation began approximately 2 weeks after the dipped petioles had been placed in the vermiculite/perlite.

Genotypes

The genotypes used in long and short-term experiments are shown in Table 1. Three genotypes were used for the short-term experiments: Biancone (non-excluder), MU27-54 (excluder) and MU26-94 (excluder) (Table 1).

Long-term experiments

For long-term experiments, petioles with sufficient root mass (approximately 3–4 weeks after dipping in indolebutyric acid) were transferred to pots containing washed river sand. Pots (0.5 L) were then irrigated with one-fifth concentration Hoagland #1 solution (Hoagland and Arnon, 1938) six times per day for 10–14 days before NaCl was added. Each irrigation

flushed pots completely. NaCl was added to a concentration of 25 mM or 50 mM, and rooted petioles were harvested 2 weeks after the introduction of NaCl. For the 50 mM treatment 25 mM NaCl was added and an additional 25 mM was added the second day. Roots, laminae, and petioles were rinsed in deionized water after their harvest. Plant material was then dried. The entire dried petiole was weighed, chopped, and put into 20 mL of 10% acetic acid/0.1 M nitric acid. Dried laminae and roots were chopped with a scissors into 3 mm pieces, and 50 mg of dry tissue was added to 5 mL of 10% acetic acid/0.1 M nitric acid. The acid mixture including plant tissue was heated to 60 °C for several hours. Chloride in the acid solution was then analyzed by using a Buchler Digital Chloridometer (Model 4-2502).

Short-term experiments

Leaves were rooted as described for long-term experiments and then transferred to one-fifth aerated Hoagland #1 solution in 50 L tanks. Rooted leaves were grown in hydroponic solution for 2–4 weeks until uptake experiments were conducted. For uptake experiments rooted leaves were transferred to beakers containing 300 mL of nutrient solution with 25 mM NaCl. Solutions in the beakers were gently aerated, and plants were left to acclimate for 30 min. $^{36}\text{Cl}^-$ (as H^{36}Cl NEN Life Science Products) was added in trace concentrations for quantification of Cl^- uptake (5.7 μm). Uptake measurements conducted under a 1000 watt metal halide lamp were allowed to proceed for 180 min. Rooted leaves were then desorbed for five minutes in 5 mM CaSO_4 , blotted dry, and then separated into roots, petioles, and laminae. Plant tissue was then dried and weighed. Dried plant material was chopped, and the Cl^- in the tissue was extracted into hot water (60 °C) overnight. An aliquot of the extracted solution was then counted using a Beckman LS3801 scintillation counter to determine the amount of Cl^- in the plant tissue. The specific activity of the uptake solution was determined for experiments by measuring 1 mL of uptake solution.

Statistical analyses were performed using JMP from SAS Institute Inc (<http://www.jmp.com/>).

Results

Long-term experiments

The long-term experiments were conducted at 25 and

Table 2. Mean ($\pm\text{SE}$) Cl^- content of rooted leaves grown in 25 mM NaCl. Chloride content is expressed as % of dry weight. Means were compared within a column by a one-way analysis of variance and p values are shown. The number of individual leaves tested for each class are shown along with the number of excluder and non-excluder genotypes tested. See Table 1 for the list of genotypes included in this analysis.

| Class | Number of individual leaves | Cl^- roots ($\pm\text{SE}$) | Cl^- in leaves (laminae and petioles) ($\pm\text{SE}$) |
|-----------------------------|-----------------------------|--|---|
| Excluder ($n = 8$) | 31 | 2.51 \pm 0.51 | 0.46 \pm 0.02 |
| Non-excluder ($n = 7$) | 29 | 2.31 \pm 0.53 | 0.65 \pm 0.04 |
| | | $p = 0.1442$ | $p < 0.0002$ |

Table 3. Mean ($\pm\text{SE}$) Cl^- content of rooted leaves grown in 50 mM NaCl. Chloride content is expressed as % of dry weight. Means were compared within a column by a one-way analysis of variance and p values are shown. The number of individual leaves tested for each class are shown along with the number of excluder and non-excluder genotypes tested. See Table 1 for the list of genotypes included in this analysis.

| Class | Number of samples | Cl^- roots ($\pm\text{SE}$) | Cl^- laminae ($\pm\text{SE}$) | Cl^- petioles ($\pm\text{SE}$) |
|-----------------------------|-------------------|--|--|---|
| Excluder ($n = 8$) | 37 | 4.01 \pm 0.11 | 0.82 \pm 0.07 | 1.20 \pm 0.07 |
| Non-excluder ($n = 6$) | 34 | 3.82 \pm 0.14 | 1.10 \pm 0.08 | 1.69 \pm 0.10 |
| | | $p = 0.28$ | $p = 0.009$ | $p < 0.0002$ |

50 mM NaCl. Plant material from 16 genotypes was used for these experiments (Table 1). For the experiment at 25 mM, the Cl^- content of root samples was not significantly different between genotypes. The analysis of the leaves (laminae and petioles) revealed that the non-excluders contained significantly more Cl^- than the excluders (Table 2). For individual genotypes the standard error was approximately 10% of the mean. At 25 mM NaCl all the excluders contained less Cl^- content in leaves than the non-excluders. For the 50 mM NaCl experiments, no significant differences in root Cl^- content were measured, but significantly higher amounts of Cl^- were found in the laminae and petioles of the non-excluders as compared to the excluders (Table 3). Standard errors for leaf Cl^- content of plants grown at 50 mM NaCl were approximately

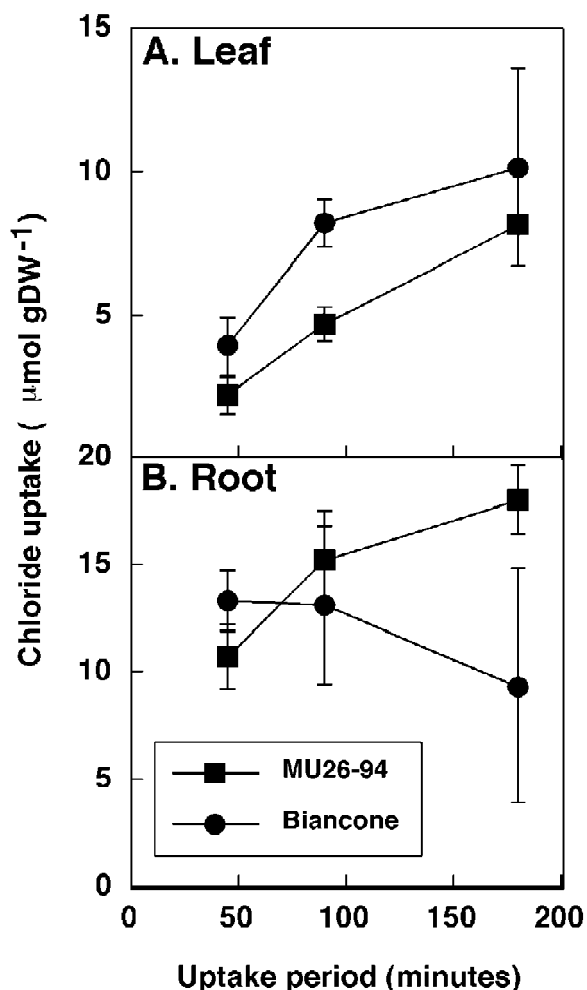


Figure 1. Mean Cl^- uptake rate by root grapevine leaves measured over time using $^{36}\text{Cl}^-$ as a tracer. Each point is the mean of three samples and the standard errors of means are shown. Genotypes were *Vitis vinifera* var. Biancone (non-excluder) and backcross progeny MU26-94 (excluder).

20%. In this experiment, several excluders had similar leaf Cl^- content as the non-excluders. This was attributed to the larger variation imposed by the greater NaCl stress. Analysis of the data showed no relationship between Cl^- accumulation in the tissue and root to shoot ratios. There was also no relationship between leaf chloride content and leaf weight.

Short-term experiments

Experiments were conducted using ^{36}Cl to measure chloride uptake. The results of two representative experiments are shown in Figure 1. In the first set of experiments, Cl^- uptake was measured at 45, 90, and

180 min for Biancone (non-excluder) and the backcross genotype MU26-94 (excluder). Leaf Cl^- content was higher for Biancone as would be expected from a non-excluder, but differences between excluder and non-excluder were only significant at the 90 min time point.

In a second experiment, three backcross genotypes were measured at a single time point (180 min) (Figure 2). The same trends as in the previous experiment were observed for Biancone (non-excluder) and MU26-94 (excluder). However, results for MU27-54 (excluder) showed that this excluder accumulated the same amount of Cl^- as the non-excluder Biancone in short term experiments (Figure 2).

Discussion

In this study, we tested whether rooted leaves could be used for studying Cl^- accumulation in place of whole plants. The results presented showed that rooted leaves maintained the same Cl^- accumulation characteristics as whole plants (Sykes, 1987) in long term experiments. Therefore, we conclude that this system should be feasible for use in a variety of studies related to ion accumulation under saline conditions.

Our results with rooted leaves were not surprising since it was known that grapevine roots, to a large extent, determine the Cl^- accumulation characteristics in grapevines (Downton, 1977). The Cl^- isotope work provided an alternative method for studying ion uptake. In the first set of experiments, it appeared that the results from using these methods closely matched the results from longer time experiments. However, in the second set of experiments the excluding genotype MU27-54 accumulated the same amount of Cl^- as the non-excluding genotype, Biancone. These results highlight some of the potentially erroneous results that may be obtained with short-term studies and suggest that they may be unreliable.

In conclusion, we have shown that the genotypic characteristics of roots from rooted grapevine leaves were similar to those of whole plants in salinity studies (Sykes, 1987) with the most consistent results obtained at 25 mM NaCl. Other applications of this system for woody perennials are conceivable. For example, it should be possible to use this system for molecular and other physiological investigations including uptake studies on additional ions. A similar system has also been successfully used to screen

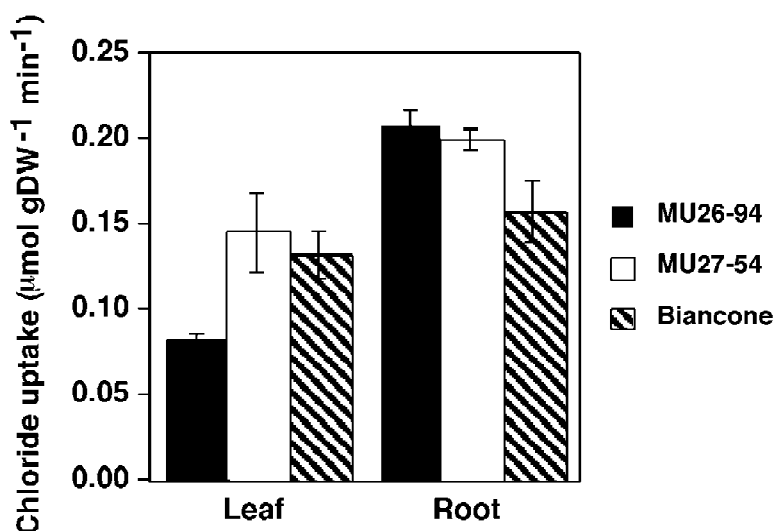


Figure 2. Mean Cl⁻ uptake rate by root grapevine leaves measured over 180 min using ³⁶Cl as a tracer. Each point is the mean of four samples and the standard errors of means are shown. Genotypes were *Vitis vinifera* var. Biancone (non-excluder) and backcross progeny MU26-94 (excluder) and MU27-54 (excluder).

grapevine genotypes for nematode resistance (Cousins and Walker, 2000).

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