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PLANT AND ENVIRONMENT INTERACTIONS

Predicting Perchlorate Uptake in Greenhouse Lettuce from Perchlorate, Nitrate, and Chloride Irrigation Water Concentrations

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Perchlorate (ClO_4^-) has been detected in edible leafy vegetables irrigated with Colorado River water. The primary concern has been the ClO_4^- concentration in lettuce (*Lactuca sativa* L. var. *capitata* L.). There has been a limited number of studies on ClO_4^- uptake, but the interactive effect of other anions on ClO_4^- uptake is not known in detail. We conducted a greenhouse ClO_4^- uptake experiment using two types of lettuce (iceberg and butterhead) to investigate the interaction of uptake of ClO_4^-, Cl^-, and NO_3^- on ClO_4^- uptake under controlled conditions. We examined three concentrations of ClO_4^-, 40, 220, and 400 $nmol_c$/L; Cl^- at 2.5, 13.75, and 25 $mmol_c$/L; and NO_3^- at 2, 11, and 20 $mmol_c$/L. Perchlorate was taken up the most in lettuce when ClO_4^- was the greatest and NO_3^- and Cl^- were lowest in concentration in the irrigation water. More ClO_4^- was detected in leafy material than in root tissue. In general, the outer leaves of iceberg and butterhead lettuce contained more ClO_4^- than did the inner leaves. The results indicate that selective ClO_4^- uptake occurs for green leaf lettuce. A predictive model was developed to describe the ClO_4^- concentration in lettuce as related to the Cl^-, NO_3^-, and ClO_4^- concentration in the irrigation water. Research results can be utilized to elucidate the effect of salts on the accumulation and uptake of ClO_4^- by edible leafy vegetables.

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PERCHLORATE SALTS have been used in rocket propellants, fireworks, and munitions (Smith et al., 2004), resulting in release into the landscape from both manufacturing and utilization. Perchlorate has been detected in surface and groundwater, beverages, baby formula, breast milk, and leafy vegetables. Perchlorate salts are very soluble in water and the ClO_4^- anion is chemically very stable, thus it persists in the environment (Herman and Frankenberger, 1998; Ellington and Evans, 2000; Urbansky et al., 2001; Urbansky, 2002; Kirk et al., 2005; El Aribi et al., 2006). The human health concern is that ClO_4^- is a competitive inhibitor for I^- and thus interrupts thyroid iodine uptake (Sanchez et al., 2007), contributing to hormone disruption and potential perturbations of metabolic activities (Yang and Her, 2011).

Perchlorate has been detected in ground and surface waters in 35 states in the United States. A drinking water standard for ClO_4^- has not been established by the USEPA, but some states have established advisory levels (maximum contaminant levels) (for example, 2 μg/L in Massachusetts, 5 μg/L in New York, 6 μg/L in California, and 14 μg/L in Arizona) (USEPA, 2005; Massachusetts Department of Environmental Protection, 2006; Zhang et al., 2007). Because of its potential toxicity, ClO_4^- was added to the Contaminant Candidate List by the USEPA in 1998 with a reference dose of 0.7 μg kg^{-1} d^{-1} (California Department of Health Services, 2005; Liu et al., 2006; Ju et al., 2008). Perchlorate in the environment is of concern due to the impact on ecosystems, ambiguities about toxicity and health effects, and as an indirect pathway for humans due to the accumulation in vegetables through irrigation water (Susarla et al., 1999; Jackson et al., 2005; Sanchez et al., 2008). Elevated concentrations of ClO_4^- have been detected in plants (Yu et al., 2004; Jackson et al., 2005; Tan et al., 2006). A number of studies have been conducted to investigate ClO_4^- in edible vegetables, especially in cabbage (*Brassica oleracea* L. var. *capitata* L.), other cole crops, lettuce, and spinach (*Spinacia oleracea* L.) affected by Colorado River water (Urbansky, 2000, 2002; Hutchinson, 2004; Sanchez et al., 2005, 2006, 2007, 2009), which is related to ClO_4^- contamination by the ClO_4^- salt manufacturing plant previously located near the Las Vegas wash in Nevada.

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Abbreviations: FW, fresh weight; GOF, goodness-of-fit.

Lettuce is of particular interest because it is the most consumed leafy vegetable in the United States (Sanchez et al., 2005; Seyfferth et al., 2008). The interaction between salts and ClO_4^- in an edible plant when ClO_4^- is taken up by the plant is not fully understood. Perchlorate uptake in three different types of lettuce (green leaf, butterhead, and crisphead lettuce) was reported by Seyfferth et al. (2008). They examined the effects of NO_3^-, SO_4^{2-}, Cl^-, pH, and HCO_3^- independently, concluding that increasing solution NO_3^- markedly decreased ClO_4^- uptake, but increasing Cl^- had a minor effect on ClO_4^- uptake in lettuce leaves. Voogt and Jackson (2010) conducted more detailed research on ClO_4^- uptake and distribution in plants under the presence of I^- and IO_3^-. The results revealed that neither I^- nor IO_3^- affected the uptake of ClO_4^- in butterhead lettuce plants, and the ratio of total I to ClO_4^- was related to the accumulated amount of ClO_4^- in the lettuce plants. When the I^- was supplied as a form of IO_3^-, the uptake of ClO_4^- was less in butterhead lettuce leaves compared with the I^- form (Voogt and Jackson, 2010).

The combined interactive effects of the NO_3^- and Cl^- ions on ClO_4^- uptake in lettuce have not been examined. This consideration is important because both NO_3^- and Cl^- are present at varying concentrations in the soil water during the growing season. Also, the accumulation pattern of ClO_4^- in root tissues has not been thoroughly investigated under the presence of Cl^- and NO_3^- salts in irrigation water. In this experiment, outer leaves were separated from inner leaves for the anion analysis for the following reasons. The outer leaves are subject to transpiration and can be expected to concentrate more salts including ClO_4^- compared with the transpiration of inner leaves, at least for head lettuce. The objectives of this study were to: (i) investigate the influence of Cl^- and NO_3^- concentrations on the uptake of ClO_4^- by lettuce, (ii) examine the ClO_4^- distribution within the plants by measuring the ClO_4^- concentration in both leaf and root parts and determine the pattern of translocation of ClO_4^- within plant materials, and (iii) develop predictive equations to represent ClO_4^- uptake in two lettuce types as related to ClO_4^-, NO_3^-, and Cl^- in irrigation water.

Materials and Methods

Greenhouse Experiment

We utilized a greenhouse facility in Riverside, CA. Thirty sand tanks (each 1.2 by 0.6 by 0.5-m depth) contained washed sand with a bulk density of 1.4 Mg/m^3. We prepared 10 different water compositions and each treatment was replicated three times (Table 1) from three randomly selected sand tanks. The water reservoirs were filled with the Half Hoagland's solution using pumps from the underground tanks to the ground, then the water was circulated through the sand several times to ensure equilibration of the solution with the sand media. Half Hoagland's solution (a plant nutrient solution) was added to each reservoir with the following concentrations: 0.17 mmol/L KH_2PO_4, 0.75 mmol/L $MgSO_4 \cdot 7H_2O$, 2.0 mmol/L KNO_3, and 0.25 mmol/L $CaSO_4$. We used deionized water and prepared the solutions with reagent-grade salts. The pH of the irrigation water ranged between 7.7 and 8.5. The electric conductivity was monitored before and during the experiment. Sorption of ClO_4^- onto the surface of the sand particles and container was determined to be negligible and thus not considered further.

Table 1. Initial concentrations of ClO_4^-, Cl^-, and NO_3^- in irrigation water.

Reservoir no.	ClO_4^-	Cl^-	NO_3^-	Replications
	nmol$_c$/L	——— mmol$_c$/L ———		no.
1	40	2.5	2	3
2	400	2.5	2	3
3	40	25	2	3
4	400	25	2	3
5	40	2.5	20	3
6	400	2.5	20	3
7	40	25	20	3
8	400	25	20	3
9	220	13.75	11	3
10	400	13.75	11	3

We used three concentrations of ClO_4^-: 40, 220, and 400 nmol$_c$/L (approximately 4, 22, and 40 μg/L, respectively); Cl^- at 2.5, 13.75, and 25 mmol$_c$/L; and NO_3^- at 2, 11, and 20 mmol$_c$/L. The experiment was designed as a classic 2^3 factorial design, with a center point and one additional nonstandard point set to high ClO_4^-, medium NO_3^-, and medium Cl^- levels. Seeds of Crispino green iceberg lettuce and Adriana green butterhead lettuce were purchased from Johnny's seeds.

The plants were irrigated twice a day at 0900 and 1300 h using the plant nutrient solution pumped from the underground tanks to the sand tanks, saturating the sand to provide a uniform root zone solution composition. Each irrigation lasted for 45 min, more than sufficient to flush the soil solution in the tanks. After each irrigation cycle, the solutions drained back into the 890-L reservoirs below the sand tanks for subsequent reuse. The ion concentrations in the reservoirs were constantly maintained by supplementing the nutrient concentrations every other week to bring them back to the initial nutrient levels. Water loss by evapotranspiration was replenished by adding deionized water to the reservoirs, thus maintaining constant volumes and osmotic potentials in the irrigation waters in each reservoir.

Iceberg and butterhead lettuce were grown for 113 and 106 d, respectively, under controlled greenhouse conditions as follows: 41% relative humidity, 18°C daytime temperature, and 15°C nighttime temperature. Inner and outer iceberg and butterhead lettuce leaves and root tissue samples from the two lettuce types were harvested from each sand tank at the end of the experiment.

Plant Tissue Processing and Perchlorate Extraction

Plant sample extraction for ClO_4^- followed the method of Seyfferth and Parker (2006). The detailed extraction methods were described by Ha et al. (2011). Perchlorate standard solutions were made from reagent-grade $NaClO_4$ (Aldrich Chemical Co.). Samples were collected in 30-mL high-density polyethylene (Nalgene) bottles and stored at 4°C until analysis.

Analysis

Perchlorate Analysis of Plant and Irrigation Water Samples

Perchlorate was analyzed with an Agilent 1100 series high-performance liquid chromatography/mass spectrometer (HPLC/MS). Detailed descriptions of this method for ClO_4^- analysis have been provided by Snyder et al. (2005) and USEPA (2007). The detailed HPLC settings were described by Ha et al. (2011). An isotopically labeled internal standard was not used.

The response variable in this study was the ClO_4^- concentration in the fresh plant tissue samples (μg/kg FW [fresh weight]). Our method detection limit with the HPLC/MS for ClO_4^- was determined as 0.5 μg/L in the plant extract, equivalent to 1.6 μg ClO_4^-/kg FW of plant tissue. As shown in Table 2, some of the inner leaf and root samples were below the 1.6 μg/kg detection limit and thus treated as left censored in the statistical analysis. Note that all ClO_4^- concentrations in iceberg and butterhead outer leaves were above the detection limit.

Nitrate and Chloride Analysis of Plant Samples and Irrigation Water

Centrifuged and filtered samples (approximately 30 mL) of the plant extracts were also analyzed for NO_3^- and Cl^-. Nitrate concentrations in the plant extract were measured by ultraviolet spectrometry (Cawse, 1967), and Cl^- was determined by coulometric–amperometric titration (Rhoades, 1982).

Statistical Methodology

We conducted a statistical analysis to evaluate the potential factors controlling ClO_4^- uptake in lettuce and additionally to develop equations relating ClO_4^-, NO_3^-, and Cl^- in irrigation water to ClO_4^- concentrations in plant tissue. The linear factorial model (with two-way interaction) was fit to both the ln-transformed leaf and root tissue data:

$$\begin{aligned}
&\ln\left(\mathrm{ClO_4^-}:\mathrm{accum}\right)= \\
&\quad \beta_0+\beta_1\ln\left(\mathrm{ClO_4^-}\right)+\beta_2\ln\left(\mathrm{NO_3^-}\right) \\
&\quad +\beta_3\ln\left(\mathrm{Cl^-}\right)+\beta_{12}\ln\left(\mathrm{ClO_4^-}\right)\ln\left(\mathrm{NO_3^-}\right) \\
&\quad +\beta_{13}\ln\left(\mathrm{ClO_4^-}\right)\ln\left(\mathrm{Cl^-}\right) \\
&\quad +\beta_{23}\ln\left(\mathrm{NO_3^-}\right)\ln\left(\mathrm{Cl^-}\right)+\varepsilon
\end{aligned} \qquad [1]$$

where ε is the error term, representing an independently, identically, and normally distributed error component, while the β parameters quantify the primary (first-order) and two-way interaction terms (Montgomery, 2002). Positive parameter estimates in this model indicate that the ln ClO_4^- levels in the plant tissue increase as the ln-transformed ClO_4^-, NO_3^-, or Cl^- water concentrations increase, while negative estimates indicate that the ln ClO_4^- concentrations decrease as the irrigation water concentrations increase.

The outer-leaf data were all above the detection limit and thus no censoring was required. Equation [1] was estimated for each lettuce variety using standard linear modeling techniques (Montgomery, 2002). For the left-censored inner-leaf and root data, Eq. [1] was estimated using maximum likelihood techniques (Lawless, 1982). All model estimation was performed using the GLM and LIFETEST procedures in SAS (SAS Institute, 1999).

Reduced forms of Eq. [1] were also fit to each plant tissue data set, based on the *p* values associated with the estimated parameters. These reduced models were estimated by removing all nonsignificant parameter estimates from the linear factorial model (at the 0.05 significance level). Goodness-of-fit (GOF) tests were calculated to assess the adequacy of each fitted equation. For the complete (i.e., uncensored) outer leaf data sets, traditional lack-of-fit *F* tests were computed (Montgomery, 2002; Myers, 1986). For the left-censored inner leaf and root data sets, asymptotic GOF tests were computed by calculating the log-likelihood (LL) score differences between the reduced and saturated models and then comparing these −2 LL scores to χ^2 distributions with the appropriate degrees of freedom.

Six statistical data analyses were performed in all, three analyses each for the iceberg and butterhead tissue samples. The primary goal in each analysis was to identify a parsimonious linear factorial model that fully described how the changing water ClO_4^-, NO_3^-, and Cl^- irrigation water concentrations influenced the plant tissue ClO_4^- concentrations.

Results and Discussion

Tan et al. (2006) considered that the sand tank environment may potentially cause ClO_4^- degradation by root bacteria in the root zone. Several researchers have thus utilized an aerated hydroponic system for laboratory-scale plant uptake experiments to minimize the rhizosphere degradation effect on ClO_4^- uptake (Tan et al., 2006; Seyfferth and Parker, 2007; Seyfferth et al., 2008). Commercial leafy vegetables, however, are grown primarily in soil and under field environments. Our experiments were designed to evaluate the combined effect of NO_3^- and Cl^- on ClO_4^- uptake in lettuce in a controlled sand tank environment to partially reflect field conditions. The concentration of ClO_4^- in the reservoirs was monitored and maintained at constant levels (4, 22, and 40 μg/L) throughout the experiment. Perchlorate degradation would not impact our results because we recirculated solutions at a constant ClO_4^- concentration and our irrigation regime was root zone concentrations equal to reservoir concentrations (similar to hydroponic systems). In addition, we found no evidence of a decrease in ClO_4^- related to a soil process, suggesting that, as expected, our rhizosphere was highly aerobic and that ClO_4^- degradation did not need to be further considered in our experiments.

We considered that the interactive effects of three independent variables on ClO_4^- in plant tissue could best be evaluated by multivariate statistical analysis. First, however, we examined a subset of the data consisting of end-member concentrations of

Table 2. Number of left-censored plant tissues for ClO_4^- measurements (i.e., measurements below the 1.6 μg/kg fresh weight method detection limit of ClO_4^-).

Treatment†	Iceberg lettuce		Butterhead lettuce	
	Inner leaves	Roots	Inner leaves	Roots
Low ClO_4^-, low NO_3^-, low Cl^-	3	0	0	0
High ClO_4^-, low NO_3^-, low Cl^-	0	0	0	0
Low ClO_4^-, low NO_3^-, high Cl^-	3	1	1	2
High ClO_4^-, low NO_3^-, high Cl^-	1	0	0	0
Low ClO_4^-, high NO_3^-, low Cl^-	3	0	2	0
High ClO_4^-, high NO_3^-, low Cl^-	2	0	0	0
Low ClO_4^-, high NO_3^-, high Cl^-	3	0	3	3
High ClO_4^-, high NO_3^-, high Cl^-	1	0	0	0
Mid ClO_4^-, mid NO_3^-, mid Cl^-	2	0	0	0
High ClO_4^-, mid NO_3^-, mid Cl^-	0	0	0	0

† Low, mid, and high ClO_4^- represent 4, 22, and 40 μg/L, respectively; low, mid, and high NO_3^- represent 2, 11, and 20 $mmol_c$/L, respectively; low, mid, and high Cl^- represent 2.5, 13.75, and 25 $mmol_c$/L, respectively.

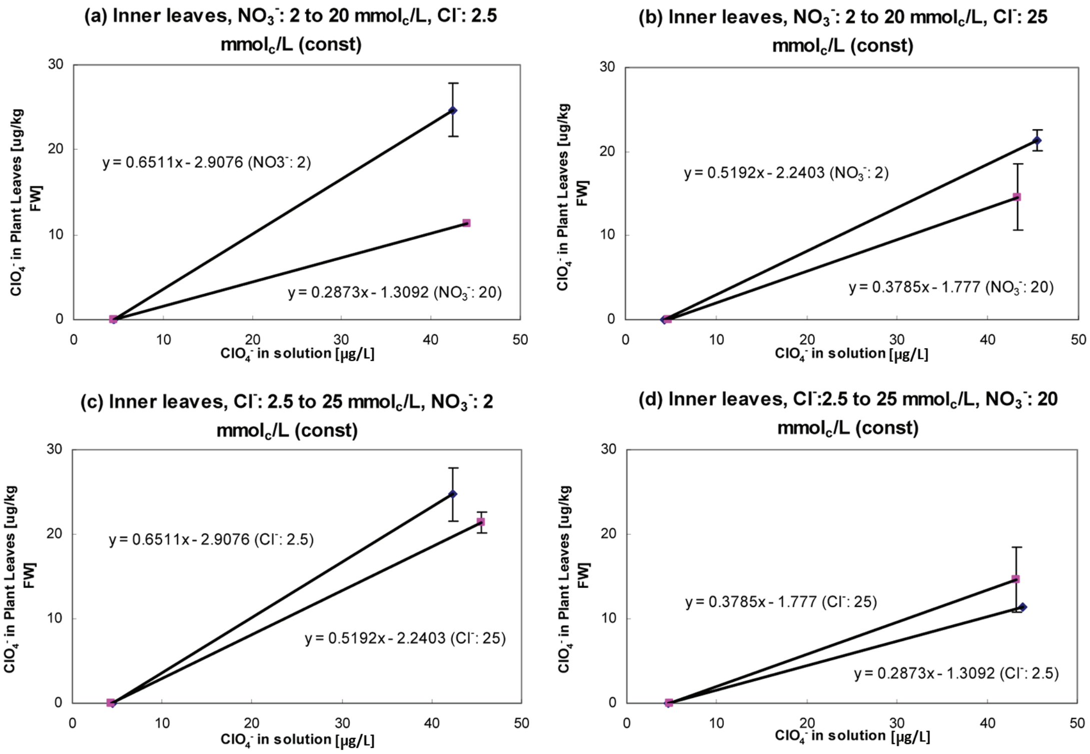

Fig. 1. Perchlorate content in iceberg inner leaves as related to irrigation water NO_3^- and Cl^- concentration at two ClO_4^- concentrations (FW, fresh weight; const, constant). Error bars indicate one standard deviation of the mean where *n* = 3.

ClO_4^- while holding the other two variables constant for both high and low concentrations of NO_3^- and Cl^-. In this manner, we could readily see the impact of the individual variables on ClO_4^- uptake.

Iceberg Lettuce

Perchlorate Uptake as Related to Chloride and Nitrate

Perchlorate uptake in plants and roots as related to the initial ClO_4^- concentration in the irrigation water is shown in Fig. 1 for iceberg lettuce. At low Cl^- and NO_3^-, the ClO_4^- concentration in the leaves accumulated greatly with increasing ClO_4^- in solution (Fig. 1a). The ClO_4^- uptake in iceberg inner leaves was greatly reduced by increasing the NO_3^- concentration from 2 to 20 mmol$_c$/L when the Cl^- concentration was low (Fig. 1a), and uptake was only moderately reduced by NO_3^- when Cl^- was high (Fig. 1b). At constant but low NO_3^-, increased Cl^- moderately reduced ClO_4^- uptake (Fig. 1c). When the NO_3^- concentration was high, an increase in Cl^- did not significantly affect ClO_4^- uptake (Fig. 1d). These results indicate that ClO_4^- uptake is affected more by the NO_3^- concentration variation than Cl^- concentration in irrigation water, but both suppress ClO_4^- uptake.

The ClO_4^- uptake characteristics of iceberg outer leaves are shown in Fig. 2. Outer leaves accumulate much more ClO_4^- than inner leaves under all conditions (compare Fig. 1a with 2a, 1b with 2b, 1c with 2c, and 1d with 2d). This was not unexpected because the outer leaves transpire and thus accumulate salts as additional water and salts continue to flow to these leaves.

Under low Cl^- and NO_3^- concentrations, ClO_4^- reached 900 µg/kg FW in the outer leaves (Fig. 2a), compared with 25 µg/kg in the inner leaves (Fig. 1a). Under the condition of a low Cl^- concentration of 2.5 mmol$_c$/L (Fig. 2a), the ClO_4^- uptake was greatly suppressed with an increase in the NO_3^- concentration from 2 to 20 mmol$_c$/L (Fig. 2a) At high Cl^- concentration, an increase in NO_3^- moderately suppressed ClO_4^- uptake, as shown in Fig. 2b. In a similar manner, when the NO_3^- concentration was low, Cl^- greatly suppressed ClO_4^- uptake (Fig. 2c) but not as much as NO_3^- (Fig. 2a). At high NO_3^-, the Cl^- concentration had no impact on ClO_4^- uptake in the outer leaves (Fig. 2d). This last observation is consistent with the inner leaf data, indicating that ClO_4^- uptake is not sensitive to the Cl^- concentration when the NO_3^- concentration reaches 20 mmol$_c$/L.

Perchlorate uptake patterns in iceberg root tissues had similar trends with those in iceberg inner leaves (data not shown). When the NO_3^- concentration was high (20 mmol$_c$/L) in the irrigation water, ClO_4^- uptake in iceberg roots did not show a difference between low Cl^- (2.5 mmol$_c$/L) and high Cl^- (25 mmol$_c$/L), which is similar to that observed in iceberg outer leaves in Fig. 2d.

Statistical Analysis

Table 3 shows the root mean square error (RMSE) estimates and *p* values associated with the individual factorial parameter estimates for Eq. [1] for each of the six leafy green lettuce data sets. The full factorial model could not be fit to the inner leaf iceberg lettuce data set due to an excessive number of censored observations. Instead, a smaller set of parameters was estimated in this model, i.e., the same set of parameters used in the reduced factorial model fit to the outer leaf iceberg lettuce data set (see Table 4).

As shown in Table 3, the ClO_4^- concentrations in the outer and inner leaves were all significantly dependent on the ClO_4^-, NO_3^-, and Cl^- concentrations in the irrigation water. Only the $\ln(NO_3^-) \times \ln(Cl^-)$ interaction estimate (β_{23}) was significant in the outer leaf iceberg model, however, and none of the interactions were significant in the inner leaf model. In contrast,

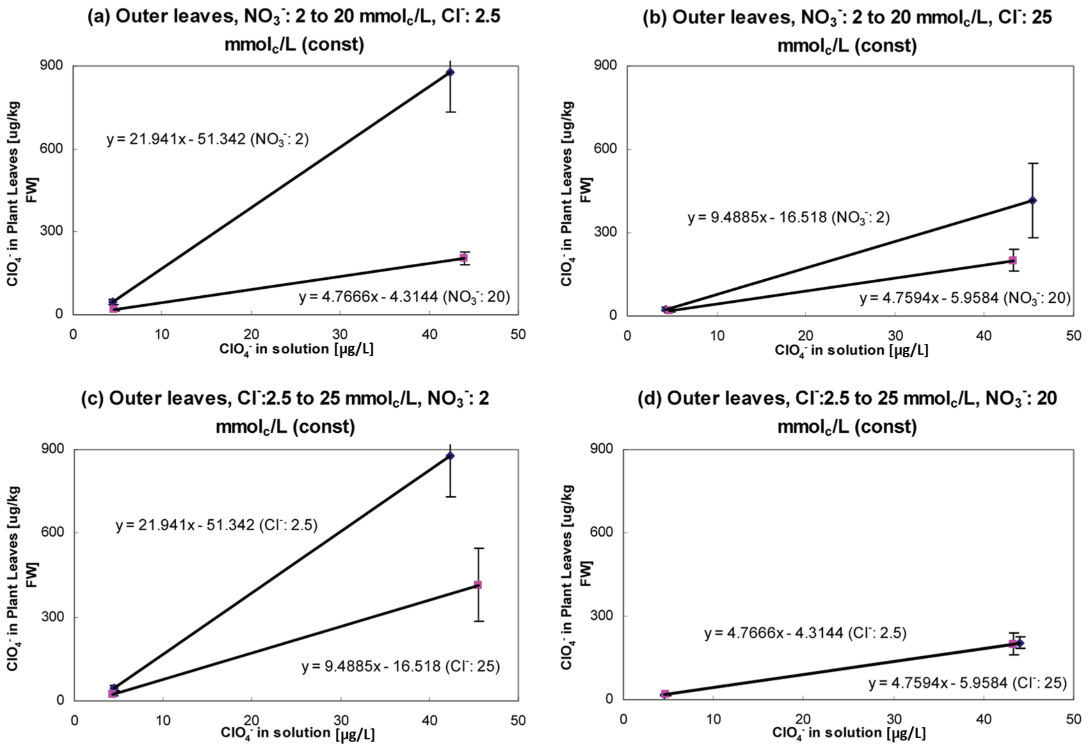

Fig. 2. Perchlorate content in iceberg outer leaves as related to irrigation water NO_3^- and Cl^- concentration at two ClO_4^- concentrations (FW, fresh weight; const, constant).

all of the root interaction estimates were significant, as was the ClO_4^- and Cl^- concentration. These results indicate that the ClO_4^- uptake or accumulation mechanism(s) in the iceberg roots were impacted by the associated NO_3^- or Cl^- concentrations.

Table 3. Summary statistics of root mean square error (RMSE) and parameter *p* values: full factorial models.

Data set	RMSE	Type III *p* values for individual parameter estimates					
		β_1	β_2	β_3	β_{12}	β_{13}	β_{23}
Iceberg lettuce, outer leaves	0.252	<0.001	0.001	0.010	0.228	0.555	0.001
Iceberg lettuce, inner leaves	1.119	0.026	0.009	0.091	NA†	NA	0.055
Iceberg lettuce, roots	0.329	<0.001	0.585	<0.001	0.001	0.017	0.039
Butterhead lettuce, outer leaves	0.328	<0.001	0.184	0.003	0.077	0.424	0.100
Butterhead lettuce, inner leaves	0.432	0.001	0.002	0.040	0.199	0.431	0.512
Butterhead lettuce, roots	0.323	<0.001	<0.001	<0.001	0.207	<0.001	0.019

† NA, not available.

Table 4. Reduced factorial model summary statistics and parameter estimates (with associated standard errors): iceberg lettuce data sets.

Model statistic	Outer leaves	Inner leaves	Roots
RMSE	0.252	1.119	0.329
r (correlation)	0.985	0.759	0.960
Goodness-of-fit *p* value	0.582	0.560	0.912
Parameter estimate			
Intercept	2.948 (0.22)†	−5.318 (4.48)	1.703 (0.46)
$\hat{\beta}_1$	1.166 (0.04)	2.656 (1.19)	0.929 (0.15)
$\hat{\beta}_2$	−0.641 (0.09)	−1.516 (0.58)	0.094 (0.17)
$\hat{\beta}_3$	−0.426 (0.08)	−0.850 (0.50)	−0.723 (0.17)
$\hat{\beta}_{12}$	NE‡	NE	−0.164 (0.05)
$\hat{\beta}_{13}$	NE	NE	0.119 (0.05)
$\hat{\beta}_{23}$	0.135 (0.04)	0.466 (0.24)	0.099 (0.05)

† Standard errors in parentheses.

‡ NE, not estimated.

The pertinent model summary statistics and reduced factorial model parameter estimates for the iceberg lettuce data sets are shown in Table 4. The summary statistics include the RMSE, the correlation between the observed and model-predicted ln(ClO_4^-) accumulation levels (r) for all uncensored observations, and the GOF test p value for each reduced model. For the outer leaf model (where no data were censored), the r statistic is equal to the square root of the usual linear model R^2 value. Additionally, all summary statistics parameter estimates shown in Table 4 correspond to the reduced factorial models; these equations generally excluded all of the nonsignificant parameter estimates (shown in Table 3); however, linear effects have been retained in these reduced models regardless of their reported Table 3 p values if any second-order interaction effects, including these linear effects, were also statistically significant.

The statistically significant estimates in the outer leaf reduced factorial model include the ln-transformed versions of the three main effects and the ln(NO_3^-) × ln(Cl^-) interaction term. The positive ln(ClO_4^-) parameter estimate indicates that increased ClO_4^- concentrations in the irrigation water resulted in higher ClO_4^- accumulation in the outer leaf plant tissue. The negative ln(NO_3^-) and ln(Cl^-) parameter estimates indicate that as NO_3^- and Cl^- levels increased, the ClO_4^- concentration in the plant tissue decreased. Additionally, the positive ln(NO_3^-) × ln(Cl^-) interaction parameter estimate implies that the NO_3^- and Cl^- anions are antagonistic. In other words, high concentrations of both anions did not decrease the ClO_4^- concentration as much as the expected additive effect of each high concentration separately.

A full factorial model could not be fit to the inner leaf data because 18 of the 30 ClO_4^- concentrations were below the detection limit (left censored, see Table 2). Instead, the same reduced factorial model used to describe the outer leaf data was refit to the inner leaf measurements; the corresponding parameter estimates are shown in the middle column of Table 4. The asymptotic standard errors associated with these parameter estimates are fairly large (due to the extensive censoring); thus the χ^2 tests were not always statistically significant (below the 0.05 level). Nonetheless, the anion parameter estimates all exhibit the same general trends (as seen in the outer leaf analysis). The ClO_4^- plant concentration increased as the ClO_4^- water concentrations increased, and plant ClO_4^- decreased as either the ln(NO_3^-) or ln(Cl^-) anion concentration increased. Likewise, the ln(NO_3^-) and ln(Cl^-) depression effects were not entirely additive but rather tended to behave in a slightly antagonistic manner. These results suggest, as expected, that the inner leaf ClO_4^- accumulation mechanism behaves in much the same way as the outer leaf mechanism, except that the ClO_4^- concentration is substantially lower in the inner leaves.

Unlike the inner and outer leaf measurements, a full factorial model needed to be used to adequately describe the ClO_4^- concentration in the root tissue samples. With respect to the main effects, the root ClO_4^- concentration increased as the irrigation water ClO_4^- concentration increased, and the concentration decreased as the Cl^- concentration increased. Additionally, all three anion concentrations interacted with one another. The ln(NO_3^-) × ln(Cl^-) interaction term is again antagonistic. The negative ln(ClO_4^-) × ln(NO_3^-) parameter estimate implies that the ClO_4^- accumulation in the root tissue due specifically to the ClO_4^- water concentration decreased as the NO_3^- concentration increased. Likewise, the positive ln(ClO_4^-) × ln(Cl^-) parameter estimate indicates that the plant ClO_4^- concentration due specifically to the ClO_4^- water concentration increased as the Cl^- concentration increased.

Finally, note that the RMSE estimate for the inner leaf model is about 4.5 times larger than the outer leaf RMSE estimate (1.119 vs. 0.252). This result indicates that the relative variation in ln(ClO_4^-) accumulation in the inner leaves was substantially greater than the relative variation in the outer leaves, even though the ClO_4^- accumulation predominantly occurred in the outer leaves. It should also be noted that all three models exhibited nonsignificant GOF test statistics; these results suggest that these fitted factorial models describe well the outer leaf and root tissue sample data and adequately describe the inner leaf data collected from iceberg lettuce.

Butterhead Lettuce

Perchlorate Uptake as Related to Chloride and Nitrate

Perchlorate concentrations in butterhead inner leaves increased with increasing ClO_4^- in solution. Concentrations were also greatest when Cl^- and NO_3^- water concentrations were low (2.5 and 2 $mmol_c$/L, respectively, Fig. 3a). Also, similar to the ClO_4^- uptake patterns in iceberg inner leaves, ClO_4^- uptake in butterhead inner leaves was more sensitive to the change in NO_3^- concentration when Cl^- was constant than when Cl^- was varied with constant NO_3^- concentration (Fig. 3). There was again little difference in ClO_4^- uptake related to an increase in Cl^- concentration when the NO_3^- concentration was high (Fig. 3d).

Butterhead outer leaves also concentrated more ClO_4^- than butterhead inner leaves (compare Fig. 4 with Fig. 3), as observed with iceberg lettuce. We noted that ClO_4^- uptake in plant leaves was consistently greatest when Cl^- and NO_3^- concentrations were low (2.5 and 2 $mmol_c$/L, respectively), as shown in Fig. 4a. When the Cl^- concentration was low (2.5 $mmol_c$/L), an increase in NO_3^- concentration in the irrigation water resulted in much less ClO_4^- accumulation in butterhead outer leaves (Fig. 4a). In contrast, under high Cl^- concentration, the increase in NO_3^- had a smaller impact on ClO_4^- accumulation, as shown in Fig. 4b. This also indicates that ClO_4^- uptake was affected by both Cl^- and NO_3^- in the irrigation water.

Similar to the suppression of ClO_4^- accumulation with increasing NO_3^- at constant Cl^-, there was suppression of ClO_4^- accumulation when the NO_3^- concentration was constant and the Cl^- concentration was increased (Fig. 4c). Perchlorate uptake patterns in butterhead outer leaves were not affected by increasing Cl^- concentration when the NO_3^- concentration was high and constant at 20 $mmol_c$/L (Fig. 4d). This trend was also observed for ClO_4^- uptake in iceberg outer leaves for high NO_3^- concentration with various Cl^- concentrations (Fig. 2d). These results indicate that ClO_4^- uptake in iceberg and butterhead outer leaves was not affected by the presence of Cl^- when the NO_3^- concentration was as high as 20 $mmol_c$/L. A similar observation was obtained for ClO_4^- uptake in iceberg and butterhead inner leaves.

Perchlorate uptake in butterhead roots was not affected by the concentration change in NO_3^- when Cl^- was high (25 $mmol_c$/L) (data not shown). This was observed for the first time because ClO_4^- uptake in plant leaves or root tissues has not been

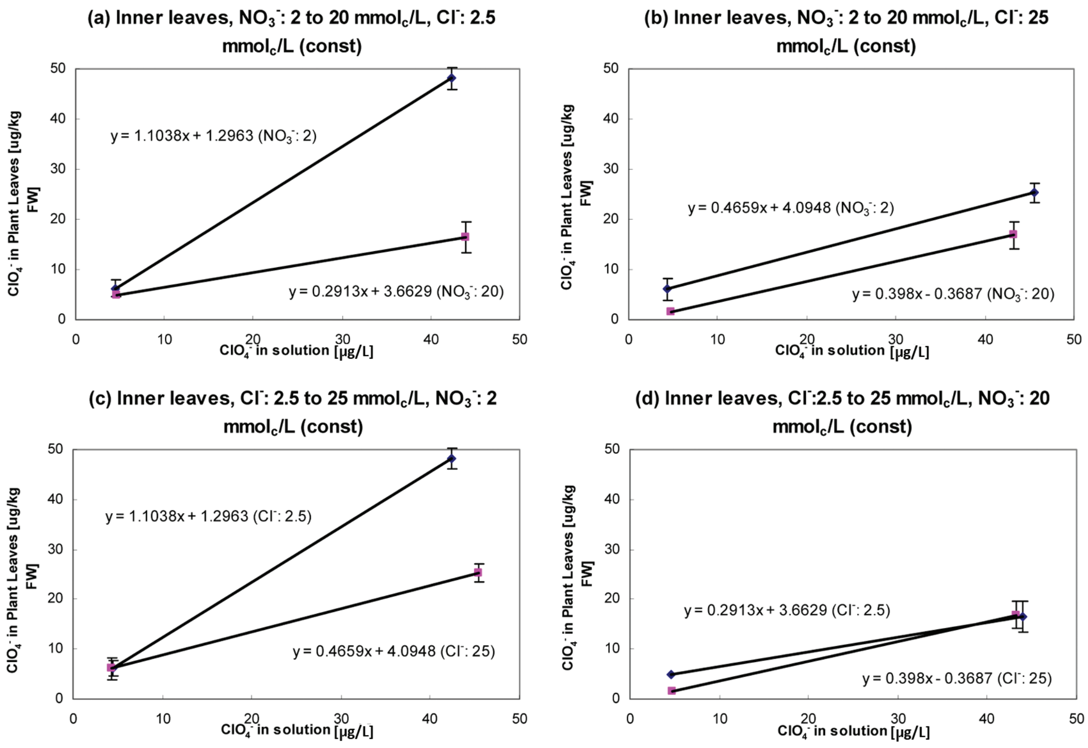

Fig. 3. Perchlorate content in butterhead inner leaves as related to irrigation water NO_3^- and Cl^- concentration at two ClO_4^- concentrations (FW, fresh weight; const, constant).

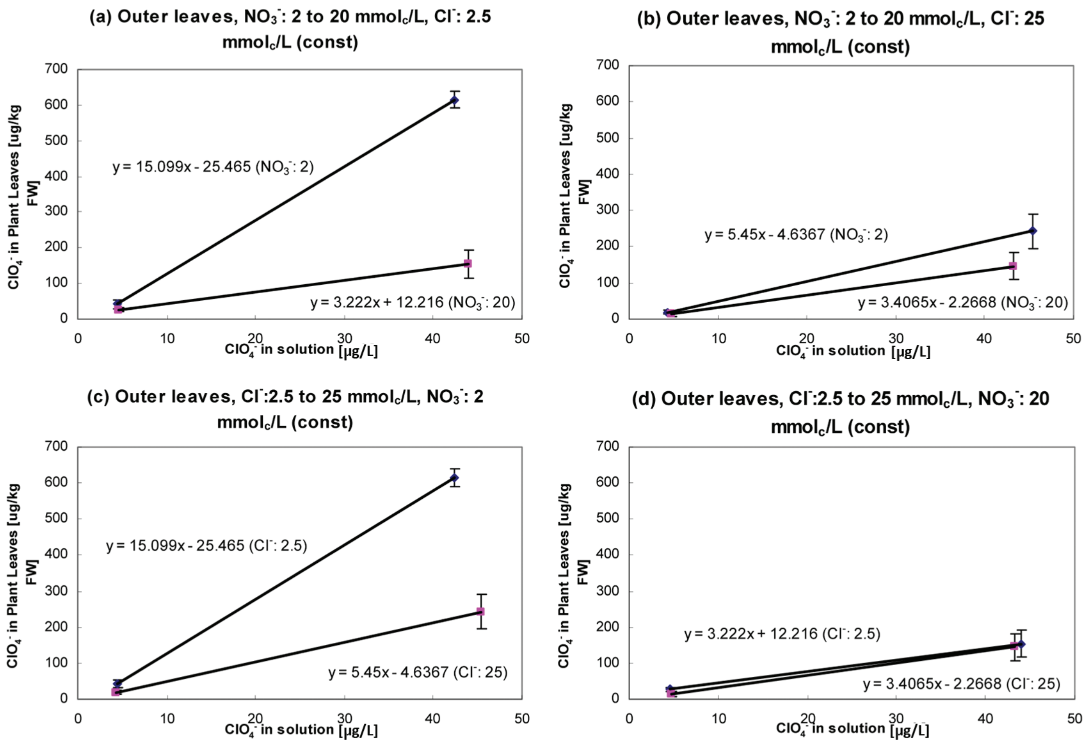

Fig. 4. Perchlorate content in butterhead outer leaves as related to irrigation water NO_3^- and Cl^- concentration at two ClO_4^- concentrations (FW, fresh weight; const, constant).

examined before under variable NO_3^- and high Cl^-. Also, ClO_4^- uptake does not appear to be sensitive to changes in the Cl^- concentration when NO_3^- is high. Therefore, just as in the case of ClO_4^- uptake in butterhead leaf tissues, ClO_4^- uptake patterns did not change with variations in Cl^- or NO_3^- concentrations when NO_3^- or Cl^- was present in high concentrations in the irrigation water (20 and 25 $mmol_c$/L, respectively).

Statistical Analysis

As shown in Table 3, none of the ion interaction estimates were significant in the butterhead leaf models, but the ClO_4^- × Cl^-

and $NO_3^- \times Cl^-$ interactions were significant for the butterhead root data. The individual ion estimates were significant for all but NO_3^- in the inner leaves, which had a *p* value of 0.184 (Table 3).

The summary statistics parameter estimates shown in Table 5 correspond to the reduced factorial models; the equations generally exclude all of the nonsignificant parameter estimates (shown in Table 3). The only statistically significant parameter estimates in the outer leaf reduced factorial model were the three main effects, as shown in Table 5. The positive $\ln(ClO_4^-)$ parameter estimate confirms that increased ClO_4^- concentrations in the irrigation water resulted in higher ClO_4^- accumulation levels in the outer leaf plant tissue. Likewise, the negative $\ln(NO_3^-)$ and $\ln(Cl^-)$ parameter estimates confirm that as either the NO_3^- or Cl^- levels increased, the ClO_4^- accumulation level in the plant tissue decreased.

The same general trends seen in the outer leaf data manifested in the inner leaf measurements; the corresponding parameter estimates are shown in the middle column of Table 5. The $\ln(ClO_4^-)$ accumulation levels increased as the $\ln(ClO_4^-)$ concentration levels increased and decreased as either the $\ln(NO_3^-)$ or $\ln(Cl^-)$ concentration levels increased. As with the outer leaf measurements, there were no statistically significant interaction effects among any of these anions. These results confirm that the inner leaf ClO_4^- accumulation mechanism behaves essentially in the same way as the outer leaf mechanism. The actual magnitude of accumulated ClO_4^- was moderately lower in the inner than the outer leaves.

As with the iceberg lettuce data, a more complicated factorial model needed to be used to adequately describe the $\ln(ClO_4^-)$ accumulation pattern in the root tissue samples. With respect to main effects, the $\ln(ClO_4^-)$ concentration increased as the $\ln(ClO_4^-)$ concentration increased, and the root ClO_4^- concentration decreased as both the $\ln(NO_3^-)$ and $\ln(Cl^-)$ concentrations increased. Additionally, $\ln(Cl^-)$ concentrations interacted with the $\ln(ClO_4^-)$ and $\ln(NO_3^-)$ concentrations. The $\ln(NO_3^-) \times \ln(Cl^-)$ interaction term was again antagonistic. Additionally, the positive $\ln(ClO_4^-) \times \ln(Cl^-)$ parameter estimate implies that the $\ln(ClO_4^-)$ accumulation in the root tissue attributed specifically to the $\ln(ClO_4^-)$ concentration level increased as the $\ln(Cl^-)$ concentration increased.

In the butterhead leaf tissue samples, the RMSE estimate for the inner leaf model was about 1.5 times as large as the outer leaf RMSE estimate (0.445 vs. 0.328). This result suggests that the relative variation in $\ln(ClO_4^-)$ accumulation in the inner leaves was marginally greater than the relative variation in the outer leaves, although once again ClO_4^- accumulation occurred more noticeably in the outer leaves. Like the iceberg models, all three butterhead models also exhibited nonsignificant GOF test statistics. These results indicate that these fitted factorial models adequately describe the outer leaf, inner leaf, and root tissue sample data collected from the butterhead lettuce crops.

Ion Uptake and Translocation

Ion competition, such as the process between Cl^- and NO_3^-, is known to be significant for plant uptake and crop production (Marschner, 1995). The ion competition of individual variables on lettuce has been previously examined. It is evident that the highest ClO_4^- concentrations in plant leaves and roots in this current study were obtained when NO_3^- and Cl^- were as low as 2 and 2.5 $mmol_c$/L, respectively, in the irrigation water. When NO_3^- and Cl^- concentrations in the irrigation water were increased to 20 and 25 $mmol_c$/L, respectively, the ClO_4^- uptake in plant leaves and roots was minimized. It also appears that the ClO_4^- concentration range selected for study affects the results. Phytoremediation studies were usually conducted at much higher solution concentration of ClO_4^- (mg/kg level, approximately between 5 and 2000 mg/kg; Nzengung et al., 1999, 2004), compared with ClO_4^- uptake experiments relevant to edible vegetables (μg/kg level; Seyfferth and Parker, 2008).

To compare uptake of different anions and evaluate ion-specific mechanisms, it is useful to calculate the ratios of concentrations in the plant to concentrations in the irrigation water. In this instance, we calculated ratios of ClO_4^- and Cl^- in the plant leaves and roots to the ion concentrations in the irrigation water.

The relative uptake of Cl^- and ClO_4^- by iceberg lettuce is shown in Fig. 5. The ratios (concentration factors) are expressed

Table 5. Reduced factorial model summary statistics and parameter estimates (with associated standard errors): butterhead lettuce data sets.

Model statistic	Outer leaves	Inner leaves	Roots
RMSE	0.328	0.445	0.330
r (correlation)	0.971	0.886	0.949
Goodness-of-fit *p* value	0.078	0.194	0.097
Parameter estimate			
Intercept	2.715 (0.23)†	0.672 (0.30)	3.102 (0.40)
$\hat{\beta}_1$	1.023 (0.06)	0.972 (0.08)	0.674 (0.12)
$\hat{\beta}_2$	−0.313 (0.06)	−0.422 (0.08)	−0.548 (0.11)
$\hat{\beta}_3$	−0.299 (0.06)	−0.231 (0.08)	−1.433 (0.18)
$\hat{\beta}_{12}$	NE‡	NE	NE
$\hat{\beta}_{13}$	NE	NE	0.251 (0.05)
$\hat{\beta}_{23}$	NE	NE	0.126 (0.05)

† Standard errors in parentheses.

‡ NE, not estimated.

as liters per kilogram FW from micrograms per kilogram FW divided by micrograms per liter for ClO_4^- and grams per kilogram FW divided by grams per liter for Cl^-. Under low Cl^-, NO_3^-, and ClO_4^- in the irrigation water, the tissue concentrated Cl^- to a somewhat greater extent than it concentrated ClO_4^-, suggesting that there may be an active Cl^- uptake or transfer mechanism (Fig. 5a). This trend was also observed with spinach leaves (Ha et al., 2011). When NO_3^- and Cl^- were set to 2 and 2.5 mmol_c/L, the range of concentration factors for the iceberg outer leaves was 10 to 20 L/kg FW, the range for the butterhead outer leaves was about 9 to 15 L/kg FW (data not shown), and 78 to 87 L/kg FW for spinach leaves (Ha et al., 2011). Spinach leaves tend to accumulate much more ClO_4^- than lettuce leaves and this reveals that lettuce leaves accumulate much less ClO_4^- than spinach under the same experimental conditions. Seyfferth and Parker (2007) reported concentration factors for whole-head crisphead lettuce as 17 to 22 L/kg FW (approximately 30–42 L/kg FW for crisphead outer leaves) and for whole-head butterhead lettuce, 8 to 18 L/kg FW (14–28 L/kg FW for butterhead outer leaves). In general, our concentration factors appeared to be less than those of Seyfferth and Parker (2007). As another example, the concentration factors for the iceberg outer leaves from the field were 0.4 to 29.0 L/kg FW and those for inner leaves from the field were 0.01 to 18.0 L/kg FW (data not shown). Concentration factors for outer leaves were always higher than those for inner leaves, as observed from the greenhouse data. Comparison of the plant/solution ratios in Fig. 5b, in contrast to Fig. 5a, shows that high Cl^- in solution suppressed both Cl^- and ClO_4^- uptake, providing evidence of both ClO_4^- and Cl^- exclusion. This was also observed with butterhead lettuce (data not shown). In the case of spinach leaves, however, increasing Cl^- concentration in the irrigation water gave much higher ClO_4^- accumulation in the spinach leaves, with concentration factors of 179 to 184, compared with the low NO_3^- and Cl^- conditions (Ha et al., 2011). In the case of relative uptake, the larger ClO_4^- ratios in Fig. 5b compared with Cl^- indicate that ClO_4^- accumulation is not as restricted as it is for Cl^-. This was also observed with the butterhead lettuce (data not shown) and spinach leaves (Ha et al., 2011). This again indicates that the uptake of these ions is not interchangeable and that different mechanisms must be operational.

Increased NO_3^- suppresses both Cl^- and ClO_4^- accumulation in lettuce leaves, as shown by a comparison of the leaf/solution ratios shown in Fig. 5a and 5c. Again, the same ClO_4^- accumulation pattern was observed for butterhead lettuce (data not shown) and spinach leaves (Ha et al., 2011). The difference in the concentration ratios between Fig. 5a and 5c suggests that NO_3^- is more effective in suppressing ClO_4^- accumulation than Cl^- at comparable concentrations. The butterhead leaves had somewhat less relative accumulation of all ions than the iceberg leaves (data not shown).

The increase in NO_3^- also suppressed the concentration factors for both ClO_4^- and Cl^- in the root (compare Fig. 6a with 6c). As with the leaf data, the concentration ratios at high NO_3^- were lower than those under low NO_3^- but the relative proportions were similar (compare Fig. 6a with 6c). These data suggest that NO_3^- suppressed Cl^- and ClO_4^- accumulation in the roots in a similar manner and that high Cl^- and high NO_3^- were about equal in terms of suppressing ClO_4^- accumulation.

Student's paired *t*-test with two-tailed distribution was conducted with the ClO_4^-/ClO_4^- and Cl^-/Cl^- ratio data. Results from treatments with high NO_3^- and high Cl^- showed no significant difference between ClO_4^-/ClO_4^- and Cl^-/Cl^- ratio data with a 95% confidence interval. All the results of the *t*-tests for Fig. 5 and 6 indicated that there were significant differences between the ClO_4^-/ClO_4^- and Cl^-/Cl^- ratio data (data not shown).

Interestingly, the accumulation ratios in butterhead lettuce roots were almost identical to those for iceberg lettuce roots

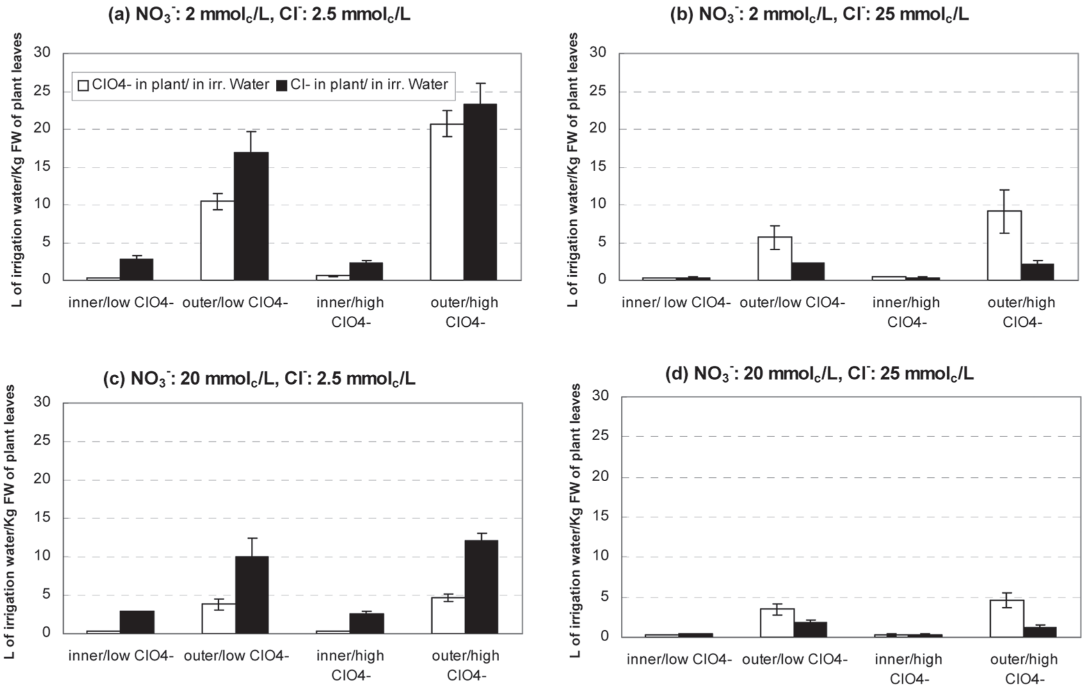

Fig. 5. Perchlorate and Cl^- concentration in inner and outer leaves of plants divided by concentration in solution (irrigation water) for iceberg lettuce leaves (FW, fresh weight). Error bars represent one standard deviation of the mean where *n* = 3.

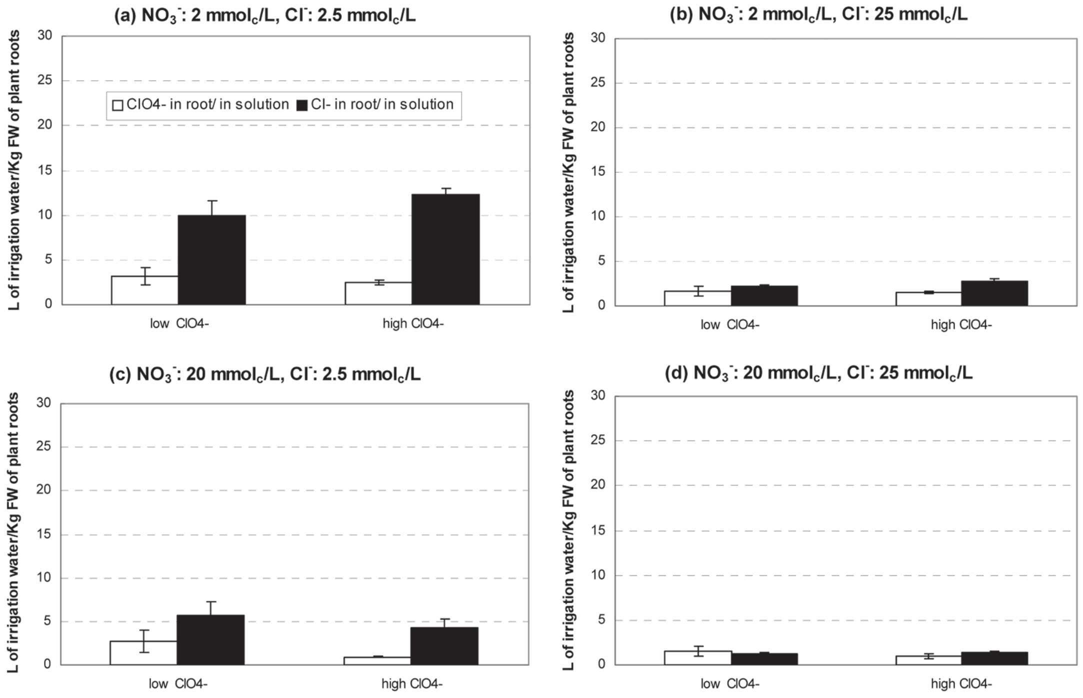

Fig. 6. Perchlorate and Cl$^-$ concentration in plants divided by concentration in solution for iceberg lettuce roots (FW, fresh weight).

(data not shown). Although it has been known that plant roots do not appear to take up ions selectively without having specific transporters for specific ions (Marschner, 1995; Seyfferth et al., 2008), our results clearly indicated that there was a different uptake pattern of ClO_4^- and Cl^- in roots among the different anion concentrations in the irrigation waters. Tan et al. (2006) also mentioned that no statistically significant effect of NO_3^- (up to 35 mmol/L) was found on ClO_4^- (1000 nmol/L) uptake in smartweed [*Persicaria hydropiper* (L.) Delarbre]. In the case of the ClO_4^- uptake in spinach roots when NO_3^- and Cl^- were set to low concentrations of 2 and 2.5 mmol$_c$/L, respectively, less ClO_4^- appeared to be taken up by spinach roots than the iceberg and butterhead lettuce roots. Concentration factors of ClO_4^- ranged from 1.6 to 2.4 L/kg FW (Ha et al., 2011), compared with 2.5 to 3.2 L/kg FW for the iceberg lettuce roots and 3.3 to 4.5 L/kg FW for the butterhead lettuce roots (data not shown). Therefore, ClO_4^- exclusion occurred in spinach roots. The concentration factors for uptake of Cl^- in spinach roots were 17 to 24 L/kg FW (Ha et al., 2011), however, but 10 to 12 and 11 to 17 L/kg FW for iceberg and butterhead lettuce roots, respectively. Spinach roots accumulated more Cl^- than lettuce roots. In this case, concentration factors of Cl^- were not reduced much by the increment of NO_3^- concentration, indicating that Cl^- uptake by spinach roots was affected more by the ClO_4^- concentration in the irrigation water. This is opposite to the uptake trend of ClO_4^- in lettuce and spinach leaves.

In this study, the amount of ClO_4^- taken up by lettuce inner leaves was the highest when NO_3^- and Cl^- had the lowest available concentration in the irrigation water according to comparisons of ClO_4^- uptake in iceberg inner leaves (Fig. 1) and butterhead lettuce inner leaves (Fig. 3). This implies that ClO_4^- uptake was affected by the presence of NO_3^- and Cl^- in the irrigation water in this study. Chloride and NO_3^- compete each other for Cl^- uptake in barley (*Hordeum vulgare* L.; Marschner, 1995). Seyfferth and Parker (2006) determined that the ClO_4^- uptake mechanism was affected by NO_3^- in plant tissues by sharing a common anion transport mechanism in higher plants. Perchlorate uptake in iceberg and butterhead lettuce was affected by NO_3^-, as reported earlier (Seyfferth et al. [2008] from their hydroponic growth chamber system); however, Cl^- suppressed ClO_4^- uptake as much as did NO_3^- in this study, in contrast to Seyfferth et al. (2008), who found a relatively minor effect. The uptake of ClO_4^- in lettuce leaves and roots was not affected much by the presence of NO_3^- and Cl^- when both NO_3^- and Cl^- were as high as 20 and 25 mmol$_c$/L, respectively. This result implies that higher NO_3^- and Cl^- concentrations in the irrigation water affect the uptake of ClO_4^- in plants, which also means that the uptake of anions in a plant transport mechanism is concentration dependent. Limited uptake channels from the roots to the plant tissues appear to exist.

General Trends and Conclusion

The following common trends emerge from the results across the six data sets for lettuce: (i) the $\ln(ClO_4^-)$ leaf accumulation trends always increased as the $\ln(ClO_4^-)$ and $\ln(Cl^-)$ anion levels increased and the $\ln(NO_3^-)$ decreased; (ii) the $\ln(ClO_4^-)$ accumulation levels were much greater in the outer leaf tissue samples of both lettuce types compared with the inner leaves; and (iii) there are relatively few statistically significant anion interactions in the leaf $\ln(ClO_4^-)$ accumulation models. In contrast, the root $\ln(ClO_4^-)$ accumulation models are more complex, exhibiting more statistically significant anion interaction parameter estimates, although the main effect trends are consistent across both the root and leaf tissue samples. We note that when statistically significant interaction effects occurred in any of the six fitted models, the signs of these parameter estimates were always consistent. This suggests that the $\ln(ClO_4^-)$ accumulation mechanisms behave in a somewhat similar and consistent manner across the two lettuce types.

The elevated ClO_4^- concentrations of the outer leaves were in contrast to the lower concentrations of the inner leaves. It appears that at least part of the large variability in lettuce ClO_4^- concentrations observed under field conditions and reported in earlier studies of ClO_4^- concentrations in market samples is related to the extent of trimming of outer leaves and the degree to which the outer leaves open and experience transpiration. As with spinach, reported in an earlier study (Ha et al., 2011), the ClO_4^- uptake was affected by the concentrations of competing ions. The extent of competition of these ions with ClO_4^- varied depending on the concentration of other ions as well. Predictive equations describing the ion interactions were in general satisfactory for representation of the data.

Acknowledgments

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