Annual Leafy Greens Research Report

For the Period April 1, 2016 to March 31, 2017

Project Title: Developing Baby Leaf Spinach with Lower Cadmium Uptake

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Abstract
The Salinas Valley has areas with anomalously high cadmium (Cd) in bedrock and agricultural soils. Spinach is an agronomically important crop in the Salinas Valley and a known hyper-accumulator of Cd (Majmundar 1980, Alia et al. 2015). Because Cd accumulation can cause health risks, understanding the genetic mechanisms by which spinach accumulates Cd so that new varieties with reduced Cd content can be developed is an important goal for spinach production. Our long-range objective is to develop superior cultivars with reduced Cd uptake. In the first year, we evaluated 625 spinach accessions from germplasm collections for Cd uptake in a soil-based greenhouse assay, with duplicate testing of 95 selections. We found a 3-fold difference in Cd accumulation ranging of 5.4 to 14.4 ppm Cd content on a dry weight basis. In addition, we identified 50 candidate genes associated with Cd accumulation and seven candidate molecular markers development, and began steps for genotyping tested accessions. Next, we will determine DNA sequence diversity in candidate genes associated with low cadmium accumulation in leaves and conduct an expression analysis to confirm the relationship between alleles at these genes and the low Cd phenotype. We will then begin hybrid development between low Cd lines and breeding populations as a first step to incorporate the trait into cultivars and to enable genetic mapping of genes associated with Cd uptake.

Objectives:
Our long-range objective of this project is to develop superior spinach cultivars that have reduced cadmium (Cd) uptake. Our immediate objectives are as follows:

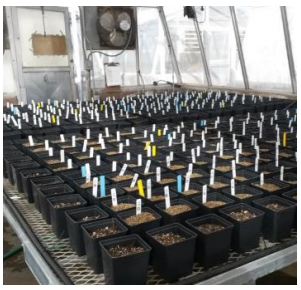
1. Evaluating ~500 spinach accessions from US and international genebanks, including commercial cultivars, for Cd uptake in a soil-based greenhouse assay.
2. Genotyping germplasm used in Objective 1 for DNA sequence diversity in candidate genes associated with low cadmium accumulation in leaves and expression analysis to confirm relationship between alleles at these genes and the low Cd phenotype
3. Developing hybrids between low Cd lines and breeding populations as a first step to incorporate the trait into cultivars and to enable genetic mapping of genes associated with Cd uptake.

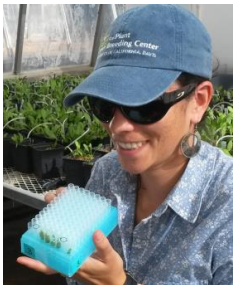
Procedures:
Objective 1
We previously obtained over 700 accessions across three germplasm collections (USDA-National

Clonal Germplasm Collection, CGN-Netherlands, and IPK-Germany) as well as commercial cultivars (F1 hybrids and open pollinated). Due to limited seed availability of the accessions, germplasm screening trials were conducted in the greenhouse rather than the field. We maintain a greenhouse dedicated to spinach breeding with space for screening and cages for making controlled crosses. Soil from two locations in the Salinas valley (north of King City and another south of Greenfield), previously shown to have high Cd levels of roughly 1.5 and 3.0 ppm, respectively, were selected for a *proof of concept* greenhouse trial in the summer of 2016. Soil was prepared as shown below by wetting, mixing, and sieving to 4mm.




Ten commercial hybrid spinach cultivars were selected based on preliminary Cd data from collaborators (Table 1) (unpublished data from Richard Smith, UC Cooperative Extension farm advisor, Monterey Co.). A split plot design was used to test two soils and 10 commercial cultivar entries in two blocks, which were split into 2 sub-blocks by soil Cd level, and randomized. Four-inch azalea pots were filled with soil (either 1.5 or 3.0 ppm Cd) and 10 seeds were sown in each pot with ¼ tsp D45 slow release fertilizer applied to the soil surface. Pots were watered daily, thinned to a maximum of 6 seeds per pot after germination, and grown for 6 weeks. Leaves and petioles were harvested, dried in a drying oven at 60C, and sent to the UC Davis Analytical Lab where samples were ground and total Cd was extracted by nitric acid/hydrogen peroxide digestion. Cd concentration was determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). Based on results from this trial, commercial cultivars Unipack 12 and Seaside were selected to use as low and high checks, respectively. The photos below show various stages of these processes.





Entry	Cultivar	Expected Cd
101	Emilia	low
102	Shelby	low
103	Sparrow	low
104	Unipack 12	low
105	Whale	low
106	Yukon	low
107	Carmel	med
108	Camaro	high
109	Seaside	high
110	Riverside	high

Table 1. Commercial hybrid cultivars included in a *proof of concept* greenhouse pre-trial with expected cadmium levels when grown in high cadmium soil.

Field soil with 3.0 ppm total Cd was used for the first spinach germplasm screening trial in the fall of 2016. Eighty gallons of soil were collected and prepared as previously described. To cover a wide range of genetic diversity, 360 accessions from three spinach germplasm collections (USDA, Germany, and Netherlands) were selected based country of origin, improvement status, and name (Tables 2 and 3). An alpha lattice block design was used with 20 blocks, each containing the two commercial checks (Seaside and Unipack 12) and 18 unique entries for a total of 20 pots per block and 400 pots in all. Commercial checks were randomized within each block. Four-inch diameter by 4.5-inch tall square pots were filled with prepared soil and 16 seeds were sown in each pot and covered with ¼ inch of vermiculite and ½ tsp D45 slow release fertilizer was applied to the surface. The trial was laid out across two 6' x 10' greenhouse benches with 10 blocks per bench. Border pots sown with a commercial cultivar were placed around all bench edges to reduce edge effects. Pots were watered as needed, thinned to a maximum of 6 seeds per pot after germination, and grown for 5 weeks. Leaf discs were collected from each plant (up to 6 per pot), pooled by entry into microtubes (70 mg each), lyophilized in a freeze dryer, and stored for DNA extraction and genotyping. Duplicate tissue samples were collected of each entry. At 5 weeks post sowing, plants (leaves with petioles) were harvested, dried in a drying oven at 60C for one week, and sent to the UC Davis Analytical Lab for cadmium analysis.

Country of Origin (No. of entries)							
18	Afghanistan	28	Germany	2	Mongolia	2	Sweden
5	Albania	6	Greece	1	Montenegro	5	Syria
1	Azerbaijan	1	Hong Kong	4	Nepal	3	Taiwan
9	Belgium	10	Hungary	23	Netherlands	1	Thailand
21	China	5	India	3	Pakistan	2	Tunisia
1	Croatia	10	Iran	1	Poland	42	Turkey
1	Denmark	3	Iraq	1	Romania	2	Turkmenistan
3	Egypt	6	Italy	7	Russian Federation	2	United Kingdom
2	Ethiopia	16	Japan	1	Serbia	27	United States
5	France	7	Korean Peninsula	1	Slovakia	1	Uzbekistan
7	Georgia	17	Macedonia	3	Spain	44	unknown

Table 2. Fall 2016 germplasm screening number of entries by country of origin as listed in germplasm collection passport data.

	Improvement Status (No. of entries)
24	advanced cultivar
83	cultivar
11	cultivated material
33	Traditional cultivar/Landrace
8	Wild material
201	unknown

Table 3. Fall 2016 germplasm screening number of entries by improvement status as listed in germplasm collection passport data.

Statistical analysis of Cd analytical results was completed in R fitting a linear mixed-effects model with entries as fixed effects and blocks as random effects. Fixed effects estimates were extracted and entries were ranked by adjusted Cd values. Statistical analysis of only checks was completed by treating checks as a randomized complete block design using the Tukey method to identify Cd levels above the 95% confidence interval (CI) for the high check and below the 95% CI for the low check. Accessions that fall outside the identified range (above or below the 95% CI of the high and low check, respectively) for Cd content were selected for re-testing. Seventy-four entries were selected below the low check and 21 entries were selected above the high check for a total of 95 entries for re-testing.

A second germplasm screening trial was completed in the spring of 2017 in the same manner with 95 selections from the fall trial plus 265 untested accessions from the 3 germplasm collections. The planting was divided into two sets with the second bench planted 2 weeks after the first bench (10 blocks per bench). The first bench was harvested at 5 weeks post sowing (as was done in the fall); however, many plants were bolting due to the increasing day length. To avoid bolting, the second bench was harvested at 4 weeks post sowing. Tissue for DNA was collected and sample preparation for cadmium analysis was completed as previously described. Samples were submitted to the analytical lab in June 2017 and results are expected in one month.

Objective 2.

The Van Deynze lab has sequenced the spinach genome *de novo* with high-accuracy assembly using PacBio whole genome sequencing (Pacific Biosciences of California, Inc., Menlo Park, CA) (unpublished, 2016). To identify candidate genes conferring reduced Cd uptake, sequestration, storage, and translocation in other plant species, a detailed literature search of Cd regulation genes across multiple species was conducted. Roughly 50 candidate genes involved in cadmium regulation, sequestration, transport, translocation, detoxification, and tolerance were identified across multiple gene families. Protein sequences were obtained for these genes across multiple species from the NCBI database (www.ncbi.nlm.nih.gov). Using the CLC Genomics Workbench 9.5.3 (www.qiagenbioinformatics.com), the tBLASTn tool was used to search for homologs of these sequences in the spinach genome. Top blast hits will be selected and used to design customized baits for a Mybaits® target enrichment genotyping array (funded by the California Spinach Program Committee, not further described here). To obtain preliminary results, tBLASTn results of two candidate genes were manually curated and used to identify seven single nucleotide polymorphisms (SNPs) within these genes using a previously developed spinach SNP discovery pipeline (Van Deynze and Ashrafi, unpublished).

DNA extractions from the 625 screened accessions are currently underway using the NucleoSpin® 96 Plant II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Protocol modifications include increasing the lysis buffer, RNase A, and binding buffer to 1.6 times the recommended volumes, and increasing the lysis incubation time from 30 minutes to two hours. DNA concentration was estimated using a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Ltd., Paisley PA4 9RF, UK) and analyzed on a BioTek Synergy H1 Multi-Mode Microplate Reader using the Gen5 Software package (BioTek Instruments Inc., Winooski, VT, USA). DNA quality was estimated by electrophoresis in 1.0% agarose gels with Bionexus 50-10,000 bp Hi-Lo DNA marker (Bionexus Inc. Oakland, CA, USA). DNAs will be sent to LGC Genomics LLC (Beverly, MA) for SNP assay development and validation using the seven identified SNPs. During the summer of 2017, we will complete an expression analysis to confirm the relationship between alleles at these genes and the low Cd phenotype. These results will allow for selection of SNPs for marker assisted selection.

Results and Discussion:

Proof of Concept Summer 2016 Trial

Total Cd content of the two soils were determined to be 1.7 and 2.8 ppm. Cadmium content of leaves and petioles ranged from 9.89 to 20.41 and 20.34 to 38.54 in plants grown in 1.7 ppm and 2.8 ppm Cd, respectively. Figure 1 shows that all cultivars grown in 2.8 ppm Cd soil contained higher Cd than when grown in 1.7 ppm Cd soil. Differences in expected and measured Cd may be in part due translocation of Cd stored in root vacuoles to flowers during bolting which was seen in some plants. Total Cd in roots averaged 15.1 and 25.95 when grown in 1.7 and 2.8 ppm Cd soil, respectively. From both soils, Seaside and Unipack 12 had higher average Cd content in roots than shoots. To determine differences between cultivars within each soil Cd level, statistical analysis will be required, however, it appears some entries with low expected Cd showed higher cadmium than some with high expected Cd (e.g. Unipack was expected to have lower Cd than Seaside, but the opposite was seen). Viroflay grown in UC mix had significantly lower Cd content (figure 3).

The *proof of concept* pre-trial was useful in planning for future greenhouse trials. It was determined that future experiments should be conducted under shorter day length (less than 14 hours) with cooler temperatures (65-75C) and in larger pots. Before results from the analytical lab were received, Unipack 12 and Seaside were selected to be low and high Cd checks to include in the first germplasm screening trial. Selection was based on preliminary data from Mao, Smith and Hartz (unpublished) as well as germination data from this pre-trial. Our results indicate that Unipack 12 and Seaside both have relatively high Cd content when grown in 2.8 ppm Cd soil during hot summer months.

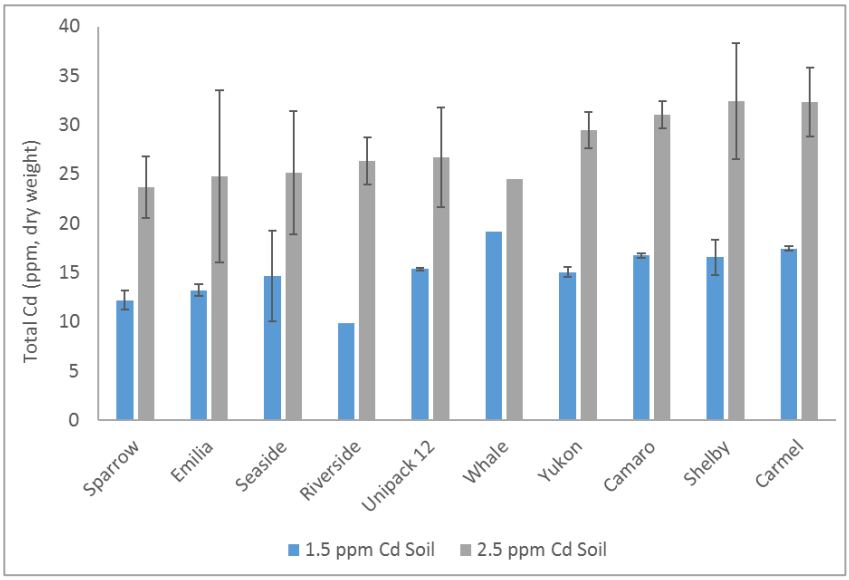


Figure 1. Total Cd of spinach leaves and petioles harvested at 6 weeks grown in two field soils with 1.7 and 2.8 ppm total Cd.

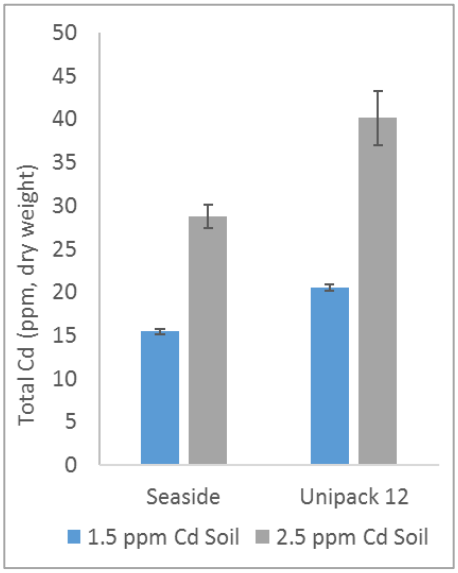


Figure 2 (left). Total Cd of spinach roots harvested at 6 weeks grown in two field soils with 1.7 and 2.8 ppm total Cd.

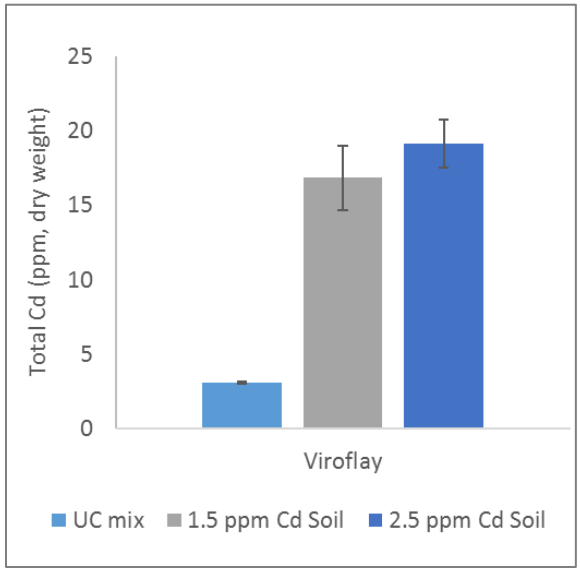


Figure 3 (right). Total Cd of spinach cultivar Viroflay harvested at 6 weeks grown in UC mix, or one of two field soils with 1.7 and 2.8 ppm total Cd.

Germplasm Screening Trials

From the fall 2016 trial, the mean total Cd of checks were 7.78 and 10.97 ppm for Unipack12 and Seaside, respectively (Table 4). These results confirm previous findings by Mou, Smith, and Hartz (unpublished), and suggest that environmental effects may have impacted results for Unipack 12 during the summer pre-trial when the greenhouse temperature was regularly above 90F. Checks in

the fall trial were relatively stable across blocks with one rank change occurring in block 20 where Unipack 12 performed anomously high (Figure 4). This datapoint was excluded from further analyses. Cd content of accessions adjusted for block effects show a normal distribution with a 3-fold difference in Cd content observed among the highest and lowest performing accessions (Figure 5). Mean Cd was 8.5 and ranged from 5.4 to 14.4 ppm. Spring 2017 trial data will be analyzed once it is received from the analytical lab.

Checks	mean	SE	df	t-ratio	lower CL	upper CL	p-value	group
Unipack 12	7.78	0.2635	17	29.54	7.23	8.34	<.0001	a
Seaside	10.97	0.2913	17	37.67	10.36	11.59	<.0001	b

Table 4. Fall 2016 trial Cd results (ppm) of checks (Unipack 12 and Seaside) using Tukey method.

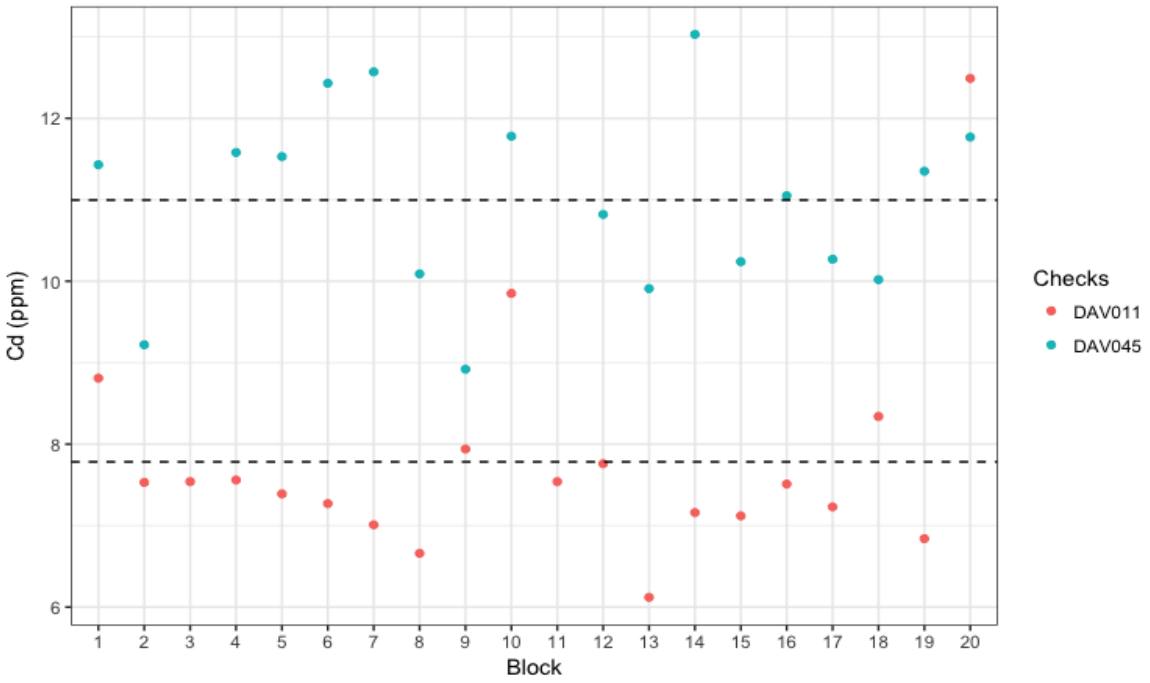


Figure 4. Cadmium content of checks Unipack 12 (DAV011) and Seaside (DAV045) with check means (dashed lines) within each block from the fall 2016 trial.

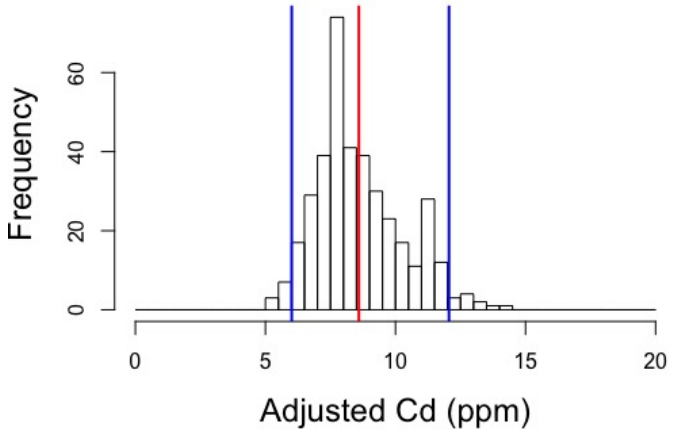


Figure 5. Number of accessions (frequency) binned by adjusted Cd content with 0.5 quantile in red, and 0.025 and 0.975 quantiles in blue.

These findings show there is a wide range of Cd uptake among screened accessions. With results from the spring trial we will make additional selections for further testing and potential use in our breeding program. Next, we will determine DNA sequence diversity in candidate genes associated with low cadmium accumulation in leaves and conduct an expression analysis to confirm the relationship between alleles at these genes and the low Cd phenotype. We will then begin hybrid development between low Cd lines and breeding populations as a first step to incorporate the trait into cultivars and to enable genetic mapping of genes associated with Cd uptake.

Citations:

Majmundar, Hasmukhrai, 1980. “Distribution of Heavy Elements Hazardous to Health, Salinas Valley Region, CA.” Special Report. Sacramento, CA: California Division of Mines and Geology.

Alia, Naz, Khan Sardar, Muhammad Said, Alam Sadia, Siddique Sadaf, Ahmed Toqeer, and Schilz Miklas, 2015. “Toxicity and Bioaccumulation of Heavy Metals in Spinach (Spinacia Oleracea) Grown in a Controlled Environment.” International Journal of Environmental Research and Public Health 12, no. 7: 7400–7416.