Project Title: Spinach Breeding and Genetics

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Abstract:

Salinity and nutrient deficiency are production constraints in all major spinach districts of California. Salt-tolerant spinach varieties were identified, which could be used by growers for cultivar selection and by breeders for crop improvement. The effects of salinity and nutrient deficiency were evaluated in sand cultures in greenhouse. Salinity significantly decreased plant growth and leaf water content, and increased chlorophyll content. Salt stress improved nutritional value by increasing carotenoid and flavonoid contents in full-strength nutrient solution. Nitrogen deficiency greatly reduced plant growth and chlorophyll content, but increased anthocyanin and phenolic contents regardless of salt treatment. Phosphorus and potassium deficiencies decreased plant growth irrespective of salinity treatment, but increased chlorophyll content under no-salt conditions. Phosphorus and potassium deficiencies increased carotenoid and flavonoid contents without salinity stress. These results suggest that reduced fertilizer application or mild salt stress might be utilized to improve the nutritional value of spinach with moderate or little yield reduction. We also identified resistant varieties and conducted experiments to breed spinach for resistance to downy mildew disease, leafminer insect, and linuron (Lorox) herbicide.

Objective 1. Screening Spinach Germplasm for Salt Tolerance

Salinity is a production constraint in all major spinach districts of California. The seawater intrusion has continued to move farther inland into groundwater aquifers beyond city limits of Castroville and Salinas because of continuing overdraft conditions for municipal and agricultural uses in the coastal regions. In the Central Valley, salts accumulate in farmland due to irrigation water from the Sacramento-San Joaquin Delta contaminated with brackish water from the San Francisco Bay, a shallow saline water table, and a lack of adequate drainage outlet. In the Imperial Valley, salts in irrigation water from Colorado River must be carefully managed to prevent yield losses.

Global warming has led to higher sea levels and farther saltwater intrusion. Rising temperatures promote water transpiration from plants and evaporation from soil, leaving more salts behind in soil. Seeds of spinach are planted ½ inch deep where soil salt levels are often highest. Spinach with shallow roots is salt-sensitive and baby spinach is especially vulnerable. Thus, there is a pressing need to mitigate the adverse effects of global warming and adapt spinach production to the changing environment in order to protect and even increase current production levels. Utilizing salt-tolerant cultivars is the most economic and sustainable means to manage salinity and enhance the spinach production in California. Once developed, cultivars with beneficial traits can be perpetually used to manage recurring problems. The project aims to screen spinach germplasm and identify salt-tolerant varieties that could be used by growers for cultivar selection and by breeders as sources of salt-tolerance for crop improvement.

Procedures

We first conducted preliminary experiments to compare the growth media suitable for the study of salt tolerance in spinach. We finally settled on rock wool cubes because they are completely sterile, have optimum air/water ratio, are ready to use, and need less labor. Different salt concentrations were also compared. We decided to use a salt solution of 4.6 ds/m for summer season and 6.5 ds/m for fall season. Water evaporates faster in summer, which increases the salt concentrations. We preliminarily screened 525 spinach varieties. Based on fresh weight, 60 varieties were selected for further screen. The 60 spinach varieties were screened in sand pots under greenhouse conditions. We conducted 2 trials, 3 replications per trial, and plants were grown for 6 weeks after seeding. Fresh and dry weight of the plants, and leaf area were measured.

Results and Discussion

The spinach plants in rock wool cubes from the preliminary screening are shown in Fig. 1.

Fig. 1. Spinach plants grown on tock wool cubes treated with salt solutions (left) and no-salt solutions (right) in a preliminary screening of 525 spinach varieties in a greenhouse.

From the preliminary screening, 60 varieties were selected based on fresh weight for further replicated screening in sand pots. Salt-treated plants looked smaller than untreated control for salt-sensitive varieties, but there is not much difference between treatments for salt-tolerant varieties (Fig. 2). PI 176371 had a 18% increase in fresh weight after salt treatment, while PI 169668 showed no weight change between salt treatment and untreated control (Table 1). NSL 4649, PI 206753, PI 171861, PI 608713, Ames 26238, and 'Sharon' had less than 20% weight reduction after the salt treatment, as compared to 35% reduction in fresh weight for some varieties. The salt-tolerant spinach varieties identified could be used by growers for cultivar selection or by breeders as source of salt-tolerance for the development of new cultivars.

Fig. 2. Salt-sensitive (top) and salt-tolerant (bottom) spinach varieties from replicated experiment in a greenhouse. Plants in sand pots were treated with no-salt (left) or salt (right) solutions.

Table 1. Fresh weight (FW) and % changes of selected spinach varieties under salt treatment and untreated control from replicated screening experiments in greenhouse.

	FW		
Varieties	Control	Salt	Change %
PI 176371	2.27	2.67	17.94
PI 169668	2.18	2.19	0.39
NSL 4649	1.73	1.63	-5.55
PI 206753	2.27	2.08	-8.47
PI 171861	1.62	1.39	-14.4
PI 608713	1.52	1.28	-15.8
Ames 26238	2.14	1.79	-16.3
Sharon	2.95	2.38	-19.3
PI 179595	2.05	1.47	-28.4
PI 647856	2.05	1.43	-30.4
PI 173131	3.14	2.17	-31
PI 379552	1.79	1.21	-32.4
PI 220686	1.76	1.14	-35.1
Ames 26247	1.22	0.79	-35.4
NSL 68263	3.3	2.13	-35.4

Objective 2. Study the Effects of Salinity and Nutrient (N, P or K) Deficiency on Spinach Growth, Physiology, and Nutritional Value

High-salinity conditions in agricultural soil and irrigation water are one of the most serious challenges faced by agricultural crops in the world. It is estimated that salt-affected soils impact nearly 10% of the land surface and 50% of irrigated land in the world (Ruan et al., 2010). The response of plants to salinity is complex and involves changes in morphology, physiology, and metabolism. Salinity effects on plants include cellular water deficit, ion toxicity, nutrient deficiencies, and oxidative stress, leading to growth inhibition, molecular damage, and even plant death (Orcutt and Nilsen, 2000). The global annual losses in agricultural production from salt-affected land are in excess of $12 billion and rising (Flowers et al., 2010; Qadir et al., 2008).

Another important limiting factor for crop production is soil nutrient deficiency. Nitrogen is considered the most common deficient nutrient, followed by P and K (Halvin et al., 2005). Nitrogen is a major constituent of proteins, nucleotides, chlorophyll, and many other metabolites

and cellular components (Lawlor, 2002), and thus N deficiency affects enzyme activities and metabolite content, hastens senescence, causes nucleic acids and proteins to break down, and therefore inhibits plant growth (Crafts-Brandner et al., 1998; Lemaitre et al., 2008; Wingler et al., 2006). Phosphorus is a macronutrient of crucial importance in signaling, photosynthesis, and other metabolism (Marschner, 1995). Potassium is an essential nutrient that affects most of the biochemical and physiological processes that influence plant growth and metabolism (Wang et al., 2013).

Spinach is an important leafy green vegetable that contains large quantities of bioactive compounds and nutrients that are not common to most other vegetables, such as r-coumaric acid derivatives that exhibit strong antioxidant activity and glucuronic acid derivatives of flavonoids (Bergman et al., 2001; Edenharder et al., 2001; Pandjaitan et al., 2005). It was identified as a moderately salt-sensitive vegetable (Shannon and Grieve, 1999). Salt stress reduced spinach germination, root elongation, seedling growth, chlorophyll content and photosynthesis, and increased membrane permeability (Delfine et al., 1998; Downton et al., 1985; Kaya et al., 2002; Robinson et al., 1983). Langdale et al. (1971) reported that the tolerance threshold for spinach is 2.0 dS m^{-1}, but irrigation with saline water of 4.0 dS_m–1 on sandy soils did not result in yield reduction (Pasternak and De Malach, 1994). Studies in solution cultures have shown that no significant growth reduction occurs up to _8 dS_m–1 (Osawa, 1963). There is limited information about the effects of micronutrient deficiency on spinach growth and physiology. With regard to nutritional quality, previous studies indicated that mild environmental stress or low fertilization could be exploited to enhance nutritional values of vegetable crops. Low N fertilization (Galieni et al., 2015; Stagnari et al., 2015) or environmental stresses, such as salt, heat, chilling, and high light intensity (Oh et al., 2009; Perez-Lopez et al., 2013), enhanced phenolic content and antioxidant capacity of lettuce (*Lactuca sativa* L.). Similarly, fruit nutritional value of tomato (*Solanum lycopersicum* L.) could be improved by low N supply (Benard et al., 2009) or salt stress (Borghesi et al., 2011). However, literature on spinach nutritional values affected by abiotic stresses is very scarce. The current study aims to assess the effects of salinity and single nutrient (N, P, or K) deficiency on spinach growth, physiology, and nutritional value.

Procedures

PLANT MATERIALS AND TREATMENTS. Two trials, each with four replications, were conducted from February 3 to March 27, 2015 and February 24 to April 17, 2015, in a greenhouse located in Salinas (36°40'40"N 121°39'20"W), CA. The greenhouse was supplemented with light of a 12-h photoperiod using Sun System 3 (Sunlight Supply Inc., Vancouver, WA, USA). Two weeks after seeded in rock wool cells (Grodan Group, Roermond, Netherlands), uniform spinach seedlings (cv. Crocodile) were transplanted into plastic pots (2.3 L) filled with sands. Plants were thinned to one plant per pot and watered every day with Hoagland nutrition solution (electric conductivity or EC: 2.5 ds/m) (Hoagland and Arnon, 1950).

Salinity and nutrient treatments were applied ten days after transplanting. Salinity stress was obtained by adding 10/5 mM NaCl/$CaCl_2$ for 2 d, and then by adding 20/10 mM NaCl/$CaCl_2$ (EC: 6.5 ds/m). Exposure of plants to increasing salt concentration allows gradual acclimation of plants to salinity conditions to avoid sudden death of plants at high salt concentration. For N, P and K deficiency, the sources of N, P and K in Hoagland's solution were replaced with

alternative chemicals. The N source, calcium nitrate and potassium nitrate, were replaced with calcium chloride and potassium sulfate, respectively. The P source, potassium dihydrogen orthophosphate, was replaced by potassium sulfate. The K sources, potassium dihydrogen orthophosphate and potassium nitrate, were replaced with sodium dihydrogen phosphate and sodium nitrate, respectively.

GROWTH AND PHYSIOLOGY MEASUREMENTS. Four weeks after treatment, leaf maximum photochemical efficiency (F_v/F_m) and photochemical yield (Y(II)) were measured with a MINI-PAM-II fluorometer (Heinz Walz, Effeltrich, Germany) on the first and third leaves from bottom of each plant. Leaf F_v/F_m was measured after leaves were adapted in darkness for 30 min.

Leaf discs were collected using a cork borer from four largest leaves of each plant to measure relative water content (RWC), specific leaf area (SLA), chlorophyll content, and nutritional values. SLA was calculated as: SLA = area / DW, where DW is leaf dry weight after drying at 65 °C for 3 d. Leaf RWC was calculated as: RWC (%) =100 × [(FW-DW) / (TW-DW)], where FW is fresh weight and TW is turgid weight after being soaked in water for 4 hours at 4 °C. Leaf pigments were extracted with methanol and absorbance of the extraction was measured at 665, 652 and 470 nm with a spectrophotometer (Spectronic Genesys, Spectronic Instruments, Rochester, NY). Chlorophyll and carotenoid contents were calculated using the formula described by Lichtenthaler (1987). For nutrition value analysis, leaf sample was soaked in liquid nitrogen immediately after harvested and stored at -80 ^{o}C.

REDUCING POWER AND CONTENTS OF PROTEIN AND AMINO ACID. For analyzing nutritional value, leaf samples were soaked in liquid nitrogen immediately after harvested and stored at -80 ^{o}C. Leaf samples were homogenized in 15 mL 0.2 M phosphate buffer (pH = 6.6) using a Polytron homogenizer (Kinematica AG, Schweiz, Switzerland). After centrifuging at 9070 g for 15 min, the supernatant was collected to measure reducing power and the content of protein and amino acid. For reducing power, 1.0 mL extract was mixed with 1.0 mL 1% potassium ferricyanide and was incubated at 50 °C for 20 min. The reaction was stopped by adding 1.0 mL 10% trichloroacetic acid. After centrifugation at 10000 rpm for 10 min, 1.0 mL supernatant was mixed with 1.0 mL water and 0.2 mL 0.1% $FeCl_3$. Absorbance at 700 nm was measured after 1.5 min. A (+)-catechin hydrate served as positive controls.

Amino acid content was determined using ninhydrin method (Yokohama and Kiramatsu, 2003). A 1% w/v ninhydrin stock solution was prepared in ethanol containing 0.025% w/v ascorbic acid. A working ninhydrin solution was prepared immediately before use by adding two parts of 0.4 M sodium acetate buffer (pH 5.0) to one part of ninhydrin stock solution. 100 µL extract or standard glutamate solution was added to 2.9 mL ninhydrin work solution and heated for 10 min. The solution was cooled, then absorbance at 570 nm was read. Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

PHYTOCHEMICAL ANALYSES. Phytochemicals were extracted with 15 mL acidified methanol (0.1% HCl) using a Polytron homogenizer, then incubated in darkness at -20 ^{o}C overnight. After centrifuging at 9,070 g for 15 min, the supernatant was collected for the analysis of nutritional value. Absorbance of extracts at 530 nm was measured for total anthocyanin content. Results were expressed as mg cyaniding 3-glucoside equivalents using a molar extinction coefficient of 29600 (Connor et al., 2002).

For total phenolics content, 0.1 mL extract was added to a mixture of 0.15 mL H_2O and 0.75 mL of 1/10 diluted Folin-Ciocalteu reagent. After 6 min, 0.60 mL of 7.5% (w/v) Na_2CO_3 was added and vortexed, then the mixture was incubated at 45 ^{o}C in a water bath for 10 min. Samples

were allowed to cool to room temperature before reading the absorbance at 765 nm (Slinkard and Singleton, 1997). A standard curve was prepared from a freshly made gallic acid solution. For total flavonoid content, 0.20 mL extract was mixed with 0.85 mL distilled water and 50 μL of 5% $NaNO_2$. After 6 min, 100 μL of 10% $AlCl_3 \cdot 6H_2O$ was added, and after another 5 min 0.35 mL of 1 M NaOH and 0.20 mL distilled water were added. The absorbance was measured immediately at 510 nm (Dewanto et al., 2002). A (+)-catechin hydrate equivalents standard curve was prepared from a freshly made solution.

Total antioxidant capacity was measured by the methods of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (Brand-Williams et al., 1995). DPPH (20 mg L^{-1}) was dissolved in 100% methanol. Extracts (20 μL) were added to 2 mL DPPH solution, and the absorbance was determined at 515 nm before and after 6 min reaction. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid) standard curve was prepared. For ferrous ion chelating ability (FICA) measurement, the reaction mixture contained 40 μL extracts, 1.48 mL methanol and 30 μL of 2 mM ferrous chloride. After vortexing, 40 μL of 5 mM ferrozine was added followed by vigorous mixing. After 4 min the absorbance of the ferrozine-ferrous ions complex was measured at 562 nm (Dinis et al., 1994). FICA was calculated as the absorbance difference between control and sample.

STATISTICAL ANALYSIS. The experiment used a complete randomized design. Each biological replicate contained one pot and each treatment contained four replicate pots for each trial. Treatment means were separated by Student's t-test at the 0.05 level of probability using the JMP program (SAS Institute Inc., Cary, NC). The interaction between the two trials was not significant so data were pooled together.

Results

GROWTH AND PHYSIOLOGY. Spinach growth was greatly inhibited by either salinity or nutrient deficiency. Without nutrient deficiency, salt stress decreased shoot FW and DW by 34 and 27% respectively (Fig. 3A, B). Both P and K deficiency similarly reduced shoot FW and DW under both control condition (by about 27 and 20% for FW and DW respectively) and salt stress (by about 20 and 14% for FW and DW respectively) (Fig. 3A, B). N deficiency greatly decreased shoot FW and DW either without (by 85 and 79% for FW and DW respectively) or with (by 68 and 61% for FW and DW respectively) salt stress. The reduction caused by nutrient deficiency was much greater under non-salt condition than under salt stress, especially for N deficiency (Fig. 3A, B).

Leaf RWC decreased under salt stress but was not affected by nutrient deficiency under either control or salt stress (Fig. 4A). Salinity decreased SLA without nutrient deficiency, and with P or K deficiency (Fig. 4B). N deficiency decreased SLA irrespective of salinity treatment while P or K deficiency similarly decreased SLA only under salt stress (Fig. 4B).

Without nutrient deficiency, salt stress increased the content of both chlorophyll a and b, total chlorophyll content, and chlorophyll a/b ratio (Fig. 5A, B, C, D). N deficiency greatly reduced chlorophyll a (by 52 and 56% under control and salt stress respectively) and b content (by 63 and 53% under control and salt stress respectively) and increased the ratio of chlorophyll a/b regardless of salinity treatment (Fig. 5A, B, D). Both P and K deficiency enhanced chlorophyll a and total chlorophyll content without salt stress, and increased chlorophyll a/b with or without salt stress (Fig. 5A, C, D).

Salinity increased spinach leaf F_v/F_m and Y(II) without nutrient deficiency (Fig. 6A, B). Without salinity stress, P deficiency increased F_v/F_m and Y(II) while N and K deficiency did not have effects on them. Under salt stress, N deficiency reduced both F_v/F_m and Y(II), P deficiency increased F_v/F_m, and K deficiency decreased Y(II) (Fig. 6A, B).

NUTRITIONAL VALUES. Without nutrient deficiency, salt stress increased carotenoid content (Fig. 7A). N deficiency greatly reduced carotenoid content either with (by 45%) or without (by 50%) salt stress while P and K deficiency increased carotenoid content without salt stress (Fig. 7A). Anthocyanin content was greatly enhanced under N deficiency by 145 and 88% without or with salt stress, respectively, but salt stress or P, K deficiency had no influences on its content (Fig. 7B).

Salt stress had no effect on total phenolic content without nutrient deficiency (Fig. 7C). N deficiency greatly enhanced phenolic content by 66 and 22% for control and salt stress respectively, P deficiency increased phenolic content with salt stress and K deficiency increased phenolic content without salt stress (Fig. 7C). Without nutrient deficiency, salt stress increased flavonoid content (Fig. 7D). N deficiency reduced flavonoid content by 14 and 36% for control and salt stress, respectively, while P or K deficiency enhanced the content without salt stress (Fig. 7D).

Total antioxidant capacity increased by N or K deficiency without salt stress (Fig. 8A). Salt stress decreased FICA without nutrient deficiency and with P or K deficiency (Fig. 8B). Nitrogen deficiency greatly reduced FICA by 80 and 46% under control and salt stress, respectively. Phosphorus deficiency decreased FICA under salt stress and K deficiency reduced FICA under no-salt conditions as well as under salt stress (Fig. 8B). Salt stress enhanced reducing power without nutrient deficiency, and also nutrient deficiency increased reducing power under both control and salt stress (Fig. 8C).

Both protein and amino acid contents were greatly reduced by N deficiency either with or without salt stress, and P deficiency decreased amino acid content under salt stress (Fig. 9A, B).

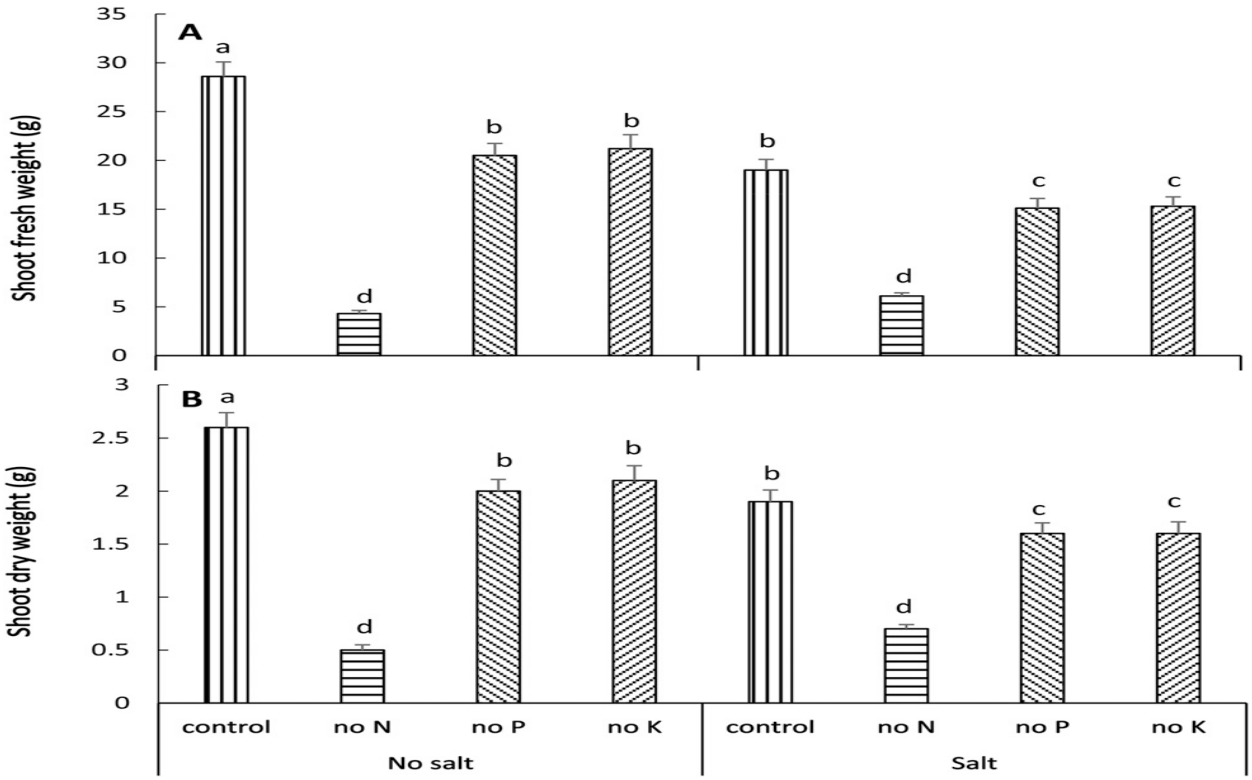

Fig. 3. Effect of salinity and nutrient deficiency on spinach shoot fresh (A) and dry (B) weight. The values are means of eight replicates ± standard error. Different letters on top of bars indicate significant different at $P \leq 0.05$ according to Student's t-test.

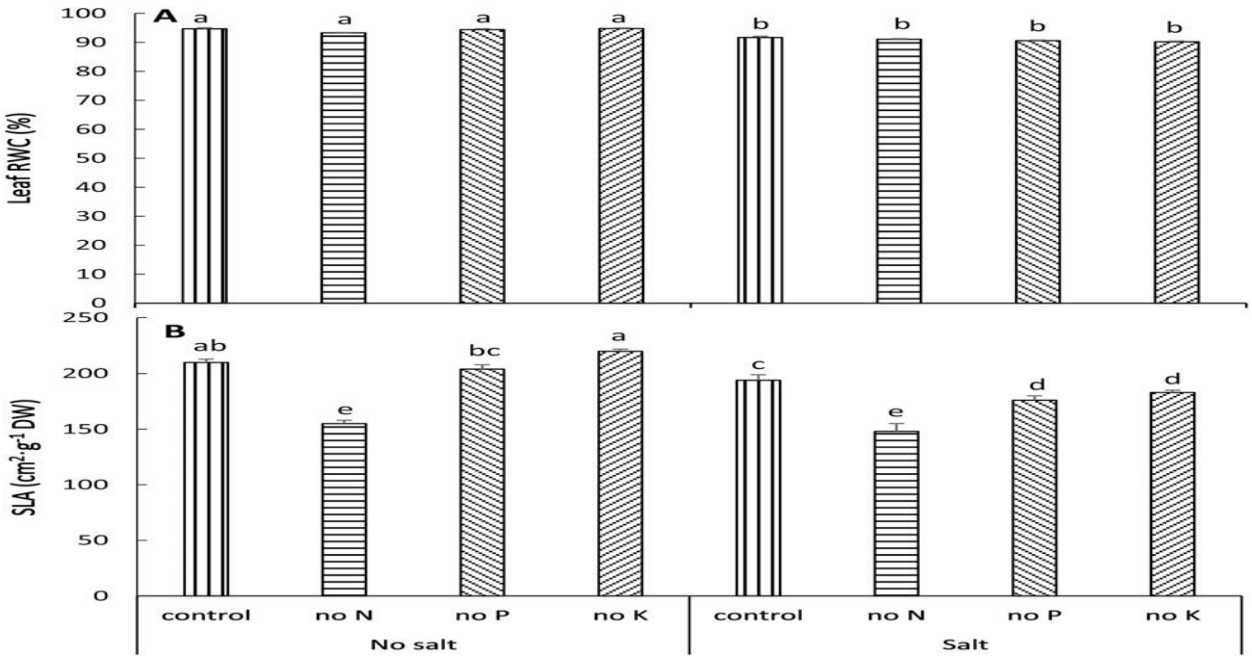

Fig. 4. Effect of salinity and nutrient deficiency on spinach leaf relative water content (RWC) (A) and specific leaf area (SLA) (B) of spinach. The values are means of eight replicates ± standard error. Different letters on top of bars indicate significant different at $P \leq 0.05$ according to Student's t-test.

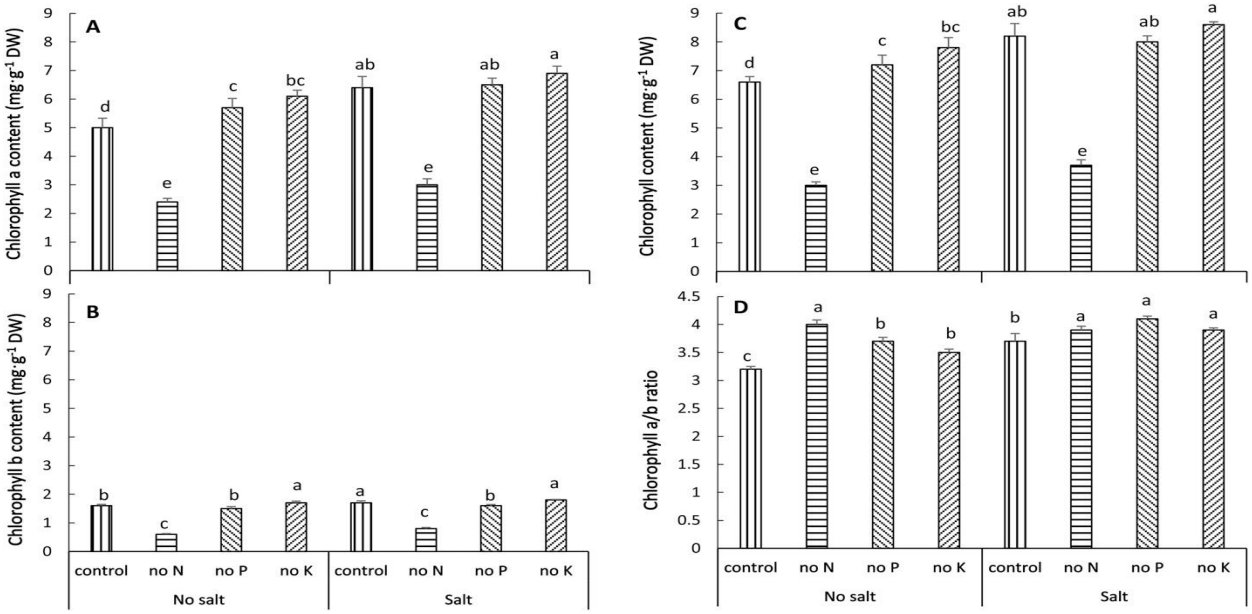

Fig. 5. Effect of salinity and nutrient deficiency on spinach leaf chlorophyll a (A), b (B), total (C) content and ratio of a/b (D). The values are means of eight replicates ± standard error. Different letters on top of bars indicate significant different at $P \leq 0.05$ according to Student's t-test.

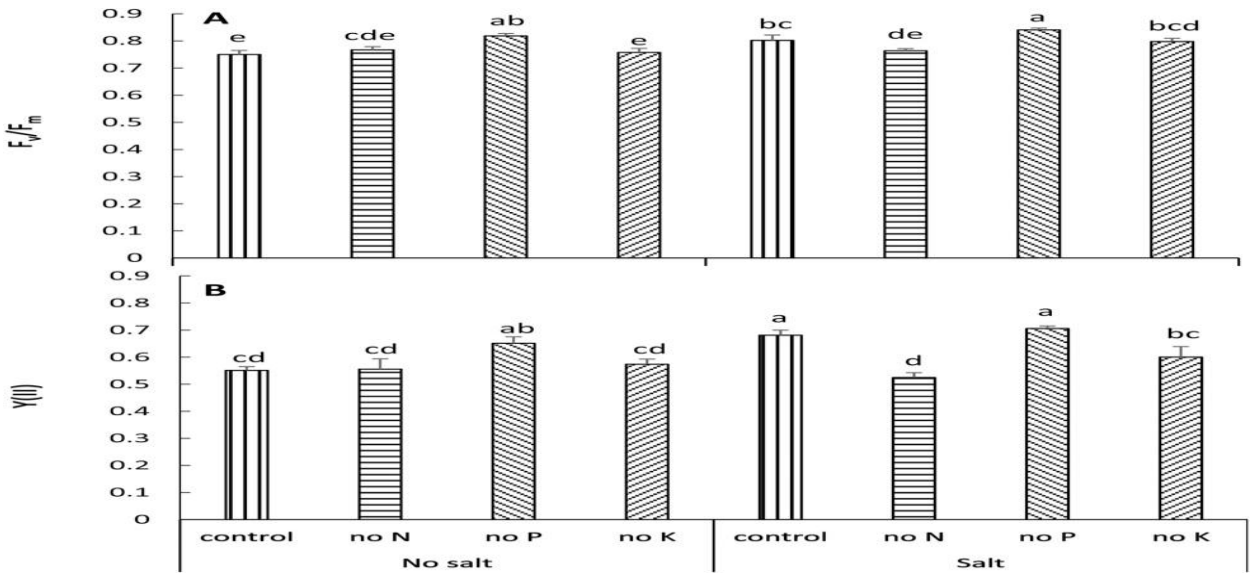

Fig. 6. Effect of salinity and nutrient deficiency on spinach leaf photochemical efficiency (F_v/F_m) (A) and photochemical yield (Y(II)) (B). The values are means of eight replicates ±

standard error. Different letters on top of bars indicate significant different at $P \leq 0.05$ according to Student's t-test.

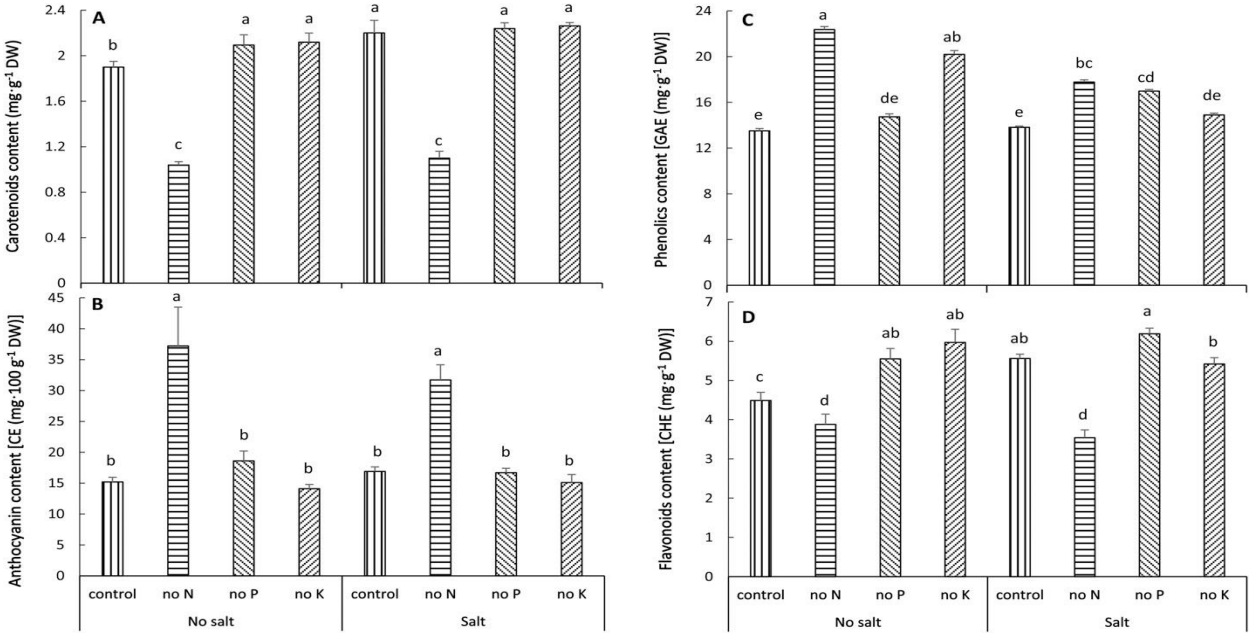

Fig. 7. Effect of salinity and nutrient deficiency on spinach leaf contents of carotenoids (A), anthocyanin (B), phenolics (C), and flavonoids (D). The values are means of eight replicates ± standard error. Different letters on top of bars indicate significant different at $P \leq 0.05$ according to Student's t-test. CE: cyaniding 3-glucoside equivalents. CHE: (+)-catechin hydrate equivalents. GAE: gallic acid equivalents.

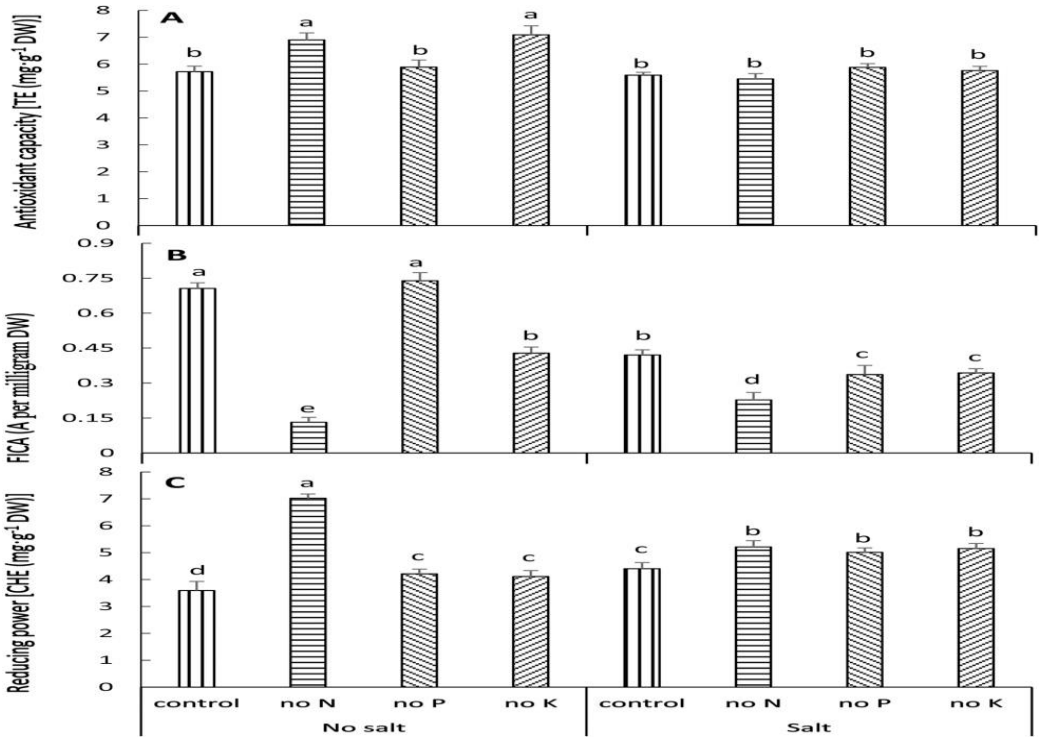

Fig. 8. Effect of salinity and nutrient deficiency on spinach leaf total antioxidant capacity (A), ferrous ion chelating ability (FICA) (B) and reducing power (C). The values are means of eight replicates ± standard error. Different letters on top of bars indicate significant different at $P \leq 0.05$ according to Student's t-test. CHE: (+)-catechin hydrate equivalents. TE: trolox equivalents.

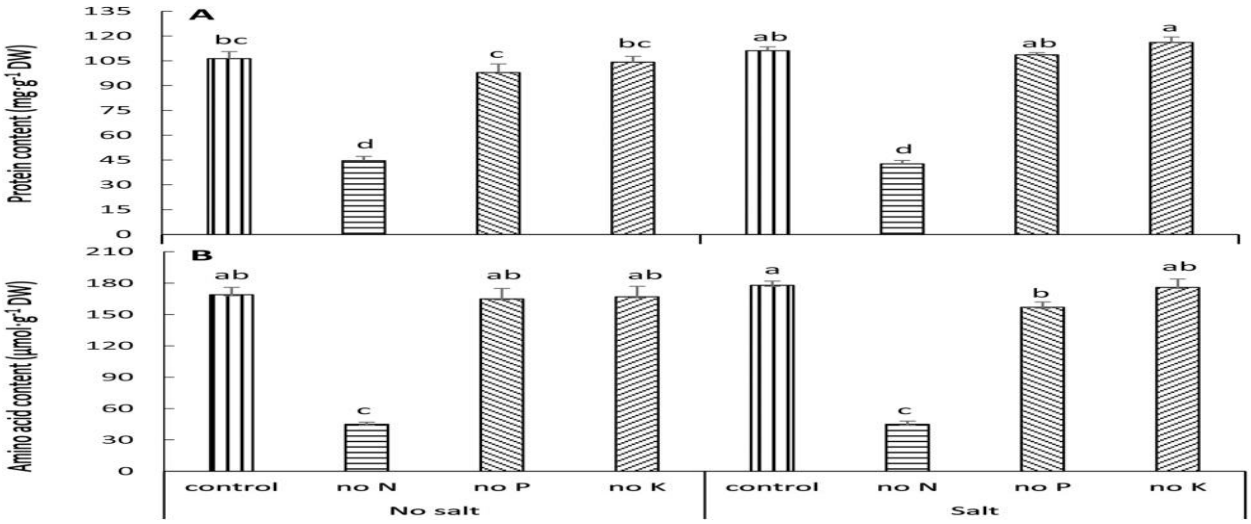

Fig. 9. Effect of salinity and nutrient deficiency on spinach leaf contents of protein (A) and amino acid (B). The values are means of eight replicates ± standard error. Different letters on top of bars indicate significant different at $P \leq 0.05$ according to Student's t-test.

Discussion

GROWTH AND PHYSIOLOGY. Spinach growth was greatly inhibited by salinity as indicated by reduced shoot FW and DW. Salinity induces an ion-specific shoot growth inhibition caused by a disturbance in mineral nutrition, which might occur either as an excess or deficiency in mineral supply. Lazof and Bernstein (1999) found that Ca^{2+} transport to young leaves is sensitive to salinity and is a key physiological response in the inhibition of lettuce leaf growth. In the present study salt stress decreased spinach leaf water content, which is consistent with previous studies in lettuce (Shannon et al., 1983; Pérez-López et al., 2013). Low leaf water content results in stomatal closure and loss of cell turgor pressure and expansion, leading to reduced photosynthetic rate and leaf area, and therefore growth inhibition (Taiz and Zeiger, 2012). Indeed, previous studies indicated that salt stress caused reduction in stomatal and mesophyll conductance which decreased the chloroplast CO_2 concentration in spinach and lettuce (Delfine et al., 1998, 1999; Robinson et al., 1983; Downton et al., 1985; Eraslan et al., 2007). This caused an inhibition of photosynthesis which was not related to changes in biochemical and photochemical capacity since salt stress did not result in decreased chlorophyll fluorescence or activity and content of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) (Brugnoli and Bjorkman, 1992; Delfine et al., 1998). Actually in the present study, mild salt stress increased spinach chlorophyll content and fluorescence. Spinach chlorophyll content was reduced by NaCl solution with high concentration of 200 or 172 mM (Delfine et al., 1998; Downton et al., 1985; Robinson et al., 1983), while chlorophyll fluorescence was not affected (Delfine et al., 1998; Robinson et al., 1983). Eraslan et al. (2008) reported that application of 50 mmol NaCl per kg as soil amendment did not altered chlorophyll content. However, Kaya et al. (2002) reported that spinach chlorophyll content was reduced by 60 mM NaCl solution. The inconsistency might be due to differences in salinity severity, period and application methods. Also in our study, salt stress increased chlorophyll a/b ratio. High chlorophyll a/b indicated more chlorophyll a-proteins of photosystem II and I and less light harvesting complex II and I (Anderson, 1986).

In the present study, salt stress decreased spinach SLA, an indicator of leaf thickness. Increased leaf thickness is often observed as a result of salt stress in spinach (Coughlan and Jones, 1980; Robinson et al., 1983; Downton et al., 1985) and other species (Longerstreth and Nobel, 1979; Heuer and Plaut, 1981), although Delfine et al. (1998) found that leaf thickness was lower in salt-stressed spinach than in control. Reduction of SLA is assumed to improve water use efficiency, because thicker leaves usually have a higher density of chlorophyll and proteins and, hence, have a greater photosynthetic capacity than thinner leaves (Craufurd et al., 1999; Liu and Stutzel, 2004; Wright et al., 1994). The reduced SLA in this study might result from reduced water content under salt stress. SLA has often been reported to decrease under drought stress (Marcelis et al., 1998; Liu and Stutzel, 2004). A decrease in SLA under water deficit may be due to the different sensitivity of photosynthesis and leaf expansion, since water deficit stress reduces leaf expansion earlier than photosynthesis (Jensen et al., 1996; Tardieu et al., 1999).

Nitrogen deficiency reduced spinach growth under either control or salt stress in the present study. The reduced plant growth was not related to water relations since N deficiency did not alter leaf RWC regardless of salinity treatment. The most general symptom of N deficiency is leaf chlorosis and growth retardation. In this study, N deficiency greatly decreased chlorophyll content (more than 50%) and increased chlorophyll a/b irrespective of salt stress, while chlorophyll fluorescence was reduced to a much less extent with salt stress. Also N deficiency can modify enzyme activities and metabolite contents (Lemaitre et al., 2008; Zhang et al., 2010).

It enhanced sugar accumulation while reduced amino acid content (Lemaitre et al., 2008; Wei et al., 2015), and hastened leaf senescence due to a high C/N ration (Gombert et al., 2006; Wingler et al., 2006). After a prolonged N starvation, breakdown of enzyme and nucleic acid occurs (Crafts-Brandner et al., 1998). Especially, breakdown of rubisco resulted in a decreased photosynthesis capacity and ultimately inhibited plant growth (Walker et al., 2001). Similarly as Stagnari et al. (2015) reported in lettuce, N deficiency decreased SLA in this study which could improve resource use efficiency.

In the present study, both P and K deficiency similarly inhibited spinach shoot growth under control or salt stress, reduced SLA under salt stress, and increased chlorophyll content without salinity. P deficiency enhanced F_v/F_m under both control and salt stress, and increased Y(II) under control condition. K deficiency only reduced Y(II) under salt stress. Previous study indicated that P deficiency reduced stomatal conductance and photosynthetic rate in spinach and peppers (Dietz and Foyer, 1986; Davies et al., 1999), and decreased chlorophyll content and photochemical efficiency in creeping bentgrass (Zhang et al., 2010). But this study indicated that photochemistry was not damaged by P deficiency in spinach. Similarly in citrus, K-deficient plant produced less biomass but did not develop leaf chlorosis (Lavon et al., 1999). In this study P or K deficiency reduced spinach growth to a much less extent with respect to N deficiency, which is consistent with other studies in citrus (Papenfus et al., 2013) and lettuce (Galieni et al., 2015). This indicates the prevalent role of N in plant energy metabolism such as photosynthesis and transpiration (Mengel and Kirkby, 1987; Galieni et al., 2015). A large portion of N in plants is associated with energy metabolism while a smaller part is incorporated in structural cell components, while P and K constitute a large part of cell components (Mengel and Kirkby, 1987).

NUTRITIONAL VALUES. Carotenoids have long been recognized as essential nutrients and important health-beneficial compounds (Fraser and Bramley, 2004). Phenolics are a class of secondary metabolites which play a key role as antioxidants. The most important group of phenolics in plants is flavonoids which have attracted considerable interest due to their broad spectrum of biological effects such as antioxidant, anti-inflammatory, vasorelaxant, antimicrobial, antiviral, anticarcinogenic and antimutagenic activities (Guo et al., 2011; Maimoona et al., 2011). Anthocyanin is a group of flavonoids with exceptionally good scavenging activities. Without nutrient deficiency, salt stress increased carotenoid and flavonoid contents, but had no effects on the content of anthocyanin and phenolics. However, total antioxidant capacity was not improved by salinity, which suggests that salt stress might reduce the content of other phytochemical antioxidants such as ascorbic acid and/or tocopherol. Application of 50 mmol NaCl per kg soil increased spinach total antioxidant capacity without changing anthocyanin content (Eraslan et al., 2008). In lettuce, salt stress was also reported to increase total antioxidant capacity (Perez-Lopez et al., 2013) and carotenoid content but did no altered phenolic content (Kim et al., 2008). The inconsistency might be due to differences in species, salinity severity or period, and application methods.

Interestingly, leaf FICA greatly decreased under salt stress in the present study. Ferrous ion chelation is another indirect mechanism of antioxidant activity. Although iron is an essential element, excess iron may lead to high levels of active oxygen species (Huang, 2003). Ferrous ion chelation can afford protection against oxidative damage (Huang, 2003). Polysaccharides, peptides, proteins, oleoresins and saponins have been reported to chelate ferrous ions (Gülçin et al., 2006; Wang et al., 2009). A previous study indicated that drought stress also reduced spinach

FICA (Xu and Leskovar, 2015). Our results suggest that spinach nutritional quality could be negatively influenced by reduced FICA under salt stress. Besides hydrogen donation, another import mechanism by which polyphenols scavenge active oxygen species is the electron transfer. In this study, salt stress enhanced reducing power, which is consistent with the increase of flavonoid content and might positively altered spinach nutritional quality.

In the present study, N deficiency increased the reducing power and the content of anthocyanin and total phenolics while decreased these of carotenoids and flavonoids, irrespectively of salinity treatment. However, it only increased antioxidant capacity without salt stress. Inconsistent effects of N starvation on lettuce nutritional value were reported. Similarly to this study, Coria-Cayupan et al. (2009) and Galieni et al. (2015) found that low N level increased phenolic compounds and antioxidant capacity. But Stagnari et al. (2015) reported that antioxidant capacity was not altered by N deficiency though it increased phenolic compounds. Low N supply was reported to increase phenolic and flavonoid compounds in tomato leaves, but no significant variation was observed in fruits (Stout et al., 1998; Stewart et al., 2001; Benard et al., 2009). The induction of many enzymes involved in phenylpropanoid pathway in plant leaves was reported in response to reduced N supply (Bongue-Bartelsman and Philips, 1995; Fritz et al., 2006).

P deficiency increased total phenolic content under salt stress and the content of carotenoids and flavonoids without salt stress in the present study. However, it had no effects on antioxidant capacity. It also reduced FICA and amino acid content under salt stress and increased reducing power regardless of salinity treatment. In lettuce, P deficiency did not alter phenolic content and antioxidant capacity (Galieni et al., 2015). But P deficiency did not increase flavonoid content in tomato leaves (Stewart et al., 2001). In this study, K deficiency increased antioxidant capacity and the contents of carotenoids, total phenolics and flavonoids, under non-salt conditions, but decreased FICA and increased reducing power regardless of salt treatment. It had no influences on the content of protein and amino acid, which is consistent with results in citrus (Lavon et al., 1999).

In summary, the mild salt stress simulated in this study inhibited spinach growth by water deficit although it enhanced photochemistry. Salt stress, when applied along with full nutrient conditions, reduced FICA, enhanced flavonoid and carotenoid contents, and increased the reducing power. Nitrogen deficiency greatly inhibited spinach growth but positively influenced nutritional quality by enhancing phenolic and anthocyanin contents, especially under no-salt condition. Phosphorus deficiency inhibited growth even though it improved photochemistry and the nutritional value by a high reducing power. Potassium deficiency appears to reduce spinach growth as well, but positively influenced nutritional value by increasing the antioxidant capacity, reducing power, and amounts of carotenoids, phenolics, and flavonoids, especially under no-salt conditions. The content of total phenolics or flavonoid does not necessarily indicate the level of antioxidant capacity in spinach. These results suggest that the nutritional value of spinach could be improved with only moderately or slightly reduced yield through cultural practices that impose either lower fertilizer levels or mild salt stress.

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Other Research Projects:

Downy Mildew Crosses were made among ten cultivars with different downy mildew-resistant genes to combine their resistances. Progenies from 26 crosses, along with resistant and susceptible controls, were planted in a field at the USDA-ARS station in Salinas, CA to test their resistance to downy mildew in conditions of natural infections in the fall of 2016. We also planted downy mildew-susceptible spinach cultivar 'Viroflay' in high density on both sides of the testing plots to attract, multiply, and spread the pathogen. Resistant plants with few downy mildew lesions were selected from each of the 26 populations and transplanted into our isolators

for open-pollination. Seeds produced have been harvested, and will be used for the next round of selection in the field.

Leafminer Predominant species of leafminers in central California is *Liriomyza langei*. They have a wide host range including broccoli, cauliflower, celery, lettuce, melons, spinach, tomato, and many weeds. Chemical control is not long-lasting, and it is well documented that leafminers can develop a high degree of resistance to insecticides. Insecticides for adult flies do not provide economic control against a moving target. Chemicals against leafminer larvae may be limited by pre-harvest spray interval (PHI) for a short-season crop like spinach or plantback restrictions for rotational crops.

A recurrent selection method was used to increase the level of resistance to leafminers in four populations of different leaf types planted in a USDA-ARS research field in Salinas in August 2016. Plants with fewer leafminer stings or mines were selected and transplanted into isolators to produce seeds for further rounds of evaluation and selection.

Herbicide Tolerance (with Steve Fennimore's Group) Few herbicides are registered for leafy vegetables in California and they are subject to cancellation due to their relatively small acreage, marginal profitability of these products, and regulatory challenges. Linuron (Lorox) is an herbicide that can provide effective control of more than 20 broadleaf weeds and grasses. Differences in tolerance to this herbicide have been observed in spinach varieties.

We screened spinach germplasm for tolerance to linuron (Lorox) herbicide in the field. Ten breeding populations were planted in the field along with 15 commercial cultivars. After the herbicide spray, surviving tolerant plants were transplanted into isolators to produce seeds for future testing and selection next year.

Publications relevant to this project in 2016-17:

Xu, C., C. Jiao, H. Sun, X. Cai, X. Wang, C. Ge, Y. Zheng, W. Liu, X. Sun, Y. Xu, J. Deng, Z. Zhang, S. Huang, S. Dai, B. Mou, Q. Wang, Z. Fei, Q. Wang. 2017. Draft genome of spinach and transcriptome diversity of 120 *Spinacia* accessions. Nature Communications 8: 15275. doi: 10.1038/ncomms15275

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