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Natural Variation of Folate Content and Composition in Spinach (*Spinacia oleracea*) Germplasm

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Supporting Information

ABSTRACT: Breeding to increase folate levels in edible parts of plants, termed folate biofortification, is an economical approach to fight against folate deficiency in humans, especially in the developing world. Germplasm with elevated folates are a useful genetic source for both breeding and direct use. Spinach is one of the well-know vegetables that contains a relatively high amount of folate. Currently, little is known about how much folate, and their composition varies in different spinach accessions. The aim of this study was to investigate natural variation in the folate content and composition of spinach genotypes grown under controlled environmental conditions. The folate content and composition in 67 spinach accessions were collected from the United States Department of Agriculture (USDA) and Asian Vegetable Research and Development Center (AVRDC) germplasm collections according to their origin, grown under control conditions to screen for natural diversity. Folates were extracted by a monoenzyme treatment and analyzed by a validated liquid chromatography (LC) method. The total folate content ranged from 54.1 to 173.2 μg/100 g of fresh weight, with 3.2-fold variation, and was accession-dependent. Four spinach accessions (PI 499372, NSL 6095, PI 261787, and TOT7337-B) have been identified as enriched folate content over 150 μg/100 g of fresh weight. The folate forms found were H_4-folate, 5-CH_3−H_4-folate, and 5-HCO-H_4-folate, and 10-CHO-folic acid also varied among different accessions and was responsible for variation in the total folate content. The major folate vitamer was represented by 5-CH_3−H_4-folate, which on average accounted for up to 52% of the total folate pool. The large variation in the total folate content and composition in diverse spinach accessions demonstrates the great genetic potential of diverse genotypes to be exploited by plant breeders.

KEYWORDS: Folate, natural variation, spinach, germplasm, composition, breeding

INTRODUCTION

Folate (vitamin B_9), a generic descriptor for a group of molecules, derivatives of tetrahydrofolate, is recently one of the most actively studied vitamins in the area of human nutrition and health. This is mainly due to its role in the prevention of some serious disorders, including neural tube defects (NTDs), such as spina bafida and anencephaly in infants, megaloblastic anemia, cardiovascular disease, and certain cancers in adults.[1] Hence, research on folate enhancement in food has gained more attention in recent years.

Folates act as co-factors in the cell cycle, carrying one carbon unit for various cellular reactions. Thus, they are engaged in the DNA biosynthesis cycle and methylation cycle, which produce vast numbers of metabolites crucial for normal cell function. Humans and animals cannot synthesize folates *de novo* and, hence, completely depend upon their dietary sources.[2,3] Therefore, plant foods are the main source of folates in the human diet. Food folate levels vary among different crops species; the staples wheat, maize, and specially, rice contain extremely low amounts of folates to meet the recommended dietary allowances (RDAs) of 400 μg/day for adults and 600 μg/day for pregnant women. However, many plants, such as leafy green vegetables (such as beans, peas, and spinach), are the best source of folate.[4,5]

Folate deficiency is a global health problem, which causes approximately 300 000 NTDs per year[6] and is responsible for 10% adult deaths from heart disease.[7] Furthermore, folate deficiency is the main cause of anemia in at least 10 million pregnant women in the developing world.[8] Some developed countries mandate a synthetic folic acid fortification program; however, it is absent in developing countries because of recurrent costs, distribution inequities, and lack of an industrial food system.[9] Moreover, concerns have arisen about the human life-threatening diseases because of chronic exposure of synthetic folic acid.[10] The overall findings indicate that it may be better to eat high folate content vegetables than to rely on supplements for the intake of the synthetic folic acid. Besides, dietary tetrahydrofolates from plant sources are predicted to have a higher margin of safety.

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Folate biofortification strategies can conceptually be categorized in two main groups: metabolic engineering and exploiting the natural variation in folate levels.[2] For the former group, the genetic modification (GM), promising tactics can rapidly increase the folate levels in food crops; however, it has been met with considerable consumer resistance amid concerns for its safety.[11] Contrary, biofortification through conventional breeding is the principal strategy to combat micronutrient deficiency advocated by most of the international research consortium and is potentially a cost-effective, sustainable, and safe intervention than other so-called interventions. Exploiting natural genetic variation of folate levels within crop species by combining genomics with conventional breeding methods is a paradigm for folate improvement of crops, and it is also useful to understand the molecular basis of folate variation in crop species. This should enable mapping of quantitative trait loci (QTLs) for folate accumulation, ultimately to be integrated in molecular marker-assisted selection.[2,12]

Spinach (*Spinacia oleracea* L.), an economically important leafy vegetable, considered to have a high nutritional value, including vitamins and minerals, as well as many health beneficial secondary metabolites and antioxidants, is mainly consumed after cooking. Spinach has enormous genetic diversity, among which might be high-folate germplasm. It might be worth identifying the spinach germplasm with elevated amounts of folate. Any such high-folate germplasm could be use as a variety without any modification or as a source of genes to introgress into crop species, which could contribute to a big proportion of RDA for folate-deficient people worldwide. Currently, very little is known about the variations of folate levels among spinach germplasm, because only very few genotypes have ever been analyzed for folate content.[13−15] Furthermore, to understand the natural genetic variation of folate levels, the genotype should be grown under control conditions. There are no comprehensive studies in which a larger number of spinach germplasm has been evaluated in controlled conditions. Other micronutrients are known to vary substantially among different spinach accessions. Among 327 accessions of spinach, leaf Fe varied 2.7-fold and leaf Zn varied 12-fold.[16] Other groups reported almost 1.9-fold variation of oxalate content among 349 accessions.[17] These data indicate that there is potential to use the genetic diversity of spinach to increase the folate levels.

Several folate vitamers account for the total folate in spinach,[15] and analyzing the different vitamers of folates in spinach is important because these compounds differ in their bioavailability.[3,18] However, the stability of folate vitamers may differ,[19,20] and therefore, genotypes with elevated proportions of bioavailable and more stable vitamers would be desirable traits for plant breeders. The extent of natural variation in folate content needs to be determined to use selection of genotypes or breeding for enhancement of folate levels.

The aim of this study was to investigate variation in folate levels and their vitamers in a comprehensive number of spinach accessions grown in controlled environmental conditions.

MATERIALS AND METHODS

Plant Materials and Culture. *Spinach Accession Collection.* A population of 67 accessions of spinach (*S. oleracea* L.) were selected from the spinach database according to their diverse geographical origin (see Supplementary Table 1 in the Supporting Information), with 57 accessions from the United States Department of Agriculture (USDA)−Agricultural Research Service (ARS) Germplasm Resources Information Network (GRAIN) (http://www.ars-grin.gov/) and 11 accessions from the Asian Vegetable Research and Development Center (AVRDC) Vegetable Genetic Resources Information System (AVGRIS) (http://203.64.245.173/avgris/index.asp). These accessions show a wide range of geographical origin, from Europe, Middle East, east Asia, to America.

Seed Germination. A total of 20 seeds of each accession were rinsed in deionized water, surface sterilized by 0.07% sodium hypochlorite (NaOCl) for 30 min, and drained. They were then placed in aerated deionized water (23 ± 3 °C) and soaked overnight. After soaking water was poured off, the seeds of each accession were placed in black plastic pots (15 × 15 × 15 cm) filled with a 1:1:1 (v/v/v) mixture of peat, perlite, and vermiculite.

Culture Condition. Accessions were grown in a controlled manner according to Gurasak et al.,[16] with slight modification. After emergence, 12 healthy plants of each accession were allowed to grow per pot, with the remaining thinned. Pots were randomly assigned in a growth chamber (made by http://www.zjuee.cn), and their position was altered every third day. Plants were maintained on a 12 h photoperiod of 200 μmol m^{-2} s^{-1} photosynthetically active radiation (incandescent and fluorescent lamps), with a 22 ± 0.5 °C/18 ± 0.5 °C day/night temperature regime. Some accessions (TOT 6497, PI 175313, and PI 604785) were grown in a 9 h photoperiod to avoid premature bolting. Relative humidity was maintained at 50 ± 5%. Plants were initially irrigated with deionized water, and following emergence, they were sub-irrigated daily with modified nutrient solution proposed by Yang et al.,[21] containing the following concentrations of mineral salts: 1.43 mM NH_4NO_3, 1.00 mM $CaCl_2$, 1.64 mM $MgSO_4$, 0.32 mM NaH_2PO_4, 1.32 mM K_2SO_4, 5.0 μM $MnCl_2$, 0.2 μM $CuSO_4$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 1.90 μM H_3BO_3, 20.0 μM Fe−HEDTA, 1.0 μM $ZnSO_4$, and 0.1 μM $NiSO_4$. Before application, nutrient solution pH was adjusted to 5.5 with 0.1 M NaOH or 0.1 M HCl.

Sampling Procedure. Plants were harvested at 5 weeks of age, when they were showing 5−6 fully expanded leaves. Plants were excised 0.5 cm above the base. Areal parts of harvested material including both mature and immature leaves (leaf blades and petioles) were weighed for fresh weight. At least six plants of each accession were cut into small pieces by sharp scissors and combined together to make a composite sample, which represented the accession for folate analysis.

Folate Analysis. *Reagents.* Liquid chromatography (LC)-grade acetonitrile, methanol L(+)-ascorbic acid (99%, crystalline), 2,3-dimercapto-1-propanol (BAL), and rat serum were purchased from Sigma-Aldrich (St Louis, MO). LC-grade phosphoric acid (85%) was purchased from Tedia Company, Inc. (Fairfield, OH). Monobasic potassium phosphate (≥99%, purity), dibasic potassium phosphate (≥99%, purity), sodium acetate (≥99%, purity), and sodium chloride (≥99%, purity) were from Merck (Darmstadt, Germany). Powdered certified reference material (BCR-485) were purchased via Sigma-Aldrich (St. Louis, MO) from the Institute for Reference Material and Measurements (Geel, Belgium) and stored as vacuum-packed sub-samples (2 g) at −80 °C until analysis. Water was purified (≤0.1 μS cm^{-1}) using a Milli-Q system (Millipore, Billerica, MA).

Folate Standards. Folic acid and the reduced forms of monoglutamyl folates, (6,*S*)-5,6,7,8-tetrahydrofolate sodium salt (H_4-folate), (6,*S*)-5-formyl-5,6,7,8-tetrahydrofolate sodium salt (5-HCO−H_4-folate), and (6,*S*)-5-methyl-5,6,7,8-tetrahydrofolate sodium salt (5-CH_3−H_4-folate), were a kind gift from Merck & Cie (Schaffhausen, Switzerland). Pteroyltri-γ-L-glutamic acid ($PteGlu_3$) and 10-formylfolic acid sodium salt (10-CHO-folic acid) were obtained from Schircks Laboratories (Jona, Switzerland). Folate standards were stored under an argon atmosphere at −80 °C until use. The actual concentrations of folate standards were assured spectrometrically according to van den Berg et al.[22] using the molar extinction coefficients reported by Baggott et al.[23] for 10-HCO-folic acid and molar extinction coefficients reported by

Eitenmiller et al.[24] for other folate forms. The standard stock solutions (200 μg/mL) and calibration solutions were prepared according to Jastrebova et al.[25] and Patring et al.,[26] with minor modification, using 1% L(+)-ascorbic acid (w/v) and 0.1% BAL (v/v) under yellow fluorescent light. The standard stock solutions were placed in 1 mL tubes, flushed with argon gas, and stored under an argon atmosphere at −80 °C maximum for 3 months. The calibration solutions were prepared immediately before use by dilution of the stock solution.

Enzyme Preparation. Folate conjugase preparation was performed as describe before.[27] Shortly, rat serum were mixed with one-tenth volume of activated charcoal to remove endogenous folates, stirred for 1 h on ice, centrifuged, and filtered through a 0.20 μm sterile syringe filter (Nalgene, Rochester, NY). Folate conjugase activity was checked using $PteGlu_3$ as described.[26] The filtered rat serum was stored in a 0.5 mL sterile tube at −80 °C for no more than 3 months. The enzyme activity of every batch of rat serum was checked prior to use.

Sample Preparation. Samples were protected against folate oxidation throughout the preparation process by argon gas, yellow fluorescence light, and cooling on ice after heating. Folates from the spinach samples were extracted following the procedure described by Zhang et al.[15] and Hefni et al.,[13] with slight modification. In brief, 5 g of composite sample (fresh weight) was immediately ground to a fine powder in liquid nitrogen. The powder was transferred to 50 mL plastic centrifuge tubes, adding 15 mL of extraction buffer [0.1 M phosphate buffer containing 1.0% of L(+)-ascorbic acid (w/v) and 0.1% BAL (v/v) at pH 6.5, freshly prepared], flushed with argon gas, and capped. The capped tube was then placed on a water bath at 100 °C for 10 min and then rapid cooled on ice. Tubes were then centrifuged at 27000*g* for 20 min at 4 °C. The supernatants were filled to an exact volume in 25 mL volumetric flasks with extraction buffer. For deconjugation of polyglutamylated folates, 175 μL of rat serum was added to the 5 mL of extraction solution to another centrifuge tube and flushed with argon gas before capping, which was then incubated on a shaking water bath at 37 °C for 2 h. An additional treatment of 5 min at 100 °C was carried out to inactivate the enzyme, again followed by cooling on ice. The samples were then centrifuged again at 27000*g* for 20 min at 4 °C for 20 min. The final solution at the centrifuge tube was then filtered through 0.45 μm pore size, 25 mm inner diameter, nylon disposable syringe filters, and the filtrates were ready for solid-phase extraction (SPE).

Sample Cleanup. Purification of the filtrates was carried out using SPE on strong anion-exchange (SAX) Isolute cartridges (3 mL/500 mg of quaternary amine N^+, counter ion Cl^-, Supelco, Bellefonte, PA) as described by Jastrebova et al.[25] and Iniesta et al.[28] A visiprep SPE vacuum manifold (Supelco, Bellefonte, PA) was used for elution under reduced pressure. The cartridges were conditioned by rinsing with methanol (2 × 2.5 mL) and water (2 × 2.5 mL), followed by purification buffer [0.01 M dibasic potassium phosphate containing 1% L(+)-ascorbic acid (w/v) at pH 7.0, 2 × 2.5 mL]. Aliquots (2.5 mL) of the sample extracts were applied to the cartridges and passed slowly with a flow rate not exceeding 1 drop/s. The elution of retained folates was performed slowly (flow rate not exceeding 1 drop/s) with 0.1 M sodium acetate containing 10% sodium chloride (w/v), 1% L(+)-ascorbic acid (w/v), and 0.1% BAL (v/v). The first portion (0.7 mL) of eluate was discarded, and the second portion (3.8 mL) was collected and weighed.

LC Conditions. The chromatographic separation method previously described by Patring et al.[26] and Jastrebova et al.[25] was used to determine individual folate, with slight modification. A LC system (Agilent 1200 Series, Agilent Technologies, Germany) was used, consisted of a gradient quaternary pump, a thermostatted autosampler, a thermostatted column compartment, an ultraviolet (UV) detector, a fluorescence detector (FLD), and a computer running Agilent Chemstation software (revision B. 04.01 SP1). The separation of folates was performed on a Zorbax SB, C_{18} column, 250 × 4.6 mm, 5 μm (Agilent Technologies, Santa Clara, CA), at 23 °C. The flow rate was 0.4 mL/min. The injection volume was 20 μL, with a total running time of 42 min. The temperature in the thermostatted autosampler was 8 °C. For the detection and quantification of folates, a FLD (excitation/emission = 290/360 nm for reduced folates and 360/460 nm for 10-HCO-folic acid) and an UV detector were used (290 nm). Peak purity and identity were confirmed by a comparison of relative peak areas in both detectors. The mobile phase was a binary gradient mixture of 30 mM potassium phosphate buffer at pH 2.3 and acetonitrile. The gradient started at 6% (v/v) acetonitrile and was maintained isocratically for the first 5 min. Thereafter, the acetonitrile content was raised linearly to 25% within 20 min and was kept constant for 2 min. Thereafter, it was decreased linearly to 6% acetonitrile for 1 min and was applied for 14 min to re-equilibrate the column.

Folate Quantification. Quantification was based on an external standard method, in which the peak area was plotted against the concentration and the least-squares regression analysis was used to fit lines to the data. Peak purity and identity were confirmed by a comparison of relative peak areas in both detectors. Quantification was based on fluorescence detection using a multi-level (n = 8) external calibration curve with a linear range over 0.5−100 ng/mL for H_4-folate, 0.3−100 ng/mL for 5-CH_3−H_4-folate, and 10−600 ng/mL for 5-HCO−H_4-folate and 10-HCO-folic acid. The calibration curves had a correlation coefficient higher than 0.9996 for all folate forms. Sensitivity was assessed by evaluating the LOD (S/N > 3) and LOQ (S/N > 10) values. Intra- and interbatch precision were determined by the relative standard deviation (RSD) obtained on 1 day and on different days at three levels. The amount of each folate form was calculated in its free acid form. Endogenous 5-CH_3−H_4-folate found in each batch of rat serum was corrected by running blank samples and subtracting the concentration from the real sample extracts. The sum of different folate forms in spinach accessions was expressed in micrograms of folic acid/100 g of fresh weight after conversion using the molecular weight of 445.4 for H_4-folate, 459.5 for 5-CH_3−H_4-folate, 473.5 for 5-HCO-H_4-folate, 469.4 for 10-HCO-folic acid, and 441.4 for folic acid.

Check of Accuracy and Quality Control. Certified reference material mixed vegetables (BCR-485) were used to check the accuracy and quality control. In BCR-485, only H_4-folate and 5-CH_3−H_4-folate were quantified and the sum of folates, expressed as folic acid, was 236.4 ± 1.2 μg/100 g (n = 2). However, 5-HCO−H_4-folate was masked and could not be quantified, and 10-HCO-folic acid was not detected, which was well in line with the data previously reported by Jastrebova et al.,[25] and Kariluoto.[29] Recovery tests were performed in duplicate by the addition of folate standards before extraction to BCR-485, and mean recovery was 97 ± 1 for H_4-folate and 98 ± 2 for 5-CH_3−H_4-folate. In addition, the total folate contents of the duplicated samples were not allowed to differ more than 10%.

Statistical Analysis. The results were presented as mean values from duplicates based on fresh weight (FW). The relationship between the total folate content and biomass yield was analyzed using correlation analysis. The Pearson correlation coefficients were calculated using SPSS 16.0.

RESULTS AND DISCUSSION

Total Folate in Diverse Spinach Accessions. Spinach is an important source of nutrients in the diet among the leafy green vegetables, ranking second behind kale in total folate.[4] However, the folate content in spinach not enough to meet the RDA from a single serving. The development of plants, such as spinach, to deliver a greater proportion of RDAs of folate (biofortification) is a prime target for plant breeders. However, very little is known about the magnitude of folate content in diverse spinach accessions. To address this question, 67 spinach accessions that were collected

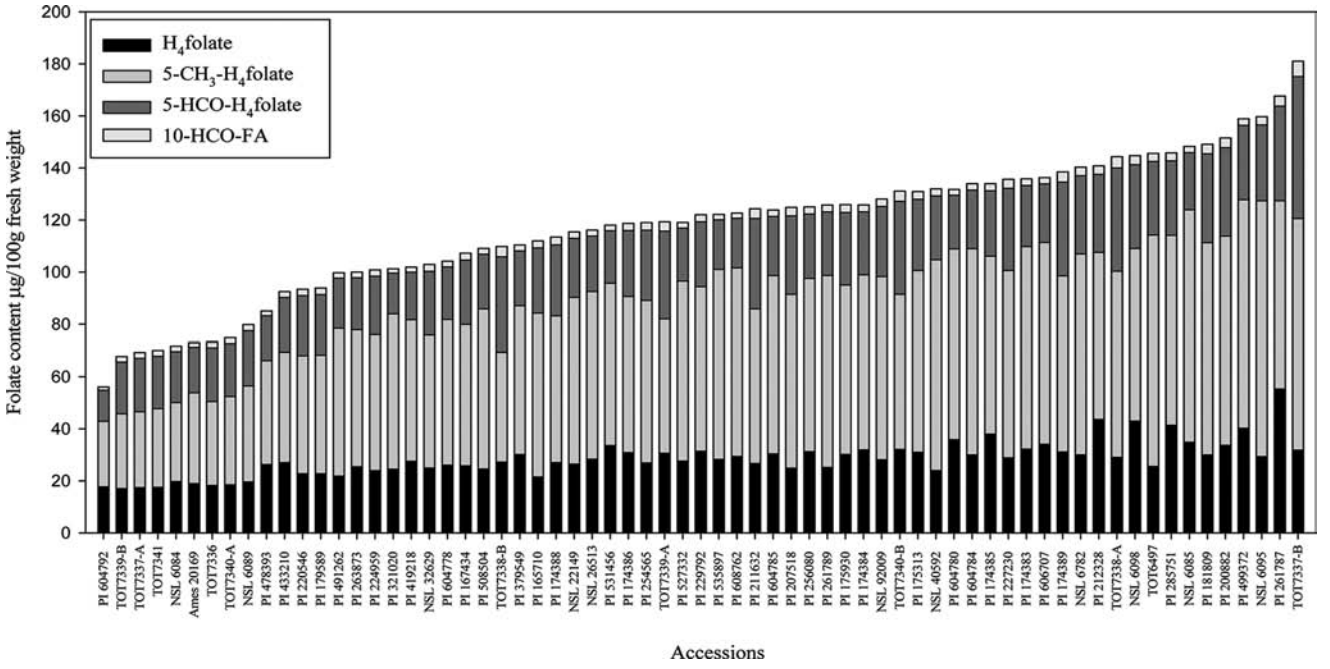

Figure 1. Folate composition measured in 67 USDA and AVRDC spinach accessions grown under a controlled manner. Folate extractions were made from at least six plants to form a composite sample, and duplicate determinations were made on each extract. Data are means of all determinations.

Table 1. Ranking of the USDA and AVRDC Spinach Accessions According to Their Total Folate Content[a]

group	folate content (µg/100 g of fresh weight)	number of accessions	name of accessions
1	<60	1	PI 604792
2	60−74	7	TOT7339-B, TOT7337-A, TOT7341, NSL 6084, Ames 20169, TOT7336, TOT7340-A
3	75−89	4	NSL 6089, PI 478393, PI 433210, PI 220546
4	90−104	10	PI 179589, PI 491262, PI 263873, PI 224959, PI 321020, PI 419218, NSL 32629, PI 604778, PI 167434, PI 508504
5	105−119	17	TOT7338-B, PI 379549, PI 165710, PI 174388, NSL 22149, local variety, PI 531456, PI 174386, PI 254565, TOT7339-A, PI 527332, PI 229792, PI 535897, PI 608762, PI 211632, PI 604785, PI 207518
6	120−134	16	PI 256080, PI 261789, PI 175930, PI 174384, NSL 92009, TOT7340-B, PI 175313, NSL 40592, PI 604780, PI 604784, PI 174385, PI 227230, PI 174383, PI 606707, PI 174389, NSL 6782
7	135−149	8	PI 212328, TOT7338-A, NSL 6098, TOT6497, PI 285751, NSL 6085, PI 181809, PI 200882
8	>150	4	PI 499372, NSL 6095, PI 261787, TOT7337-B

[a] In each group, the accessions are given in order of increasing folate content.

from diverse geographical origin were grown under controlled conditions and screened for their natural diversity of folate levels.

The folate concentration among the spinach accessions ranged from 54.1 to 173.2 µg/100 g of FW, which was a 3.2-fold difference from the lowest to highest folate concentrations, with a mean content being 113 µg/100 g of FW (Figure 1). When the range in the total folate content was divided into intervals of 14 µg/100 g of FW, giving eight groups, the results showed that each of the groups include 1−17 accessions (Table 1). This comparison thus showed that most of the accessions were distributed near their mean content. However, the highest number of accessions (17 accessions) fell within the interval of 106−119 µg/100 g of FW, which mean that 25% of accessions had the folate contents in the range of 106−119 µg/100 g of FW.

The second highest group (16 accessions) within the interval of 120−134 µg/100 g of FW means that 23% of accessions had folate contents in the range of 120−134 µg/100 g of FW. This second group covered 50% of the accessions, which were the medium sources of folate content, with the range of 105−134 µg/100 g of FW. Above and below the range of 105−134 µg/100 g of FW, 8 and 10 accessions fall into 135−149 µg/100 g and 90−104 µg/100 g of FW, respectively. A total of 8 accessions, falling into the range of 135−149 µg/100 g of FW, were the moderate sources of folate and accounted for 12% of the total accessions analyzed. A total of 10 accessions accounted for 14% of total accessions, falling into the range of 90−104 µg/100 g of FW, and were the moderate low folate content accessions. Furthermore, 12 accessions fall within the interval of below 60−89 µg/100 g of FW, among them 4 accessions fell within the interval of 74−89 µg/100 g of FW, 7 accessions fell within the range of 60−74 µg/100 g of FW, and 1 accession fell below 60 µg/100 g of FW. This group was the poorest

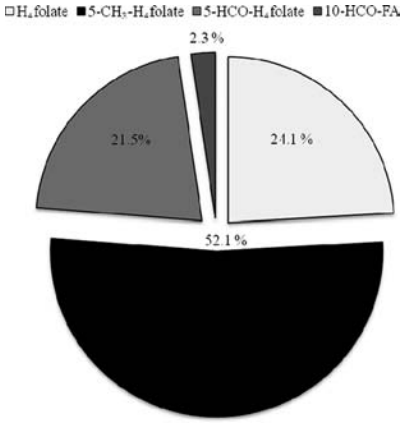

Figure 2. Folate vitamer distribution in AVRDC and USDA spinach (*S. oleracea*) accessions.

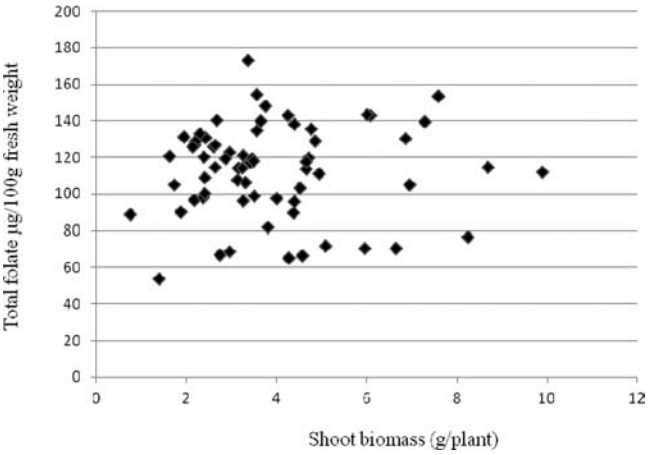

Figure 3. Correlation of total folate contents and fresh weight in spinach accessions (not significant).

folate-containing accession, accounting for 17% of total accessions screened. Finally, 4 accessions exceed the range 150 μg/100 g of FW. These were the highest folate content accessions, accounting for 6% of the total accessions screened.

The highest folate content of 173.2 $\pm$ 1.1 μg/100 g of FW was found in accessions number TOT 7337-B, which originated from India. Other accessions in the topmost group were from France (PI 261787), the United States (NSL 6095), and the former Soviet Union (PI 499372). On the other hand, the lowest folate content of 54.1 $\pm$ 2.5 μg/100 g of FW was found in accession number PI 604792, which was originated from Germany. Accessions in the bottom group, however, originated from Bangladesh, India, the United States, China, Pakistan, and Afghanistan. Overall, a comparison of total folate contents of the accessions to the information of on their origin thus showed that the highest and lowest folate sources were evenly distributed among their geographical origin.

The total folate content of spinach was reported in lots of studies, varying from 194−364 μg/100 g of FW for the microbiological assay[4,30] to 48−177 μg/100 g of FW for the high-performance liquid chromatography (HPLC) method;[14,31] however, no study was reported with large accessions in control conditions. In general, the folate content determined by HPLC was 20−52% lower than that for the microbiological assay.[29,32,33] Confirmation of the method validation is also essential, and analysis of certified reference materials is essential to reduce methodological differences. In this study, we checked the BCR-485 for method validation. The certified value of total folate by the microbiological assay was 315 $\pm$ 28 μg/100 g, and the certified value of total folate from our study was 236.4 $\pm$ 1.2 μg/100 g, which was 25% lower than the certified value, in line with previous studies.[13,25] However, all of the previous studies were mainly focused only on the amount of folate in spinach, and there are no previous studies with such a marked number of accessions as screened in this study. In addition, all of the accessions were grown in similar conditions, as required for true comparisons of natural genetic variation. Thus, this study resulted in valuable new information on the extent of natural variation in folate levels of spinach.

Folate Vitamer Distribution. Folate vitamer distribution was analyzed (Figure 2), because each of the vitamers differs in their stability and bioavailability. 5-CH_3−H_4-folate was the most abundant vitamer found in spinach and varied from 25.2 to 98 μg/100 g of FW, contributing on average 52.1% of all of the analyzed vitamers, varying from 46 to 63% depending upon the accessions screened. On the other hand, H_4-folate, 5-HCO−H_4-folate, and 10-HCO-folic acid were contributing 24.1, 21.5, and 2.3% of the analyzed vitamer, respectively, which were 47% of the total vitamers. However, in terms of stability, 5-HCO-H_4-folate and 5-CH_3−H_4-folate were known to be more stable folate vitamers,[19,20] which were contributing 73% of the total folate pool in spinach. Furthermore, 5-CH_3−H_4-folate is the first choice of enhancement, because it is the only form found in human circulation.[3] Some of the previous reports with different sample preparation techniques and detection systems showed some extent of variation of the folate vitamer distribution in spinach[13,14,31,32] and also reported that 5-CH_3−H_4-folate was the main vitamer in spinach. However, our results consistent with data from Zhang et el.,[15] demonstrating that H_4-folate, 5-CH_3−H_4-folate, 5-HCO−H_4-folate, and 10-HCO−folic acid constituted 28, 53, 20 and 7.3% of the total folate pool of different batches of spinach, respectively.

Association of the Total Folate Content with Biomass Yield. A large number of spinach accessions were compared in this highly controlled experiment. Interactions of the biomass yield and total folate content were also compared (Figure 3). There was no or little correlation (r = 0.029) between this two parameters and was statistically insignificant (p = 0.813). This suggested that the biomass yield and folate content might be independent traits; these two traits might be possible to improve independently. Hence, it might be possible to combine a high biomass yield with a high folate content in a domestic cultivar. Ward et al.[34] reported that the folate content did not clearly correlate with either 100 grain weight (r = 0.137; p = 0.095) or bran yield (r = −0.127; p = 0.121) when 175 wheat lines were considered. Similar trends was also observed in leaf yield and oxalate content in diverse spinach accessions.[17]

Spinach is a widely consumed vegetable in the world and encompasses broad genetic diversity. The main objective of this experiment was to explore the natural variation of folate content among the spinach accessions for the biofortification program and whether folate-rich accessions could be identified that could be a source of spinach genes useful to increase the future folate content in spinach by smart breeding. Discovering the high and low folate spinach accessions may prove useful toward

understanding the molecular basis of folate variation in spinach. In this controlled study, we found that the folate content varied greatly between the accessions evaluated. A 3.2-fold variation in the total folate concentration was observed between the highest and lowest accessions, and a 3.8-fold variation was found in the main vitamer, 5-CH_3−H_4-folate, concentration. However, it might be possible that crossing a high folate line with most stable and bioavailable vitamers could be effective, because no breeding efforts have previously been directed toward specifically increasing folate in spinach. Besides a transgenic approach, molecular marker assistant breeding might be one such approach, exemplified by the production of broccoli with a 100-fold increase in the ability of anticarcinogenic marker enzyme quinone reductase.[35] Several approaches, such as QTL characterization, may also be possible, for example, the improvement of carotenoid levels in carrots and tomatoes.[36,37] Besides, there are many more accessions extant that what we have examined in this study, and we cannot preclude that natural genetic variation is substantially higher than what we observed.

This experiment was a first step toward folate biofortification in a widely consumed vegetable, spinach. However, the highest folate content found in spinach accessions was as high as 173.2 μg/100 g of FW, which was nearly the same as transgenic tomato (180.5 μg/100 g)[38] and lettuce (188.5 μg/100 g).[39] Cooking loss according to literature data demonstrates ∼40% of spinach folate loss after 30 s of boiling.[40] Thus, the highest folate-rich spinach accession serving alone can provide about 49.4% of the dietery reference intake (DRI) for an adult (400 μg/day) and 32.9% of the DRI for a pregnant woman (600 μg/day) without any modification (NDB 11458, 190 g cooked, according to the USDA National Nutrient Database for Standard Reference, Release 23).

In conclusion, this study provides new data on natural variation in the folate content in spinach. The large number of accessions from diverse geographical origin and controlled experimental conditions provide a unique opportunity to obtain reliable information on folates in spinach accessions. The highest and lowest folate content accessions were evenly distributed among their geographical origin. The biomass yield and folate content could be improved independently, and it might be possible to combine both traits together. These screening data provide the basis for breeding folate-rich spinach varieties.

■ ASSOCIATED CONTENT

Supporting Information. Spinach (*Spinacia oleracea*) accessions used in this study (Supplementary Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ DISCLOSURE

Safety: General guidelines for work with organic solvents and acids were considered. 2,3-Dimercapto-1-propanol (BAL) has been indicated to be toxic by European Union regulatory information.

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