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Spinach (Spinacia oleracea L.) Seed Germination and Whole Plant Growth Response to Heat Stress and Association Mapping of Bolting, Tallness and Erectness for Use in Spinach Breeding

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Spinach (*Spinacia oleracea* L.) Seed Germination and Whole Plant Growth Response to Heat Stress and Association Mapping of Bolting, Tallness and Erectness for Use in Spinach Breeding

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by

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Abstract

The effect of temperature on spinach seed germination was evaluated using a total of nine spinach genotypes and seven temperatures: 10, 15, 20, 25, 30, 32, and 35 ^{o}C in growth chambers. Genetic variation was observed. 'Donkey', 'Marabu', and 'Raccoon' showed higher seed germination percentage with over 70% at 30 and 32 ^{o}C, indicating the three spinach genotypes had heat-tolerance for germination. However, all spinach genotypes except 'Ozarka II' had lower germination percentages of less than 30% while 'Ozarka II' had 63% at 35 ^{o}C, indicating 'Ozarka II' may be a source of heat-tolerance for seed germination.

Seed germination may be useful as an early screening method for heat-tolerant spinach genotypes. The whole-plant experiment was conducted using two temperatures, 20 and 32 ^{o}C, in growth chambers. Variation was observed among spinach cultivars for leaf area and shoot dry weight at 32 ^{o}C. 'Samish' was the only cultivar to have no significant difference between 20 and 32 ^{o}C for leaf area, while the leaf area for 'Ozarka II', 'Donkey', and 'Marabu' decreased from 20 to 32 ^{o}C. This may indicate that 'Samish' is heat-tolerant. However, significant differences were not observed among cultivars for shoot dry weight. The results from whole-plant growth at high temperature did not parallel those of the seed germination study, and the evidence does not indicate that seed germination can be used as a screening tool for heat tolerant cultivars.

Bolting, tallness, and erectness are important morphological traits in spinach breeding. A total of 288 USDA spinach accessions were used as the association panel in this research. Single nucleotide polymorphisms (SNPs) discovered through genotyping by sequencing (GBS) were used for genotyping. Three SNP markers, AYZV01001038_398, AYZV01031624_1060, and AYZV01088923_95 were found to be associated with bolting. Eight SNP markers, AYZV01011130_540, AYZV01180397_2162, AYZV01069590_19842, AYZV01105690_376, AYZV01058838_64, AYZV01152613_2532, AYZV01113619_2197, and AYZV01003134_248 were associated with tallness. Four SNP markers,

AYZV01137843_229, AYZV01158294_79, AYZV01023368_256, and AYZV01097131_197 were associated with erectness. These SNP markers may be used in spinach molecular breeding to select spinach bolting, tallness and erectness through marker-assisted selection.

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1. Chitwood, J., A. Shi, B. Mou, M. Evans, J. Clark, D. Motes, P. Chen, and D. Hensley. 2016. Population structure and association analysis of bolting, plant height, and leaf erectness in spinach. HortScience (In press.)

Chapter 1: Introduction and Literature Review

Introduction

Spinach (*Spinacia oleracea* L.) is a leafy green, cool-season vegetable that is known for its nutritive value, and is considered one of the most popular vegetables in the U.S (Naeve, 2014). The economic importance of spinach, especially fresh-market spinach, continues to grow in the U.S. as well as globally. In 2014, the annual per capita consumption of fresh market spinach was 0.77 kg per person in the U.S, and has been as high as 1.27 kg (Naeve, 2014). In order to meet demands, production of spinach has risen as well. Harvested acres of spinach has risen from 13,937 ha in 2012 to 15,191 ha in 2014 (USDA, 2015). Spinach production in the U.S. was valued over $250 million in 2014 (USDA, 2015).

Spinach is a member of the subfamily Chenopodioideae and is related to swiss chard, quinoa, and sugar beet (Yamamoto et al., 2014). It is believed to have originated from Persia, where the earliest references to spinach occurred between 200 and 600 A.D., and was transported to India and Asia and then later to the Mediterranean countries and Europe (Wright, 2001). Spinach was introduced to North America by early European colonists, and documented cultivation of three recognized cultivars took place by 1806 (Morelock and Correll, 2008).

Spinach is an annual, cool-season, flowering vegetable that produces a rosette of leaves during its vegetative stage. The vegetative state is harvested for consumption. Leaf shape ranges from round to pointed and leaf texture can be flat, savoy (crinkled), or semi-savoy (an intermediate). It is a diploid 2n=12 and typically dioecious (Morelock and Correll, 2008). Bolting, or flowering, of spinach results in either male or female plants and is photosensitive. Seeds can be round or spiny, although the standard for spinach seeds in the U.S. is round (Morelock and Correll, 2008). Spinach plants are open-pollinated and wind pollinated.

Cultivation of spinach is highly mechanized and takes place primarily in open field settings where it is direct seeded (Koike et al., 2011; Morelock and Correll, 2008) although greenhouse-grown spinach is gaining in importance (Brandenberger et al., 2007). Greenhouse and high tunnel food crops

have been an increasing interest within the industry in the U.S. Cultivating various types of leaf vegetables has potential both in the U.S. and globally for being a very valuable market. Spinach is an excellent choice for this niche because of its high nutritive content and short-duration production cycles (Brandenberger et al., 2007). Local spinach production around many parts of the U.S. is dependent upon season, and when local spinach is unavailable, it is primarily shipped from California or Arizona which decreases its quality (Both et al., 1996). In warmer climates, the sensitivity of spinach to higher temperatures is a limiting factor to its production (Ikeda et al., 1995). The ability to produce spinach in a greenhouse year-round increases the productivity of greenhouse farms in addition to increasing the seasons in which locally grown, fresh spinach is available.

Spinach is grown commercially for fresh market, frozen, and canned products (Naeve, 2015). Fresh market spinach may be sold as clipped, bagged "baby leaf" spinach or in bunches, and spinach produced for fresh market made up 90% of all spinach production in the U.S. in 2014 (Naeve, 2015). California is the leading state for spinach production, with 10,521 ha of spinach harvested in 2014 (USDA, 2015). Arizona, Texas, and New Jersey follow with 3,237, 607, and 526 ha harvested, respectively (USDA, 2015). Spinach is produced virtually year-round in the cool, coastal valleys of California (Koike et al., 2011) and in the cool, dry winter months in Arizona and Texas (CFAIT, 2014).

Temperature Requirements

Spinach is a cool-season crop and has a specific growing season determined by optimal temperatures for growth. Experiments have shown that spinach seeds will germinate in soil temperatures from 5 ^{o}C to 30 ^{o}C with germination percentages highest at 20 ^{o}C and dropping abruptly between 25 and 30 ^{o}C (Atherton and Farooque 1983). Substantial seedling root development requires temperatures above 18.9 ^{o}C, and top growth will be limited at temperatures below 12.3 ^{o}C and above

23.3 °C (Wilcox and Pfeiffer, 1990). Air temperatures above 35 °C affect the efficiency of metabolism in spinach plants, resulting in reduced yields (Lefsrud et al., 2005).

Studies have been done on the heat-shock response of spinach, both with whole plants and detached leaf tissue. Thebud and Santarius (1982) found that chloroplasts in whole spinach leaves were more sensitive to high temperatures than mitochondria, and heat injury is due mostly to irreversible damage to the photosynthesis systems. Heat-shock proteins (HSPs) are an important response by plants to temperature stress, and Somers et al. (1989) found that the lowest temperature to induce HSPs in whole spinach plants was 28 °C. In a study by Lefsrud et al. (2005), higher than optimum air temperatures were found to significantly reduce the accumulation of secondary plant compounds in spinach, including lutein and β-carotene, carotenoids that serve important functions in photosynthesis. Further studies on how high temperature stress affects spinach plants found that, in a study using whole spinach plants as well as detached spinach leaves, after being exposed to heat shock (35 – 50 °C) for 30 min, CO_2 assimilation decreased and pigment proteins in thylakoid membranes aggregated, slowing down the plant's ability to photosynthesize (Tang et al. 2007).

Their findings indicate that spinach has quite specific growing needs concerning temperatures. Therefore, a specific growing season is required for spinach production. The ideal growing season for spinach is affected by the changing climate as temperature increases, thereby shortening the time period of optimal temperatures for spinach.

There has been extensive research on climate change both globally and within the U.S., and the overall pattern, from data including air temperature records, ice core samples, ocean temperature records, and greenhouse gas emissions, indicates a world-wide warming trend (Walthall et al. 2013). Regions in the U.S. have experienced shifts in average temperatures, excluding the southeast, and the most notable temperature shifts have occurred during the spring and summer (Walthall et al. 2013), thus indicating that the number of days with cardinal temperatures for spinach are growing smaller.

Although spinach is not known for growing well in the summer, it is possible to select and develop heat-resistant spinach which can grow year-round and withstand the high temperatures of summer in a greenhouse or high tunnel setting.

In order to characterize heat tolerance in spinach, a reasonable place to begin is with germination. A spinach cultivar that is able germinate with a high percentage in higher soil/medium temperatures has the potential to extend the growing season of spinach. Rapid and uniform germination is also necessary for efficient crop production, both in field and greenhouse practices. While it has been reported that seed treatments may be effective for increasing germination of spinach at higher temperatures (Katzman et al., 2001), managing this trait via breeding heat-tolerant cultivars is a more manageable practice for spinach producers.

Germination under heat stress may also play a significant role in selecting heat-tolerant spinach. Historically, mass selection was the primary method for developing cultivars, with hybrid breeding becoming popular in recent years, but all are based on field testing (Morelock and Correll, 2008). While field testing is necessary in many cases, there are numerous environmental effects that contribute to germination beyond that of temperature. Therefore, it may be useful to reduce the number of genotypes planted by prior testing, improving the statistical approach to reduce error and estimate genotype by environment interaction. Using germination as this pre-test has been successful in other crops, such as *Sorghum bicolor* (L.) (Tiryaki and Andrews, 2001), and would allow quicker and more efficient selections to be made. Tiryaki and Andrews (2001) found a high correlation between sorghum seed germination measurements in growth chamber and seedling emergence in the field at similar temperatures. Performing germination tests in petri dishes under controlled conditions is a relatively simple technique that could screen large numbers of genotypes with lower costs, and this has potential to be a very useful tool for spinach breeders.

Spinach Breeding

Spinach breeding programs in the U.S. have focused on a variety of traits, depending on location of the program and the intended market. Disease resistance, especially to *Albuigo occidentalis* (white rust) and *Peronospora farinosa* f. sp. *spinaciae* (downy mildew) has been a primary focus as well as other important agronomic traits (Morelock and Correll, 2008).

Bolting is an important trait to consider in relation to developing spinach cultivars for year-round production because of its sensitivity to photoperiod (Chun et al., 2000). Long-day exposure induces bolting in spinach, rendering the plant unmarketable (Goreta and Leskovar, 2006). Because some commercially grown spinach is cut multiple times (Morelock and Correll, 2008), overwintered spinach that is susceptible to bolting in the spring will reduce the number of harvests that may be taken and therefore reduce overall yield. Chun et al. (2000) found that bolting can be affected by modification of light during transplant production, but this is impractical for large-scale production where spinach is almost entirely direct seeded (Koike et al., 2011). Genetic variation among spinach for bolting has been documented for many years, and therefore, late-bolting cultivars can be developed through breeding efforts (Goreta and Leskovar, 2006).

Plant growth habit traits are also important to consider for spinach production. Commercial spinach cultivation is highly mechanized (Koike et al., 2011; Morelock and Correll, 2008), and traits such as tallness and erectness effect the ability to harvest the spinach leaves. Plant height in spinach is a complex trait with which generally large QTL regions are associated (Bezant et al. 1996), and a range of phenotypic values often occur. Spinach plant erectness refers to how to close to or far away from the ground the spinach leaves lie on a mature plant.

Molecular Breeding Techniques

Molecular markers have become of increasing importance in plant breeding. For many major crop species, potential genetic variation for important agronomic traits already exists with varying degrees of accessibility (Thomson et al. 2010). DNA markers for genes of interest allow breeders to make selections when otherwise the gene for the trait may have been masked by heterozygosity. Association mapping is relatively recent technology which identifies quanitative trait loci (QTLs) associated with phenotypic characteristics (Zhu et al., 2008), and provides the link for breeders to make selections based on genetic information.

Molecular markers and marker assisted selection (MAS) have been successfully used to select specific genes/alleles in plant breeding, and as cost decreases along with rapid improvement of the technology, these methods are becoming more widely used (Kumar et al., 2012; Morelock and Correll, 2008; Thomson et al., 2010). Genetic research across many disciplines, from human genomic studies to marker assisted breeding of livestock, utilize single nucleotide polymorphisms (SNPs) as the current marker of choice for various reasons, but especially their abundance within any genome and cost efficiency (Zhu et al., 2008). The use of SNPs has become a powerful tool for gaining a better understanding of plant genomics by mapping chromosomes via association mapping and tagging important genes, as well as diversity analysis and other studies (Kumar et al. 2012). Association mapping has been used to successfully identify markers and loci associated with major agronomic traits (Lakew et al., 2013) such as *Colletotrichum sublineolum* (anthracnose) resistance in sorghum (Upadhyaya et al., 2013), growth habit and days to flowering in *Phaseolus vulgaris* L**.** (common bean) (Nemli et al., 2013), and heat-tolerance in *Vigna unguiculata* (cowpea) (Lucas et al., 2013).

To date, knowledge of the spinach genome is limited and few reports have been published on the use of molecular markers in spinach with none reporting the use of SNP markers. Khattak et al. published a genetic linkage map in 2006 with six linkage groups, constructing the map with 101

amplified fragment length polymorphisms (AFLPs) and nine simple sequence repeats (SSRs). This genetic map has a total length of 585 cM, and with an average distance of 5.18 cM between markers (Khattak et al., 2006), it does not offer a great amount of detail about the linkage groups and may contain errors. AFLPs and SSRs, while useful, are less specific than SNP markers. The identification of SNP markers for spinach traits of interest, including bolting, tallness, and erectness, will provide breeders with powerful tools to develop improved spinach cultivars more efficiently. Thus far, no SNP markers and no SNP genetic maps are publically available for spinach. Therefore, the development of robust SNP markers and SNP genetic maps would be valuable a resource for spinach breeding efforts.

Genotyping by sequencing (GBS) is one of the next-generation sequencing platforms that utilizes a simple, highly-multiplexed system for constructing reduced representation libraries, which uses inexpensive barcoding, reduces sample handling, requires fewer PCR and purification steps, and includes no size fractionation (Elshire et al., 2011). GBS can be applied to a wide array of organisms including plants for genome sequencing and SNP discovery, and is a rapid and less expensive approach for trait mapping and association. With GBS, plant breeders can utilize techniques of molecular breeding by conducting genomic selection on any germplasm or species with or without prior knowledge of the genome in the species (Elshire et al., 2011; Sonah et al., 2013). The GBS platform is an advantageous approach for genome-wide SNP discovery, genetic map construction, linkage mapping, and genome-wide association in spinach.

Genetic diversity forms the raw material of plant breeding and is crucial for successful breeding programs (Jansen et al., 2006). Understanding the genetic diversity in one's crop allows a breeder to make informed choices when making crosses and when incorporating more variation into a program. Historically, the importance of genetic diversity can be highlighted by the Irish potato blight famine of 1850 and the epidemic of the southern corn leaf blight in the U.S. in 1970 (Ulstrap, 1972; Xu, 2012). In both cases, genetic uniformity resulted in the decimation of crops by a pathogen, and the value of

understanding the genetic variation of a crop has become well known (Xu et al., 2012). Genetic diversity also plays an important role in association mapping by providing population structure information (kinship matrix) to analyze loci association with traits (Khan, 2013).

A variety of molecular markers may be used for analyzing genetic diversity including restricted fragment length polymorphisms, AFLPs, SSRs, and SNPs (Jansen et al., 2006; Wurschum et al., 2013). Software programs such as STRUCTURE and MEGA6 use polymorphisms of the chosen marker among individuals in the tested population to draw conclusions of population structure and diversity (Pritchard et al. 2000; Tamura et al., 2013). The most common type of sequence variation in the genome are SNPs, and this type of marker provides a powerful tool for investigating and understanding the genetic diversity in a crop (Wurschum et al., 2013).

Because the use of molecular markers in spinach has been limited up to this point, diversity studies have, too, been limited (Hu et al., 2007). Kuwahara et al. (2013) analyzed 250 individuals from West Asia, East Asia, Japan, Europe and the U.S. using SSR markers for six loci and found an overall significant genetic differentiation among spinach from different geographical regions. Diversity has also been observed among Iranian landraces of spinach, where high variation in morphological traits such as leaf shape, pedicle length, and the percentage of female plants was correlated to the variation in genotypes (Sabaghnia et al., 2014). Further contributions to the understanding of genetic diversity in spinach will be useful for spinach breeding efforts.

Literature Cited

Anderson, C.R. 2014. University of Arkansas Division of Agriculture. Home Gardening series: spinach. FSA 6077. Web. 13 Jan. 2014. http://www.uaex.edu/Other_Areas/publications/PDF/FSA-6077.pdf.

Association of Official Seed Analysts. 1993. Rules for Testing Seeds. Assn. Offic. Seed Analysts. Bozeman, MT.

Atherton, J.G., and A.M. Farooque. 1983. High temperature and germination in spinach. I. The role of the pericarp. Scientia Hort. 19:25-32.

Bezant, J., D. Laurie, N. Pratchett, J. Chojecki, and M. Kearsey. 1996. Marker regression mapping of QTL controlling flowering time and plant height in a spring barley (*Hordeum vulgare* L.) cross. Heredity. 77:64-73.

Both, A.J., A.R. Leed, E. Goto, L.D. Albright, and R.W. Langhans. 1996. Greenhouse spinach production in a NFT system. Proc. Int. Sym. Acta Hort. 440:187-192.

Brandenberger, L.P., T. Cavins, M. Payton, L. Wells, and T. Johnson. 2007. Yield and quality of spinach cultivars for greenhouse production in Oklahoma. HortTechnology 17:269-272.

Brandenberger, L.P., J.C. Correll, T. E. Morelock, and R.W. McNew. 1991. Characterization of resistance of spinach to white rust (*Albugo occidentalis*) and downy mildew (*Peronospora farinosa* f. sp. *spinaciae*). Phytopathology 84:431-437.

California Foundation for Agriculture in the Classroom. 2014. Commodity fact sheet: spinach. CFAITC, Sacramento, CA.

Chun, C., A. Watanabe, H. Kim, T. Kozai, and J. Fuse. 2000. Bolting and growth of *Spinacia oleracea* L. can be altered by modifying the photoperiod during transplant production. HortScience 35:624–626.

Correll, J.C., B.H. Bluhm, C. Feng, K. Lamour, L.J. du Toit, and S.T. Koike. 2010. Spinach: better management of downy mildew and white rust through genetics. Eur. J. Plant Pathol. 129:193-205.

Elshire, R.J., J.C. Glaubitz, and Q. Sun. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PloS One 6:19379.

Goreta, S. and D. Leskovar. 2006. Screening spinach cultivars for white rust and bolting. HortTechnology 16:162-166.

Heydecker, W., and P.I. Orphanos. 1968. The effect of excess moisture on the germination of *Spinacia oleracea* L. Planta 83:237-247.

Hu, J., B. Mou, and B. Vick. 2007. Genetic diversity of 38 spinach (*Spinacia oleracea* L.) germplasm accessions and 10 commecial hybrids assessed by TRAP markers. Genet. Resources and Crop Evol. 54:1667-1674.

Hum-Musser, S.M., T.E. Morelock, and J.B. Murphy. 1999. Relation of heat-shock proteins to thermotolerance during spinach seed germination. Horticultural Studies. Ark. Agri. Expt. Sta. Res. Ser. 466:103-105.

Ikeda, H., T. Wada, T. Mirin, K. Okabe, A. Tazuke, and H. Furukawa. 1995. Year-round production of spinach by NFT and DFT in the greenhouse. Acta Hort. 396:257-264.

Jansen, J., H. Verbakel, J. Peleman, and T. van Hintum. 2006. A note on the measurement of genetic diversity within genebank accessions of lettuce (*Lactuca sativa* L.) using AFLP markers. Theor. Appl. Genet. 112:554-561.

Katzman, L., A. Taylor, and R. Langhans. 2001. Seed enhancements to improve spinach germination. HortScience. 36:979-981.

Khattak, J., A.M. Torp, and S.B. Andersen. 2006. A genetic linkage map of *Spinacia oleracea* and localization of a sex determination locus. Euphytica 148:311-318.

Koike, S., M. Cahn, M. Cantwell, S. Fennimore, M. Lestrange, E. Natwick, R. Smith, and E. Takele. 2011. Spinach production in California. Univ. California Agri. Natural Resources. Publ. 7212.

Kumar, S., T. W. Banks, and S. Cloutier. 2012. SNP discovery through next-generation sequencing and its applications. Intl. J. of Plant Genomics 2012:1-15.

Kuwahara, K., R. Suzuki, Y. Ito, T. Mikami, and Y. Onodera. 2012. An analysis of genetic differentiation and geographical variation of spinach germplasm using SSR markers. Plant Genet. Resources 12:185-195.

Lakew, B., R. Henry, S. Ceccarelli, S. Grando, J. Eglinton, and M. Baum. 2012. Genetic analysis and phenotypic associations for drought tolerance in *Hordeum spontaneum* introgression lines using SSR and SNP markers. Euphytica 189:9–29

Lefsrud, M., D.A. Kopsell, D.E. Kopsell, and J. Curran-Celentano. 2005. Air temperature effects biomass and carotenoid pigment accumulation in spinach and kale grown in a controlled environment. HortScience. 40:2026-2030.

Leskovar, D. and V. Esensee. 1999. Pericarp, leachate, and carbohydrate involvement in thermoinhibition of germinating spinach seeds. J. Amer. Soc. Hort. Sci. 124:301-306.

Lucas, M., J. Ehlers, B. Huynh, N. Diop, P. Roberts, and T. Close. 2013. Markers for breeding heat-tolerant cowpea. Mol. Breeding. 31:529-536.

Machado, J., M. Souza, D. Oliveira, A. Cargnin, A. Pimentel, and J. Assis. 2010. Recurrent selection as breeding strategy for heat tolerance in wheat. Crop Breeding and App. Biotechnol. 10:9-15.

Morelock, T. E., and J.C. Correll. (2008). Spinach, p 189-218 in: J. Prohens and F. Nuez, (eds.). Vegetables I: Asteraceae, Brassicaceae, Chenopodiaceae, and Cucurbitaceae. Springer, New York.

Naeve, L. 2015. Spinach. Agr. Mktg. Resource Ctr.

Nemli, S., T. Asciogul, H. Kaya, A. Kahraman, D. Esiyok, and B. Tanyolac. 2014. Association mapping of five agronomic traits in the common bean (*Phaseolus vulgaris* L.). J. Sci. Food Agr. 94:3141-3151.

Pritchard J.K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945—959.

Sabaghnia, N., H. Asadi-Gharneh, and M. Janmohammadi. 2014. Genetic diversity of spinach (*Spinacia oleracea* L.) landraces collected in Iran using morphological traits. Acta. Agri. 103:101-111.

Somers, D., W.R. Cummins, and W.G. Filion. 1989. Characterization of the heat-shock response in spinach (*Spinacia oleracea* L.). Biochem. Cell Biol. 67:113-120.

Sonah H., M. Bastien, E. Iquira, A. Tardivel, and G. Legare. 2013. An improved genotyping by sequencing (GBS) approach offering increased versatility and efficiency of SNP discovery and genotyping. PLoS ONE 8:54603.

Tamura K., G. Stecher, D. Peterson, A. Ailipski, and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. and Evol. 30:2725-2729.

Tang, Y., X. Wen, Q. Lu, Z. Yang, Z. Cheng, and C. Lu. 2007. Heat stress induces and aggregation of the light-harvesting complex of photosystem II in spinach plants. Plant Physiol. 143:629-638.

Thebud, R. and K. Santarius. 1982. Effects of high-temperature stress on various biomembranes of leaf cells *in situ* and *in vitro*. Plant Physiol. 70:200-205.

Thomson, M., A. Ismail, S. McCouch, and D. Mackill. 2010. Marker assisted breeding, p. 451-469. In: Abiotic stress adaptation in plants: Physiological, molecular, and genetic foundation. Springer Netherlands.

Tiryaki, I. and D. Andrews. 2001. Germination and seedling cold tolerance in sorghum: I. Evaluation of rapid screening methods. Agron. J. 93:1386-1391.

Ullstrup AJ. 1972. The impacts of the southern corn leaf blight epidemics of 1970-1971. Annu. Rev. Phytopathol. 10:37-50.

U.S. Department of Agriculture, National Agriculture Statistics Service. 2015. Vegetables 2014 Summary. U.S. Dept. Agr. Natl. Agr. Stat. Serv., Washington, D.C.

Upadhyaya, H., Y. Wang, R. Sharma, and S. Sharma. 2013. Identification of genetic markers linked to anthracnose resistance in sorghum using association analysis. Theor. Appl. Genet. 126:1649-1657.

Walthall, C.L., J. Hatfield, P. Backlund, L. Lengnick, E. Marshall, M. Walsh, S. Adkins, M. Aillery, E.A. Ainsworth, C. Ammann, C.J. Anderson, I. Bartomeus, L.H. Baumgard, F. Booker, B. Bradley, D.M. Blumenthal, J. Bunce, K. Burkey, S.M. Dabney, J.A. Delgado, J. Dukes, A. Funk, K. Garrett, M. Glenn, D.A.

Grantz, D. Goodrich, S. Hu, R.C. Izaurralde, R.A.C. Jones, S-H. Kim, A.D.B. Leaky, K. Lewers, T.L. Mader, A. McClung, J. Morgan, D.J. Muth, M. Nearing, D.M. Oosterhuis, D. Ort, C. Parmesan, W.T. Pettigrew, W. Polley, R. Rader, C. Rice, M. Rivington, E. Rosskopf, W.A. Salas, L.E. Sollenberger, R. Srygley, C. Stöckle, E.S. Takle, D. Timlin, J.W. White, R. Winfree, L. Wright-Morton, L.H. Ziska. 2013. Climate change and agriculture in the U.S.: Effects and adaptation. USDA Tech. Bull. 1935. Washington, DC.

Waters, E.R., J.L. Garrett, and E. Vierling. 1996. Evolution, structure, and function of the small heat-shock proteins in plants. J. Exp. Bot. 47:325-338.

Wilcox, G.E., and C.L. Pfeiffer. 1990. Temperature effect on seed germination, seedling root development, and growth of several vegetables. J. Plant Nutr. 13:1393-1403.

Wright, C. A. 2001. Mediterranean vegetables: A cook's ABC of vegetables and their preparation in Spain, France, Italy, Greece, Turkey, the Middle East, and North Africa, with more than 200 authentic recipes for the home cook. Harvard Common Press, Boston, MA.

Wurschum, T., S. Langer, C. Friedrich, H. Longin, V. Korzum, E. Akhunov, E. Ebmeyer, R. Schachschneider, J. Schacht, E. Kazman, and J. Reif. 2012. Theor. Appl. Genet. 126:1477-1486.

Xu, Y. 2012. Molecular plant breeding. CABI, Oxfordshire, UK.

Yamamoto, K., Y. Oda, A. Haseda, S. Fujito, T. Mikami, and Y. Onodera. 2014. Molecular evidence that the genes for dioecism and monoecism in *Spinacia oleracea* L. are located at different loci in a chromosomal region. Hered. 112:317-324.

Zhu, C., M. Gore, E. Buckler, and J. Yu. 2008. Status and prospects of association mapping in plants. Plant Genome. 1:5-20.

Chapter 2. Effect of Temperature on Seed Germination in Spinach (*Spinacia oleracea* L.)

*Abstract***.** The economic value of spinach (*Spinacia oleracea* L.) continues to grow in the U.S. as well as globally. Breeding heat-tolerant spinach is a challenge project to meet the demand of increasing spinach production to grow in heat conditions. Seed germination is the early stage to test, screen, and develop heat-tolerant spinach cultivars. The objective of this research was to determine temperature effect on the seed germination percentage and to select heat-tolerant spinach genotypes. Nine spinach genotypes were used in this research. The germination experiment was conducted using seven temperatures: 10, 15, 20, 25, 30, 32, and 35 ^{o}C in growth chambers. The temperature trials were conducted using a completely randomized design (CRD) with three replicates. Variation in spinach seed germination percentage was observed among the nine spinach genotypes across the seven temperatures. 'Donkey', 'Marabu', and 'Raccoon' showed higher seed germination percentage compared to other genotypes with over 70% at 30 and 32 ^{o}C, indicating possible heat-tolerance for germination. However, all spinach genotypes except 'Ozarka II' had greatly reduced germination percentages compared to 'Ozarka II' at 35 ^{o}C, indicating 'Ozarka II' could be a very good source of heat-tolerance. The performance of 'Ozarka II', 'Donkey', 'Marabu', and 'Raccoon' with higher germination percentages at temperatures above 30 ^{o}C may indicate their potential as donors of heat tolerance in spinach breeding.

The economic value of spinach (*Spinacia oleracea* L.) continues to grow in the U.S. as well as globally. Its high nutritive content has made it of increased importance to health-conscious diets and thus the demand for fresh spinach has increased, driving production up as well. Spinach production was valued at over $250 million in the U.S. in 2014 (USDA, 2015).

Spinach is a cool-season vegetable and has a specific growing season determined by optimal temperatures. Depending on climate, it is typically grown in the early spring or late fall when cool temperatures are experienced (Anderson, 2014). Prior experiments have shown that spinach seeds will germinate in soil temperatures from 5 to 30 ^{o}C with germination percentages highest at 20 ^{o}C and dropping abruptly between 25 and 30 ^{o}C (Atherton and Farooque, 1983). Spinach seed germination has been reported to cease entirely at 35 ^{o}C (Levoskar et al., 1999). Substantial spinach seedling root development requires temperatures above 18.9 ^{o}C, and top growth will be limited at temperatures below 12.3 ^{o}C and above 23.3 ^{o}C (Wilcox and Pfeiffer, 1990). Studies have been done on the heat shock response of spinach, both with whole plants and detached leaf tissue. It has been reported that after exposure to heat shock (35 – 50 ^{o}C) for 30 min, CO_2 assimilation decreased and pigment proteins in thylakoid membranes aggregated, slowing the plant's ability to photosynthesize (Tang et al., 2007). In addition, the first heat-shock proteins (HSPs) in spinach leaf tissue were induced when the temperature reached 28 ^{o}C and a full range of HSPs were produced at 36 ^{o}C (Somers et al., 1989).

Spinach is grown for fresh market, freezing and canning, and 90% of the spinach grown in the U.S. is for fresh market (Naeve, 2014). California, Arizona, Texas and New Jersey grow about 98% of the commercial fresh-market spinach (Naeve, 2014). Nearly half of California's spinach is grown in Monterey County, and although spinach can be grown there nearly year-round, production is limited to the regions and seasons that meet the temperature requirements of spinach (Koike, 2011). In Arizona and Texas, production mainly takes place in the winter. (CFAIT, 2014).

Due to increasing demand, spinach production needs to extend growing seasons and production areas such as in early summer or as a year-round vegetable crop to grow in greenhouse or high tunnel settings. Breeding heat-tolerant spinach is a challenge to meet demand. In order to characterize heat-tolerance in spinach, a reasonable place to start is with germination. A spinach genotype that is able to germinate with a high percentage at high temperatures has the potential to play a role in extending the growing season of spinach (Katzman et al., 2001). Rapid and uniform germination is also necessary for efficient crop production, both in field and greenhouse practices. While it has been reported that seed treatments may be effective for increasing germination of spinach at higher temperatures (Katzman et al., 2001), managing this trait via selecting heat-tolerant genotypes shows more promise for spinach producers.

Germination under heat stress may also play a significant role in selecting heat-tolerant cultivars. Historically, mass selection was the primary method for developing spinach cultivars, with hybrid breeding becoming popular in recent years (Morelock and Correll, 2008). While field testing is often necessary for selecting traits in spinach breeding, there are numerous environmental effects that contribute to germination performance beyond that of temperature. Therefore, it may be useful to reduce the number of genotypes planted by prior testing, improving the statistical approach to reduce error and estimate genotype by environment interaction. Using germination as this pre-test has been successful in other crops, such as sorghum (*Sorghum bicolor* L.) (Tiryaki and Andrews, 2001), and would allow quicker and more efficient selections to be made.

The objectives of this study were to determine how temperature affects spinach seed germination and evaluate potential genetic variation for germination under heat stress.

Materials and Methods

Nine spinach genotypes were used to study the effect of temperature on seed germination in this research. 'Donkey', 'Marabu', 'Tyee', 'Samish', and 'Raccoon' are commercially available cultivars which were ordered from Snow Seed Company in the spring of 2014. These cultivars were selected because of their popularity and heat-tolerant qualities. 'Donkey', 'Marabu', and 'Tyee' have been marketed as being heat-tolerant or good for summer spinach growing (Swallowtailgardenseed.com, 2014). 'Samish' and 'Raccoon' are not described as heat-tolerant but were chosen as comparison cultivars. Four genotypes from the University of Arkansas (U of A) spinach breeding program, 'Ozarka II', 'Fallgreen', F88-310, and F88-354 (Brandenberger et al., 1991) were also included. 'Ozarka II' and 'Fallgreen' are cultivars that were released in 1984 and 1987, respectively, while F88-310 and F88-354 are breeding lines of the U of A spinach breeding program (Morelock and Correll, 2008). Most of the seeds from these genotypes were harvested in 2008 and stored at -20 ^{o}C. These genotypes were selected either because of their frequent use in past spinach production in Arkansas, or because these are advanced lines with very high white rust resistance in the spinach breeding program and will likely be used as parents.

The experiment was conducted in growth chambers at the Harry R. Rosen Alternative Pest Control Center of the U of A. There were seven temperature treatments: 10, 15, 20, 25, 30, 32, and 35 ^{o}C. The previously reported optimum for spinach germination is 20 ^{o}C (Atherton and Farooque, 1983), therefore two temperatures below the optimum, 10 and 15 ^{o}C, were used in the experiment. Previous studies have shown that germination is inhibited at 30 ^{o}C (Atherton and Farooque, 1983; Katzman et al., 2001) and suppressed altogether at 35 ^{o}C (Leskovar et al., 1999). Therefore, 30, 32, and 35 ^{o}C were chosen as the heat stress treatment temperatures for the experiment.

Seeds were surface-sterilized prior to the germination tests following methods of Sauer and Burroughs (1986). Seeds were first soaked for 1 h in distilled water, then rinsed for 30 sec in 100%

ethanol (C_2H_6O). Next, seeds were soaked in a 2% NaOCl solution for 10 min, and finally rinsed with autoclaved water three times.

The germination tests followed the procedures listed by the Association of Official Seed Analysts (1993). For each genotype, 50 seeds were placed on top of two sheets of blotter paper in 9 cm petri dishes. Dishes were pre-moistened with 2 mL autoclaved water (Heydecker and Orphanos, 1968) and placed into zip-sealing bags to prevent water loss. Seeds were allowed to germinate at the designated temperature for 21 d (AOSA, 1993). Germinated seeds were counted and removed on 7 d intervals beginning the seventh day after sowing. Seeds were considered germinated when 1 mm of the radicle had protruded through the seed coat. The experimental design was a completely randomized design (CRD) with three replicates.

Total germination was tallied after 7 d, and percentages were calculated and analyzed with Microsoft Excel and JMP Pro 11 (SAS, 2014). Microsoft Excel was used for data organization and drawing plots to display the germination percentage by temperature. Analysis of Variance in JMP was used for statistical analysis. The least squared mean (LSM) was calculated for each genotype by temperature as well as for each temperature by cultivar. The student's T-test is used to analyze the significant differences of the data.

Results and Discussion

The germination percentage of the nine spinach genotypes across the seven temperatures (Tables 1 and 2) decreased as temperature increased (Fig. 1). Although the highest germination percentage was observed at 15 ^{o}C , the germination percentages at 15 and 20 ^{o}C were not significantly different at P=0.01 level (Table 1), which confirmed the optimal temperature for spinach germination of 20 ^{o}C reported by Atherton and Farooque (1983). The overall germination percentage was lower below

15 °C or higher than 20 °C, and dropped abruptly above 30 °C with a particularly sharp drop at 35 °C (Fig. 1).

Variation was observed among the nine genotypes for germination under the seven temperatures tested (Table 2). 'Donkey', 'Tyee', F88-310, 'Raccoon', and 'Samish' had the highest germination percentages ['Ozarka II', 'Marabu', and F88-354 had intermediate germination percentages] while at 10 °C. 'Fallgreen' had the lowest germination percentage of the nine genotypes at 10 °C. At 15 °C, 'Raccoon' and F88-354 had the highest germination percentages (98 and 97.7%, respectively), although they were not significantly different from F88-310, 'Donkey', and 'Tyee' with 92 to 96%. Seven of the nine genotypes had similar germination percentages (80 to 89%) at 20 °C, which was expected because 20 °C is the optimal temperature for spinach seed germination (Atherton and Farooque, 1983). At 25 °C, 'Donkey', 'Marabu', and 'Raccoon' had the highest germination percentages, between 86 to 93%. 'Ozarka II', F88-354, and 'Samish' made up the next group with 83 to 81% germination. F88-310 followed with 76%, then 'Tyee' with 74% and finally 'Fallgreen' with the lowest of 66%.

Germination percentages remained unexpectedly high at 30 °C. Although seed priming has been reported to result in higher germination for spinach seeds germinated at 30 °C (Katzman et al., 2001), it has been observed that germination inhibition begins at temperatures exceeding 20 °C, and germination is totally suppressed by 35 °C (Leskovar et al., 1999). I observed that 'Raccoon' and F88-310 had above 80% germination, and 'Ozarka II', 'Donkey', 'Marabu', and F88-354 had germination percentages above 70%. Further, at 32 °C, three genotypes, 'Donkey', 'Marabu', and 'Ozarka II', had germination percentages above 75%. Finally, all genotypes except Ozark II dropped their germination percentage sharply at 35 °C with less than 30%, while Ozark II still had high germination with 63% (Fig. 2).

Variation among genotypes may result from several factors including the production of HSPs (Hum-Musser et al., 1999). Organisms produce HSPs in response to heat stress (Somers et al., 1989). These proteins function as molecular chaperones and may be crucial for cell survival under heat stress

(Waters et al., 1996). Hum-Musser et al. (1999) evaluated HSP amounts in seeds germinated under heat stress for several cultivars, including the U of A cultivar Fallgreen, and found a higher accumulation of HSPs in the cultivars with higher germination percentages under heat stress. 'Ozarka II' may have the ability to produce greater amounts of HSPs than the other cultivars we tested.

Comparing 'Donkey', 'Marabu', and 'Tyee', cultivars marketed as heat-tolerant, to 'Raccoon' and 'Samish', cultivars not marketed as heat-tolerant, at 35 ^{o}C did not offer a clear illustration of accuracy in these heat tolerance claims (Fig. 3). 'Donkey', 'Tyee', and 'Marabu' were expected to maintain higher germination percentages at higher temperatures, but 'Tyee' had the lowest germination percentage at 30 and 32 ^{o}C of the five cultivars. 'Raccoon' had the highest germination percentage of 84% at 30 ^{o}C. At 35 ^{o}C, 'Donkey', 'Marabu', and 'Tyee' as a group had higher germination than 'Raccoon' and 'Samish' (Fig. 3).

U of A germplasm has been incorporated into many cultivars over the years (Morelock and Correll, 2008), and several of these have been used in studies of spinach seed germination under heat stress (Hum-Musser et al., 1999; Leskovar et al., 1999). Interestingly, the U of A-based cultivars seem to be among the more heat-tolerant cultivars studied. In my study, three of the four U of A genotypes had higher germination percentages than the commercially available cultivars (Fig. 2). This may be the result of selection-based breeding in Arkansas, where spinach breeding lines are planted at the U of A Vegetable Research Station in Alma, AR in the early fall and selected throughout the winter and spring. Average high temperatures for Alma between August and October range from 34 to 24 ^{o}C respectively (usclimatedata.com, 2015), and recurrent selection of lines that are able to germinate and tolerate these or higher temperatures may have resulted in better heat tolerance among U of A spinach germplasm. Recurrent selection has been shown to be a successful strategy in breeding for heat tolerance in wheat (Machado et al., 2009) and unintended but nevertheless present selection pressure for heat tolerance in U of A spinach lines may result in future releases of heat-tolerant cultivars.

Conclusions

Temperature had an effect on spinach seed germination. Germination percentages increased from 10 to 20 ^{o}C and reached their maximum at 20 ^{o}C, which is the reported optimum for spinach seed germination. Above 20 ^{o}C, germination percentages decreased, with a sharp drop at 35 ^{o}C. Variation among spinach genotypes was observed, suggesting that breeding for heat tolerance is a possibility. The U of A cultivar Ozarka II was more heat-tolerant than the other genotypes tested and may be used as a donor of heat tolerance in the U of A spinach breeding program.

Literature Cited

Anderson, C.R. 2014. University of Arkansas Division of Agriculture. Home Gardening series: spinach. FSA 6077. Web. 13 Jan. 2014. http://www.uaex.edu/Other_Areas/publications/PDF/FSA-6077.pdf.

Association of Official Seed Analysts. 1993. Rules for Testing Seeds. Assn. Offic. Seed Analysts. Bozeman, MT.

Atherton, J.G., and A.M. Farooque. 1983. High temperature and germination in spinach. I. The role of the pericarp. Scientia Hort. 19:25-32.

Brandenberger, L.P., T. Cavins, M. Payton, L. Wells, and T. Johnson. 2007. Yield and quality of spinach cultivars for greenhouse production in Oklahoma. HortTechnology 17:269-272.

Brandenberger, L.P., J.C. Correll, T. E. Morelock, and R.W. McNew. 1991. Characterization of resistance of spinach to white rust (*Albugo occidentalis*) and downy mildew (*Peronospora farinosa* f. sp. *spinaciae*). Phytopathology 84:431-437.

California Foundation for Agriculture in the Classroom. 2014. Commodity fact sheet: spinach. CFAITC, Sacramento, CA.

Correll, J.C., B.H. Bluhm, C. Feng, K. Lamour, L.J. du Toit, and S.T. Koike. 2010. Spinach: better management of downy mildew and white rust through genetics. Eur. J. Plant Pathol. 129:193-205.

Heydecker, W., and P.I. Orphanos. 1968. The effect of excess moisture on the germination of *Spinacia oleracea* L. Planta 83:237-247.

Hum-Musser, S.M., T.E. Morelock, and J.B. Murphy. 1999. Relation of heat-shock proteins to thermotolerance during spinach seed germination. Horticultural Studies. Ark. Agri. Expt. Sta. Res. Ser. 466:103-105.

Katzman, L., A. Taylor, and R. Langhans. 2001. Seed enhancements to improve spinach germination. HortScience. 36:979-981.

Koike, S., M. Cahn, M. Cantwell, S. Fennimore, M. Lestrange, E. Natwick, R. Smith, and E. Takele. 2011. Spinach production in California. Univ. California Agri. Natural Resources. Publ. 7212.

Leskovar, D. and V. Esensee. 1999. Pericarp, leachate, and carbohydrate involvement in thermoinhibition of germinating spinach seeds. J. Amer. Soc. Hort. Sci. 124:301-306.

Machado, J., M. Souza, D. Oliveira, A. Cargnin, A. Pimentel, and J. Assis. 2010. Recurrent selection as breeding strategy for heat tolerance in wheat. Crop Breeding and Applied Biotechnol. 10:9-15.

Morelock, T. E., and J.C. Correll. 2008. Spinach, p 189-218 in: J. Prohens and F. Nuez, (eds.). Vegetables I: Asteraceae, Brassicaceae, Chenopodiaceae, and Cucurbitaceae. Springer, New York.

Naeve, L. 2015. Spinach. Agr. Mktg. Resource Ctr.

Sauer, D.B. and R. Burroughs. 1986. Disinfection of seed surfaces with sodium hypochlorite. Phytopathol. 76:745-749.

Snow Seed Company. 2013. Products List: Spinach. 12 Nov. 2013. http://www.snowseedco.com/vegetables/spinach/

Somers, D., W.R. Cummins, and W.G. Filion. 1989. Characterization of the heat-shock response in spinach (*Spinacia oleracea* L.). Biochem. Cell Biol. 67:113-120.

Swallow Tail Garden Seeds. 2015. Spinach seeds. 29 Nov. 2015. http://www.swallowtailgardenseeds.com/veggies/spinach.html.

Tang, Y., X. Wen, Q. Lu, Z. Yang, Z. Cheng, and C. Lu. 2007. Heat stress induces and aggregation of the light-harvesting complex of photosystem II in spinach plants. Plant Physiol. 143:629-638.

Tiryaki, I. and D. Andrews. 2001. Germination and seedling cold tolerance in sorghum: I. Evaluation of rapid screening methods. Agron. J. 93:1386-1391.

U.S. Climate Data. 2015. Climate Fort Smith – Arkansas. 30 Nov. 2015. http://www.usclimatedata.com/climate/fort-smith/arkansas/united-states/usar0197.

U.S. Department of Agriculture, National Agriculture Statistics Service. 2015. Vegetables 2014 Summary. U.S. Dept. Agr. Natl. Agr. Stat. Serv., Washington, D.C.

Waters, E.R., J.L. Garrett, and E. Vierling. 1996. Evolution, structure, and function of the small heat-shock proteins in plants. J. Exp. Bot. 47:325-338.

Wilcox, G.E., and C.L. Pfeiffer. 1990. Temperature effect on seed germination, seedling root development, and growth of several vegetables. J. Plant Nutr. 13:1393-1403.

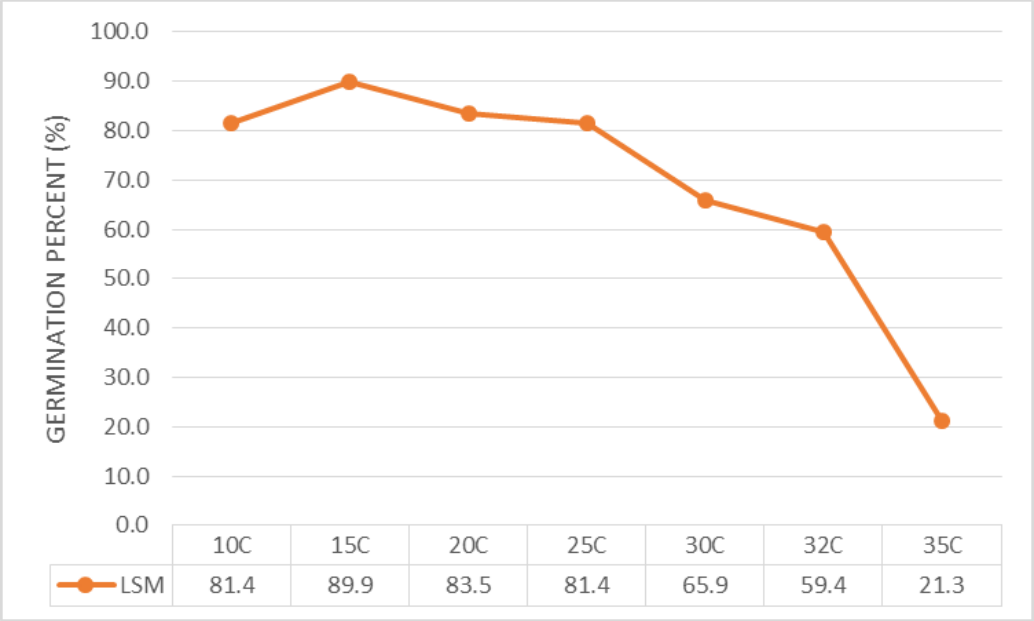

Fig. 1. The germination percentages at seven temperatures: 10, 15, 20, 25, 30, 32, and 35 °C. LSM = least square mean calculating by JMP Genomics for the nine tested spinach genotypes and the figure was drawn using MS Excel.

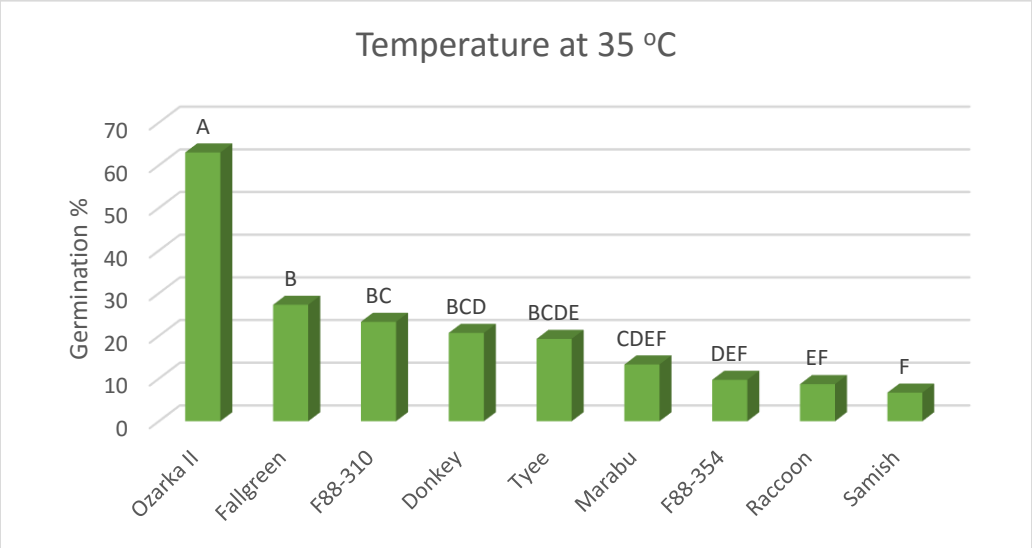

Fig. 2. The germination percentage of each spinach variety at 35 °C. The letters A, B, BC, BCD, BCDE, CDEF, DEF, EF, and F represent the statistical significant test at P=0.01 level by JMP Genomics.

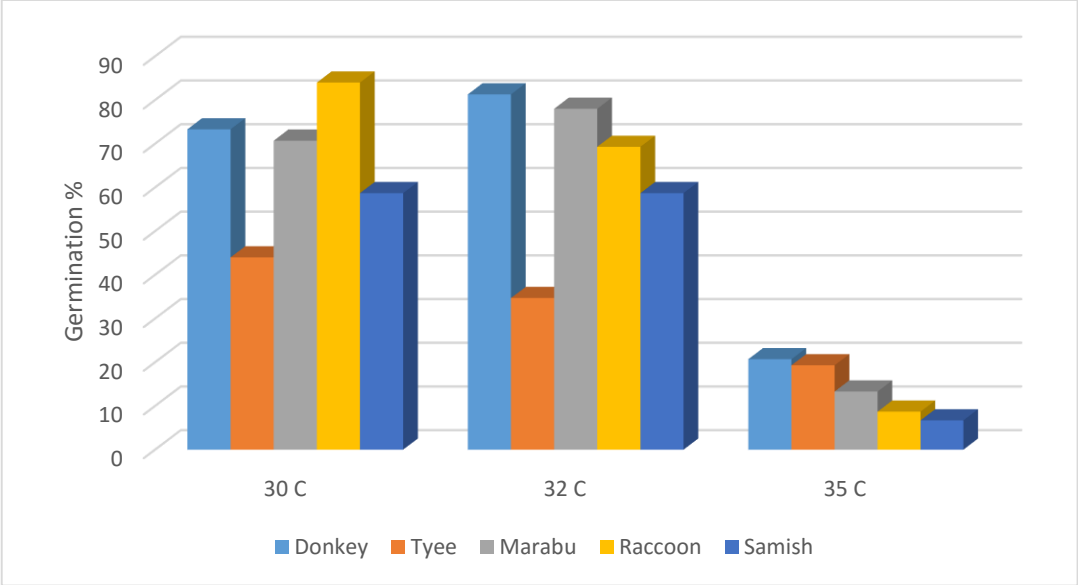

Fig. 3. Comparison of seed germination percentages among five spinach cultivars, 'Donkey' (light blue), 'Tyee' (orange), 'Marabu' (grey), 'Raccoon' (yellow), and 'Samish' (dark blue) at 30, 32 and 35 ^{o}C.

Table 1. Germination percentages at seven temperatures across nine spinach genotypes.

Temp	Ozarka II	Donkey	Raccoon	Marabu	F88-310	F88-354	Samish	Tyee	Fallgreen	LSM
10C	68.7 CD	96.0 A	94.0 AB	72.7 B	91.0 A	66.7 C	91.3 A	96.0 A	56.7 B	81.4 B
15C	90.7 A	92.7 AB	98.0 A	90.0 A	94.7 A	97.7 A	80.0 A	96.7 A	68.7 AB	**89.9 A**
20C	87.3 A	86.7 BC	84.0 C	80.0 AB	68.0 BC	96.7 A	85.3 A	89.3 AB	74.3 A	83.5 AB
25C	81.7 AB	93.3 A	88.7 BC	86.7 A	76.0 ABC	83.3 B	82.0 A	74.7 B	66.7 AB	81.4 B
30C	78.0 B	73.3 D	84.0 C	70.7 B	80.0 AB	70.7 C	58.7 B	44.0 C	33.3 C	65.9 C
32C	75.0 BC	81.3 C	69.3 D	78.0 AB	54.3 C	49.3 D	58.7 B	34.7 CD	34.0 C	59.4 C
35C	63.0 D	20.7 E	8.7 E	13.3 C	23.3 D	9.7 E	6.7 C	19.3 D	27.3 C	21.3 D

[z]Significant test of the seed germination percentage under nine temperatures for each spinach genotype and also for all nine spinach combined at P=0.01 level listed in column of the table.

Table 2. Germination percentages of nine spinach genotypes across seven temperatures.

Genotypes	10C	15C	20C	25C	30C	32C	35C	LSM
Ozarka II	68.7 B	90.7 BC	87.3 ABC	81.7 BCD	78.0 A	75.0 AB	63.0 A	77.8 A
Fallgreen	56.7 C	68.7 E	74.3 BC	66.7 E	33.3 C	34.0 E	27.3 B	51.6 B
F88-310	91.0 A	94.7 ABC	68.0 C	76.0 CD	80.0 A	54.3 CD	23.3 BC	69.6 A
Donkey	96.0 A	92.7 ABC	86.7 ABC	93.3 A	73.3 A	81.3 A	20.7 BCD	77.7 A
Tyee	96.0 A	96.7 AB	89.3 AB	74.7 DE	44.0 C	34.7 E	19.3 BCDE	65.0 AB
Marabu	72.7 B	90.0 C	80.0 ABC	86.7 AB	70.7 AB	78.0 A	13.3 CDEF	70.2 A
F88-354	66.7 B	97.7 A	96.7 A	83.3 BC	70.7 AB	49.3 DE	9.7 DEF	67.7 A
Raccoon	94.0 A	98.0 A	84.0 ABC	88.7 AB	84.0 A	69.3 ABC	8.7 EF	75.2 A
Samish	91.3 A	80.0 D	85.3 ABC	82.0 BCD	58.7 B	58.7 BCD	6.7 F	66.1 AB

[z]Significant test of seed germination percentages of the nine spinach genotypes for each temperature and also for the nine temperature combined at P=0.01 level listed in the column of the table.

Chapter 3. Plant Growth Response of Spinach Under Heat Stress

*Abstract***.** The economic value of spinach (*Spinacia oleracea* L.) continues to grow in the U.S. as well as globally. Breeding heat-tolerant spinach cultivars is needed to meet the demand for increasing spinach production by expanding spinach growing regions and seasons. Genetic variation for growth at high temperatures is critical and seed germination may be useful as an early screening method for heat-tolerant spinach genotypes. The objective of this research was to investigate whether or not genetic variation exists for spinach whole-plant growth at high temperatures and to determine whether germination can be used as a screening tool. The whole plant experiment was conducted using two temperatures, 20 and 32 ^{o}C, in growth chambers. The trials were conducted using a completely randomized block design (RCBD) with dependent variables leaf area and shoot dry weight. Variation was observed among spinach cultivars for leaf area and shoot dry weight at high temperature. 'Samish' was the only cultivar to have no significant difference between 20 and 32 ^{o}C for leaf area, while the leaf area for 'Ozarka II', 'Donkey', and 'Marabu' decreased from 20 to 32 ^{o}C. This may indicate that 'Samish' is heat-tolerant. However, significant differences were not observed within any cultivars for shoot dry weight. The results from whole-plant growth at high temperature did not parallel results from the seed germination study, and the evidence does not indicate that seed germination can be used as a screening tool for heat-tolerant cultivars.

The economic value of spinach (*Spinacia oleracea* L.) continues to grow in the U.S. as well as globally. Its high nutritive content has made it of increased importance to health-conscious diets and thus the demand for fresh spinach has increased, driving production up as well. Spinach production has been valued over $250 million in the U.S. in 2014 (USDA, 2015).

Spinach is a cool-season, leafy vegetable and has a specific growing season determined by optimal temperatures. Spinach growth occurs between 5 and 30 ^{o}C with an optimum temperature around 20 ^{o}C (Naeve, 2014). Substantial seedling root development requires temperatures above 18.9 ^{o}C, and top growth is limited at temperatures below 12.3 ^{o}C and above 23.3 ^{o}C (Wilcox and Pfeiffer, 1990). Studies have been done on the heat-shock response of spinach, both with whole plants and detached leaf tissue. It has been reported that after being exposed to heat shock (35 – 50 ^{o}C) for 30 min, CO_2 assimilation decreased and pigment proteins in thylakoid membranes aggregated, slowing down the plant's ability to photosynthesize (Tang et al., 2007). In addition, the first heat-shock proteins (HSPs) in spinach leaf tissue were induced when the temperature reached 28 ^{o}C and a full range of HSPs were produced at 36 ^{o}C (Somers et al., 1989).

Spinach is grown for fresh market, freezing and canning, and 90% of the spinach grown in the U.S. is for fresh market (Naeve, 2014). California, Arizona, Texas and New Jersey grow up to 98% of the commercial fresh-market spinach (Naeve, 2014). Nearly half of California's spinach is grown in Monterey County, and although spinach can be grown there nearly year-round, production is limited to the regions and seasons that meet the temperature requirements of spinach (Koike, 2011). In Arizona and Texas, production mainly takes place in the winter (CFAIT, 2014).

To meet increasing demand, spinach production will need to expand to more regions and extended seasons for growing spinach. Breeding for heat-tolerant spinach cultivars is necessary to achieve this. However, breeders face several challenges in developing heat-tolerant spinach cultivars, including the need for genetic variation and extraneous factors that influence plant growth at high

temperatures. Genetic variation for growth under heat stress is critical to improving the trait. Hum-Musser et al. (1999) found variation in spinach genotypes for germination and HSPs at high temperatures, but there is little understanding of whole plant growth response to high temperature. Additionally, extraneous factors influencing crop growth may make selection of heat-tolerant breeding lines difficult (Tiryaki and Andrews 2001).

Historically, mass selection was the primary method for developing cultivars, with hybrid breeding becoming popular in recent years, but all are based on field testing (Morelock and Correll 2008). While field testing is necessary in many cases for selecting traits, there are numerous environmental effects that contribute to performance of a spinach breeding line beyond that of temperature. Therefore, it may be useful to reduce the number of genotypes planted by prior testing, improving the statistical approach to reduce error and estimate genotype by environment interaction. Using germination as this pre-test has been successful in other crops, such as sorghum (*Sorghum bicolor*) (Tiryaki and Andrews 2001), and would allow quicker and more efficient selections to be made.

In the previous chapter, I found genetic variation for spinach seed germination at high temperatures and identified at least one spinach genotype, 'Ozarka II', that was heat-tolerant with respect to seed germination. The objective of this research was to investigate genetic variation for whole-spinach-plant growth at high temperatures and to determine whether germination could be used as an initial screening tool for selecting heat-tolerant spinach cultivars.

Materials and Methods

This experiment is a continuation of the previous work done on how temperature affects spinach seed germination. Two temperatures were selected for evaluating heat tolerance in whole spinach plants. The temperature of 20 ^{o}C has been previously described as the optimal temperature for spinach germination and growth (Atherton and Farooque, 1983; Wilcox and Pfeiffer, 1990). Thus, 20 ^{o}C

served as the control treatment. There has been no published evaluation of whole-plant spinach growth above 25 °C to date. Based on data observed in germination testing, 35 °C was chosen as the temperature for the heat stress treatment. However, initial testing resulted in death of all plants. Instead, 32 °C was used as the heat stress temperature at which germination percentages varied from 81% to 34% and five of the nine genotypes tested had less than 50% germination.

Four cultivars were selected for testing based on the data from the germination experiment in chapter 2 above. 'Ozarka II' was developed by the University of Arkansas (U of A) spinach breeding program that was very successfully used in spinach production in Arkansas. 'Ozarka II' had 63% germination at 35 °C in the prior study and may be a donor of heat-tolerant traits in the U of A spinach breeding program. 'Donkey' is a commercially available cultivar that has been marketed as heat tolerant and is popular among home gardeners as well as large scale production. 'Donkey' had just 20% germination at 35 °C but did much better at 32 °C, with 83% germination. 'Marabu' is also a commercially available cultivar that is marketed as preferable for early summer production. In germination tests at 35 °C, 'Marabu' exhibited 13% germination. The last genotype selected was the commercially available cultivar, 'Samish'. Not marketed as a heat tolerant cultivar, 'Samish' was chosen for its poor performance in previous germination testing. At 35 °C, it had only 6% germination.

Experiments were conducted in Percival 41L-2 (Percival Scientific, Inc., Perry, IA) growth chambers in the U of A Division of Agriculture Harry R. Rosen Alternative Pest Control Center. The experimental design was a randomized complete block design (RCBD). For each growth chamber experiment, two trays were used, top and bottom shelf, to block against variation within the growth chamber. Each tray was divided into three blocks with six individual plants of each genotype per block.

Black plastic trays containing 72 cells were filled with pre-moistened Sun Gro Horticulture Mix #1 (LC-1) (Sun Gro Horticulture, Agawam, MA). Cell size was 4 cm wide by 2 cm deep. Two seeds were sown per cell, and after germination, each cell's plants were culled to one plant per cell (Fig. 1). In

commercial production of baby spinach, plants are harvested between 21 and 50 d after planting (CFAITC, 2014). The temperature tests lasted a total of 35 d, an average of the length of time for commercial harvest. Two weeks were allowed for germination and seedling establishment at 20 ^{o}C with day zero being the day of sowing. On the 14th d after sowing, plants were either transferred to the test temperature of 32 ^{o}C or maintained at 20 ^{o}C for the control. Plants were then allowed to grow for 21 d. Plants were watered every other day as needed using a measuring cup to administer water to the top of the soil surfaces in order to prevent the soil from becoming over-saturated in the bottom of the cells. On the day of harvest, the leaf area of each plant was measured using a LI-COR leaf area meter (LI-COR, Lincoln, NE). Shoots were then placed in paper sacks and allowed to dry in a drying oven for six to eight weeks. Dry weights were then taken.

Data was organized using Microsoft Excel and statistically analyzed using JMP Pro 11 (SAS, 2014). Analysis of variance (ANOVA) was used to analyze effects and interactions, and Student's t test was used for mean separation.

Results and Discussion

The ANOVA showed that while temperature as a main effect was not significant for leaf area or shoot dry weight, the interaction of temperature and genotype was significant (Table 1). Least-squared means (LSM) of leaf area and shoot dry weight were compared using Student's t test with an alpha of 0.05 (Table 2). Within each cultivar, temperature had a significant effect on leaf area. LSM values for 'Ozarka II', 'Donkey', and 'Marabu' (17.75 cm^2, 18.32 cm^2, and 16.49 cm^2, respectively) were greater at the optimal temperature of 20 ^{o}C than those at the heat stress temperature of 32 ^{o}C (14.45 cm^2, 14.73 cm^2, and 12.87 cm^2, respectively). Temperature did not have a significant effect on shoot dry weight for 'Donkey', 'Marabu', or 'Ozarka II', but 'Samish' had a significantly higher shoot weight at 32 ^{o}C (99.2 mg) compared to 20 ^{o}C (84.2 mg) (Table 2). Blocking and temperature by block also had significant effects on

leaf area (Table 1), indicating there was an effect of location within the growth chamber, but there was no significant effect of block by spinach cultivar or temperature by block by spinach cultivar (Table 1), showing the spinach genotypes had stable results. When analyzed individually, block did not change the outcome of performance of the cultivars with respect to leaf area and shoot dry weight.

'Donkey', 'Marabu', and 'Ozarka II' all had significantly greater mean leaf areas at 20 ^{o}C than at 32 ^{o}C (Table 2). This was expected because temperatures above 23 ^{o}C have been reported to limit top growth (Wilcox and Pfeiffer 1990). The 32 ^{o}C temperature is well above this value and should have exhibited definite heat stress on the plants. 'Samish' was the only cultivar that did not have significant differences in mean leaf area between 20 ^{o}C and 32 ^{o}C (Table 2). This could indicate that 'Samish' is tolerant to heat stress, if indeed its growth was unaffected by the high-temperature treatment.

The mean shoot dry weights of 'Donkey', 'Marabu', and 'Ozarka II' at 20 ^{o}C were not significantly different than those at 32 ^{o}C. However, the mean shoot dry weight of 'Samish' at 20 ^{o}C was unexpectedly lower than the mean shoot dry weight at 32 ^{o}C. Dry weights may not be a reliable measurement of spinach growth under heat stress (Lefsrud et al., 2005). In a study by Lefsrud et al. (2005), fresh weights of spinach and kale (*Brassica oleracea* L.) were significantly affected by changes in air temperature but dry weights of the spinach biomass were not. Morphological traits, rather than dry weights, have been used for understanding heat stress in other crops. Jenni and Yan (2009) observed tip burn and rib discoloration in order to study the effects of heat stress in lettuce (*Lactuca sativa*). Gan et al. (2004) recorded fertile pods and seed weight of mustard (*Brassica juncea*) as responses to high temperature stress.

When comparing across cultivars, there were no significant differences between the means of the four cultivars for either leaf area or shoot dry weight at 20 ^{o}C (Table 2). This was expected, because 20 ^{o}C is the optimum temperature for spinach growth. At 32 ^{o}C, 'Samish' had a greater mean leaf area than the other three cultivars, among which there were no significant differences. There were also

significant differences between the cultivars in mean shoot dry weight at 32 ^{o}C (Table 2). ‘Samish’ was significantly different from the other three cultivars. ‘Ozarka II’ and ‘Donkey’ were not significantly different, although ‘Ozarka II’ was significantly different from ‘Marabu’. Finally, ‘Donkey’ and ‘Marabu’ were not significantly different.

Differences were calculated between leaf area and shoot dry weight measurements by subtracting means of values measured at 20 ^{o}C from means of values measured at 32 ^{o}C as another way to examine variation among the cultivars. The ANOVA test for leaf area differences showed significant differences among the cultivars (Table 3), and ‘Samish’ was the only cultivar different from the other three (Table 4). For the change in shoot dry weight, the ANOVA showed no significant difference among the cultivars (Table 3).

Conclusions

Genetic variation was observed for leaf area and shoot dry weight at high temperature of 32 °C, although shoot dry weight within the tested cultivars was not observed as expected. ‘Samish’ was the only cultivar to have no significant changes in both leaf area and shoot dry weight, and may be considered heat-tolerant. However, the pattern of growth from this experiment does not parallel the data from the previous study on seed germination at high temperature of Chapter 2, and therefore it cannot be stated that seed germination may be used as a screening tool for heat tolerance. Instead, the two traits should be considered separate and bred for independently.

Literature Cited

Atherton, J.G., and A.M. Farooque. 1983. High temperature and germination in spinach. I. The role of the pericarp. Scientia Hort. 19:25-32.

California Foundation for Agriculture in the Classroom. 2014. Commodity fact sheet: spinach. CFAITC, Sacramento, CA.

Gan, Y., S. Angadi, H. Cutforth, D. Potts, V. Angadi, and C. McDonald. 2004. Canola and mustard response to short periods of temperature and water stress at different developmental stages. Can. J. Plant Sci. 84:697-704.

Hum-Musser, S.M., T.E. Morelock, and J.B. Murphy. 1999. Relation of heat-shock proteins to thermotolerance during spinach seed germination. Horticultural Studies. Ark. Agri. Expt. Sta. Res. Ser. 466:103-105.

Jenni, S., and W. Yan. 2009. Genotype by environment interactions of heat stress disorder resistance in crisphead lettuce. Plant Breeding 128:374-380.

Koike, S., M. Cahn, M. Cantwell, S. Fennimore, M. Lestrange, E. Natwick, R. Smith, and E. Takele. 2011. Spinach production in California. Univ. California Agri. Natural Resources. Publ. 7212.

Lefsrud, M., D.A. Kopsell, D.E. Kopsell, and J. Curran-Celentano. 2005. Air temperature effects biomass and carotenoid pigment accumulation in spinach and kale grown in a controlled environment. HortScience 40:2026-2030.

Morelock, T. E., and J.C. Correll. 2008. Spinach, p 189-218 in: J. Prohens and F. Nuez, (eds.). Vegetables I: Asteraceae, Brassicaceae, Chenopodiaceae, and Cucurbitaceae. Springer, New York.

Naeve, L. 2015. Spinach. Agr. Mktg. Resource Ctr.

Somers, D., W.R. Cummins, and W.G. Filion. 1989. Characterization of the heat-shock response in spinach (*Spinacia oleracea* L.). Biochem. Cell Biol. 67:113-120.

Tang, Y., X. Wen, Q. Lu, Z. Yang, Z. Cheng, and C. Lu. 2007. Heat stress induces and aggregation of the light-harvesting complex of photosystem II in spinach plants. Plant Physiol. 143:629-638.

Tiryaki, I. and D. Andrews. 2001. Germination and seedling cold tolerance in sorghum: I. Evaluation of rapid screening methods. Agron. J. 93:1386-1391.

U.S. Department of Agriculture, National Agriculture Statistics Service. 2015. Vegetables 2014 Summary. U.S. Dept. Agr. Natl. Agr. Stat. Serv., Washington, D.C.

Wilcox, G.E., and C.L. Pfeiffer. 1990. Temperature effect on seed germination, seedling root development, and growth of several vegetables. J. Plant Nutr. 13:1393-1403.

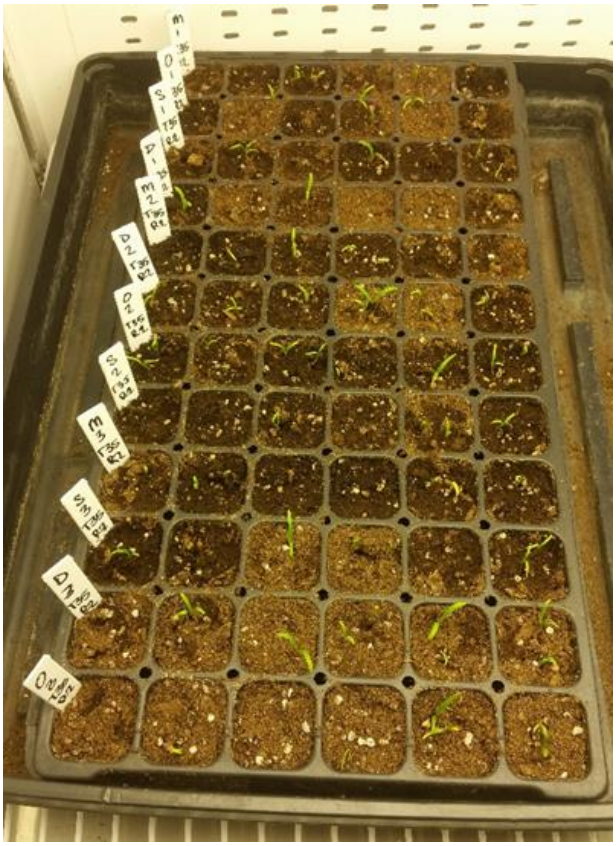

Fig. 1. Spinach seedlings emerging in optimum conditions prior to being transferred to heat stress treatment. Cells that have two seedlings were culled so that all cells contained one seedling only.

Table 1. ANOVA of leaf area and shoot dry weight of spinach.

Y	Source	DF	Sum of Squares	Prob > F
Leaf area	Spinach cultivar	3	174.8	0.0001
	Temperature	1	156.0	<.0001
	Block	3	391.4	<.0001
	Temperature X spinach cultivar	3	84.1	0.0118
	Temperature X block	3	601.4	<.0001
	Block X spinach cultivar	9	76.0	0.3152
	Temperature X block X spinach cultivar	9	64.5	0.4404
Shoot dry weight	Spinach cultivar	3	0.00262	0.0013
	Temperature	1	0.00005	0.559
	Block	3	0.04121	<.0001
	Temperature X spinach cultivar	3	0.00161	0.0174
	Temperature X block	3	0.00134	0.0365
	Block X spinach cultivar	9	0.00169	0.2697
	Temperature X block X spinach cultivar	9	0.00198	0.1706

Table 2. Least square means of leaf area and shoot dry weight for each cultivar grown at 20 °C and 32 °C. Mean separation is shown for the interaction of temperature and cultivar.

	Leaf area (cm^2)			Shoot dry weight (mg)	
	20 °C	32 °C		20 °C	32 °C
Samish	18.12 AB	18.79 A		84.2 BC	99.2 A
Ozarka II	17.75 AB	14.45 CD		88.3 B	89.2 B
Donkey	18.32 AB	14.37 CD		86.7 B	80.8 BC
Marabu	16.49 BC	12.87 D		80.0 BC	75.8 C

[z]Means connected by the same letter, for both columns and rows are not significantly different (α=0.05).

Table 3. ANOVA for differences calculated for means of leaf area and shoot dry weight of both temperatures tested.

Y	Source	DF	Sum of squares	F ratio	Prob > F
Difference in leaf area	Spinach cultivar	3	168.2	3.83	0.0165*
	Block	3	1202.8	27.40	<.0001*
Difference in shoot dry weight	Spinach cultivar	3	0.0025	2.45	0.077
	Block	3	0.0024	2.38	0.0838

Table 4. LSM for differences in leaf area and differences in shoot weight (20 °C means subtracted from 32 °C means) for each spinach cultivar with mean separation by Student's t test.

Y	Spinach cultivar	LSM
Difference in leaf area	Samish	0.67 A
	OzarkaII	-3.30 B
	Marabu	-3.62 B
	Donkey	-3.95 B
Difference in shoot dry weight	Samish	0.01358 A
	OzarkaII	0.00071 AB
	Marabu	-0.00370 B
	Donkey	-0.00446 B

[z]Means connected by the same level are not significantly different (α=0.05).

Chapter 4. Association Analysis of Bolting, Tallness, and Erectness in Spinach

Abstract. Spinach (*Spinacia oleracea* L.) is an important vegetable worldwide with high nutritional and health-promoting compounds. Bolting is an important trait to consider in order to grow spinach in different seasons and regions. Plant height and leaf erectness are important traits for machine-harvesting. Breeding slow-bolting, taller and more erect spinach cultivars is needed for improved spinach production. A total of 288 USDA spinach accessions were used as the association panel in this research. Single nucleotide polymorphisms (SNPs) discovered through genotyping by sequencing (GBS) were used for genotyping. Two structured populations and the admixtures were inferred for the 288 spinach panel using STRUTURE and MEGA. Association mapping was conducted using single marker regression (SMR), general linear model (GLM), and mixed linear model (MLM) built in TASSEL. Three SNP markers, AYZV01001038_398, AYZV01031624_1060, and AYZV01088923_95 were found to be associated with bolting. Eight SNP markers, AYZV01011130_540, AYZV01180397_2162, AYZV01069590_19842, AYZV01105690_376, AYZV01058838_64, AYZV01152613_2532, AYZV01113619_2197, and AYZV01003134_248 were associated with plant height. Four SNP markers, AYZV01137843_229, AYZV01158294_79, AYZV01023368_256, and AYZV01097131_197 were associated with erectness. These SNP markers may provide breeders with a tool in spinach molecular breeding to select spinach bolting, plant height and erectness through marker-assisted selection.

Molecular markers have become of increasing importance in plant breeding. For many major crop species, potential genetic variation for important agronomic traits already exists with varying degrees of accessibility (Thomson et al., 2010). DNA markers for genes of interest allow breeders to make selections when otherwise the gene for the trait may have been masked by heterozygosity. Association mapping is relatively recent technology development which identifies quantitative trait loci (QTLs) associated with phenotypic characteristics (Zhu et al., 2008), and provides the link for breeders to make selections based on genetic information.

Molecular markers and marker assisted selection (MAS) have been successfully used to select specific genes/alleles in plant breeding, and as cost decreases along with rapid improvement of the technology, these methods are becoming more widely used (Morelock and Correll, 2008; Thomson et al., 2010; Kumar et al., 2012). Genetic research across many disciplines, from human genomic studies to marker assisted breeding of livestock and plants, utilizes single nucleotide polymorphisms (SNPs) as the marker of choice for various reasons, but especially their abundance within any genome and cost efficiency (Zhu et al., 2008). The use of SNPs has become a powerful tool for gaining a better understanding of plant genomics by mapping chromosomes via association mapping and tagging important genes, as well as diversity analysis and other studies (Kumar et al., 2012). Association mapping has been used to successfully identify markers and loci associated with major agronomic traits (Lakew et al., 2013) such as anthracnose resistance in sorghum (*Sorghum bicolor*) (Upadhyaya et al., 2013), growth habit and days to flowering in common bean (*Phaseolus vulgaris*) (Nemli et al., 2014), and heat-tolerance in spinach (Lucas et al., 2013).

Some of the major agronomic traits of interest in spinach are bolting, plant height, and leaf erectness. Bolting is an important trait to consider in relation to developing spinach cultivars for year-round production because of its sensitivity to photoperiod (Chun et al., 2000). Long-day exposure induces bolting in spinach, rendering the plant unmarketable (Goreta and Leskovar, 2006). Because

some commercially grown spinach is cut multiple times (Morelock and Correll, 2008), overwintered spinach that is susceptible to bolting in the spring reduces the number of harvests that may be taken and therefore reduces overall yield. Genetic variation among spinach for bolting has been documented for many years, and therefore, late-bolting cultivars can be developed through breeding efforts (Goreta and Leskovar, 2006).

Commercial spinach cultivation is highly mechanized (Morelock and Correll, 2008; Koike et al., 2011), and traits such as plant height and erectness affect the ability to harvest the plants. Plant height in spinach is a complex trait with which generally large QTL regions are associated (Bezant et al., 1996), and a range of phenotypic values often occur. Spinach erectness refers to how close to or far away from the ground the spinach leaves lie on a mature plant. In the U.S., erect leaves are generally preferred to accommodate high-density spinach production and mechanical harvesting.

To date, knowledge of the spinach genome is limited and few reports have been published on the use of molecular markers in spinach with none on the use of SNP markers. Khattak et al. (2006) published a genetic linkage map with six linkage groups, constructing the map with 101 amplified fragment length polymorphisms (AFLPs) and nine simple sequence repeats (SSRs). This genetic map has a total length of 585 cM, and with an average distance of 5.18 cM between markers (Khattak et al., 2006), but does not offer a great amount of detail about the linkage groups. AFLPs and SSRs, while useful, are less specific than SNP markers. Recently, Chan-Navarrete et al. (2015) first reported a SNP genetic maps of six linkage groups (P01-P06) consisted of 283 SNP markers, ranging in size from 46 to 116 cM and identified 39 QTLs related to nitrogen use efficiency (NUE) in spinach. The identification of SNP markers for spinach traits of interest, including bolting, plant height, and erectness, will provide breeders with powerful tools to develop improved spinach cultivars more efficiently. Therefore, the development of robust SNP markers and SNP genetic maps would be a valuable resource for spinach breeding efforts.

Genotyping by sequencing (GBS) is one of the next-generation sequencing platforms that utilizes a simple, highly-multiplexed system for constructing reduced representation libraries. It also utilizes inexpensive barcoding, reduces sample handling, requires fewer PCR and purification steps, and includes no size fractionation (Elshire et al., 2011). GBS can be applied to a wide array of organisms including plants for genome sequencing and SNP discovery, and is a rapid and inexpensive approach for trait mapping and association. With GBS, plant breeders can utilize techniques of molecular breeding by conducting genomic selection on any germplasm or species with or without prior knowledge of the genome in the species (Elshire et al., 2011; Sonah et al., 2013). The GBS platform is an advantageous approach for genome-wide SNP discovery, genetic map construction, linkage mapping, and genome-wide association in spinach.

Genetic diversity forms the raw material of plant breeding and is crucial for successful breeding programs (Jansen et al., 2006). Understanding the genetic diversity in one's crop allows a breeder to make informed choices when making crosses and when incorporating more variation into their program. Genetic diversity also plays an important role in association mapping by providing population structure information (kinship matrix) to analyze loci association with traits (Khan and Korban, 2012; Khan, 2013). Because the use of molecular markers in spinach has been limited up to this point, molecular diversity studies have also been limited (Hu et al., 2007). Kuwahara et al. (2012) analyzed 250 individuals from West Asia, East Asia, Japan, Europe, and the U.S. using SSR markers for six loci and found overall significant genetic differentiation among spinach from the different geographical regions. Diversity has also been observed among Iranian landraces of spinach, where high variation in morphological traits such as leaf shape, pedicle length, and the percentage of female plants was correlated to the variation in genotypes (Sabaghnia et al., 2014). Further contributions to the understanding of genetic diversity in spinach will be useful for spinach breeding efforts.

The objectives of this study was to perform association analysis for bolting, plant height, and leaf erectness in the 288 accessions of USDA spinach collection.

Materials and Methods

Plant material and phenotyping

A total of 288 spinach accessions were used for the association analysis in this study (Table S1). All seeds were kindly provided by Dr. David Brenner at the North Central Regional Plant Introduction Station, USDA-ARS, Iowa State University, Ames, IA, originally collected from 30 countries.

Phenotypic data of spinach bolting, plant height, and erectness of the 288 accessions were observed at the USDA-ARS research station in Salinas, CA and can be downloaded from USDA-GRIN web site at https://npgsweb.ars-grin.gov/gringlobal/method.aspx?id=492382. For each accession, there were 10 plants grown in plastic pots (10 x 10 x 10 cm) with 2 sand: 1 soil (by volume) in a greenhouse. Plant height was measured as the height from soil/medium surface to the highest leaf tip of the plant 55 d after planting. For leaf erectness, leaves were rated "semi-upright" if they were about 45^{o} from horizontal level and "upright" if they were closer to the upright position. An accession was deemed "early bolting" if any plant started stem elongation earlier than 60 d after planting, "intermediate" if bolting between 60 to 70 d, and "late bolting" after the 70 d.

Phenotypic data for plant height were analyzed using Microsoft (MS) Excel 2013 for the average, range, standard deviation (SD), standard error, and coefficient of variation (CV)., The CV, also known as relative standard deviation (RSD), is a standardized measure of dispersion of a probability distribution or frequency distribution, where CV = SD / mean x 100. The distributions of bolting, plant height, and erectness were also drawn using MS Excel.

DNA extraction, GBS, and SNP discovery.

Genomic DNA was extracted from fresh leaves of greenhouse-grown spinach plants using the CTAB (hexadecyltrimethyl ammonium bromide) method (Kisha et al., 1997). DNA sequencing was done by next-generation sequencing technologies using genotyping by sequencing (GBS) (Elshire et al., 2011; Sonah et al., 2013). GBS was done using Illumina HiSeq 2000 at the Beijing Genome Institute (BGI), Hong Kong, China. Sequence assembly, mapping, and SNP discovery of GBS data were analyzed using SOAP family software (http://soap.genomics.org.cn/). The GBS data provided by BGI averaged 3.26 M short-read and 283.74 Mbp data-points for each spinach sample. The short reads of the GBS data were aligned to spinach genome reference Viroflay-1.0.1 (AYZV01) (http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AYZV01#contigs) using SOAPaligner/soap2 (http://soap.genomics.org.cn/) and SOAPsnp v 1.05 was used for SNP calling (Li, 2011; Li et al., 2009).

Approximately one half-million SNPs were discovered from the GBS data among the 288 spinach germplasm accessions and the original SNP data were also provided by BGI. The SNP information was updated to spinach genome reference Spinach-1.0.3 (AYZV02) (http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AYZV02) using BLAST after AYZV02 was released on July 07, 2015. The spinach accessions and SNPs were filtered before conducting genetic diversity and association analyses. If the spinach accession had greater than 35% missing SNP data, the genotype was removed from the panel. The SNP data was filtered by minor allele frequency (MLF) > 2%, missing data < 25%, and heterozygous genotype < 50%. After filtering, 1,733 SNPs for 288 spinach accessions were used for genetic diversity and association analysis.

Population structure and genetic diversity

The model-based program STUCTURE 2.3.4 (Pritchard et al., 2000) was used to assess the population structure of the 288 spinach accessions based on 1,733 loci. In order to identify the number of populations (K) making up the structure of the data, the burn-in period was set at 10,000 with the

Markov Chain Monte Carlo iterations and the run length set at 10,000 in an admixture model. The analysis then correlated allele frequencies independent for each run (Lv et al., 2012). Ten runs were performed for each simulated value of K, which ranged from 1 to 10. For each simulated K, the statistical value delta K was calculated using the formula described by Evanno et al. (2005). The optimal K was determined using Structure Harvester (Earl and von Holdt, 2012; http://taylor0.biology.ucla.edu/structureHarvester/). After the optimal K was determined, a Q-matrix was obtained and was used in Tassel 5 for association analysis. Each spinach accession was then assigned to a cluster (Q) based on the probability determined by the software that the genotype belonged in the cluster. The cut-off probability for assignment to a cluster was 0.50. Based on the optimum K, a Bar plot with 'Sort by Q' was obtained to show the visual of the population structure among the 288 spinach accessions.

Genetic diversity was also assessed and the phylogeny trees were drawn using MEGA 6 (Tamura et al., 2013) based on the Maximum Likelihood tree method with the following parameters. Test of Phylogeny: Bootstrap Method, No. of Bootstrap Replications: 500, Model/Method: General Time Reversible model, Rates among Sites: Gamma distributed with Invariant sites (G + I), Number of Discrete Gamma Categories: 4, Gaps/Missing Data Treatment: Use all sites, ML Heuristic Method: Subtree-Pruning-Regrafting-Extensive (SPR level 5), Initial Tree for ML: Make initial tree automatically (Neighbor Joining), and Branch Swap Filter: Moderate. In order to compare the results from the two software programs, during the drawing of the phylogeny trees by MEGA, the colored shape and branch of each spinach genotype was drawn using the same color which was located at the cluster (Q) from STRUCTUR. For sub-tree of each Q (cluster), the shape of 'Node/Subtree Marker' and the 'Branch Line' was drawn with the same color as in the figure of the Bar plot of the population clusters from the STRUCTURE analysis.

Association analysis

Association analysis was performed using TASSEL 5 software, in which general linear model (GLM), and mixed linear model (MLM) were used and compared (Bradbury et al., 2007; http://www.maizegenetics.net/tassel). GLM analysis incorporated population structure (*Q*-matrix), and MLM used both population structure (*Q*-matrix) and kinship (*K*-matrix) in the association analysis (Bradbury et al., 2007; Shi et al., 2015). *Q*-matrix was estimated using STUCTURE 2.3.4 (Pritchard et al., 2000) as described above section in detail. Kinship (*K*-matrix) was estimated by the tool Kinship built in Tassel 5 with Scald_IBS method. The QGene 4.3.10 was used to conduct single marker regression (SMR) for all SNPs (Joehanes & Nelson 2008). Although QGene was developed for QTL mapping, it can also be used in association analysis through SMR. SMR for each SNP was estimated using QGene with 1,733 SNP loci in 288 genotypes without Q and without K matrices.

Results and Discussion

Phenotyping

Phenotypic data for bolting was classified as early, intermediate, or late. In this research, we only selected early and late bolting types: 173 early and 115 late accessions were included (Supplement Table S1; Fig. 1). Phenotypic data of plant height was measured in centimeters and they showed a near normal distribution (Supplement Table S1; Fig. 2). The range of plant height was from 4.5 to 16.2 cm with a median of 8.6 cm and an average of 8.8 cm; the standard deviation of plant height was 1.9 with the standard deviation error 0.0065. The CV was 21.3%, indicating there were significant genetic differences of plant height among the 288 spinach accessions (Supplement Table S1). Phenotypic data for erectness was classified as SEMI or UP. Of the 288 accessions, 230 were SEMI and 58 were UP (Supplement Table S1; Fig. 3).

Population structure

The population structure of the 288 spinach accessions was inferred using STRUCTURE 2.3.4 and the optimum K was K=2 with the online tool Structure Harvester at http://taylor0.biology.ucla.edu/structureHarvester/ (Earl and von Holdt, 2012), as indicated by the highest delta K value (Fig. 4 A). This indicated the presence of two main population clusters (Q1 and Q2) within the 288 spinach accessions. Fig. 4 B is the bar plot drawn to visualize the population structure where Q1 is red and Q2 is green. Each spinach accession was assigned to one of the two populations based on probabilities (P) given by STRUCTURE. Because some spinach accessions had similar P values between the two clusters, we defined the accession as Q1Q2 of admixture. Two clusters in Fig. 4 C were also divided by Maximum Likelihood (ML) using MEGA 6 (Tamura et al., 2013), which were corresponded as the two structure populations as Q1 and Q2 with same color, indicating there were two well-differentiated genetic populations in the spinach panel plus the admixture Q1Q2 with the empty black square shape in the Fig. 4 C. There are 93 accessions in Q1 (32.3%), 129 accessions in Q2 (44.8%), and 66 accessions in the admixture Q1Q2 (22.9%) (Supplement Table S1).

Genetic diversity was further analyzed using the Maximum Likelihood (ML) method by MEGA 6 (Tamura et al., 2013). Several phylogenetic trees were drawn based on interpretation of results. We defined Q1 and Q2 as the two clusters and used the same colors as the population structure Q1 (red) and Q2 (green) from STRUCTURE 2.3.4 (Fig. 4B) to draw the subtrees of the phylogenetic tree in MEGA 6 plus the admixture Q1Q2 (Fig. 4C). The phylogenetic tree (Fig. 4C) from MEGA 6 was consistent with the structure populations (Q1 and Q2) from STRUCTURE 2.3.4, indicating that there were two well-differentiated genetic populations and admixture in the spinach panel.

In order to view the phylogenetic trees easily, the spinach accession numbers were combined (the accession original country, the accession geography region, and the structure population) into one taxon name for each spinach accession to draw the combined tree. For example, the taxon name,

Ames23662_Afghanistan_Asia_Q1 includes the accession number – Ames23662, which was originally collected from Afghanistan in Asia; and assigned to cluster Q1. The combining taxon name for each spinach accession is shown in the Supplement Table S1, and Supplement Fig. S1. Because of the large size of the table and figures, they are included in the supplementary material. Viewing from Figs. 4 and Supplement figure S1, the 288 spinach accessions showed a clear division when they were organized into two structured populations. Therefore, we used the Q matrix with two structures in the association mapping in TASSEL below.

STUCTURE 2.3.4 (Pritchard et al., 2000) was used to determine population structure and pick up the k when the delta K value was highest. MEGA 6 (Tamura et al., 2013) was also used to analyze the genetic diversity and draw the phylogenetic trees for the same association panel. If both analyses from STRUCTURE and MEGA matched, I thought there were well-differentiated genetic populations and admixture in the panel. Q-matrix with k vector was used in TASSEL for association analysis. STUCTURE software has been widely used program for association mapping in plants (Jin et al. 2010; Price et al., 2010; Pritchard et al., 2000; Shi et al., 2016; Upadhyaya et al., 2013; Zhu et al. 2008) and provide an effective correction for population stratification (Price et al., 2010). Population stratification is an issue to affect association mapping and many different methods and models of correcting for stratification have been developed (Freedman et al., 2004; Price et al., 2010; Pritchard et al., 2000). There have been varied reviews on the impact of population stratification in association mapping (Freedman et al., 2004; Price et al., 2010). The Mixed Models by Price et al. (2010) may be a good one for us to use in our future study of spinach association mapping.

Association analysis

SNP markers were identified for bolting, plant height and erectness using three models, SMR, GLM, and MLM.

SMR, GLM, and MLM approaches all identified three SNP markers, AYZV02001321_398, AYZV02041012_1060, and AYZV02118171_95 (AYZV01001038_398, AYZV01031624_1060, and AYZV01088923_95), as having association with bolting with a P-value <0.0001 (Table 1). The percentages of R^2 for the three SNP markers AYZV01001038_398, AYZV01031624_1060, and AYZV01088923_95 were 8.5%, 6.6%, and 6.6%, respectively, based on SMR. The GLM produced R^2 values of 8.7%, 6.8%, and 6.3%, respectively, and MLM was similar with 7.8%, 6.5%, and 6.1%, respectively. The smaller P-value with and not low R^2 indicated that the three SNP markers were good markers which may be validated for use in spinach breeding to select late bolting through marker-assisted selection.

Eight SNP markers, AYZV01011130_540, AYZV01180397_2162, AYZV01069590_19842, AYZV01105690_376, AYZV01058838_64, AYZV01152613_2532, AYZV01113619_2197, and AYZV01003134_248, were associated with spinach plant height with P-values <0.001 except AYZV01113619_2197 and AYZV01003134_248 based on MLM (Table 1). The percentages of R^2 ranged from 3.9 to 10.4% (Table 1). The SNP markers AYZV01011130_540 and AYZV01180397_2162 was excellent markers with P-value < 0.000001, < 0.00001, and < 0.0001 from SMR, GLM, and MLM, respectively. The R2 was greater than 8.8%, 8.2%, and 7.0% from SMR, GLM, and MLM, respectively (Table 1), indicating the two SNP markers were strongly associated with spinach plant height and may be excellent markers for selection of plant height in spinach breeding through MAS after validation.

Four SNP markers, AYZV01137843_229, AYZV01158294_79, AYZV01023368_256, and AYZV01097131_197, were associated with erectness (Table 1). SMR, GLM, and MLM did not show similar results for the four SNP markers. AYZV01137843_229 and AYZV01158294_79 were good markers with P value < 0.001 except P = 0.00153 at MLM analysis for AYZV01158294_79 (Table 1). AYZV01023368_256 and AYZV01097131_197 showed weak association with P value < 0.006 except P =

0.01109 at SMR analysis for AYZV01097131_197. Therefore, the four SNP markers may provide a tool for selecting erectness in spinach molecular breeding.

In my study, the association studies were performed by using compressed mixed linear model (Zhang, 2010) implemented in TASSEL 5 (Bradbury et al., 2007). The model incorporates population structure as fixed effects and cryptic relationship among individuals to define the variance structure of random individual genetic effects to control false positives. The analysis of population structure was conducted by using STRUCTURE software package to derive the Q matrix (Pritchard et al., 2000). The Qgene 4.3.10 software was used for single marker regression (SMR), although QGene was developed for QTL mapping, it can also be used in association analysis through SMR. Although different models, SMR, GLM, and MLM did not give us the same results, three SNP markers for bolting, eight markers for plant height, and four markers for erectness were found constantly to be associated with the traits. Current, the available spinach genome reference is Spinach-1.0.3 (AYZV02) (http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AYZV02) as released on July 07, 2015, which represent approximately half of the spinach genome (Dohm et al., 2014; Minoche et al., 2015). A more comprehensive version of the spinach genome assembly may be made available publicly in 2016 (van Deynze, 2014; van Deynze et al., 2015; Allen van Deynze, personal communication), but unfortunately, the spinach whole genome sequences with physical maps are not available publicly yet. After available, we will map QTLs for bolting, plant height, or leaf erectness on chromosome.

Spinach bolting, plant height, and leaf erectness are important agronomic traits. Slowing-bolting or late-bolting spinach can be used for year-round production because of its un-sensitivity to photoperiod (Chun et al., 2000). Slowing-bolting is also good for commercially grown spinach with multiple times cutting during grown season (Morelock and Correll, 2008) and increases the number of harvests, thus increasing overall yield. Commercial spinach cultivation is highly mechanized (Morelock and Correll, 2008; Koike et al., 2011), and spinach cultivars with taller plant height and erect leaves are

generally preferred to accommodate high-density spinach production and mechanical harvesting. From this research, eight accessions, PI103063, PI169678, PI169684, PI171863, PI171865, PI174386, PI175929, and PI648963 are late-bolting and up leaf erectness with 9 cm plant height and they are good sources as parents in spinach breeding program.

Conclusions

Three SNP markers, AYZV02001321_398, AYZV02041012_1060, and AYZV02118171_95 (AYZV01001038_398, AYZV01031624_1060, and AYZV01088923_95), were identified to be associated with bolting. Eight SNP markers, AYZV02014270_540, AYZV02250508_2162, AYZV02091523_19842, AYZV02141794_376, AYZV02077023_64, AYZV02210662_2532, AYZV02153224_2197, and AYZV02003975_248 (AYZV01011130_540, AYZV01180397_2162, AYZV01069590_19842, AYZV01152613_2532, AYZV01105690_376, and AYZV01058838_64), were found to be associated with plant height. Four SNP markers, AYZV02188832_229, AYZV02219088_79, AYZV02030116_256, and AYZV02129827_197 (AYZV01137843_229, AYZV01124048_231, AYZV01158294_79, and AYZV01023368_256), were associated with erectness. These SNP markers may provide a tool utilized in spinach molecular breeding to select bolting, plant height, and erectness through MAS.

Literature Cited

Bezant, J., D. Laurie, N. Pratchett, J. Chojecki, and M. Kearsey. 1996. Marker regression mapping of QTL controlling flowering time and plant height in a spring barley (*Hordeum vulgare* L.) cross. Heredity 77:64-73.

Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler. 2007. TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics 23:2633-2635.

Chan-Navarrete, R., O. Dolstra, M. van Kaauwen, E.T.L. van Bueren, C.G. van der Linden. 2015. Genetic map construction and QTL analysis of nitrogen use efficiency in spinach (*Spinacia oleracea* L.). Euphytica (DOI 10.1007/s10681-015-1618-6).

Chun, C., A. Watanabe, H. Kim, T. Kozai, and J. Fuse. 2000. Bolting and growth of *Spinacia oleracea* L. can be altered by modifying the photoperiod during transplant production. HortScience 35:624–626.

Dohm, J.C. et al. 2014. The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*). Nature 505:546–549.

Earl, D.A., and B.M. von Holdt. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genet. Resources 4:359-361.
Elshire, R.J., J.C. Glaubitz, and Q. Sun. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PloS One 6(5):19379.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14:2611–2620.

Freedman, M.L., D. Reich, K.L. Penney, G.J. McDonald, A.A. Mignault, N. Patterson, S.B. Gabriel, E.J. Topol, J.W. Smoller, C.N. Pato, M.T. Pato, T.L. Petryshen, L.N. Kolone, E.S. Lander, P. Sklar, B. Henderson, J.N. Hirschhorn, and D. Altshuler. 2004. Assessing the impact of population stratification on genetic association studies. Nature Genet. 36:388-393.

Goreta, S. and D. Leskovar. 2006. Screening spinach cultivars for white rust and bolting. HortTechnology 16:162-166.

Hu, J., B. Mou, and B. Vick. 2007. Genetic diversity of 38 spinach (*Spinacia oleracea* L.) germplasm accessions and 10 commercial hybrids assessed by TRAP markers. Genet. Resources and Crop Evol. 54:1667-1674.

Jansen, J., H. Verbakel, J. Peleman, and T. van Hintum. 2006. A note on the measurement of genetic diversity within genebank accessions of lettuce (*Lactuca sativa* L.) using AFLP markers. Theor. Appl. Genet. 112:554-561.

Joehanes, R., and J.C. Nelson. 2008. QGene 4.0, an extensible Java QTL-analysis platform. Bioinformatics 24:2788-2789.

Khan, M.A. 2013. Association Mapping Using TASSEL Software. Available online at: http://articles.extension.org/pages/62755/association-mapping-using-tassel-software (verified Dec 31, 2015).

Khan, M.A. and S. Korban. 2012. Association mapping in forest trees and fruit crops. J. Exp. Bot. 63:4045-4060.

Khattak, J., A.M. Torp, and S.B. Andersen. 2006. A genetic linkage map of *Spinacia oleracea* and localization of a sex determination locus. Euphytica 148:311-318.

Kisha T., C.H. Sneller, and B.W. Diers. 1997. Relationship between genetic distance among parents and genetic variance in populations of soybean. Crop Sci. 37:1317–1325.

Koike, S., M. Cahn, M. Cantwell, S. Fennimore, M. Lestrange, E. Natwick, R. Smith, and E. Takele. 2011. Spinach production in California. Univ. California Agri. Natural Resources. Publ. 7212.

Kumar, S., T.W. Banks, and S. Cloutier. 2012. SNP discovery through next-generation sequencing and its applications. Intl. J. Plant Genomics, Article ID 831460, 15 pages http://dx.doi.org/10.1155/2012/831460.

Kuwahara, K., R. Suzuki, Y. Ito, T. Mikami, and Y. Onodera. 2012. An analysis of genetic differentiation and geographical variation of spinach germplasm using SSR markers. Plant Genet. Resources 12:185-195.

Jin, L., Y. Lu, P. Xiao, M. Sun, H. Corke, J. Bao. 2010. Genetic diversity and population structure of a diverse set of rice germplasm for association mapping. Theor. Appl. Genet. 121:475-487.

Lakew, B., R. Henry, S. Ceccarelli, S. Grando, J. Eglinton, and M. Baum. 2012. Genetic analysis and phenotypic associations for drought tolerance in *Hordeum spontaneum* introgression lines using SSR and SNP markers. Euphytica 189:9–29.

Li, H. 2011. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics 27:2987-2993.
Li, R., C. Yu, Y. Li, T. Lam, S. Yiu, K. Kristiansen, and J. Wnag. 2009. SOAP2: An improved ultrafast tool for short read alignment. Bioinformatics 25:1966-1967.

Lucas, M., J. Ehlers, B. Huynh, N. Diop, P. Roberts, and T. Close. 2013. Markers for breeding heat-tolerant cowpea. Mol. Breeding 31:529-536.

Lv, J., J. Qi, Q. Shi, D. Shen, S. Zhang, A. Zhang, G. Shao, H. Li, Z. Sun, Y. Weng, Y. Shang, X. Gu, X. Li, X. Zhu, J. Zhang, R. van Treuren, W. van Dooijeweert, Z. Zhang, and S. Huang. 2012. Genetic diversity and population STRUCTURE of cucumber (*Cucumis sativus* L). PLoS ONE 7:46919.

Minoche, A.E., J.C. Dohm, J. Schneider, D. Holtgrawe, P. Viehover, M. Montfort, T.R. Sorensen, B. Weisshaar, and H. Himmelbauer. 2015. Exploiting single-molecule transcript sequencing for eukaryotic gene prediction. Genome Biol. 16:184.

Morelock, T. E., and J.C. Correll. 2008. Spinach, p 189-218 in: J. Prohens and F. Nuez, (eds.). Vegetables I*: Asteraceae, Brassicaceae, Chenopodiaceae*, and *Cucurbitaceae*. Springer, New York.

Nemli, S., T. Asciogul, H. Kaya, A. Kahraman, D. Esiyok, and B. Tanyolac. 2014. Association mapping of five agronomic traits in the common bean (*Phaseolus vulgaris* L.). J. Sci. Food Agr. 94:3141-3151.

Price, A.L., N.A. Zaitlen, D. Reich, and N. Patterson. 2010. New approaches to population stratification in genome-wide association studies. Nat Rev Genet. 11(7): 459–463.

Pritchard J.K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945—959.

Sabaghnia, N., H. Asadi-Gharneh, and M. Janmohammadi. 2014. Genetic diversity of spinach (*Spinacia oleracea* L.) landraces collected in Iran using morphological traits. Acta. Agri. Slovenica 103(1):101-111.

Shi, A., B. Buckley, B. Mou, D. Motes, J.B. Morris, J. Ma, H. Xiong, J. Qin, W. Yang, J. Chitwood, Y. Weng, and W. Lu. 2016. Association analysis of cowpea bacterial blight resistance in USDA cowpea germplasm. Euphytica 208:143–155.

Sonah H., M. Bastien, E. Iquira, A. Tardivel, and G. Legare. 2013. An improved genotyping by sequencing (GBS) approach offering increased versatility and efficiency of SNP discovery and genotyping. PLoS ONE 8:54603.

Tamura, K., G. Stecher, D. Peterson, A. Ailipski, and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evolution 30:2725-2729.
Thomson, M., A. Ismail, S. McCouch, and D. Mackill. 2010. Marker assisted breeding, p. 451-469. In: Abiotic stress adaptation in plants: Physiological, molecular, and genetic foundation. Springer Netherlands.

Upadhyaya, H., Y. Wang, R. Sharma, and S. Sharma. 2013. Identification of genetic markers linked to anthracnose resistance in sorghum using association analysis. Theor. Appl. Genet. 126:1649-1657.
van Deynze, A. 2014. A De Novo Draft Assembly of Spinach Using Pacific Biosciences Technology. Plant & Animal Genomics XXII conference, January 10-15, 2014, San Diego, CA (http://aa314.gondor.co/webinar/a-de-novo-draft-assembly-of-spinach-using-pacific-biosciences-technology/, accessed on March 21, 2016).

van Deynze, A., H. Ashrafi, L. Hickey, P. Peluso, D. Rank, J. Chin, N. Rapicavoli, J. Drake, T. Garvin, and M. Schatz. 2015. Using spinach to compare technologies for whole genome assemblies. Plant & Animal Genomics XXIII conference, January 10-14, 2015, San Diego, CA.

Zhang, Z., E. Ersoz, C.Q. Lai, R.J. Todhunter, H.K. Tiwari, M.A. Gore, P.J. Bradbury, J. Yu, D.K. Arnett, J.M. Ordovas, and E.S. Buckler. 2010. Mixed linear model approach adapted for genome-wide association studies. Nature Genet. 42: 355-360.

Zhu, C., M. Gore, E. Buckler, and J. Yu. 2008. Status and prospects of association mapping in plants. Plant Genome 1:5-20.

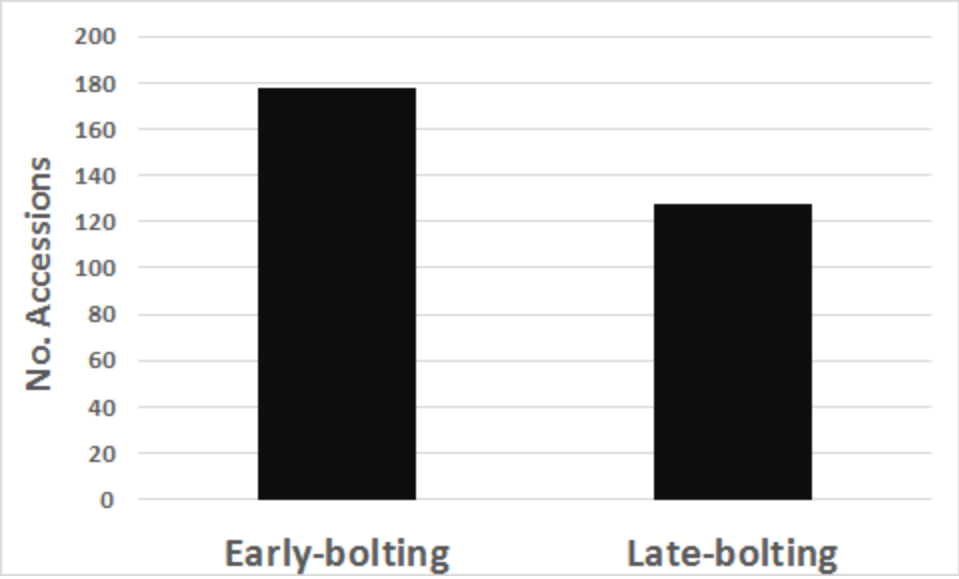

Fig. 1. The distribution of spinach bolting in 288 spinach accessions (an accession was deemed “early bolting” if any plant started stem elongation earlier than 60 d after planting, and “late bolting” after the 70 d mark).

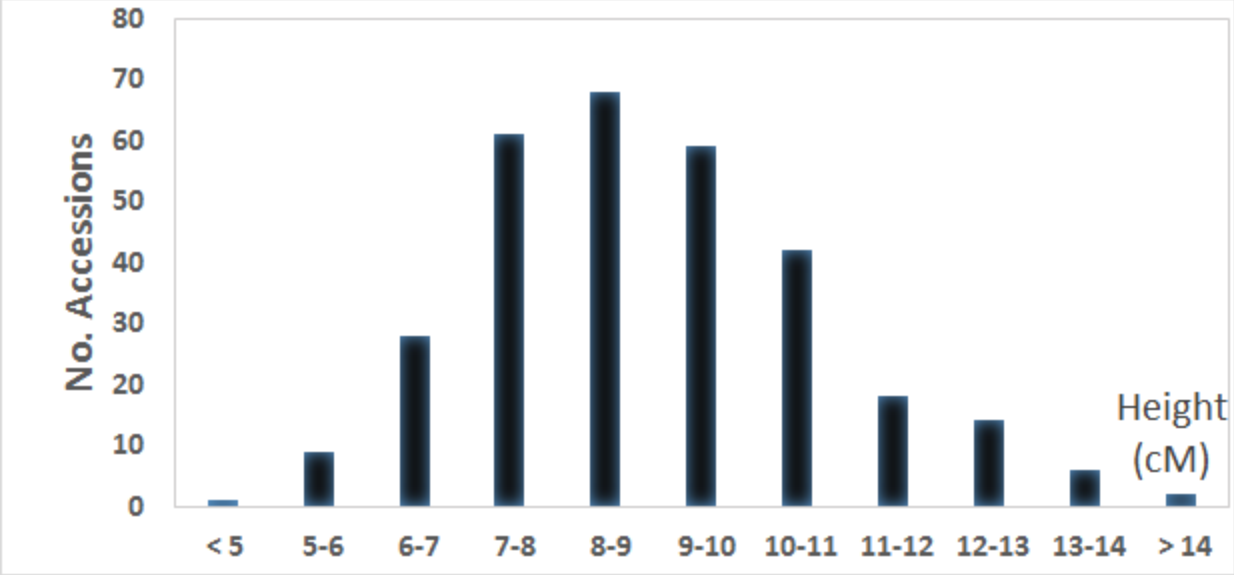

Fig. 2. The distribution of spinach plant height in 288 spinach accessions (Plant height was measured as the height (cM) from ground to the highest leaf tip of the plant 55 d after planting).

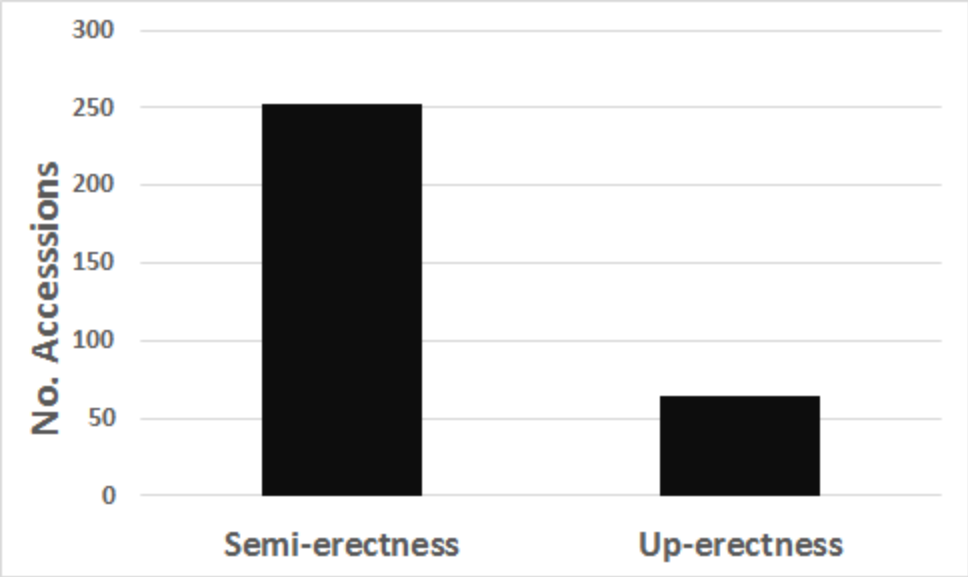

Fig. 3. The distribution of spinach erectness in 288 spinach accessions (For leaf erectness, leaves were rated “semi-upright” if they were about 45◦ from horizontal level and “upright” if they were closer to the upright position).

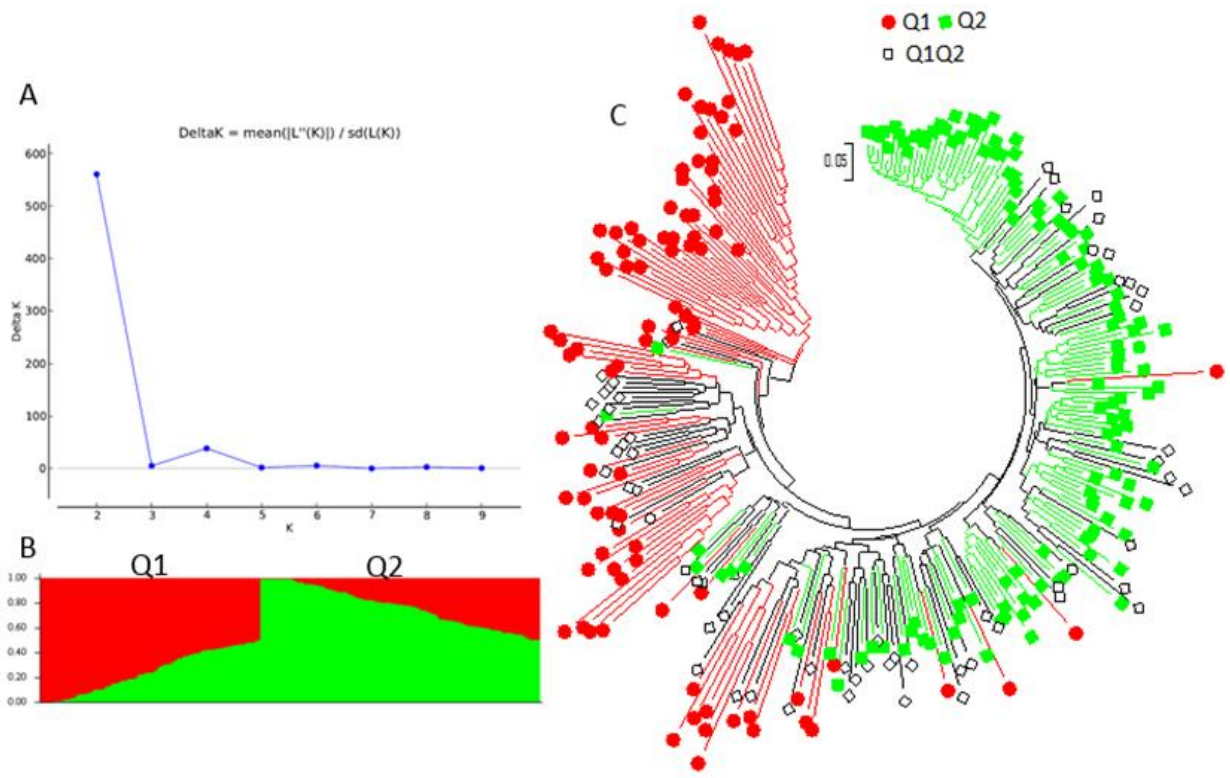

Fig. 4. Model-based populations in the association panel (A) Delta K values for different numbers of populations assumed (K) in the STRUCTURE analysis (B) Classification of spinach accessions into two populations using STRUCTURE 2.3.4. The distribution of the accessions to different populations is indicated by the color code (Q1: red and Q2); (C) Maximum Likelihood (ML) tree of the 288 accessions drawn by MEGA 6. The color codes for each population are consistent in the figure B and C, and the empty black square as the admixture Q1Q2.

Table 1. Fifteen SNP markers associated with bolting, plant height, and leaf erectness identified from three modes using QGene4 and Tassel in 288 spinach accessions.

Spinach genome Spinach-1.0.3 information				Viroflay-1.0.1		P value			R^2 (%)			Trait
SNP name[a]	SNP Type	Contig at AYZV02 project	SNP Position	Contig at AYZV01 project	SNP Position	SMR[b]	GLM[b]	MLM[b]	SMR	GLM	MLM	
AYZV02001321_398	C/A	AYZV02001321	398	AYZV01001038	398	1.39E-06	1.13E-06	1.05E-05	8.5	8.7	7.8	Bolting
AYZV02041012_1060	G/A	AYZV02041012	1060	AYZV01031624	1060	5.28E-05	4.04E-05	9.93E-05	6.6	6.8	6.5	
AYZV02118171_95	G/A	AYZV02118171	95	AYZV01088923	95	1.75E-04	2.93E-04	5.15E-04	6.6	6.3	6.1	
AYZV02014270_540	A/G	AYZV02014270	540	AYZV01011130	540	9.87E-07	2.62E-06	5.32E-05	8.9	8.3	7.1	Plant height
AYZV02250508_2162	T/A	AYZV02250508	2162	AYZV01180397	2162	1.23E-07	6.53E-07	7.24E-05	10.4	9.2	7.1	
AYZV02091523_19842	A/G	AYZV02091523	19842	AYZV01069590	19842	5.76E-06	1.21E-05	3.65E-04	8	7.5	6.1	
AYZV02141794_376	A/G	AYZV02141794	376	AYZV01105690	376	1.61E-04	2.05E-04	4.00E-04	6.4	6.1	6.1	
AYZV02077023_64	G/T	AYZV02077023	64	AYZV01058838	64	3.84E-04	3.93E-04	4.67E-04	6.3	6.2	6.3	
AYZV02210662_2532	A/G	AYZV02210662	2532	AYZV01152613	2532	4.46E-05	1.36E-04	5.56E-04	6.6	5.8	5.2	
AYZV02153224_2197	T/C	AYZV02153224	2197	AYZV01113619	2197	3.74E-04	4.93E-04	0.00157	4.4	4.2	3.9	
AYZV02003975_248	T/A	AYZV02003975	248	AYZV01003134	248	2.61E-04	5.63E-04	0.00165	6	5.4	4.9	
AYZV02188832_229	G/T	AYZV02188832	229	AYZV01137843	229	2.34E-04	0.00107	7.15E-04	6.1	5	6.7	Erectness
AYZV02219088_79	C/A	AYZV02219088	79	AYZV01158294	79	1.72E-04	1.63E-04	0.00153	6.1	6.1	5.2	
AYZV02030116_256	T/C	AYZV02030116	256	AYZV01023368	256	0.00125	5.25E-04	0.00202	4.9	5.5	5.5	
AYZV02129827_197	A/C	AYZV02129827	197	AYZV01097131	197	0.01109	3.87E-05	0.00678	3	6.6	3.6	

[z]SNP name is defined as the contig name plus the SNP position on the contig.
[y]SMR = single marker regression using the QGene 4.3.10 (Joehanes & Nelson 2008); GLM = regression linear model and MLM = mixed linear model using TASSEL 5 (Bradbury et al. 2007; http://www.maizegenetics.net/tassel).

Conclusions

Temperature had an effect on spinach seed germination. Germination percentages increase 10 to 20 °C and reach their maximum at 20 °C, which is the optimum for spinach seed germination. Above 20 °C, germination percentages decrease, with a sharp drop at 35 °C. Variation among spinach genotypes was observed, suggesting that breeding for heat tolerance is a possibility. The U of A cultivar Ozarka II was more heat-tolerant than the other genotypes tested and may be used as a donor of heat tolerance in the U of A spinach breeding program.

There was genetic variation among cultivars for both leaf area and shoot dry weight at 32 °C. Significant differences within 'Ozarka II', 'Donkey', and 'Marabu' for leaf area are indicative that growth for these three cultivars slowed at 32 °C, while 'Samish' had no significant changes leaf area and may be considered heat-tolerant. There were no significant differences for shoot dry weight observed within any of the cultivars, and shoot dry weight may not be a useful measurement for spinach heat tolerance. The pattern of growth from this experiment does not align with the data from the previous study on seed germination at high temperature of chapter 2, and therefore it cannot be stated that seed germination may be used as a screening tool for heat tolerance. Instead, the two traits should be considered separate and bred for independently.

Three SNP markers, AYZV01001038_398, AYZV01031624_1060, and AYZV01088923_95, were identified to be associated with bolting. Six SNP markers, AYZV01011130_540, AYZV01180397_2162, AYZV01069590_19842, AYZV01152613_2532, AYZV01105690_376, and AYZV01058838_64, were found to be associated with tallness. Four SNP markers, AYZV01137843_229, AYZV01124048_231, AYZV01158294_79, and AYZV01023368_256, were associated with erectness. These SNP markers can be used in spinach molecular breeding to select bolting, tallness, and erectness through MAS.