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Phenotypic relationships among oil, protein, fatty acid composition and seed size traits in *Cucurbita pepo*

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ABSTRACT

Pumpkin seed (*Cucurbita pepo* L.) is high in oil, protein and total unsaturated fatty acids (TUFA), and provides an important source of nutrition and income globally. Use of pumpkin seed in the snacking and vegetable oil industry in the U.S. is expected to rise as the market for healthy foods increases. Currently, most of the pumpkin seed consumed in the U.S. is imported, and there is need to breed high-yielding and nutritious cultivars that are locally adapted to various agro-ecological growing zones in the country. In the current study, phenotypic variation of key seed nutrition traits among 35*C. pepo* accessions of different seed phenotypes (hulled, semi-hulled, thin layer and 'naked') was determined with the primary goal of identifying the best parental material for breeding as well as elucidating phenotypic relationships among the traits. Seed oil percentage ranged from 29.33% to 48.41% and was significantly ($P < 0.05$) negatively correlated (−0.51) with seed protein percentage, which ranged from 19.48% to 31.35%. Linoleic acid ($\bar{x} = 51.19\%$) was the major fatty acid in the seed, followed by oleic ($\bar{x} = 30.77\%$), palmitic ($\bar{x} = 9.84\%$), and stearic ($\bar{x} = 5.63\%$) acid. Significant negative correlations were found between linoleic and oleic acid (−0.96), linoleic and stearic acid (−0.37), and seed size and seed protein percentage (−0.39). Conversely, significant positive correlations were found between seed size and oil content (0.56–0.70), seed size and palmitic acid (0.49–0.65), seed size and stearic acid (0.38–0.46), palmitic acid and seed oil percentage (0.50), and stearic acid and seed oil percentage (0.31). Hulled seed accessions were significantly lower in seed oil percentage, palmitic acid and seed size than 'naked' seed accessions. On the other hand, 'naked' seed accessions had significantly lower seed protein percentage than semi-hulled seed accessions. Collectively, this data suggests a wide variation in seed nutrition within *C. pepo* and provides insight into the phenotypic relationships among important seed traits. Several accessions high in oil, protein and TUFA were identified and will be useful in breeding for enhanced pumpkin seed nutrition.

1. Introduction

The cultivated species of squash (*Cucurbita pepo*, *C. moschata*, *C. maxima*, *C. argyrosperma* and *C. ficifolia*) constitute a major crop grown in the U.S. primarily for culinary, processing and ornamental purposes with an annual value exceeding 230 million dollars (United States Department of Agriculture, 2016). Although currently of minor value in the U.S., use of pumpkin seed (*C. pepo*) in confectionery and vegetable oil industry is expected to rise as the market for healthy foods increases. Currently, pumpkin seed snacks are popular in retail stores across the country in trail mixes with various nuts, seeds, dried fruit as well as an ingredient in breakfast cereal and bread (Baxter et al., 2012; Loy, 2004). In addition, pumpkin seed oil can be purchased by the bottle or as formulated capsules in health-food stores (Stevenson et al., 2007). In Europe, Asia, Africa and Australia, pumpkin seeds provide a significant source of nutrition and income (Baxter et al., 2012; Fruhwirth and Hermetter, 2007; Nakić et al., 2006) where they are utilized in vegetable oils, snacks, and high-protein animal feed (Lazos et al., 1992).

Pumpkin seed is a nutritious food with high oil (50% w/w) and protein (35%) content, which varies depending on cultivar (Fruhwirth and Hermetter, 2007). Palmitic (≤15%), stearic (≤8%), oleic (≤47%) and linoleic (≤61%) fatty acids are the main components of the oil (Bavec et al., 2007), while albumins and globulins make up approximately 60% of the crude protein. The oil content and fatty acid composition of pumpkin seed is comparable to that of soybean (*Glycine max*) (Panthee et al., 2005), sunflower (*Helianthus annuus*) (Baboli and Kordi, 2010; Tang et al., 2006), safflower (*Carthamus tinctorius*) (Yermanos et al., 1967) and watermelon (Jarret and Levy, 2012; Meru and McGregor, 2014). The high levels of unsaturated fatty acids (oleic and linoleic acids) in pumpkin seed oil provide health benefits that

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reduce risks of arteriosclerosis and heart-related ailments (Wassom et al., 2008). Pumpkin seed contains significant levels of antioxidants (tocopherols and tocotrienols) which have been associated with a reduced risk of gastric, breast, lung, and colorectal cancer (Lelley et al., 2009; Nesaretnam et al., 2007; Stevenson et al., 2007). Furthermore, phytosterols present in pumpkin seed play a key role in lowering cholesterol levels and treatment of enlarged prostate (benign prostate hyperplasia) (Fruhwirth and Hermetter, 2007; Thompson and Grundy, 2005).

Currently, most of the pumpkin seed consumed in the U.S. is imported, and only a few dual-purpose cultivars are locally grown for processing and seed consumption (Baxter et al., 2012). To meet the current and projected demand for pumpkin seed in the U.S., it is critical for growers to have access to pumpkin cultivars with optimized seed yield, seed size and seed nutrition. Cultivars with the hull-less ('naked') seed phenotype are ideal for snacking and allow efficient oil extraction since they eliminate the need for manual de-hulling prior to use (Lelley et al., 2009). The 'naked' seed trait is conferred by a single recessive mutation that leads to significant reduction in the amounts of lignin and cellulose in the hypodermis, sclerenchyma and parenchyma tissues of the seed coat (Fruhwirth and Hermetter, 2007).

To improve desirable traits in pumpkin seed such as seed size and nutritive value, it is important to evaluate the phenotypic variation of these traits in diverse pumpkin germplasm and identify the best parental material for breeding. In addition, an understanding of the phenotypic relationships among seed size and nutrition traits can assist breeders identify the best selection strategy for improvement of target traits in pumpkin. For example, breeders for oil crops such as sunflower (*Helianthus annuus*) (Tang et al., 2006) and safflower (*Carthamus tinctorius*) (Yermanos et al., 1967) exploit the negative correlation between seed size and seed oil content to indirectly improve the latter by selecting for smaller seeds. Indirect selection for seed nutrition traits is not only cost effective but also rapid and non-destructive.

The aim of the current study was to determine oil, protein, fatty acid composition and seed size traits among 35 *Cucurbita pepo* accessions and elucidate the phenotypic relationships among the traits.

2. Materials and methods

2.1. Plant material and seed size determination

Thirty-five *C. pepo* plant introductions (PI) (17) and cultivars (18) were sourced from the United States Department of Agriculture-Agricultural Research Service germplasm collection and commercial seed companies, respectively. These accessions represent zucchini, crookneck, straightneck, acorn and pumpkin groups of *C. pepo* and are open pollinated, except for Honey Bear, which is a commercial hybrid. Hereafter, the PI's and cultivars will be referred to as accessions, regardless of their wild or cultivated status. The seeds were stored at 7 °C in a seed cooler facility at the University of Florida-Tropical Research and Education Center, Homestead, FL until further analysis. Seed size was determined by measuring the weight of ten seeds (10SWT) on a STX 123 portable weighing balance (Ohaus Corporation, Parsippany, NJ), as well as the average seed length (SL) and seed width (SWD) of five randomly chosen seeds measured using a digital electronic caliper (Marathon, Richmond Hill ON, Canada). The type of seed coat for each accession was recorded as hulled, semi-hulled, thin layer or 'naked' (Fig. 1).

2.2. Seed oil and protein analysis

Determination of seed oil and protein percentage was carried out using a nuclear magnetic resonance instrument [NMR (MiniSpec MQ20 NMR analyzer; Bruker Optics, Billerica, MA)] as described by Burke et al. (2005) and Wills et al. (2010). For each accession, seeds were divided in two batches to form biological replicates, and the average value was used for data analysis.

2.3. Fatty acid analysis

Five seeds from each accessions were manually de-hulled with a steel blade to obtain kernels. The kernels for each sample were crushed with a mortar and pestle and 300 mg of the powder was weighed and transferred into 2.2-mL 96-well plates (Fisher Scientific, Pittsburgh, PA). The standard method (Ce 1-62) for analyses of fatty acid composition in fats and oils recommended by the American Oil Chemist's Society was used to prepare fatty acid methyl esters (FAMEs) (American Oil Chemist's Society, 2005), following protocols developed for watermelon seed (Meru and McGregor, 2014). Briefly, the oil was extracted by incubating each sample in 2 mL of hexane (Fisher Scientific) at room temperature (25 °C) for 15 min. The supernatant (0.5 mL) from each sample was transferred to a new 96-well plate and evaporated to dryness with a stream of N2 at 50 °C on a ZipVap 96-well evaporator (GlasCol, Terre Haute, IN). To each well, 0.2 mL of ethyl ether (BDH, Poole, U.K.) was added to solubilize the lipids followed by addition of 0.2 mL 0.1 M KOH and incubation at 50 °C for 10 min to convert the lipids into FAMEs. The methylation reactions were stopped by adding 0.2 mL of 0.15 M HCl. The samples were incubated at room temperature for 15 min, and an aliquot (0.5 mL) of the organic layer containing FAMEs was transferred to 2 mL autosampler vials (Fisher Scientific, Pittsburgh, PA) for gas chromatography (GC) analysis. A GC (6890 Series, Agilent, Wilmington DE) using a DB-225MS column (30 m × 0.25 mm × 0.25 μm) at a flow rate of 0.8 mL/min was used to separate 1 μL of injected FAMEs. The temperature program began with an initial temperature of 120 °C then the temperature was increased at a rate of 4 °C/min to a final temperature of 220 °C with a hold time of 35 min. A standard (Supelco® 37 Component FAME Mix, Sigma-Aldrich, St. Louis, MO) with known concentrations for palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) FAMEs were used to identify peaks. The profile for the fatty acids was estimated from chromatograms using Turbochrom Workstation 6.1.1 Software (Perkin Elmer, Waltham, MA). Fatty acid extraction was carried out using two biological replicates and the average value was used in data analysis.

2.4. Correlations, trait distribution and mean separation

Pearson correlations between the values of the phenotypic traits were calculated using JMP (Version 11; SAS Institute, Cary, NC). Phenotypic distribution for each trait was determined in Excel (Microsoft Corporation, Tigard, OR), while trait means for the different seed phenotypes were separated using Fisher's protected least significant difference test (Ott and Longnecker, 2001) in PROC GLM procedure of SAS (SAS Institute Inc., Cary, NC).

3. Results

3.1. Phenotypic analysis of traits

Variation in seed size and nutrient traits was observed among the 35 *C. pepo* accessions (Table 1).

3.2. Seed size

Seed length ranged from 9.94 mm to 19.33 mm (Fig. 2) and was significantly higher in accessions with 'naked' seed phenotype compared to those with hulled seed phenotype (Table 2). On the other hand, 10SWT (0.16 g–2.87 g) and SWD (6.74 mm–10.38 mm) (Fig. 2) were similar in accessions with 'naked' and semi-hulled seed phenotype, but significantly higher compared to those with hulled seeds (Table 2).