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Genotype and Growing Environment Interaction Shows a Positive Correlation between Substrates of Raffinose Family Oligosaccharides (RFO) Biosynthesis and Their Accumulation in Chickpea (*Cicer arietinum* L.) Seeds

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Supporting Information

ABSTRACT: To develop genetic improvement strategies to modulate raffinose family oligosaccharides (RFO) concentration in chickpea (*Cicer arietinum* L.) seeds, RFO and their precursor concentrations were analyzed in 171 chickpea genotypes from diverse geographical origins. The genotypes were grown in replicated trials over two years in the field (Patancheru, India) and in the greenhouse (Saskatoon, Canada). Analysis of variance revealed a significant impact of genotype, environment, and their interaction on RFO concentration in chickpea seeds. Total RFO concentration ranged from 1.58 to 5.31 mmol/100 g and from 2.11 to 5.83 mmol/100 g in desi and kabuli genotypes, respectively. Sucrose (0.60−3.59 g/100 g) and stachyose (0.18−2.38 g/100 g) were distinguished as the major soluble sugar and RFO, respectively. Correlation analysis revealed a significant positive correlation between substrate and product concentration in RFO biosynthesis. In chickpea seeds, raffinose, stachyose, and verbascose showed a moderate broad sense heritability (0.25−0.56), suggesting the use of a multilocation trials based approach in chickpea seed quality improvement programs.

KEYWORDS: *chickpea, Cicer arietinum, raffinose family oligosaccharides (RFO), myo-inositol, galactinol, raffinose, stachyose, verbascose, genotype × environment (G × E)*

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the second most important pulse crop after dry beans, cultivated over 11.98 million hectares with a total production of 1.09 million tonnes around the world during 2010.[1,2] Chickpea is broadly classified into two clusters, (a) the kabuli type (white flower and large, cream-colored seeds) is usually grown in temperate regions, whereas (b) the desi type (purple flower and small, dark, angular seeds) is mainly produced in semiarid tropical regions of the world.[3,4] Chickpea seeds make an important nutritional contribution to the population of developing countries as they are excellent sources of carbohydrate (40−59%), protein (13.5−31.7%), vitamins, and minerals. In addition, chickpea seed constituents such as polyunsaturated fatty acids (PUFA), saturated fatty acids (<1%), and dietary fibers (about 10%) have been associated with several beneficial health-promoting properties.[5] Hence, chickpea is considered as part of a health-promoting diet.[6] However, the presence of some antinutritional factors such as raffinose family oligosaccharides (RFO) or α-galactosides reduce chickpea's acceptability in food products, particularly in Western countries.[7] In legume seeds, total α-galactosides vary from 0.4 to 16.1% of dry matter and in chickpea seeds range from 2.0 to 7.6%.[8] Raffinose is the first member of this family followed by stachyose and verbascose.[9] Some alternative RFO such as lychnose and manninotriose have been recently reported from Caryophyllacean[10] and Lamiaceae[11] plants, respectively, but their presence in chickpea seeds has not yet been reported. RFO represent a class of soluble but nonreducing and nonstructural oligosaccharides having $\alpha(1\rightarrow6)$ linkage between sucrose and galactosyl subunits.[12] Therefore, these sugars are indigestible in human and monogastric animals as they lack α-galactosidase, a hydrolyzing enzyme responsible for RFO breakdown.[13,14] Consequently, RFO escape digestion and absorption in the small intestine, but large intestinal microflora metabolize RFO and produce carbon dioxide, hydrogen, and small quantities of methane, causing flatulence, diarrhea, and stomach discomfort in humans.[15−17] As RFO act as substrate for intestinal bacteria, they are also considered as prebiotics. These oligosaccharides also participate in important plant processes such as desiccation during seed maturation, carbon sourcing in the early stages of germination, translocation of photoassimilates, and abiotic stress tolerance.[8,18−20] Utilization of RFO may also support the growth of root nodulating bacteria (e.g., *Rhizobium meliloti*) in the rhizosphere of legume plants, thus helping in nitrogen fixation.[21] Therefore, to increase the acceptability of chickpea in human and animal diets, RFO concentration needs to be reduced without affecting their physiological role in plants and beneficial effect on human health. Different treatments such as soaking, enzyme treatment, and γ-radiation exposure can be used to reduce RFO in legume seeds.[22−24] Exposure to such mechanical and chemical treatments can reduce the nutritional quality of seeds. Therefore,

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it is desirable to develop genetic strategies to reduce RFO concentration in chickpea seeds. In this study we show that there is natural variation in RFO concentrations in chickpea seeds. Both genotype and environment affect the accumulation of RFO concentration in chickpea seeds.

MATERIALS AND METHODS

Plant Material and Growing Conditions. A set of 171 chickpea genotypes (116 desi types and 55 kabuli types, Supporting Information Tables 1 and 2) was selected from the gene bank of International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India (ICRISAT) on the basis of geographic origin. These genotypes represented eight different geographic regions including chickpea's center of origin and center of diversity (Table 1). These genotypes were grown in the field as well as under greenhouse conditions in two biological replications. The field trials were conducted at ICRISAT (17° 53′ N latitude, 78° 27′ E longitude and 545 m altitude, Patancheru, India) for two seasons: 2008−2009 and 2009−2010 (from October to mid-March). For 2008−2009, the mean daily minimum and maximum temperatures were 15.0 and 31.1 °C, respectively. The average bright sunshine hours were 8.9 with approximately 352.1 μM m^{-2} s^{-1} of solar radiation. The daily mean minimum and maximum temperatures during 2009−2010 were 16.2 and 30.0 °C, respectively, along with an average of 8.1 h of bright sunshine and approximately 333.4 μM m^{-2} s^{-1} of solar radiation. These genotypes were also grown under controlled greenhouse (GH) conditions at the University of Saskatchewan (52° 07′ N latitude, 106° 38′ W longitude and 481.5 m altitude, Saskatoon, SK, Canada) from March to July 2010. In the greenhouse, the average daily minimum and maximum temperatures were 18 and 23 °C with an 18 h photoperiod and 385 μM m^{-2} s^{-1} of photosynthetically active radiation.

Table 1. Geographical Origin of Chickpea Genotypes Used in the Study

	no. of genotypes	
region	desi	kabuli
1. Europe	10	8
2. meso-America	4	1
3. North Africa	9	10
4. North America	1	0
5. South America	0	2
6. South Asia	68	18
7. southwestern Asia	13	11
8. sub-Saharan Africa	11	5

Total RFO Determination. Total RFO concentration in chickpea seed meal (500 ± 5 mg) was determined by stepwise enzymatic hydrolysis of complex RFO into D-galactose, D-fructose, and D-glucose molecules using α-galactosidase (from *Aspergillus niger*) and invertase (from yeast) using a commercial assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland). The resulting D-glucose concentration was determined using glucose oxidase/peroxidase reagent (GOPOD) that produced a red quinoneimine, the concentration of which was determined at $A_{510\ nm}$ using a spectrophotometer (DU 800, Beckman Coulter Inc., Fullerton, CA, USA). This method determined all oligosaccharides including raffinose, stachyose, and verbascose concentration as a group. Total RFO concentration was calculated on a molar basis as 1 mol of each oligosaccharide contains 1 mol of D-glucose.

HPAEC-PAD Analysis of Chickpea Seeds' Soluble Sugars. Soluble sugars from chickpea seed meal (500 ± 5 mg) were extracted using a method described by Frias et al.[25] and Sanchez-Mata et al.[26] with some modifications.[27] For quantification of each member of the raffinose family, a recently optimized analytical method was followed using high-performance anion exchange chromatography [ion chromatography system (ICS 5000), Thermo Fisher Scientific, Stevens Point, WI, USA] coupled with a disposable gold electrode, a Ag/AgCl reference electrode, and a CarboPac PA100 (4 × 250 mm) analytical column (unpublished). Raffinose (16.1 min), stachyose (17.0 min), and verbascose (19.5 min) were determined along with *myo*-inositol (1.7 min), galactinol (2.0 min), glucose (7.4 min), fructose (8.8 min), and sucrose (10.8 min) within 20 min of run time.

Data and Statistical Analysis. Box plot analysis was employed to represent variation among geographical regions for selected seed constituents (Figures 1 and 2). Shannon−Weaver diversity index (SDI) was calculated to analyze the diversity present in each geographical region (Tables 2 and 3). For both SDI and box plot analysis, pooled data from all three growing environments were used.

General linear model was applied to calculate analysis of variance (ANOVA) using MINITAB 14 statistical software (Minitab Inc., State College, PA, USA). Mean sum of squares (MSS) from ANOVA was utilized to calculate heritability (h^2).[28] To determine the SDI, the following formula was used:[29]

$$\mathrm{SDI} = (-\sum_{i=1}^{n} P_i \times \log_e P_i)/\log_e n$$

n represents the total number of phenotypic classes, and P_i is the proportion of total number of entries in the ith class. Phenotypic classes were prepared by using MINITAB 14 statistical software.

RESULTS AND DISCUSSION

Diversity Pattern among Geographical Regions. On the basis of their origin, desi and kabuli genotypes were grouped into seven geographical regions. In desi genotypes, the South Asian region showed the highest diversity index (0.33−0.87) for all of the selected seed constituents, as this region has maximum representation (68 genotypes contributing about 59% to total desi genotypes) in the germplasm collection (Figure 1). Consequently, South Asian genotypes showed the highest variation in seed constituents, ranging from 0.01 to 0.10 g/100 g, from 0.03 to 0.31 g/100 g, from 0.03 to 0.42 g/100 g, from 0.01 to 0.05 g/100 g, from 0.60 to 2.93 g/100 g, from 0.09 to 1.19 g/100 g, from 0.18 to 2.36 g/100 g, and from 0.01 to 0.13 g/100 g for *myo*-inositol, galactinol, glucose, fructose, sucrose, raffinose, stachyose, and verbascose, with average values of 0.05, 0.17, 0.22, 0.01, 1.72, 0.74, 1.33, and 0.06 g/100 g of chickpea seed meal, respectively (Figure 1). Southwestern Asia is one of chickpea's primary centers of origin, whereas sub-Saharan Africa contained genotypes from Ethiopia considered to be a secondary center of genetic diversity for chickpea. Therefore, the second highest SDI values for all traits were expressed by genotypes either from southwestern Asia or sub-Saharan Africa. SDI ranged from 0.29 to 0.76, from 0.13 to 0.68, from 0.15 to 0.68, from 0.27 to 0.68, and from 0.23 to 0.51 for southwestern Asia, sub-Saharan Africa, North Africa, Europe, and meso-America, respectively. This germplasm collection had no desi genotype from South America, whereas only one and four from North America.

In kabuli genotypes, the South Asian region showed the highest SDI values for most chickpea seed constituents, such as fructose (0.67), raffinose (0.86), stachyose (0.89), verbascose (0.89), and total RFO (0.92). In South Asian genotypes, concentrations of fructose, raffinose, stachyose, verbascose, and total RFO varied from 0.01 to 0.05 g/100 g, from 0.48 to 1.13 g/100 g, from 0.80 to 2.28 g/100 g, from 0.02 to 0.12 g/100 g, and from 2.27 to 5.83 g/100 g with mean values of 0.01, 0.79, 1.46, 0.07, and 3.96 g/100 g (mmol/100 g for total RFO) of chickpea seed meal, respectively (Figure 2). The highest SDI values for *myo*-inositol (0.88) and sucrose (0.77) were observed for North African genotypes with concentrations ranging from 0.02 to 0.09 g/100 g and from 1.29 to 3.59 g/100 g with mean values of 0.05 and 2.41 g/100 g of chickpea seed meal, respectively. Galactinol

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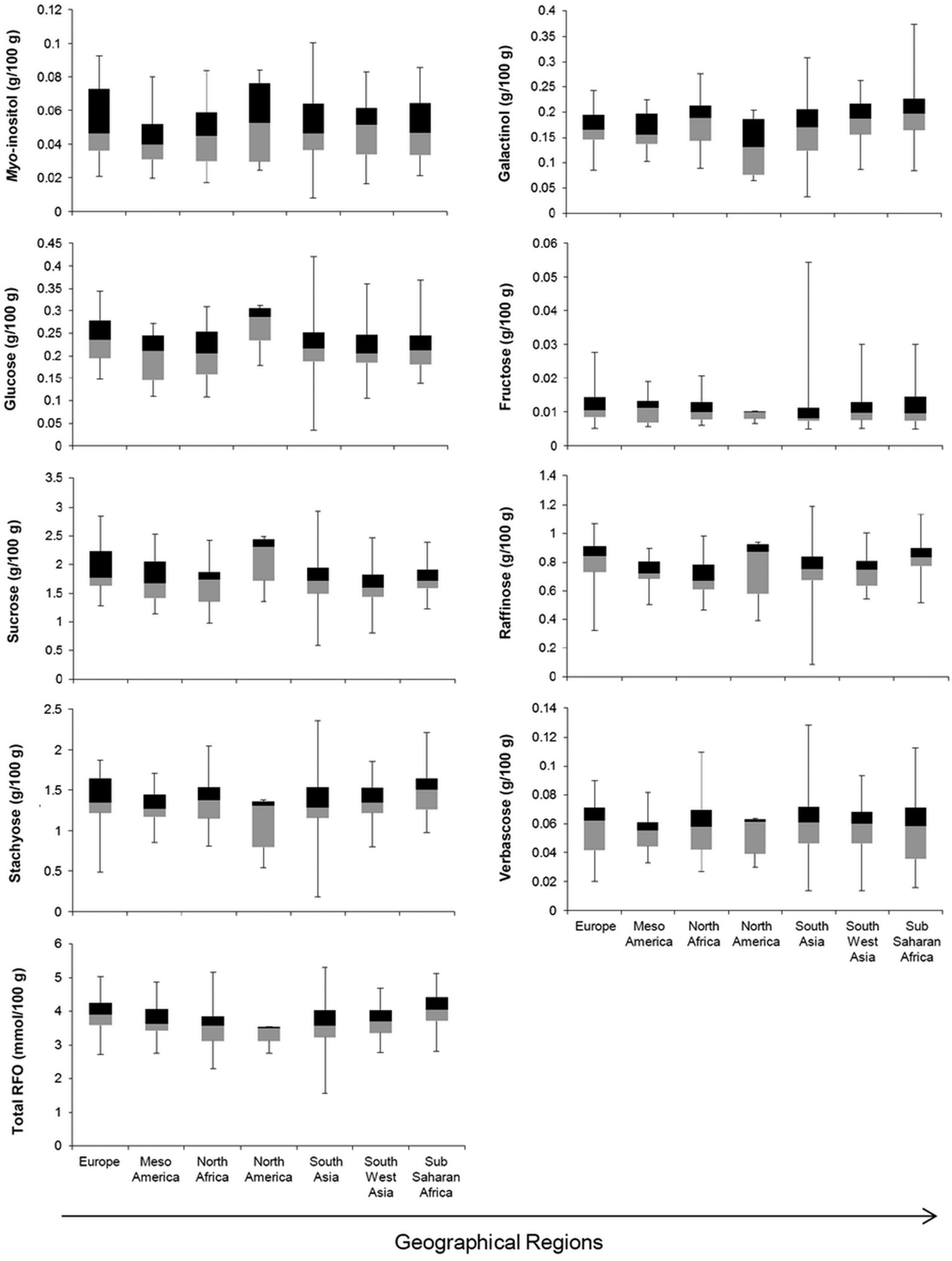

Figure 1. Box plot analysis for desi genotypes showing variation for selected chickpea seed constituents in different geographical regions using pooled data from different growing environments. Upper and lower error bars represent the lowest and highest concentrations. Black and gray boxes indicate third and second quartiles, whereas the middle line shows the median of the data set.

concentration ranged from 0.05 to 0.30 g/100 g in European genotypes with a mean concentration of 0.17 g/100 g of chickpea seed meal that resulted in the highest SDI of 0.89 among all geographical regions. However, the highest SDI for glucose

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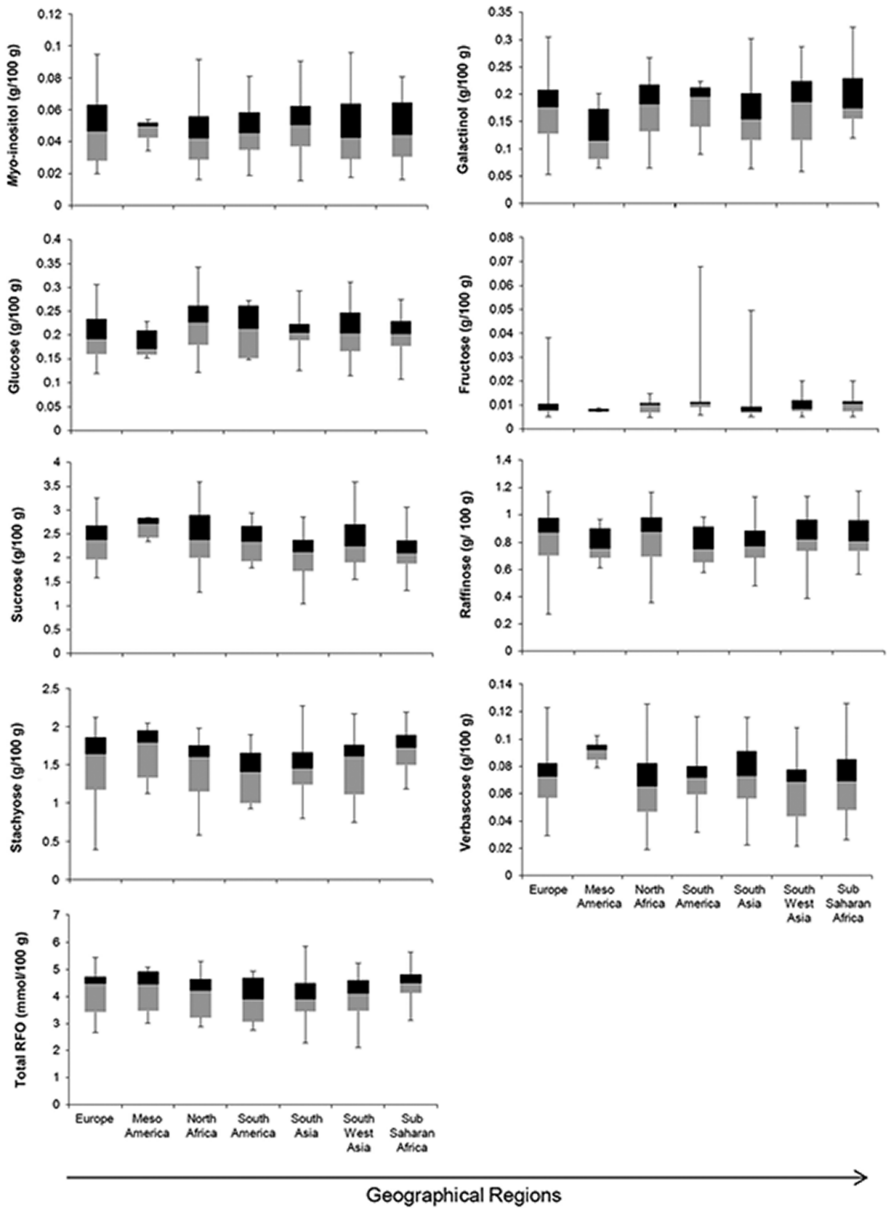

Figure 2. Box plot analysis for kabuli genotypes showing variation for selected chickpea seed constituents in different geographical regions using pooled data from different growing environments. Upper and lower error bars represent the lowest and highest concentrations. Black and gray boxes indicate the third and second quartiles, whereas the middle line shows the median of the data set.

Table 2. Shannon−Weaver Diversity Index (SDI) of Selected Chickpea Seed Constituents in Different Geographical Regions for Desi Genotypes

	SDI as per geographical region					
seed constituent	Europe	meso-America	North Africa	South Asia	southwestern Asia	sub-Saharan Africa
myo-inositol	0.59	0.29	0.61	0.76	0.62	0.38
galactinol	0.58	0.26	0.43	0.75	0.46	0.67
glucose	0.51	0.50	0.68	0.85	0.76	0.68
fructose	0.27	0.23	0.15	0.33	0.29	0.13
sucrose	0.68	0.51	0.64	0.80	0.56	0.68
raffinose	0.54	0.21	0.48	0.74	0.68	0.62
stachyose	0.56	0.38	0.64	0.68	0.67	0.46
verbascose	0.57	0.39	0.64	0.87	0.56	0.62
total RFO	0.61	0.42	0.67	0.74	0.69	0.66

(0.75) was calculated for southwestern Asian genotypes with concentrations ranging from 0.11 to 0.31 g/100 g with a mean value of 0.21 g/100 g of chickpea seed meal. South Asian genotypes had the highest representation in the germplasm

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Table 3. Shannon−Weaver Diversity Index (SDI) of Selected Chickpea Seed Constituents in Different Geographical Regions for Kabuli Genotypes

	SDI as per geographical region					
seed constituent	Europe	South America	North Africa	South Asia	southwestern Asia	sub-Saharan Africa
myo-inositol	0.64	0.33	0.88	0.68	0.80	0.46
galactinol	0.89	0.36	0.87	0.75	0.86	0.35
glucose	0.63	0.32	0.54	0.65	0.75	0.43
fructose	0.62	0.36	0.33	0.67	0.58	0.00
sucrose	0.71	0.32	0.77	0.66	0.73	0.61
raffinose	0.60	0.32	0.71	0.86	0.82	0.61
stachyose	0.60	0.33	0.65	0.89	0.80	0.51
verbascose	0.62	0.36	0.73	0.89	0.78	0.35
total RFO	0.65	0.30	0.70	0.92	0.56	0.41

Table 4. Analysis of Variance and Heritability of Chickpea Selected Seed Constituents

	mean sum of squares[a]				
seed constituent	genotype (G)	environment (E)	replication	G × E	heritability (h^2)
			Desi		
myo-inositol	3.3×10^{-4}***	7.5×10^{-2}***	5.7×10^{-6}ns	2.4×10^{-4}***	0.10
galactinol	5.8×10^{-3}***	0.5***	1.8×10^{-3}ns	1.5×10^{-3}***	0.55
glucose	5.2×10^{-3}***	0.2***	4.4×10^{-5}ns	3.2×10^{-3}***	0.16
fructose	1.5×10^{-4}***	1.8×10^{-3}***	2.8×10^{-5}ns	1.2×10^{-4}***	0.05
sucrose	0.4***	7.2***	2.8×10^{-4}ns	0.1***	0.37
raffinose	0.1***	1.3***	6.0×10^{-4}ns	1.0×10^{-2}***	0.56
stachyose	0.2***	10.3***	7.1×10^{-4}ns	4.6×10^{-2}***	0.52
verbascose	8.0×10^{-4}***	3.7×10^{-2}***	1.4×10^{-4} ns	3.7×10^{-4}***	0.25
total RFO	1.3***	35.4***	4.2×10^{-2}ns	0.2***	0.61
			Kabuli		
myo-inositol	3.8×10^{-4}***	4.0×10^{-2}***	7.0×10^{-7}ns	2.7×10^{-4}***	0.10
galactinol	6.2×10^{-3}***	0.3***	1.2×10^{-3} ns	2.5×10^{-3}***	0.31
glucose	3.5×10^{-3}***	0.1***	1.6×10^{-4}ns	3.3×10^{-3}***	0.02
fructose	5.4×10^{-5}***	1.1×10^{-4}***	1.5×10^{-5}ns	4.1×10^{-5}***	0.07
sucrose	0.8***	10.1***	7.9×10^{-3}ns	0.2***	0.53
raffinose	5.5×10^{-2}***	2.2***	2.4×10^{-3}ns	1.8×10^{-2}***	0.39
stachyose	0.2***	13.2***	3.2×10^{-3}ns	6.0×10^{-2}***	0.39
verbascose	9.5×10^{-4}***	4.1×10^{-2}***	3.1×10^{-5}ns	2.9×10^{-4}***	0.39
total RFO	1.1***	47.1***	0.4×10^{-3}ns	0.3***	0.45

[a] ***, significant at $P \leq 0.001$; ns, nonsignificant.

collection, sharing about 32.7% of total kabuli genotypes followed by genotypes from southwestern Asia (20%), North Africa (18.2%), Europe (14.5%), and sub-Saharan Africa (9%), respectively. On the basis of SDI, these genotypes were conjointly considered as a diverse collection and used further to study variation in chickpea seed constituents.

Impact of Genotype and Environment Influencing Seed Constituents' Concentration. Analysis of variance (ANOVA) showed significant effect ($P \leq 0.001$) of genotype (G) and growing environment (E) on concentrations of *myo*-inositol, galactinol, glucose, fructose, sucrose, raffinose, stachyose, verbascose, and total RFO in both desi and kabuli genotypes. The interaction between genotype and growing environment (G × E) also exhibited a significant effect ($P \leq 0.001$) on these seed constituents (Table 4). These results concur with the conclusions of Kumar et al.[14] showing a significant effect ($P \leq 0.05$) of genotype × location on sucrose, raffinose, and stachyose concentration in seven soybean genotypes. Recently, Tahir et al.[27] reported a significant ($P \leq 0.0001$) effect of cultivar, environment, and their interaction on glucose, sucrose, and RFO concentrations in lentil seeds.

Variation for Selected Seed Constituents in Desi and Kabuli Genotypes. HPAEC-PAD analysis revealed the highest concentration of sucrose among soluble sugars in chickpea seeds. Stachyose was the predominant RFO found in chickpea seeds followed by raffinose, whereas verbascose was present only as a small fraction. Previously, Frias et al.,[30] El-Adawy,[31] Aguilera et al.,[32] and Berrios et al.[33] also reported stachyose as a major RFO in chickpea seeds. In desi type (Figure 3), genotypes grown in GH showed significantly lower ($P \leq 0.001$) total RFO concentration (1.58−4.67 mmol/100 g) compared to genotypes grown in field conditions during 2009 (1.88−5.31 mmol/100 g) and 2010 (2.80−4.95 mmol/100 g). GH-grown genotypes had total RFO with a mean concentration of 3.32 mmol/100 g, whereas in the field in 2009 and 2010 it was 4.09 and 3.66 mmol/100 g, respectively. A similar pattern of total RFO was observed in kabuli type (Figure 4) showing lower concentration (2.11−4.56 mmol/100 g) in GH-grown genotypes than in field-grown genotypes during 2009 (3.46−5.83 mmol/100 g) and 2010 (3.01−5.35 mmol/100 g).

Individual RFO members also accumulated at significantly lower concentration in GH-grown genotypes than their field-grown counterparts. In GH-grown desi type, raffinose (0.27−

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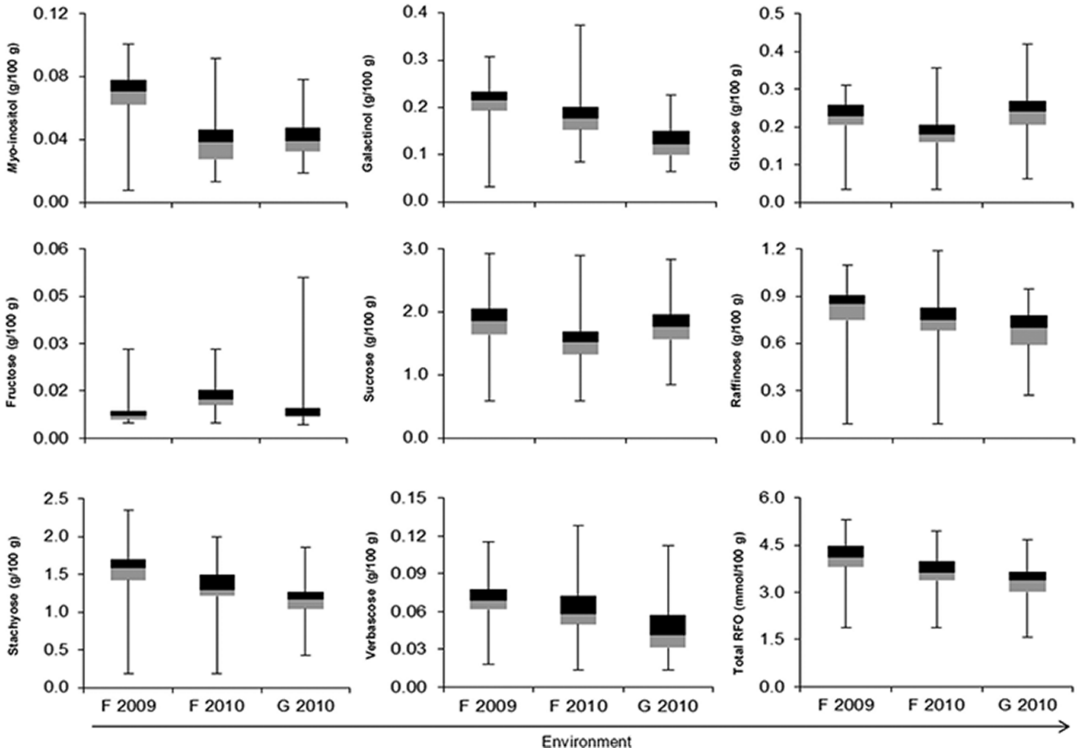

Figure 3. Box plot analysis for selected chickpea seed constituents of desi genotypes in different growing environments. Genotypes grown in the field during 2008−2009 and 2009−2010 are represented as F 2009 and F 2010, respectively, whereas G 2010 represents greenhouse genotypes grown in 2010. Upper and lower error bars represent the lowest and highest concentration. Black and gray boxes indicate third and second quartiles, whereas the middle line shows the median of the data set.

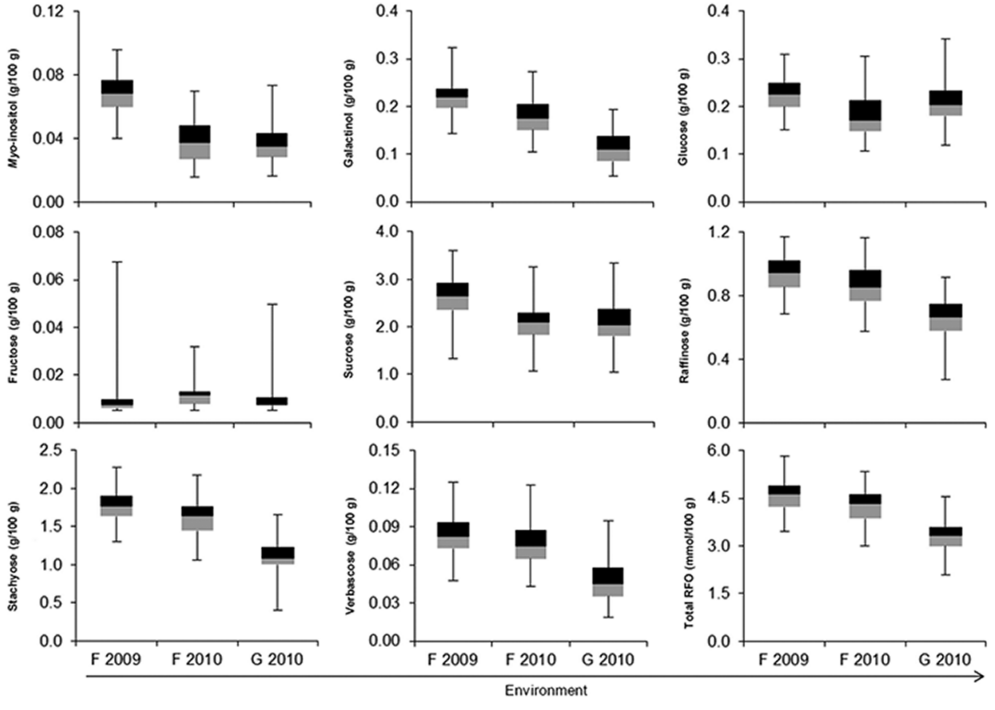

Figure 4. Box plot analysis for selected chickpea seed constituents of kabuli genotypes in different growing environments. Genotypes grown in the field during 2008−2009 and 2009−2010 are represented as F 2009 and F 2010, respectively, whereas G 2010 represents the greenhouse genotypes grown in 2010. Upper and lower error bars represent the lowest and highest concentrations. Black and gray boxes indicate third and second quartiles, whereas the middle line shows the median of the data set.

0.95 g/100 g), stachyose (0.43−1.86 g/100 g), and verbascose (0.01−0.11 g/100 g) had mean values of 0.68, 1.15, and 0.05 g/100 g, respectively (Figure 3). Genotypes grown in the field during 2009 had average values of 0.85, 1.57, and 0.07 g/100 g for raffinose, stachyose, and verbascose with ranges of 0.09−1.10, 0.18−2.36, and 0.02−0.11 g/100 g, respectively, whereas

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Table 5. Correlation among Chickpea Selected Seed Constituents in Desi and Kabuli Genotypes[a]

	myo-inositol	galactinol	glucose	fructose	sucrose	raffinose	stachyose	verbascose
				Desi				
galactinol	0.64***							
glucose	0.39***	0.00ns						
fructose	−0.03ns	0.07ns	0.01ns					
sucrose	0.36***	0.03ns	0.56***	−0.07ns				
raffinose	0.40***	0.39***	0.12**	0.07ns	0.15***			
stachyose	0.50***	0.53***	−0.01 ns	0.07ns	0.09*	0.78***		
verbascose	0.49***	0.40***	−0.03 ns	0.08ns	0.18***	0.50***	0.64***	
total RFO	0.46***	0.47***	−0.01 ns	0.04ns	0.08*	0.85***	0.91***	0.60***
				Kabuli				
galactinol	0.68***							
glucose	0.47***	0.12*						
fructose	0.04ns	0.15**	−0.01ns					
sucrose	0.33***	0.23***	0.39***	−0.08ns				
raffinose	0.42***	0.55***	0.11ns	0.05ns	0.41***			
stachyose	0.44***	0.64***	0.01ns	0.07ns	0.35***	0.89***		
verbascose	0.47***	0.49***	0.09ns	0.05ns	0.41***	0.66***	0.72***	
total RFO	0.44***	0.62***	0.01ns	0.06ns	0.33***	0.89***	0.92***	0.69***

[a] ***, **, and * are significant at P ≤ 0.001, P ≤ 0.01, and P ≤ 0.05, respectively; ns, nonsignificant.

genotypes grown in the field during 2010 showed variation from 0.40 to 1.19 g/100 g, from 0.78 to 1.99 g/100 g, and from 0.01 to 0.13 g/100 g for raffinose, stachyose, and verbascose with mean values of 0.75, 1.35, and 0.06 g/100 g, respectively (Figure 3). Kabuli type chickpea genotypes followed the same pattern for variation among RFO members. In GH-grown kabuli type, raffinose (0.27−0.95 g/100 g), stachyose (0.40−1.65 g/100 g), and verbascose (0.01−0.11 g/100 g) showed mean values of 0.66, 1.12, and 0.05 g/100 g, respectively (Figure 4). Kabuli genotypes grown in the field during 2009 contained raffinose, stachyose, and verbascose with mean values of 0.94, 1.79, and 0.08 g/100 g that ranged from 0.69 to 1.17 g/100 g, from 1.31−2.38 g/100 g, and from 0.05 to 0.13 g/100 g, respectively. However, genotypes grown in the field during 2010 ranged from 0.58 to 1.08 g/100 g, from 1.06 to 2.17 g/100 g, and from 0.04 to 0.12 g/100 g for raffinose, stachyose, and verbascose with mean values of 0.84, 1.59, and 0.08 g/100 g, respectively (Figure 4). The lower concentration of RFO in controlled growing environment (GH with less temperature variation, longer photoperiod, and higher photosynthetically active radiation) supports physiological roles of these oligosaccharides in providing tolerance against abiotic stresses.[8,34] RFO act as reactive oxygen species scavengers, signaling molecules, and osmo-protectants, thus providing protection against oxidative, freezing, salinity, and drought stress.[35−40]

In desi genotypes, sucrose concentration varied from 0.84 to 2.84 g/100 g in GH-grown genotypes with a mean value of 1.79 g/100 g, whereas in field-grown genotypes it ranged from 0.60 to 2.93 g/100 g and from 0.81 to 2.64 g/100 g during 2009 and 2010, having average values of 1.87 and 1.52 g/100 g, respectively. However, sucrose varied from 1.05 to 3.33 g/100 g, from 1.33 to 3.59 g/100 g, and from 1.07 to 2.94 g/100 g in kabuli genotypes grown under GH and field conditions (2009 and 2010) with mean values of 2.11, 2.62, and 2.03 g/100 g, respectively. Higher sucrose concentration can be due to its role as a universal molecule to transport carbon and a substrate for raffinose biosynthesis.[41−43] Sosulski et al.[44] estimated sucrose content in hull-free chickpea seeds with mean value of 2.69 g/100 g, which was about 32% of total sugars and highest among soluble sugars. Later, Xiaoli et al.[45] reported the amount of sucrose, raffinose, stachyose, and verbascose in seeds of 19 chickpea cultivars varied from 1.80 to 5.22 g/100 g, from 0.46 to 0.92 g/100 g, from 1.60 to 3.10 g/100 g, and from 0.27 to 0.70 g/100 g, respectively. The variations for important chickpea seeds' constituents described in the present study concur with the range reported in previous studies conducted by Sanchez-Mata et al.,[46] Frias et al.,[30] Alajaji and El-Adawy,[23] Aguilera et al.,[32] and Berrios et al.[33] concluding varying ranges of mean values for sucrose, raffinose, and stachyose from 0.79 to 3.53 g/100 g, from 0.32 to 1.45 g/100 g, and from 0.74 to 2.56 g/100 g, respectively.

Other minor components of chickpea seeds, such as *myo*-inositol, galactinol, glucose, and fructose were also determined. In desi type (Figure 3), *myo*-inositol and galactinol ranged from 0.01 to 0.10 g/100 g and from 0.03 to 0.37 g/100 g with mean values of 0.05 and 0.17 g/100 g, respectively. Similarly, *myo*-inositol in kabuli type (Figure 4) varied from 0.02 to 0.10 g/100 g but with a relatively higher mean value of 0.03 g/100 g. Kabuli genotypes showed variation from 0.05 to 0.32 g/100 g for galactinol, having a mean concentration of 0.1 g/100 g. Desi and kabuli genotypes showed variation from 0.03 to 0.42 g/100 g and from 0.11 to 0.34 g/100 g for glucose concentration with averages of 0.22 and 0.10 g/100 g, respectively, whereas fructose concentration varied from 0.001 to 0.03 g/100 g and from 0.003 to 0.07 g/100 g in desi and kabuli genotypes with mean values of 0.01 and 0.006 g/100 g, respectively (Figures 3 and 4). Sosulski et al.[44] and Jukanti et al.[4] also reported low concentrations of galactinol in chickpea seeds with mean values of 0.50 and 0.39% of chickpea seed dry matter, respectively. These results correspond to the concentrations of glucose (0.05−0.10% of dry matter) and fructose (0.1−0.3% of dry matter) in chickpea seeds reported earlier.[32,33]

Correlation among Chickpea Seed Components. Total RFO showed a positive correlation with raffinose (r = 0.85/0.89), stachyose (r = 0.91/0.92), and verbascose (r = 0.60/0.69) in chickpea genotypes (desi/kabuli) significant at $P \leq 0.001$ (Table 5). Raffinose, stachyose, and verbascose were collectively determined during total RFO assay; hence, the resulting correlation confirmed the accuracy and precision of the HPAEC-PAD method for the concentration of RFO members with enzymatic assay for total RFO determination.

myo-Inositol was significantly ($P \leq 0.001$) and positively correlated with galactinol ($r = 0.64/0.68$), glucose ($r = 0.39/0.47$), sucrose ($r = 0.36/0.68$), raffinose ($r = 0.40/0.42$), stachyose ($r = 0.50/0.44$), and verbascose ($r = 0.49/0.47$) in desi/kabuli genotypes. Galactinol also showed a significant ($P \leq 0.001$) positive correlation with raffinose ($r = 0.39/0.55$), stachyose ($r = 0.53/0.64$), and verbascose ($r = 0.40/0.49$) in chickpea genotypes (desi/kabuli). In desi genotypes, sucrose was positively correlated with raffinose ($r = 0.15$; $P \leq 0.001$), stachyose ($r = 0.09$; $P \leq 0.05$), and verbascose ($r = 0.18$; $P \leq 0.001$), whereas in kabuli types, sucrose showed positive correlations with raffinose ($r = 0.41$), stachyose ($r = 0.35$), and verbascose ($r = 0.41$) significant at $P \leq 0.001$. In previous studies also, sucrose showed significant positive correlations with raffinose and stachyose concentrations in soybean seeds.[47,48]

A significant positive correlation was observed between substrate and product concentrations in RFO biosynthetic pathway in chickpea seeds. The first committed step in RFO biosynthesis is galactinol formation in which *myo*-inositol and UDP-galactose act as substrates. Furthermore, galactinol in conjunction with sucrose, raffinose, and stachyose participates in the biosynthesis of raffinose, stachyose, and verbascose, respectively. Correlation analysis suggested substrate concentration as one of the main regulating factors for varying RFO concentration in different chickpea genotypes. The other regulatory factors might be expression of genes encoding RFO biosynthetic enzymes and/or their activities that still need to be studied. Such studies would be utilized to identify the key step of RFO biosynthesis. As in the case of *Brassica napus*,[49] antisense technology was used to down-regulate galactinol synthase, which resulted in substantial reduction in galactinol and stachyose concentrations in mature transgenic seeds. Such transgenic approaches can also be followed in chickpea to develop varieties with reduced RFO concentration.

Heritability of Important Chickpea Seed Constituents. The significant impact of environment and genotype × environment on the performance of a particular genotype suggests complex genetic regulation of traits.[48,50] Broad sense heritability (h^2) was estimated on the basis of the pooled ANOVA of genotypes grown in the field and greenhouse environments (Table 4). Ayele[51] described high, medium, and low heritability as ≥0.6, 0.3−0.6, and <0.3, respectively. The h^2 of important chickpea seed constituent was estimated with a maximum of 0.61 for total RFO and a minimum of 0.05 for fructose in desi genotypes, whereas h^2 in kabuli genotypes showed a minimum of 0.02 for glucose and a maximum of 0.53 for sucrose. The results for h^2 are in agreement with the heritability ranges reported for sucrose (0.43−0.87), raffinose (0.42−0.56), and stachyose (0.30−0.74) in soybean seeds.[48,52,53] McPhee et al.[50] also estimated narrow sense heritability for sucrose, raffinose, and stachyose in common bean seeds with values of 0.22, 0.54, and 0.44, respectively.

The present study revealed significant impacts of genotype (G), environment (E), and G × E on concentrations of raffinose family oligosaccharides, suggesting their complex genetic regulation in chickpea seeds. Sucrose and stachyose were identified as predominant soluble sugars and RFO in chickpea seeds. A significant positive correlation was observed between substrate and product concentration in the RFO biosynthetic pathway. Among all of the genotypes screened, some were identified as having low RFO concentration. Desi genotypes ICCV 07115, ICCV 07116, and ICCV 07117 showed the lowest total RFO (1.58−2.46 mmol/100 g), raffinose (0.27−0.52 g/100 g), and stachyose (0.43−1.05 g/100 g) in the field- as well as GH-grown environments. Accession ICC 16528 performed stably in different environmental conditions, and it is one of the kabuli genotypes with low total RFO (2.11−3.84 mmol/100 g), raffinose (0.39−0.74 g/100 g), stachyose (0.90−1.46 g/100 g), and verbascose (0.02−0.06 g/100 g). These genotypes can be utilized in chickpea improvement programs to develop cultivars with reduced RFO concentration. The moderate heritability of RFO trait suggested the use of a multilocation trial based approach while using germplasms for chickpea improvement programs.

■ ASSOCIATED CONTENT

Supporting Information

Details of desi and kabuli type chickpeas used in the study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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