RAFFINOSE FAMILY OLIGOSACCHARIDES (RFO) BIOSYNTHESIS IN CHICKPEA (*Cicer arietinum* L.) SEEDS

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Plant Sciences, University of Saskatchewan, Saskatoon, Saskatchewan

By

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ABSTRACT

To increase the global acceptability of chickpea by improving its nutritional quality, seed RFO (Raffinose Family Oligosaccharides) concentration needs to be reduced without affecting their role during seed development and positive impact on human health. To achieve this objective, the key regulating step(s) of RFO biosynthesis needs to be identified. The three main objectives of the thesis were: (1) to optimize an analytical method to determine soluble sugars concentration in chickpea seeds including RFO, (2) to determine chickpea genotypes with contrasting seed RFO concentration, and (3) to optimize and validate RFO biosynthetic enzyme activity assays. These three objectives of the thesis provided basis of the fourth objective. For the first objective, a modified HPAEC-PAD (High performance anion exchange chromatography with pulsed amperometric detector) based gradient approach was optimized to study the concentration and composition of soluble sugars in chickpea seeds. The optimized method separated all the soluble sugars within 20 min of run time with higher accuracy, sensitivity and precision compared to previously reported methods. Therefore, the optimized method was utilized to study the natural variation in RFO concentration of 171 chickpea genotypes. Sucrose (0.60 - 3.59 g/100 g) and stachyose (0.18 − 2.38 g/100 g) were predominant among soluble sugars and RFO, respectively. Analysis of variance revealed a significant impact ($P \leq 0.001$) of genotype (G), environment (E), and their interaction (G×E) on seed RFO concentration in chickpea. A significant positive correlation was observed between substrate and product concentration in RFO biosynthesis. Raffinose, stachyose and verbascose showed moderate broad sense heritability (0.25 − 0.56) suggesting the quantitative nature of the RFO trait in chickpea seeds. Desi (ICC 1163, ICC 1471, ICC 9562, ICCV 07115, ICCV 07116 and ICCV 07117) and kabuli (ICC 5270, ICC 10674, ICC 16216, ICC 16528, ICCV 3 and ICCV 91302) chickpea genotypes with high and low RFO concentrations (high RFO genotypes are underlined) were identified. RFO biosynthetic enzymes activities were optimized for substrate and protein concentration, temperature (25 °C), time (10 min for galactinol synthase and 60 min for other biosynthetic enzymes) and pH (7.0). These assays were validated at different seed developmental stages of two released varieties: CDC Vanguard and CDC Frontier. Simultaneously, RFO accumulation at different seed developmental stages was also studied. During 18 - 38 DAF (days after flowering), about a 75 % decrease in seed moisture was observed coinciding with the accumulation of RFO providing desiccation tolerance to maturing seeds. The initial substrates *viz. myo*-inositol and sucrose were observed throughout

seed development process having maximum accumulation at 18 - 20 (0.50 – 0.57 g/100 g) and 20 - 22 (9.94 - 11.17 g/100 g) DAF that decreased afterwards supporting the biosynthesis of galactinol and raffinose, respectively. Galactinol is considered as the universal galactosyl donor, it showed the highest concentration at 30 DAF and this was later utilized for increased RFO accumulation till 36 DAF. Activity of RFO biosynthetic enzymes was observed 2 - 6 days prior to first detection of their corresponding products whereas the highest activities were determined 2 - 4 days prior to maximum accumulation of RFO. However, maximum GS (galactinol synthase) activity was observed at 36 DAF but this did not correspond to amount of galactinol accumulation in seeds. This indicated that galactinol was synthesized in higher amount even after 30 DAF but directed towards RFO biosynthesis thus could not necessarily accumulate in seeds. A galactinol independent pathway was also found operative in chickpea seeds. These results suggested that substrate concentration and GS activity might be the possible factors regulating seed RFO concentration in chickpea.

The fourth objective utilized the information, material and methods from the previous three objectives. Chickpea genotypes with contrasting RFO concentration were compared for seed size and weight, germination capacity and RFO biosynthesis (accumulation and biosynthetic enzymes activities during seed development). Sucrose concentration showed a significant positive (r = 0.728, $P \leq 0.05$) correlation with seed size/weight. RFO concentration was a facilitator of seed germination rather than regulating factor. Higher accumulation of *myo*-inositol and sucrose in high RFO genotypes during early seed developmental stages suggested that initial substrates concentrations may influence seed RFO concentration. High RFO genotypes expressed about 2 - 3 fold higher activity for all RFO biosynthetic enzymes compared to those with low RFO concentration. The enzyme activity data corresponded with the accumulation of individual RFO during chickpea seed development. In conclusion, regulating galactinol synthase activity is a potential strategy to reduce seed RFO concentration in chickpea.

The present study can be extended to study RFO biosynthesis at the transcript level and the influence of RFO biosynthetic enzymes on seed size and weight, germination, RFO concentration, yield, and stress tolerance.

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ABBREVIATIONS

ATFCC	:	Australian Temperate Field Crops Collection
cDNA	:	Complementary Deoxyribonucleic Acid
cv.	:	Cultivar
DAF	:	Days After Flowering
DM	:	Dry Matter
DP	:	Degree of Polymerization
DW	:	Dry Weight
EC	:	Enzyme Commission number
FAO STAT	:	Food and Agriculture Organization Statistics
G×E	:	Genotype by Environment Interaction
GGT	:	Galactan:galactan Galactosyl Transferase
GS	:	Galactinol Synthase
H^2	:	Heritability
h^2	:	Broad sense heritability
HPAEC-PAD	:	High Performance Anion Exchange Chromatography with Pulsed Amperometric Detector
HPLC-RI	:	High Performance Liquid Chromatography with Refractive Index detector
ICARDA	:	International Center for Agricultural Research in the Dry Areas
ICRISAT	:	International Crop Research Institute of the Semi-Arid Tropics
Kat	:	Katal
K_m	:	Michaelis-Menten constant
Mbp	:	Mega base pairs
MUFA	:	Monounsaturated Fatty Acid
PGR	:	Plant Genetic Resources
PSV	:	Protein Storage Vacuoles
PUFA	:	Polyunsaturated Fatty Acid
RDS	:	Readily Digestible Starch
RFO	:	Raffinose Family Oligosaccharides
ROS	:	Reactive Oxygen Species
RS	:	Resistant Starch
RS/RFS	:	Raffinose Synthase
SCFA	:	Short Chain Fatty Acid
SFA	:	Saturated Fatty Acid
STS	:	Stachyose Synthase
UDP	:	Uridine Diphosphate
USDA	:	United States Department of Agriculture
VS	:	Verbascose Synthase

1. INTRODUCTION

1.1 Background

Chickpea (*Cicer arietinum* L.) is the second most widely cultivated pulse crop after dry beans (FAO STAT 2012). It is an excellent source of protein, carbohydrate, vitamins, minerals, polyunsaturated fatty acids, dietary fibers and other bioactive compounds. Being nutritionally balanced and economically accessible, chickpea has the potential of making a higher nutritional contribution to the world's growing population. However, presence of compounds like raffinose family oligosaccharides (RFO) in chickpea seeds reduces its acceptability as food and feed mainly in the western countries.

RFO represent a group of soluble but non-reducing and non-structural sugars characterized by the presence of α(1→6) glycosidic linkages. Raffinose is the first member of this family followed by stachyose and verbascose (Martínez-Villaluenga et al. 2008). Chickpea seeds contain about 2.0 – 7.6 % of RFO. Humans and monogastric animals due to lack of α-galactosidase enzyme in the digestive tract, can not digest RFO. Hence, RFO remain intact in stomach and small intestine but are fermented by the large intestinal microflora. The microbial RFO breakdown results in production of deleterious gases causing metabolic discomfort and flatulence when consumed in higher concentration (Swennen et al. 2006; Kumar et al. 2010). RFO are also considered as part of prebiotic and functional food when present in low concentration, thus attributed for various health promoting characteristics (Roberfroid 2002). In plants, RFO participate in important physiological mechanisms like seed germination (Blöchl et al. 2008), stress tolerance (Nishizawa-Yokoi et al. 2008; Keunen et al. 2013), seed desiccation (Angelovici et al. 2010) and translocation of photoassimilate (Sprenger and Keller 2000). RFO also support the growth and survival of nitrogen fixing bacteria in root rhizosphere of legumes (Gage 1998). Therefore, to improve chickpea's acceptability globally, RFO concentration needs to be reduced without affecting their role in plants and positive impact on human health. Various physico-mechanical treatments have been reported to reduce RFO concentration significantly in seeds of chickpea and other legumes (Aguilera et al. 2009; Devindra et al. 2011). Such treatments are expensive, time consuming and also reduce the concentration of other nutrients (Alajali and El-Adawy 2006). Alternatively, genetic approaches along with biochemical studies and plant breeding strategies are being employed to develop chickpea varieties with low seed RFO concentration (Polowick et al. 2009; Bock et al. 2009).

To explore RFO biosynthesis in chickpea seeds, an accurate determination of individual

RFO and their substrates is essential. Although Sánchez-Mata et al. (1998) and Xioli et al. (2008) reported chromatographic methods to determine RFO but these methods are incapable of simultaneous quantification of verbascose and substrates of RFO biosynthesis (*myo*-inositol and galactinol), respectively. It reflects the need of a precise and accurate analytical method to determine individual RFO (raffinose, stachyose and verbascose) and their biosynthetic substrates (*myo*-inositol, galactinol, UDP-galactose and sucrose) simultaneously in chickpea seeds.

Chickpea seed constituents (protein, starch and amylose) and other plant characteristics (grain yield, seed weight, plant height etc.) are significantly influenced by genotype (G), environment (E) and their interaction (G×E) (Rubio et al. 1998; Sirohi et al. 2001; Frimpong et al. 2009; ALwawi et al. 2010; Dehghani et al. 2010). In legumes like, soybean (*Glycine max* L. Merr.; Cicek et al. 2006; Jaureguy et al. 2011) and lentil (*Lens culinaris* Medikus subsp. Culinaris; Tahir et al. 2011), significant impact of G, E and G×E on seed RFO concentration was observed. Studies regarding natural variation and environmental effect assist plant breeders in selecting genotypes to develop strategies for genetic and molecular studies. Therefore, such studies must be performed for RFO concentration in chickpea seeds as there is no report available till date.

RFO biosynthesis is initiated with the formation of galactinol followed by raffinose (Martínez-Villaluenga et al. 2008). Higher members of raffinose family are synthesized by either galactinol-dependent or -independent pathway. The former utilizes galactinol as galactosyl unit donor whereas in the latter, already present raffinose family member acts as a donor. The main RFO biosynthetic enzymes are galactinol synthase (GS), raffinose synthase (RS), stachyose synthase (STS), verbascose synthase (VS) and galactan:galactan galactosyl transferase (GGT). GS and RS catalyze the biosynthesis of galactinol and raffinose, respectively. In galactinol dependent pathway, STS and VS play important role while GGT is the core component of galactinol independent pathway (Peterbauer and Richter 2001; Karner et al. 2004; Bachmann et al. 1994; Haab and Keller 2002). There is still controversy on the key regulation step of RFO biosynthesis. Some reports support GS as the key enzyme whereas others consider substrate accumulation as regulating step elucidating the dependency of key step on type of crop/plant (Peterbauer et al. 2001; Karner et al. 2004; Lahuta et al. 2005; Bock et al. 2009). Consequently, to reduce RFO concentration in chickpea seeds, RFO biosynthetic mechanism and key regulating step must be explored in chickpea seeds.

1.2 Research objectives

Based on the background information, present research proposal has following objectives:

1. To optimize an analytical method to determine concentration of soluble sugars in chickpea seeds.
2. To study natural variation and effect of genotype, environment and their interaction on chickpea seeds RFO concentration.
3. To study RFO biosynthesis during different stages of chickpea seed development.
4. To compare RFO biosynthesis among contrasting RFO chickpea genotypes.

2. REVIEW OF LITERATURE

2.1 Chickpea: an introdction

2.1.1 Taxonomy and botanical specifications

Chickpea (*Cicer arietinum* L.; 2n = 2x = 16) is an important pulse crop throughout the world, especially in developing countries (Tekeoğlu et al. 2002). It is a self-pollinating diploid annual crop with a life cycle of 3 – 4 months, and a genome size of about 740 Mbp (Rajesh et al. 2008; Varshney et al. 2013). Chickpea is a member of legume (Latin *legumen* describing seeds harvested from pods) family – Fabaceae or Leguminosae which includes about 730 genera and more than 19400 species worldwide. Hence, Leguminosae is the third largest family of angiosperms after Orchidaceae and Asteraceae whereas second only to Poaceae in agricultural and economic importance (Wojciechowski et al. 2004). Chickpea belongs to genus *Cicer* L., tribe *Cicereae* Alef. and subfamily *Papilionoideae*. Out of total 44 species of the genus *Cicer*, only one species named chickpea (*C. arietinum* L.) is currently being cultivated all over the world. *C. arietinum* along with *C. echinospermum* and *C. reticulatum* constitutes primary gene pool, whereas *C. bijugum*, *C. judaicum* and *C. pinnatifidum* collectively institute secondary gene pool and all other *Cicer* species come under tertiary gene pool (Van der Maesen et al. 2007).

Chickpea plant has an indeterminate growth habit and usually attains a height of 20 to 100 cm (Singh 1997; Van der Maesen et al. 2007). It has a tap root system (1.5 – 2.0 m deep) with some lateral branches. These roots also contain bacteria filled nodules that help in nitrogen fixation in soil. The root system supports an erect, branched (primary, secondary and tertiary branches), green and solid stem. Leaves in chickpea are green, one at each node with alternate phyllotaxy and unipinnately compound (11 to 13 leaflets/leaf) having stipules (3 - 5 mm long and 2 - 4 mm wide) at their origin from stem (Singh 1997). The outer surface of chickpea plant is mostly covered with glandular or non-glandular hairs. Chickpea flowers are typically papilonaceous and contain calyx (5 sepals), corolla (5 petals), androecium (10 stamens) and one hairy ovary. In corolla, petals are arranged in a 1+2+2 arrangement, the biggest petal is known as “standard” while others are termed as “wings” and “keel” in decreasing order of their sizes. Stamens follow a 9+1 (nine stamens have fused filament while the tenth is separate) arrangement that makes chickpea flower “diadelphous”. Both stamens and pistil are covered by “keels” (Singh and Diwakar 1995; Singh 1997). Chickpea flowers are cleistogamous in nature, *i.e.* flowers open only after fertilization that supports self-fertilization (only up to 1.9 % outcrossing; Srinivasan and Gaur 2012) (Figure 2.1).

2.1.2 Classification

Chickpea is mainly divided into two groups (Figure 2.1) discerned on the basis of distinct morphological characteristics (Cobos et al. 2007). Desi type (microsperma) represents the first group having small pods, seeds, leaves and stature with anthocyanin pigmented stem, purple or pink flowers and dark-colored, angular seeds. Geographically, desi types are predominant in semi-arid tropical regions of the world (Indian subcontinent, east Africa, central Asia and to a limited extent in the Mediterranean Basin). Second group of chickpea, named as kabuli type or macrosperma is characterized by large pods, leaflets and taller stature having stem without anthocyanin pigmentation, white flower and larger, smooth surface, creamy/beige-colored seeds. Kabuli types are usually grown in temperate regions (Mediterranean basin and central Asia) of the world (Redden and Berger 2007; Cobos et al. 2007; Agarwal 2012). Desi type has significantly thicker seed coat in comparison to kabuli (Wood et al. 2011) but the adherence of seed coat to cotyledon is good in both types. Being members of the same gene pool, both types have full cross compatibility (Redden and Berger 2007; Cobos et al. 2007).

2.1.3 Origin and geographical distribution

Chickpea is a member of "Founder Crop Package" or West Asian Neolithic crop assemblage associated with crop domestication and evolution of agriculture in the Fertile Crescent about 10,000 years ago (Singh 1997; Abbo et al. 2003; Kerem et al. 2007; Upadhyaya et al. 2011). Besides chickpea, this crop package consists of einkorn wheat (*Triticum monococcum* L.), emmer wheat (*T. turgidum* L.), barley (*Hordeum vulgare* L.), lentil (*Lens culinaris* Medic.), pea (*Pisum sativum* L.), bitter vetch (*Vicia ervilia* L. Willd.) and flax (*Linum usitatissimum* L.) (Abbo et al. 2003). Based on analysis resulted from seed protein electrophoresis, interspecific hybridization, karyotype and isozyme pattern, *C. reticulatum* Ladiz. is considered as wild progenitor of cultivated chickpea and its distribution is restricted to only about 18 locations (37.3 - 39.3° N, 38.2 - 43.6° E) in south-eastern Turkey (Ladizinsky and Adler 1976; Singh 1997; Kerem et al. 2007).

The major change due to chickpea domestication is the transformation from winter habit with autumn sowing to a spring habit (vernalization-insensitive; Redden and Berger 2007). Although this transformation resulted into about 10 to 90 % reduction in crop yield but helped in reducing or avoiding Ascochyta blight infestation that may cause total crop loss during winter (Kerem et al. 2007). Other changes during domestication include loss of dormancy, reduced pod dehiscence, larger seed and plant size and reduced anthocyanin

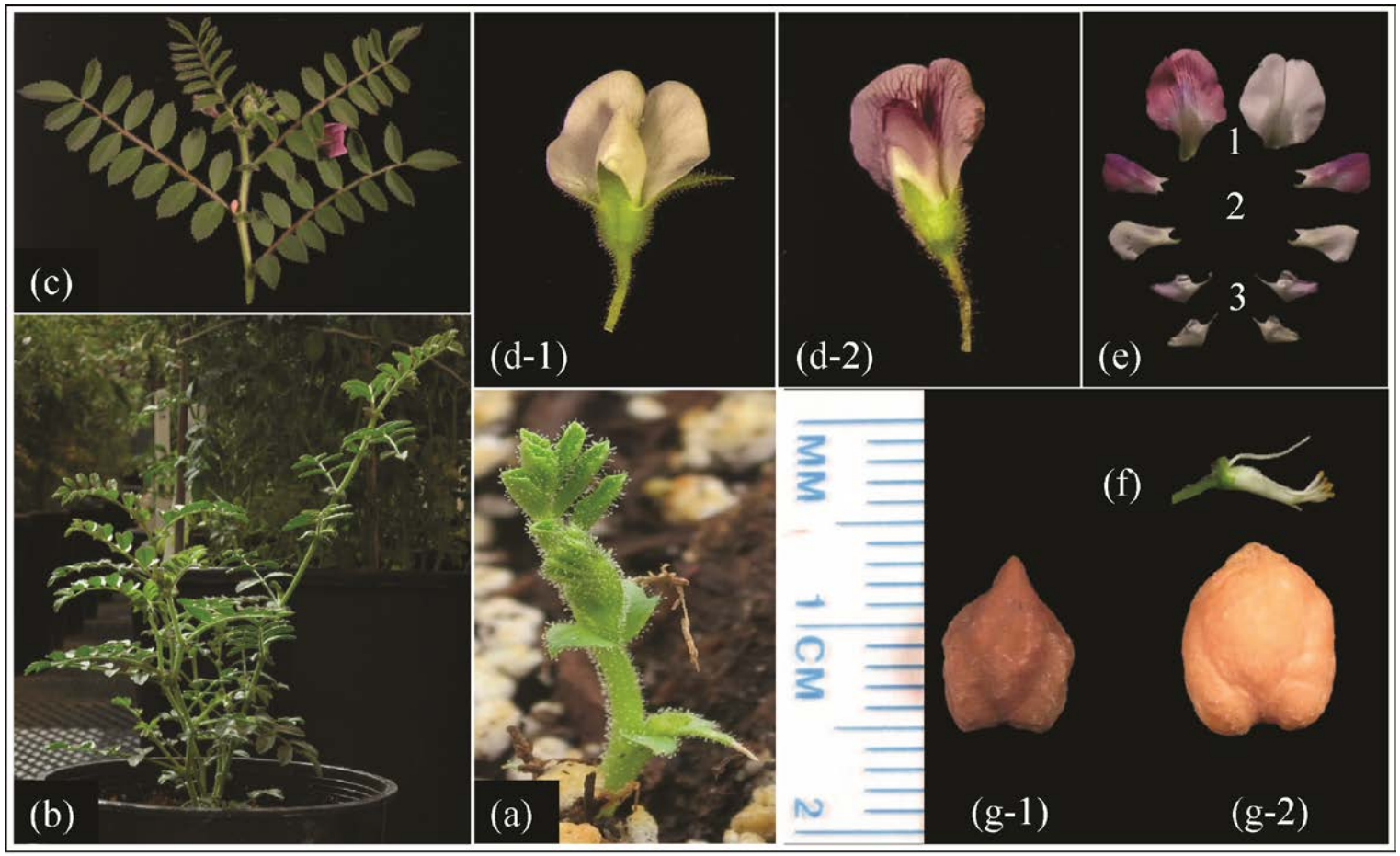


Figure 2.1 Chickpea plant, flowers and seeds.

Photographs showing, (a) germinated chickpea seedling; (b) chickpea plant; (c) adaxial view of apical portion of chickpea plant having unipinnate compound leaves; (d-1) flower of desi type chickpea; (d-2) flower of kabuli type chickpea; (e) 1+2+2 arrangement of corolla showing (1) standard, (2) wing and (3) keel of desi and kabuli type; (f) diadelphous anthers; (g-1) seed of desi type chickpea and (g-2) seed of kabuli type chickpea.

pigmentation (Redden and Berger 2007). On the basis of genetic variation and distribution pattern of two different chickpea variants, microsperma/desi is proposed to be more primitive while macrosperma/kabuli type is derived from the former through selection process (Singh 1997).

Chickpea is believed to be originated in south-eastern Turkey and adjoining areas of Syria. The Southwest Asia and Mediterranean regions are considered as chickpea's primary centers of diversity whereas Ethiopia is the secondary center of diversity (Singh 1997; Redden and Berger 2007). Macrosperma type was predominant around Mediterranean basin while microsperma was abundant in the eastern part. Hence, chickpea is known as "chana" which is derived from Sanskrit word "chennuka" and microsperma is called as "desi" in Hindi which means "local" in English. As linguistic evidence, macrosperma was introduced in India from Kabul (capital of Afghanistan) through "Silk Route" and therefore acquired the present name "kabuli" in Hindi (Van der Maeson 1972). Chickpea is also known as Gram (derived from Portuguese "grâo" means grain; Van der Maeson 1972), Bengal Gram (English), Nohud or Lablabi (Turkey), Shimbra (Ethiopia), Garbanzo (Latin America) and Hamaz (Arabic) in different parts of world (Hannan et al. 2001).

Chickpea was introduced in the new world by Spaniard and Portuguese. Hence, Mexico is one of major chickpea producing countries today. Chickpea was also distributed to Australia, South Asia, North Africa and Subsaharan Africa where it is being cultivated till date (Van der Maeson 1972; Redden and Berger 2007).

2.1.4 Current statistics

Chickpea is the second most important pulse crop after dry beans in terms of area harvested and total production (FAO STAT 2012). During 2012, total world chickpea production was 11.6 Million Tonnes (MT) over a total harvested area of 12.3 Million Hectare (MHa) that contributed about 16.3 % to total world pulse production (71.3 MT). From 1960 to 2012, an increment of 11.7 % was observed in total world chickpea area harvested whereas total world production was increased by 73.3 % (Figure 2.2). Presently, chickpea is being cultivated in >50 countries across the globe. India is the largest producer of chickpea and shared about 66.2 % (7.7 MT) of total world chickpea production during 2012. In the same year, Australia ranked second followed by Turkey contributing 5.8 (0.67 MT) and 4.6 % (0.54 MT) to total world chickpea production, respectively. Other major chickpea producing countries (rank) were Myanmar (4^{th}), Ethiopia (5^{th}), Iran (6^{th}) and Pakistan (7^{th}) and Mexico (8^{th}). Canada ranked 9^{th} having a total production of 0.16 MT that shared about 1.35 % of

total world chickpea production. Saskatchewan and Alberta are two main chickpea producing provinces of Canada. Chickpea statistics has been summarized in Figure 2.2.

2.1.5 Germplasm, core, minicore and composite collections

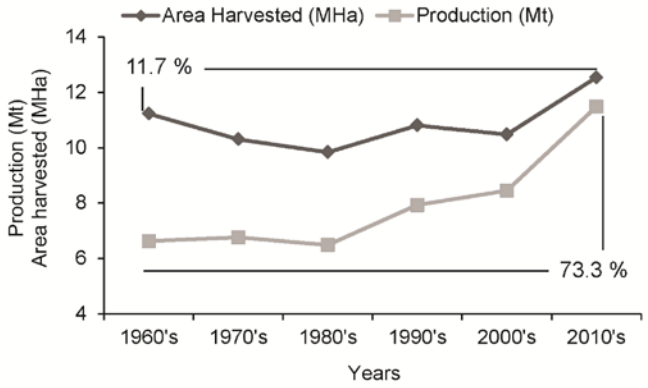
Plant genetic resources (PGR) are the reservoir of genetic diversity that make platform for plant breeding programs to increase yield and improve quality, adaptability and stress tolerance of various crop species (Ulukan 2011). PGR includes primitive forms, landraces, cultivars, related wild species, weed species, breeder's elite lines and mutants (Van et al. 2011). Conservation of more than 6.1 million accessions in >1300 gene-banks reflects the importance of PGR in crop improvement programs (Redden and Berger 2007).

ICRISAT (International Crop Research Institute of the Semi-Arid Tropics, Patancheru, India) and ICARDA (International Center for Agricultural Research in the Dry Areas, Beirut, Lebanon) are two major gene-banks for chickpea. ICRISAT gene-bank preserves 20,268 chickpea accessions collected from India (7677; 37.9 %), Iran (5295; 26.1 %), Turkey (971; 4.8 %), Ethiopia (960; 4.7 %), Afghanistan (734; 3.6 %), Pakistan (723; 3.6 %) and other countries (3908; 19.3 %) (ICRISAT 2013). ICARDA gene-bank has 12,070 accessions mainly from Iran (1780; 14.7 %), Turkey (970; 8.0 %), India (410; 3.4 %) and Chile (340; 2.8 %). Other important chickpea gene-banks are USDA (United States Department of Agriculture) and ATFCC (Australian Temperate Field Crops Collection) having about 900 and 670 accessions, respectively (Redden and Berger 2007).

To use the genetic diversity of large germplasm collections in plant breeding approaches, their size needs to be reduced so that it can be managed easily during replicated trials and evaluation for economically important traits. In 1984, Frankel proposed the concept of "Core Collection" which was further extended by Frankel and Brown (1984) and Brown (1989a). The main idea was to minimize the repetitiveness of the collection while maintaining the genetic diversity and the accessions omitted from the core would be considered as reserve collection (Brown 1989b). Core collection represents at least 70 % diversity with total entries of about 10 % (max 3000 per species) of the total collection. Upadhyaya et al. (2001) developed a core collection of 1956 chickpea accessions (11.5 % of total collection) from ICRISAT collection (total 16,991 accessions) based on geographical distribution and 13 quantitative traits. Desi type shared 74.9 % (1465 accessions) of the core collection whereas kabuli and intermediates contributed 22.1 (433 accessions) and 3.0 % (58 accessions), respectively.

To utilize genetic diversity in more effective and manageable manner, Upadhyaya and

(a)



(b)

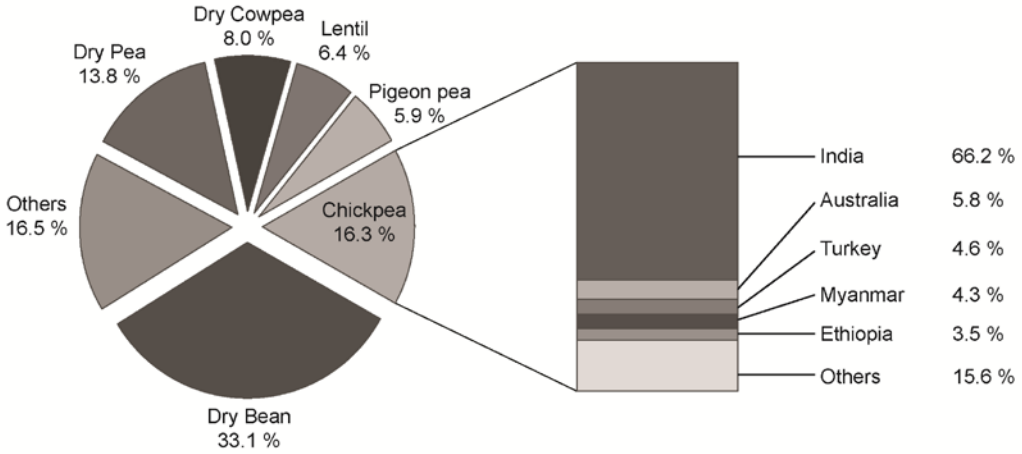


Figure 2.2 Graphical representations of chickpea statistics.

(a) increase in production and harvested area of chickpea from 1960's to 2010's and (b) chickpea's share in total pulse production and major chickpea producing countries during 2012.

Ortiz (2001) selected 211 accessions from chickpea core collection based on the data of 22 morphological and agronomic traits. This new small collection is designated as "Minicore Collection". Minicore subset represented 10.8 % accessions from core collection with no significant variation in terms of organization (proportion of desi, kabuli and intermediate types) and scores (mean, variances, frequency distribution, correlation and Shannon-Weaver diversity index) of selected chickpea traits. Minicore collection is also helpful for those crop species that have very large germplasm collections. Later, Upadhyaya et al. (2006) developed a "global composite collection" to enhance the utilization of chickpea accessions both from ICRISAT and ICARDA gene-banks. Composite collection includes total 3,000 accessions: 1956 from chickpea core collection, 709 from ICARDA gene-bank, 39 advanced breeding lines and released cultivars, 35 distinct morphological variants, 20 wild species (*Cicer echinospermum* and C. *reticulatum*) accessions and 241 accessions carrying specific traits (stress tolerance or resistance and important agronomic characters like early maturity, multi-seeded pods, double podded, large-seed size, high seed protein, nodulation and responsiveness to high-input conditions).

2.2 Nutritional quality of chickpea seeds

Chickpea (*Cicer arietinum* L.) is one of the potential crops that can make an important nutritional contribution to the population of developing countries. It is not only nutritionally balanced but also within the economic accessibility of people (Jukanti et al. 2012). In a mature chickpea seed, cotyledon shares about 83 and 92 % of seed weight in desi and kabuli types, respectively whereas seed coat covers about 15 % in desi type and 6.5 % in kabuli type. Embryo occupies only about 1.5 – 2.0 % of chickpea seed. Cotyledon is rich in carbohydrates and protein. Seed coat is mainly composed of minerals and dietary fibers while embryo mainly contains lipids and vitamins (Chibbar et al. 2010; Table 2.1). The amount of each seed constituent is highly influenced by genotype, environment and their interactions (Kumar et al. 2010; Tahir et al. 2011). These nutrients are attributed for many beneficial health-promoting properties defining chickpea as a functional and health promoting diet (Martínez-Villaluenga et al. 2008; Jukanti et al. 2012).

2.2.1 Protein

Proteins are required for proper growth and development in humans. Protein concentration in chickpea seeds varies from about 20 to 28 % in desi types and 18 to 31 % in kabuli types (Table 2.1). In pulse crops, most of the protein is found as storage protein among

which globulin is the major class followed by albumin whereas prolamins and glutelins (predominant in cereals) are present as minor components. Globulin represents salt-water soluble proteins and mainly comprises of legumin (11S) and vicilin (7S) family (Boye et al. 2010; Roy et al. 2010). Chickpea seeds have an albumin to globulin ratio of 1:4 and legumin:vicilin of 4 - 6:1 (Gupta and Dhillon 1993; Boye et al. 2010). A protein molecule is composed of linked amino acids. Out of total 20 amino acids, ten amino acids are required from the diet and they are termed as essential amino acids for humans. The remaining ten amino acids can be synthesized by human body hence known as non-essential amino acids. Chickpea protein has good proportion of amino acids (Table 2.2) and contains a relatively higher concentration of essential amino acid lysine (4.9 – 7.7 g/100 g protein) as compared to cereal grains (~2.8 g/100 g protein). However, sulphur-containing amino acids methionine and cysteine are in lower concentration in chickpea as compared to cereals. Therefore, consumption of pulses with cereals (like rice and wheat) in 2 - 4:1 provides balanced proportion of amino acids (Leterme and Muñoz 2002). In addition to composition of proteins, its digestibility is also very important for human nutrition (Wang et al. 2010). Protein digestibility is affected by various factors such as inhibitors of enzymatic breakdown of proteins. The enzyme inhibitors can be inactivated during processing or cooking but chickpea type and genotype also affect protein digestibility and in chickpea, it varies from 34 – 76 % (Boye et al. 2010; Jukanti et al. 2012). Protein in kabuli type has higher digestibility than in desi type (Wang et al. 2010; Jukanti et al. 2012).

2.2.2 Lipid

Fat concentration in chickpeas varies from 3.1 to 5.9 % in desi and 5.1 to 6.9 % for kabuli types (Table 2.1) and can be considered high when compared to other pulse crops (Jukanti et al. 2012). Polyunsaturated- (PUFA), monounsaturated- (MUFA) and saturated- (SFA) fatty acids share about 66, 19 and 15 % of the total fat content in chickpea seeds. Among PUFA, linoleic acid (42.25 – 65.25 % of oil) is the most prevalent fatty acid in chickpea seed followed by oleic acid (18.4 – 42.5 % of oil) and palmitic acid (8.5 – 20.4 % of oil) (Table 2.3).

2.2.3 Minerals

In chickpea, potassium is the predominant mineral followed by phosphorous and calcium (Table 2.4). On average, a 100 g serving of chickpea can meet significant requirement of daily allowances of Fe (75/33 % in males/females), Zn (48/66 %), Ca (13/13

Table 2.1 Nutritional composition of chickpea seeds

Seed constituents	Khan et al. (1995)		Rincón et al. (1998)		Wang and Daun 2004		Others*
	g/100 g DM*		g/kg DM		g/100 g DM		g/100 g DM
	Desi	Kabuli	Desi	Kabuli	Desi	Kabuli	
Carbohydrate	47.4	55.8	474.2	475.7	-	-	54 - 66
Protein	25.4	24.4	215	217	20.3 - 27.5	17.9 - 30.8	16 - 28
Fat	3.7	5.1	30.7	46	4.4 - 5.9	5.5 - 6.9	3.1 - 7.0
Crude fibre	11.2	3.9	216.7	195.1	22.8 - 27.1	7.2 - 13.4 (ADF+NDF)*	18.4 (IDF+SDF)*
Ash	3.2	2.8	32.2	34.2	2.7 - 3.5	2.9 - 3.8	3.6

* Dalgetty and Baik (2003); Alajali and El-Adawy (2006); Iqbal et al. (2006); DM = dry matter; ADF = Acid Detergent Fibre; NDF = Neutral Detergent Fibre; IDF = Insoluble Dietary Fibre; SDF = Soluble Dietary Fibre

Table 2.2 Amino acid profile of chickpea seeds

Amino acids	Dhawan et al. (1991)	Khan et al. (1995)		Wang and Daun (2004)		Alajali and El-Adawy (2006)	Iqbal et al. (2006)	Zia-Ul-Haq et al. (2007)
	g/100 g protein	g/100 g protein		g/16 g N		g/16 g N	g/100 g protein	g/100 g protein
		Desi	Kabuli	Desi	Kabuli			
Essential								
Arginine	9.36 - 9.53	-	-	8.3 - 13.6	8.3 - 13.7	10.3	8.3	8.0 - 8.5
Histidine	2.26 - 2.59	-	-	1.7 - 2.7	1.7 - 2.4	3.4	3	2.9 - 3.2
Isoleucine	4.13 - 4.39	3.60 - 4.25	3.50 - 4.16	2.5 - 4.4	2.6 - 3.9	4.1	4.8	4.5 - 4.8
Leucine	6.98 - 7.38	7.81 - 8.25	7.15 - 8.17	5.6 - 7.7	5.6 - 7.2	7	8.7	8.1 - 8.5
Lysine	6.87 - 7.01	6.15 - 6.55	6.25 - 6.86	5.2 - 6.9	4.9 - 6.7	7.7	7.2	6.7 - 7.0
Methionine	1.42 - 1.63	1.15 - 1.20	1.22 - 1.26	1.1 - 1.7	1.1 - 2.1	1.6	1.1	0.8 - 1.1
Phenylalanine	5.39 - 5.61	5.36 - 5.68	4.90 - 6.10	4.5 - 5.9	4.5 - 6.2	5.9	5.5	5.0 - 5.3
Threonine	3.69 - 4.09	3.00 - 3.45	3.15 - 3.23	3.7 - 4.7	3.3 - 5.1	3.6	3.1	2.7 - 3.0
Tryptophan	-	-	-	0.8 - 1.1	0.7 - 1.6	1.1	0.9	0.8 - 0.9
Valine	4.03 - 4.48	4.07 - 4.72	4.26 - 4.38	2.8 - 4.7	2.9 - 4.6	3.6	4.6	4.1 - 4.6
Non-essential								
Alanine	3.99 - 4.54	-	-	3.6 - 4.5	3.5 - 4.7	4.4	4.97	4.7 - 5.2
Aspartic acid	11.35 - 12.10	-	-	11.1 - 15.9	11.2 - 12.9	11.4	11	10.9 - 11.5
Cystine	-	1.07 - 1.15	1.09 - 1.10	1.1 - 1.6	0.8 - 2.0	1.3	0.6	0.4 - 0.7
Glutamic acid	16.37 - 16.96	-	-	13.4 - 18.7	13.1 - 17.5	17.3	17.3	17.3 - 17.8
Glycine	3.96 - 4.20	-	-	3.3 - 4.2	3.2 - 4.5	4.1	3.7	3.4 - 3.7
Proline	3.98 - 4.76	-	-	4.0 - 6.3	3.8 - 6.5	4.6	3.8	3.8 - 4.1
Serine	4.87 - 5.13	-	-	5.5 - 6.9	5.2 - 6.7	4.9	3.7	3.2 - 3.7
Tyrosine	3.03 - 3.37	2.89 - 3.25	2.75 - 3.50	1.4 - 3.1	2.2 - 3.3	3.7	2.8	2.6 - 3.1

Table 2.3 Fatty acid composition of chickpea seeds

Fatty Acid	Fatty acid concentration (% of total oil)			
	Wang and Daun (2004)		Zia-Ul-Haq et al. (2007)	Campos Vega et al. (2010)
	Desi	Kabuli		
Lauric (C12:0)	0.00 - 0.10	ND	-	-
Myristic (C14:0)	0.17 - 0.32	0.19 - 0.26	-	-
Palmitic (C16:0)	8.56 - 11.05	8.52 - 10.30	18.9 - 20.4	10.87
Palmitoleic (C16:1)	0.23 - 0.30	0.27 - 0.34	0.3 - 0.5	0.23
Margaric (C17:0)	-	-	-	0.06
Stearic (C18:0)	1.04 - 1.60	1.21 - 1.68	1.3 - 1.7	1.85
Oleic (C18:1)	18.44 - 28.51	27.70 - 42.46	21.6 - 22.2	33.51
Linoleic (C18:2)	53.13 - 65.25	42.25 - 56.59	54.7 - 56.2	49.74
Linolenic (C18:3)	2.54 - 3.65	2.23 - 3.91	0.5 - 0.9	2.41
Arachidic (C20:0)	0.45 - 0.74	0.59 - 0.76	1.0 - 1.4	0.6
Gadoleic (C20:1)	0.41 - 0.59	0.48 - 0.70	-	0.39
Eicosadienoic (C20:2)	0.08 - 0.15	0.00 - 0.09	-	-
Behenic (C22:0)	0.30 - 0.42	0.29 - 0.48	-	0.21
Erucic (C22:1)	0.00 - 0.21	0.00 - 0.16	-	Tr*
Lignoceric (C24:0)	ND*	0.00 - 0.29	-	-

ND = Not Detected; Tr = Traces

Table 2.4 Mineral composition of chickpea seeds

Minerals	Ibáñez et al. (1998)		Cabrera et al. (2003)	Wang and Daun (2004)		Alajali and El-Adawy (2006)	Iqbal et al. (2006)	Zia-Ul-Haq et al. (2007)	Dilis and Trichopoulou (2009)
	mg/100 g		µg/g edible portion	mg/100 g DW*		mg/100 g DW	mg/100 g	mg/100 g	mg/100 g
	Desi	Kabuli		Desi	Kabuli				
Na	22.9	21.07	-	-	-	121	101	96 - 107	39
K	878	926	-	1027.6 - 1479.1	816.1 - 1580.1	870	1155	1109 - 1272	1000
P	NA	NA	-	276.2 - 518.6	294.1 - 828.8	226	251	239 - 263	310
Ca	210	154	-	115.0 - 226.5	80.5 - 144.3	176	197	185 - 219	160
Fe	4.51	4.46	65.00 - 70.20	4.6 - 7.0	4.3 - 7.6	7.72	3	2.4 - 4.1	5.5
Cu	1.25	1.2	3.2 - 4.9	0.5 - 1.4	0.7 - 1.4	1.1	11.6	10.7 - 12.2	-
Zn	3.57	3.5	37.40 - 42.80	2.8 - 5.1	3.6 - 5.6	4.32	6.8	3.5 - 6.0	3
Mn	1.72	1.65	-	2.8 - 4.1	2.3 - 4.8	2.11	1.9	1.2 - 2.3	-
Mg	128	122	-	143.7 - 188.6	152.9 - 212.8	176	4.6	4.3 - 5.0	130
Cr			0.09 - 0.25						
Al			2.7 - 18.0						
Ni	-	-	0.20 - 0.35	-	-	-	-	-	-
Pb			0.40 - 0.69						
Cd			0.004 - 0.015						

*DW = dry weight

%), Mg (34/45 %), K (21/21 %) Cu (90/90 %) and P (48/48 %) (Thavarajah and Thavarajah 2012; Jukanti et al. 2012). However, the mineral concentration can show large variations depending on genotype and growth conditions, and in particular soil environment. For example, chickpea seeds grown in North America have a high selenium concentration (15.3 - 56.3 µg/100 g) that is adequate to fulfill 61 % of the recommended daily allowance in humans (Campos-vega et al. 2010).

2.2.4 Vitamins

Vitamins are an integral component of daily human diet. They are required in small quantity but crucial for proper growth and various metabolic events (Ciftci et al. 2010). Chickpea has a good complement of vitamins; the predominant being tocopherol (Table 2.5). Chickpea seeds also contain antioxidants/pigments such as carotenoids, which give bright colors to plant tissues. The important carotenoids in chickpea are β-carotene, lutein, zeaxanthin, beta-cryptoxanthin, lycopene and alpha-carotene. With the exception of lycopene, wild accessions of chickpea contain higher concentrations of carotenoids than cultivated varieties (Abbo et al. 2005). In plants, the most prevalent carotenoid is β-carotene, which can easily be converted in to vitamin A. Chickpea seeds are rich in β-carotene and on a dry weight basis contain more than Golden rice or red-colored wheat (Jukanti et al., 2012).

2.2.5 Other bioactive compounds

Besides major nutritional constituents, chickpea seeds also contain phytic acid, saponins, oxalic acid, tannin, phenolics and enzyme inhibitors (Table 2.6). Phytic acid constitutes about 0.4 to 1.7 % of chickpeas and has an important cellular function for plant and seed development. The component has a negative effect on nutrition by chelating mineral nutrients, thereby lowering their bioavailability (Dilis and Trichopoulou 2009). Thus, about 60 - 90 % of total phosphorous present in legume seeds is unavailable for uptake and high presence of phytic acid is thought to exacerbate iron, calcium and zinc malnutrition in developing countries. The saponins (0.4 – 5.0 %) and inhibitors of trypsin, chymotrypsin and α-amylase have been reported to reduce the bioavailability of other nutrients in chickpea seeds (Jukanti et al. 2012).

2.2.6 Carbohydrates

The main energy provided by chickpea in human diet and animal feed is derived from carbohydrates, which constitute about 47 - 66 % of chickpea seed weight. Dietary

Table 2.5 Vitamin profile of chickpea seeds

Vitamin	Chavan et al. (1986)	Wang and Daun (2004)		Dilis and Trichopoulou (2009)	Ciftci et al. (2010)
	mg/100 g	mg/100 g		mg/100 g	µg/g
		Desi	Kabuli		
Retinol (A)	-	ND*	ND	ND	-
Carotene	-	-	-	6000	46.3 (β form)
Ascorbic acid (C)	2.15 - 6.00	0.67 - 3.01	0.28 - 2.40	Tr*	-
D	-	ND	ND	-	115.4 (D_2+D_3)
Thiamine (B1)	0.028 - 0.40	0.22 - 0.34	0.39 - 0.78	0.39	-
Riboflavin (B2)	0.15 - 0.30	0.16 - 0.24	0.10 - 0.34	0.24	-
Niacin (B3)	1.60 - 2.90	1.43 - 2.28	0.48 - 1.49	-	-
Pentathenic acid (B5)	-	0.85 - 1.65	0.72 - 1.19	-	-
Pyridoxine (B6)	0.55	0.27 - 0.36	0.19 - 0.51	0.53	-
Cyanocobalamin (B12)	-	ND	ND	-	-
Biotin	-	ND	ND	-	-
α-tocopherol	-	1.65 - 2.17	1.43 - 2.80	2.88 (Vitamin E)	22
γ-tocopherol	-	7.85 - 11.55	9.16 - 13.62		6.9
Folic acid	0.15	0.11 - 0.29	0.15 - 0.49	-	
K	1.2	-	-	-	23.2

ND = Not Detected; Tr = Traces

Table 2.6 Important bioactive compounds of chickpea seeds

Compounds	Champ (2002)	Alajali and El-Adawy (2006)	Zia-Ul-Haq et al. (2007)	Dilis and Trichopoulou (2009)	Mittal et al. (2012)
	% DM*	mg/g DM	mg/100 g	% DM	
Trypsin inhibitor activity	1 - 15 TIU units/mg DM	11.9 TIU/mg protein*	-	-	64.58 - 107.22 TIU/g
Haemagglutinin activity	0	6.22 HU/mg*	-	-	-
Phytate	0.4 - 1.1	1.21	138 - 171	0.5	12.82 - 13.28 µmol/g
Oxalate	0.07	-	-	0.07	0.16 - 0.39 %
Total phenolic acid	0.1 - 0.6	-	-	90 mg GAE/100 g*	-
Tannin	0.0 - 0.1	4.85	740 - 763	0.0 - 0.1	0.38 - 5.63 mg/g
Saponin	0.4	0.91		5	0.34 - 0.44 %

*DM = Dry Matter; TIU = Trypsin Inhibited Units; HU = Haemagglutinin Unit; GAE = Gallic Acid Equivalents

carbohydrates are also attributed for various structural, biochemical and physiological properties. On the basis of their degree of polymerization (DP), carbohydrates are divided into four main classes: mono- (DP 1), di- (DP 2), oligo- (DP 3 - 9) and poly-saccharides (DP > 10; Asp 1995; Cummings and Stephen 2007).

Starch granule, the major polysaccharide in chickpea seeds represent the major energy source and comprises 36.9 - 54.9 % of seed weight (Wood and Grusak, 2007). Two large glucan polymers, amylose and amylopectin, combined with minute amounts of proteins and minerals make up the granules. The amylose molecules are linear α(1→4)-linked glucan polymers that are sparsely branched through α(1→6) linkages. Amylopectin polymers, in contrast, are heavily branched as a result of α(1→6) linkages positioned at every 20 - 30 glucose residue on the α(1→4) glucan backbone. For desi and kabuli chickpeas, the amylose concentration varies from 20 - 42 % and 20.7 - 46.5 %, respectively; thus many chickpea genotypes have considerable higher amylose concentration than cereal starches, which are in the 25 - 28 % range (Chibbar et al., 2010). In humans, carbohydrates when consumed are acted on by enzymes which degrade the complex molecules in to progressively smaller molecules and finally into glucose to be absorbed by the blood stream. The ease by which food carbohydrates are broken down and delivered into blood stream is of great importance for human health (Chibbar et al. 2010). For starches, the ratio of amylose to amylopectin concentration in grains and seeds affects digestibility, where the less branched amylose molecules are more resistant to degradation in the digestive tract than the heavily branched amylopectin. Based on *in vitro* enzymatic hydrolysis assays, starch can be classified as readily digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). The RDS fraction is broken down to constituent glucose molecules within 20 minutes, whereas it takes 100 min to break down the SDS and the amylose-rich RS fraction remains undigested after 120 min. In the human body, RDS and SDS are completely digested within the small intestine by enzymatic digestion, whereas RS needs to reach the large intestine before degradation is initiated by bacterial fermentation. Similar to RS, dietary fibers of the cell wall are largely resistant to digestion in the small intestine, but undergo fermentation in the large intestine.

Sucrose is the predominant soluble sugar in chickpea seeds followed by RFO. Chickpea seeds also contain other mono- and di- saccharides listed in Table 2.7.

Dietary fibers (DF) are defined as 10 or more monomeric units that cannot be digested or absorbed in human small intestine (Chibbar et al. 2010). On the basis of their fermentation in large intestine, DF can be grouped as; (a) Insoluble fibres are metabolically inert thus

Table 2.7 Carbohydrates composition in chickpea seeds

Compounds	Quemener and Brillouet (1983)	Sánchez-Mata et al. (1998)	Alajali and El-Adawy (2006)	Han and Baik (2006)	Aguilera et al. (2009)	Dilis and Trichopoulou (2009)
	% DM*	g/100 g	g/100 g DW	mg/g	g/kg DM	% DM
myo-inositol	0.3	-	-	-	-	-
Galactinol	0.08	-	-	-	-	-
Ribose	-	0.03 - 0.19	-	-	-	-
Fructose	-	0.23 - 0.29	-	-	3.1	-
Glucose	-	0.07	-	-	0.5	-
Galactose	-	0.05	-	-	0.1	-
Sucrose	3.5	1.09 - 2.28	1.89	-	15.2	-
Maltose	-	0.57 - 0.61	-	-	3.3	-
Raffinose	0.7	0.57 - 0.63	1.45	50.2	3.2	0.4 - 1.2
Stachyose	2.4	0.74 - 1.17	2.56	27	17.7	2 - 3.6
Verbascose	ND	-	0.19	ND*	-	0.6 - 4.2
Ciceritol	2.8	2.51 - 2.79	-	67.7	27.6	-
Starch (g/100 g)*	36.91 - 54.9					

*- Dalgetty and Baik (2003); Frimpong et al. (2009); DM = Dry Matter; ND = Not Detected

supports gastrointestinal movement (e.g. cellulose, hemicelluloses and lignin) while, (b) Soluble (prebiotic, viscous) fibres are fermented in the large intestine and support the growth of intestinal bacteria (e.g. pectins, β-glucans, and galactomanan gums). DF constitutes about 18 - 22 % of total chickpea seed weight (Table 2.8) which is a relatively higher when compared to cereals such as wheat (12 %), rice (2 - 4 %) and other pulse crops such as peas (5.1 %) and beans (2.7 %) (Chibbar et al. 2010; Tosh and Yada 2010; Jukanti et al. 2012).

2.3 Alpha-Galactosides and raffinose family oligosaccharides (RFO)

Among different oligosaccharides, *α*-galactosides are considered as an important group of low molecular weight non-reducing sugars that are soluble in water and water-alcohol solutions (Martínez-Villaluenga et al. 2008). These oligosaccharides are ubiquitous in plant kingdom and ranks next to sucrose among soluble sugars (Frias et al. 1999). *α*-Galactosides get accumulated in higher amount in storage organs like seeds during later stages of development and maturation (Peterbauer et al. 2001).

2.3.1 Chemical structures of RFO

Alpha-galactosides are considered as sucrosyl galactosides and characterized by the presence of α(1→6) linkage between the galactosyl residues and the C-6 of the glucose moiety of sucrose (Tapernoux-Luthi et al. 2004). Alpha-galactosides can further be classified into two groups (Han and Baik 2006). Raffinose family oligosaccharides (RFO) constitute the first group, and the first member of this group, raffinose [*α*-D-galactopyranosyl-(1→6)-*α*-D-glucopyranosyl-(1→2)-*β*-D-fructofura- noside; degree of polymerization (DP) = 3] is the main RFO in most monocotyledon seeds, while its higher homologues, stachyose [*α*-D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)-*α*-D-glucopyranosyl-(1→2)-*β*-D-fructofuranoside; DP = 4], verbascose [*α*-D-galactopyranosyl-(1→6)-[*α*-D-galactopyranosyl-(1→6)]2-*α*-D-glu- copyranosyl-(1→2)*β*-D-fructofuranoside; DP = 5] and ajugose [*α*-D-galactopyrano- syl-(1→6)-[*α*-D-galactopyranosyl-(1→6)]3-*α*-D-glucopyranosyl-(1→2)-*β*-D-fructof- uranoside; DP = 6], accumulate predominantly in seeds of dicotyledons (Sprenger and Keller 2000). Higher members like ajugose are generally found in trace quantities in seeds (Peterbauer and Richter 2001). The second group of α-galactosides includes galactosyl cyclitols (Lahuta et al. 2010). Ciceritol [*α*-D-galactopyran osyl-(1→6)-*α*-D-galactopyranosyl-(1→2)-4-O-methyl-*quiro*-inositol] is the most common galactosyl cyclitols and was first reported from chickpea (*Cicer aurenticum*; Quemener and Brillouet 1983) followed by lentil (*Lens culinaris*; Bernabe et al. 1993; Martínez-Villaluenga et al. 2008). The chemical

Table 2.8 Concentration of dietary fibres in chickpea seeds

Name	Rincón et al. (1998)		Dalgetty and Baik (2003)	Aguilera et al. (2009)	Tosh and Yada (2010)
	g/kg DM*		% DM	g/kg DM	g/100 g
	Desi	Kabuli			
Total dietary fibre	216.7	195.1	-	214.4	18 - 22
Soluble dietary fibre	39.3	37.7	8.4	9.6	4 - 8
Insoluble dietary fibre	178.1	157.4	10	204.8	10 - 18

***** DM = Dry Matter

structures of important α-galactosides are presented in Figure 2.3.

2.3.2 RFO concentration in crops

Alpha-galactosides concentration varies widely among different crop species as summarized in Table 2.9. The concentration and composition of α-galactosides depend on type of crop, growing environment and also on the genotype (Reddy and Salunkhe 1980; Sosulski et al. 1982; Quemener and Brillouet 1983; Andersen et al. 2005; Martin-Cabrejas et al. 2008; Martínez-Villaluenga et al. 2008; Dilis and Trichopoulou 2009; Huynh et al. 2008; Wang et al. 2010). Sosulski et al. (1982) studied the variation in α-galactoside concentration in 11 legumes and reported stachyose as the major component in chickpea and lentil flours. They also reported verbascose as the predominant α-galactoside in mung bean and fababean. In case of cowpea and soybean, RFO accounted for more than 50 % of total soluble sugars (Martin-Cabrejas et al. 2008). Vidal-Valverde et al. (1998) observed higher amount of verbascose (2.29 % of dry matter) followed by stachyose (1.10 % of dry matter) and raffinose (0.28 % of dry matter) in fababean. Quemener and Brillouet (1983) detected ciceritol in chickpea (2.80 % per dehulled seed), lentil (1.60 %), white lupin (0.65 %), soybean (0.08 %) and bean (traces). Andersen et al. (2005) studied the compositional variations of α-galactosides in barley and different species of leguminosae and brassicaceae. The highest concentration of total RFO was reported in lupin (9.1 ± 2.6 g/100 g seeds), while *Brassica* samples contained 1.4 ± 0.5 g RFO (only raffinose and stachyose) per 100 g of seeds. Barley, *H. vulgare* L. cv. Vega, contained 0.5 g raffinose per 100 g of seeds, which was the only RFO component present in the *H. vulgare* seeds. They also observed a wide variation in concentration and composition among lupin species. Lupin was reported to have 0.30 – 1.90, 2.30 – 8.60 and ND – 3.50 % of raffinose, stachyose and verbascose, respectively (Martínez-Villaluenga et al. 2008). Ajugose was present exclusively in lupin seeds. *L. albus* and *L. mutabilis* contained the lowest ajugose levels (0.2 – 0.5 % and 0.2 %, respectively) followed by *L. angustifolius* (1.7 – 2.6 %) and, *L. luteus* (0.6 – 4.6 %; Andersen et al. 2005; Martínez-Villaluenga et al. 2008). Reddy and Salunkhe (1980) reported RFO concentration from long grain polished rice and black gram. They found absence of RFO in rice but found higher concentration of verbascose (3.44 %) followed by stachyose (0.89 %) and raffinose (trace) in black gram. Huynh et al. (2008) reported the presence of only raffinose in wheat (0.30 %) and barley (0.59 %) among α-galactosides. Faba bean has been reported to contain higher amount of verbascose (2.7 %) while field pea was found to have higher amount of stachyose (2.7 %). Saini and Knights (1984) studied the variation for total oligosaccharides in desi

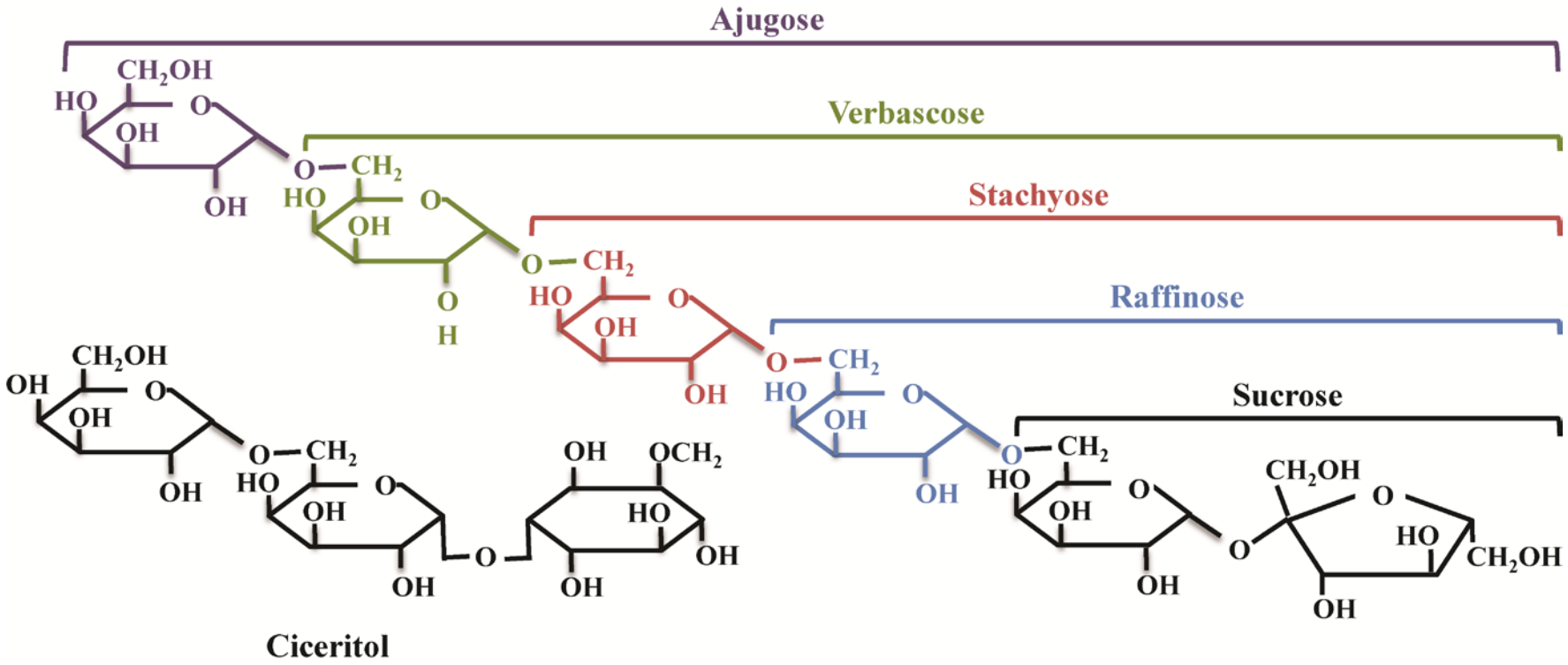


Figure 2.3 Chemical structures of important α-galactosides.

Table 2.9 Variation in α-galactoside concentration (% of dry matter) among different crop species

Crop	Raffinose	Stachyose	Verbascose	Ajugose	Ciceritol	References
Chickpea (*Cicer arietinum*)	0.45 – 2.1	1.72 – 6.15	ND - 4.50	ND*	~2.8	1,3,4,5,6,7,12, 13,14
Soybean (*Glycine max*)	0.67 - 1.15	2.75 - 2.85	ND - 0.30	ND	0.05 - 0.08	1,2,3,6,7
Lupin (*Lupinus albus, L. luteus, L. angustifolius* and *L. mutabilis*)	0.30 - 1.90	2.30 - 8.60	ND - 3.50	0.20 - 4.60	0.65	1,3,6,7
Cow pea (*Vigna unguiculata*)	0.41	3.22 - 4.44	0.48	ND	0.04	1,2,3
Lentil (*Lens culinaris*)	0.31 - 1.00	1.47 - 3.10	0.47 - 3.10	ND	1.6	1,3,4,6,7
Field pea (*Pisum sativum*)	0.60 - 1.40	1.71 - 2.70	2.3	ND	ND	1,3,6,7,9,10
Mung bean (*Vigna radiata*)	0.23	0.95	1.83	ND	ND	1,
Fababean (*Vicia faba*)	0.10 - 0.30	0.67 - 1.50	1.45 - 3.10	ND	ND	1,3,4,6,7,9,11
Black gram (*Vigna mungo*)	Trace	0.89	3.44	ND	ND	8
Bean (*Phaseolus vulgaris*)	<0.05 - 2.50	0.20 - 4.20	0.06 - 4.00	ND	Trace	3,4,5,6,7
Mustard (*Brassica campestris*)	0.2	0.7	ND	ND	ND	6,7
Rapeseed (*B. napus*)	0.20 - 0.40	0.70 - 1.70	ND	ND	ND	6,7
Black mustard (*B. nigra*)	0.6	1.3	ND	ND	ND	6,7
Barley (*Hordeum vulgare*)	0.59	ND	ND	ND	ND	6,7,9
Rice, polished long grain (*Oryza sativa*)	ND	ND	ND	ND	ND	8
Wheat (*Triticum aestivum*)	0.3	ND	ND	ND	ND	9

[1]Sosulski et al. 1982; [2]Martin-Cabrejas et al. 2008; [3]Quemener and Brillouet 1983; [4]Dilis and Trichopoulou 2009; [5]Wang et al. 2010; [6]Andersen et al. 2005; [7]Martínez-Villaluenga et al. 2008; [8]Reddy and Salunkhe. 1980; [9]Huynh et al. 2008; [10]Vidal-Valverde et al. 2003; [11]Vidal-Valverde et al. 1998; [12]Saini and Knights 1984; [13]Alajaji and El-Adawy 2006; [14]Frias et al. 2000; *ND = Not Detected

and kabuli chickpeas (7 varieties of each). They concluded that on an average kabuli chickpeas (1.47, 5.30 and 0.12 g/100 g of raffinose, stachyose and verbascose, repectively) indicated 3.2 % higher levels of total oligosaccharides than desi types (1.48, 5.06 and 0.15 g/100 g of raffinose, stachyose and verbascose, repectively). Andersen et al. (2005) reported individual *α*-galactoside concentration in *Brassica* spp. that was lesser compared to legumes. *B. campestris* and *B. nigra* contained 0.2 and 0.6 % of raffinose, respectively whereas *B. napus* contained 0.2 – 0.4 %. In Brassica species, stachyose concentration ranged from 0.7 to 1.7 % of seed weight. The higher RFO like verbascose and ajugose were not detected in *Brassica* species (Martínez-Villaluenga et al. 2008). Total α-galactoside concentrations of 18 pea varieties varied from 2.26 to 6.34 % of dry matter (Vidal-Valverde et al. 2003). Stachyose (1.07 – 2.67 % of dry matter) was found in higher amount than that of raffinose (0.41 – 1.03 % of dry matter), while verbascose was present in 15 varieties ranging from 0.17 – 2.67 % of dry matter.

2.3.3 Biosynthesis of RFO

RFO biosynthesis has been summarized as Figure 2.4. Sucrose is formed as a major output of photosynthesis in higher plants. The biosynthesis of *α*-D-galactosyl derivatives of sucrose are initiated by enzyme galactinol synthase (GS, UDP-*α*-D-galactose:1-L-myo-inositol-*O*-*α*-galactopyranosyltransferase, EC 2.4.1.123). GS catalyzes the transfer of galactosyl unit from UDP-D-galactose (derived from the common nucleotide pathway *via* UDP-D-galactose 4-epimerase; Joersbo et al. 1999) to *myo*-inositol generating galactinol. The biosynthesis of RFO proceeds by reversible transfer of the galactosyl residue from donor galactinol to sucrose that results in synthesis of raffinose (trisaccharide) and inositol is released. This reaction is catalyzed by raffinose synthase (RS; EC 2.4.1.82; Martínez-Villaluenga et al. 2008). Two pathways have been reported for stachyose and higher homologues' biosynthesis: 1) galactinol -dependent and, 2) -independent. In galactinol dependent pathway, raffinose serves as an acceptor for another galactosyl residue from galactinol, yielding tetrasaccharide stachyose in the presence of stachyose synthase (STS; EC 2.4.1.67) enzyme (Karner et al. 2004). Likewise, verbascose is synthesized from stachyose in the presence of enzyme verbascose synthase (VS). However, existence of verbascose synthase (VS; EC 2.4.1.x) has yet to be proven (Lahuta 2006). Activity of verbascose synthase was observed in purified stachyose synthase from seeds of pea (Peterbauer et al. 2002), while stachyose synthase from adzuki bean seeds was devoid of verbascose synthase activity (Peterbauer and Richter 1998). Therefore a new galactinol-independent pathway has

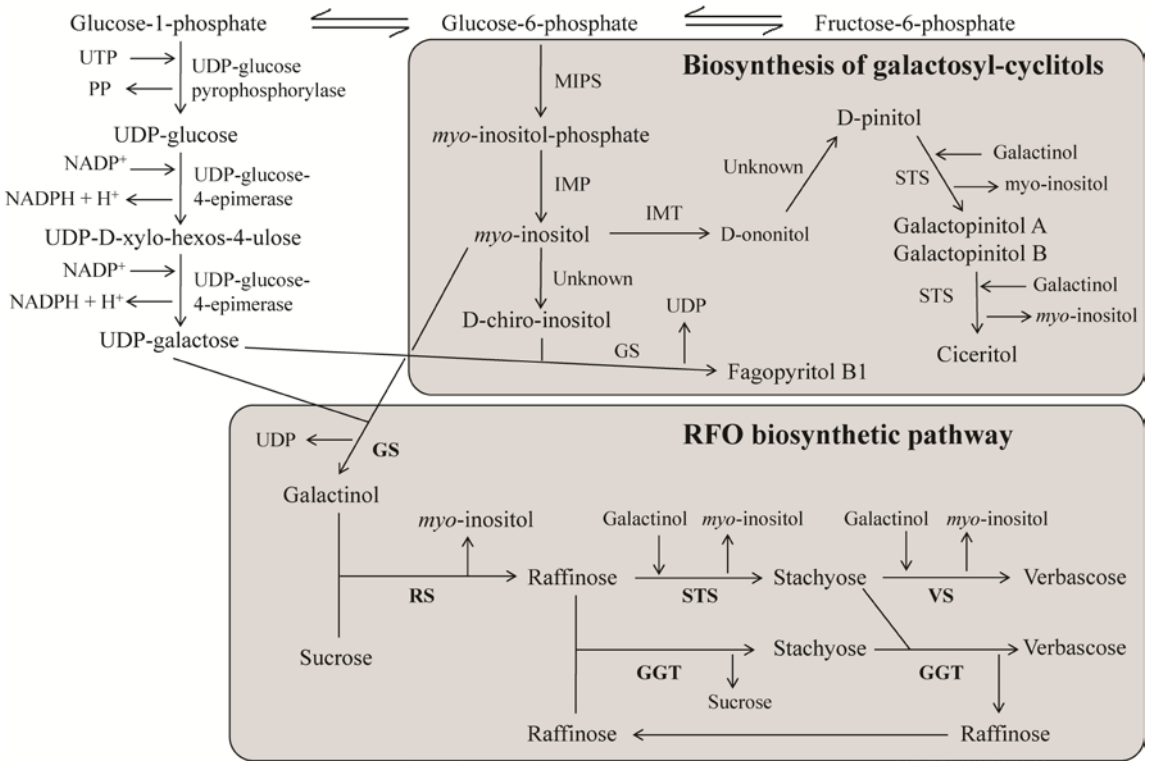


Figure 2.4 Biosynthsis of raffinose family oligosaccharides and cyclitols

MIPS, *myo*-inositolphosphate synthase (EC 5.5.1.4); IMP, *myo*-inositol-phosphate monophosphatase (EC 3.1.3.25); IMT, *myo*-inositol 4-methyltransferase (EC 2.1.1.129); GS, galactinol synthase (EC 2.4.1.123); RS, raffinose synthase (EC 2.4.1.82); STS, stachyose synthase (EC 2.4.1.67); VS, verbascose synthase; GGT, galactan:galactan galactosyltransferase (Peterbauer and Richter 2001; Ueda et al. 2005; Velíšek and Cejpek 2005; Ma et al. 2005; Martínez-Villaluenga et al. 2008; Obendorf et al. 2009).

been proposed for the biosynthesis of higher members of raffinose family (Bachmann et al. 1994; Haab and Keller 2002). According to this, an already present RFO molecule transfers its terminal galatosyl residue to other yielding a higher member of raffinose family. Galactan:galactan galactosyltransferase (GGT) has been proposed to catalyze this reaction (Haab and Keller 2002; Tapernoux-Luthi et al. 2004).

Galatosyl cyclitols are also described to support stachyose biosynthesis by acting as a galactosyl donor (Peterbauer et al. 2001). Ciceritol, galactopinitol and fagopyritol are important cyclitols found in plants. Ciceritol and galactopinitol are STS catalyzed galactosylated products of ononitol and pinitol, while fagopyritol is synthesized as a product of GS reaction between UDP-galactose and D-chiro-inositol (Ueda et al. 2005; Obendorf et al. 2009).

In brief, Galactinol synthase (GS) and raffinose synthase (RS) catalyze the initial consecutive committed steps in RFO biosynthesis (Keller, 1992) whereas STS, VS and GGT are responsible for the synthesis of higher members of RFO. RFO biosynthetic genes and corresponding enzymes have been studied in various crops (Holthaus and Schmitz 1991; Downie et al. 2003; Dierking and Bilyeu 2008)

2.3.3.1 Galactinol synthase

GS (or GolS) is the most studied enzyme of RFO biosynthetic pathway. GS is described as an extravacuolar enzyme, most probably cytosolic (Keller 1992). It could play a key regulatory role in carbon partitioning between sucrose and RFO (Cunningham et al. 2003). It is reported from a variety of plant species listed in Table 2.10. Sprenger and Keller (2000) found two distinct *GolS* in *Ajuga reptans*. *GolS-1* (AJ237693) contained an open reading frame of 333 codons (total 1251 nucleotides) and *GolS-2* (AJ237694) was partially isolated. cDNA of *Medicago sativa GolS* was 1326 bp in length encoding a polypeptide of 325 amino acids having molecular mass of 37.6 kDa and pI of 5.7. *Arabidopsis thaliana* contained seven *GolS*-related genes (Accession No. AC002337, AC009323, AC003970, AC002292, AC005244, AL049171AL161564, AC004473; Taji et al 2002) named as *ATGolS1* to *7*. All sequences were characterized to have hydrophobic pentapeptide (APSAA) at carboxyl terminal end. Their expression profile revealed that *ATGolS1* and *2* were induced by drought and high salinity stresses, while cold stress induced *ATGolS3*. Similar conclusions were reported by Downie et al. (2003). They found up-regulated tomato *GolS* (Accession no. AF447452) activity in vegetative tissues/seeds during cold/dehydration. Tomato *Gol*S had total 3,789 nucleotides having three exons (cDNA of 1247 bp) coding for a protein sequence

Table 2.10 RFO biosynthetic pathway enzymes and their genes

Enzyme	Molecular mass	pH	pI	Opt. Temp. (°C)	Plant	Accession No./work done	References
Galactinol	36-66 kDa	7	4.1-8.11	50	*Cucurbita pepo*	Purified protein	Smith et al. 1991
synthase					*Phaseolus vulagaris*	Purified protein	Liu et al. 1995
					Cucurbita pepo	Purified protein	Liu et al. 1995
					Ajuga reptans	AJ237693 and AJ237694	Sprenger and Keller 2000
					Arabidopsis thaliana	AC002337, AC009323, AC003970, AC002292, AC005244, AL049171AL161564 and AC004473	Taji et al. 2002
					Medicago sativa L.	AY126615	Cunningham et al. 2003
					Lycopersicon esculentum Mill.	AF447452	Downie et al. 2003
					Zea mays L.	AF497507, AF497508 and AF497509	Zhao et al. 2004
					Glycine max	AY126715	Obendorf et al. 2004
					Fagopyrum esculentum	AY126718, AY126716 and AY126717	Ueda et al. 2005
					Verbascum phoeniceum	EF494114 and EF494115	McCaskill and Turgeon 2007
					Xerophyta viscosa	EF017945	Peters et al. 2007
					Coptis japonica	AB353350	Takanashi et al. 2008
					Boea hygrometrica	FJ222452	Wang et al. 2009
					Populus trichocarpa x deltoides	EU305718, EU305721and EU305723	Philippe et al. 2010
					Coffea arabica L.	GQ497218, GQ497220 and GQ497219	dos Santos et al. 2011
					Brassica napus L.	FJ407183	Li et al. 2011
					Salvia miltiorrhiza Bunge	GQ245764, JF937200 and JF937201	Wang et al. 2012
					Gossypium hirsutum cv. Y18	JF813792	Zhou et al. 2012
					Medicago falcata	FJ607306	Zhuo et al. 2013
Raffinose	80-88.7 kDa	7	NA	42-45	*Vicia faba*	Purified protein	Lehle and Tanner 1973
synthase					*Pisum sativum* L.	AJ426475	Peterbauer et al. 2002
					Oryza sativa L.	Q5VQG4 (for protein)	Li et al. 2007
					Glycine max (L.) Merr.	E25448 and E24424	Dierking and Bilyeu 2008
					Cucumis sativus L.	DQ414725	Sui et al. 2012

Table 2.10 contd. Genes and enzymes of RFO biosynthetic pathway

Enzyme	Molecular mass	pH	pI	Opt. Temp. (°C)	Plant	Accession No./work done	References
Stachyose synthase	88.6-95 kDa	6.5-7	4.7-5.39	32-35	*Cucurbita pepo*	Purified protein	Gaudreault and Webb 1981
					Cucumis melo L.	Purified protein	Huber et al 1990
					Cucumis melo	Purified protein	Holthaus and Schmitz 1991
					Vigna angularis Ohwi et Ohashi	Purified protein	Peterbauer and Richter 1998
					Vigna angularis Ohwi et Ohashi	Y19024	Peterbauer et al. 1999
					Lens culinaris L.	Purified protein	Hoch et al. 1999
					Pisum sativum L.	AJ311087 and Purified protein	Peterbauer et al. 2002
					Zea mays L.	NM001158819	Alexandrov et al 2009
GGT	48 kDa	5/7	4.7	20-40	*Ajuga reptans* L.	Purified protein	Haab and Keller 2002
					Ajuga reptans L.	AY386246	Tapernoux-Luthi e al. 2004

of 318 amino acids. Obendorf et al. (2004) heterologously expressed recombinant soybean *GolS* (Accession no. AY126715) in *E. coli* and confirmed the role of this protein in catalyzing the synthesis of fagopyritol B1 and galactinol but not galactopinitol. This important role of GS was further explored by Ueda et al. (2005). They reported three isoforms of GS from *Fagopyrum esculentum* (*FeGolS-1*, *-2* and *-3*; accession no. AY126718, AY126716 and AY126717) and associated *FeGolS-1* with fagopyritol B1 synthesis, while *FeGolS-2* catalyzed biosynthesis of fagopyritol A1 and B1 in a 1:4 mole ratio. The cDNAs of *FeGolS-1* and *FeGolS-2* were 1269 and 1326 bp long encoding proteins of 38.3 and 40.7 kDa, respectively. *FeGolS-3* was partially isolated from the plant. Peters et al. (2007) isolated GolS (Accession No. EF017945) gene from leaves of *Xerophyta viscosa* plants subjected to water deficit and studied RFO level during stress. Accumulation of RFO during water deficit was also supported by Wang et al. (2009). They characterized a *GolS* gene from *Boea hygrometrica* (*BhGolS1*; Accession No. FJ222452). Conserved pentapeptide (APSAA) at carboxyl terminal ends in many GolS proteins was not present in *BhGolS1,* suggesting that this sequence may not be essential for enzyme activity. *BhGolS1* was found to encode 334 amino acids along with a conserved region common to glycosyltransferase family 8 proteins, manganese binding motif and a serine phosphoprylation site. They also concluded that ABA and dehydration induced accumulation of *BhGolS* was triggered independently in each organ. While working on berberine tolerance gene, Takanashi et al. (2008) isolated a cDNA encoding a putative protein of 336 amino acids and this sequence shared a high similarity with proteins of GolS family. Philippe et al. (2010) sequenced seven full length cDNA of GolS: three from *Populus trichocarpa* (EU305718, EU305721and EU305723) and four from *Populus trichocarpa* x *Populus deltoides* (EU305719, EU305720, EU305722 and EU305724). These genes contained nucleotide sequences from 1269 to 1454 bp encoding protein sequences from 336 to 338 amino acids. APSAA region was partly conserved in these sequences but manganese binding motif was present in all reported sequences.

GS is characterized as monomeric polypeptide of 36 - 66 kDa with an isoelectric point of 4.1. The purified enzyme had an optimum pH and temperature of 7.0 and 50 °C, respectively and its activity was enhanced by dithiothreitol (DTT) and $MnCI_2$. It showed K_m values of 6.5 and 1.8 mM for *myo*-inositol and UDP-galactose, respectively (Smith et al. 1991; Liu et al. 1995; Peterbauer and Richter 2001). dos Santos et al. (2011) reported three isoforms of *GolS* from *Coffea arabica* L. These isoforms were composed of 1402, 1445 and 1702 nucleotides coding for 388, 334 and 344 amino acid residues, respectively. These isoforms were further characterized to have molecular mass of 38.54, 38 and 39.59 kDa along with pI of 4.93, 8.11

and 5.07, respectively. Li et al. (2011) cloned GS from *Brassica napus* L. seeds (*BnGOLS-1*; Accession no. FJ407183) having total 1384 bp with open reading frame of 1029 bp encoding 342 amino acids. *BnGOLS-1* showed accumulation concomitant with desiccation tolerance. In 2012, GS was reported from *Salvia miltiorrhiza* Bunge (3 isoforms having accession no. GQ245764, JF937200 and JF937201) and *Gossypium hirsutum* cv. Y18 (Accession no. JF813792) by Wang et al. and Zhou et al., respectively. Zhuo et al. (2013) isolated a cold responsive GS gene from *Medicago falcate* (*MfGolS1*; accession no. FJ607306). Overexpression of *MfGolS1* in transgenic tobacco showed improved tolerance against freezing, chilling, drought and salt stress.

2.3.3.2 Raffinose synthase

Raffinose synthase (RS/RFS) channels sucrose into RFO biosynthetic pathway (Peterbauer et al. 2002). RS has been reported from very few plant species as listed in Table 2.10. Lehle and Tanner (1973) purified RFS protein from seeds of *Vicia faba* having molecular weight of 80 kDa. Purified RFS showed a pH and temperature optima at 7.0 and 42 °C, respectively. Later in 2002, Peterbauer et al. cloned full length cDNA of RFS from seeds of *Pisum sativum* L. Pea *RFS* (Accession no. AJ426475) was comprised of total 2652 nucleotides encoding a protein of 798 amino acids having a calculated molecular mass of 88.7 kDa. They also purified the enzyme partially showing pH optima at 7.0. Galactinol and sucrose were characterized for K_m value of 7.3 mM and 22.9 mM, respectively for raffinose synthesis. Equlibrium constant of 4.1 was detected for raffinose synthesis reaction, while 1-deoxygalactonojirimycin was identified as potent inhibitor of this enzyme. Li et al. (2007) cloned a putative RFS gene from rice seedling (*Oryza sativa* L.). They expressed the gene in *E. coli* and purified enzyme from recombinant protein having molecular mass of 85 kDa (820 amino acids) with optimum activity at 45 °C and pH 7.0. An enhanced RFS activity was observed with 5 mM DTT. Soybean [*Glycine max* (L.) Merr.] contained four *RS* genes out of which RS2 was found to be more responsible for raffinose synthesis (Dierking and Bilyeu 2008). Out of six putative *RS* genes in Arabidopsis, Egert et al. (2013) revealed that *RS 5* is solely responsible for abiotic stress induced accumulation of raffinose in leaves. They further proposed the presence of at least one more copy of seed specific raffinose synthase.

Higher members of raffinose family oligosaccharides can be synthesized either following galactinol dependent pathway or galactinol independent pathway. In galactinol dependent pathway, STS and VS are proposed to play important role. In this pathway, galactinol acts as a galactosyl unit donor leading to synthesis of higher members of raffinose family like

stachyose, verbascose and ajugose. On the other hand, already present lower DP (degree of polymerization) RFO acts as galactosyl unit donor in galactinol independent pathway yielding next higher molecule of raffinose family. In this reaction, GGT catalyzes the galactosyl transfer.

2.3.3.3 Stachyose synthase and verbascose synthase - galactinol dependent pathway

STS is studied in different plant species as summarized in Table 2.10. Stachyose synthase has been purified from *Cucurbita pepo* (Gaudreault and Webb 1981), *Cucumis melo* (Huber et al. 1990; Holthaus and Schmitz 1991), *Vigna angularis* (Peterbauer and Richter 1998), *Lens culinaris* (Hoch et al. 1999), *Pisum sativum* (Peterbauer et al. 2002) and *Zea mays* (Alexandrov et al. 2009). Peterbauer et al. (1999) cloned and studied the expression of STS from adzuki bean. The complete cDNA of adjuki bean STS (Accession no. Y19024) was composed of 3046 nucleotides encoding a polypeptide of 857 amino acids. Later in 2002, Peterbauer et al. described STS as a multifunctional enzyme. They cloned, purified and characterized STS from pea seeds (Accession no. AJ311087) and found that pea STS had activities to synthesize stachyose as well as verbascose. Pea *STS* had a total of 2727 nucleotides having an open reading frame coding for 853 amino acid residues. STS might be a monomeric (Hoch et al. 1999) or dimeric (Holthaus and Schmitz 1991) poypeptide. Purified STS had a molecular weight of 88.6 to 95 kDa having an isoelectric point of 4.7 - 5.39. STS showed an optimum pH and temperature range from 6.5 to 7.0 and from 32 to 35 °C, respectively (Peterbauer and Richter 1998; Peterbauer et al. 2002b).

VS catalyzes the reaction of verbascose synthesis as mentioned in some articles (Peterbauer and Richter 2001; Peterbauer et al. 2001; Obendorf et al. 2009) but no report is available regarding its cloning or purification till date.

2.3.3.4 Galactan:galactan galactosyl transferase - galactinol independent pathway

Bachmann and Keller (1995) reported GGT as an enzyme responsible for the biosynthesis of high molecular weight members of raffinose family. To date, GGT (Galactan:galactan galactosyltransferase) has been reported only from leaves of *Ajuga reptans* (Haab and Keller 2002; Table 2.10). Purified GGT had a native molecular mass of 212 kDa which separated into three bands (48 kDa, 66 kDa, and 60 kDa) on SDS-PAGE. GGT showed an isoelectric point of 4.7 and pH optima around 5.0. On the basis of this

sequence information, Tapernoux-Luthi et al. (2004) cloned GGT gene (Accession no. AY386246) from leaves of *Ajuga reptans*. GGT sequence was composed of total 1391 nucleotides corresponding to a protein of 404 amino acids.

Peterbauer et al. (2001) studied the enzyme activities synthesizing verbascose in pea seeds. They found the activity of VS in assays where galactinol was used but considerable amount of verbascose was also formed in control assays that were performed without galactinol. Hence they concluded that galactinol dependent as well as galactinol independent pathway was operative in pea seeds. Therefore, there is need to focus more on this part of RFO biosynthesis as neither VS nor GGT (except *Ajuga reptans*) is well characterized from any of the crop species.

RFO are ubiquitous in legume seeds (Blöchl et al. 2008). High level of RFO has several negative effects including flatulence, metabolic discomfort and diarrhea, decreased metabolizable energy and improper digestion (Martínez-Villaluenga et al. 2008). However, they participate in important physiological mechanisms in plants like desiccation tolerance, seed longevity (Koster 1991), detoxification of reactive oxygen species (ROS; Bolouri-Moghaddam et al. 2010) and tolerance against biotic and abiotic stresses (Liu et al. 2007; Cho et al. 2010). In humans, RFO stimulate growth of certain remedial bacteria, *viz.* lactic acid bacteria (Peterbauer and Richter 2001) including bifidobacterium (Trojanova et al. 2006) in large intestine.

2.4 Physiological role of RFO in plants

RFO represent a class of non-structural carbohydrates that are widely distributed in a wide variety of species of the plant kingdom (Toldi et al. 2009). These non-reducing and soluble oligosaccharides play important role in plant growth and development. RFO participates in different physiological mechanisms including desiccation tolerance (Martínez-Villaluenga et al. 2008), seed storability (Horbowicz and Obendorf 1994), stress (biotic and abiotic) tolerance (Nishizawa-Yokoi et al. 2008), photoassimilate translocation (Dinant and Lemoine 2010) and seed germination (Blöchl et al. 2007) (Figure 2.5).

2.4.1 Seed development and desiccation tolerance

Water is essential for plants. It not only plays important role in many biochemical reactions but also helpful in maintaining the structure of a plant cell *via* hydrophilic and hydrophobic interactions (Koster and Leopold 1988). During the process of seed maturation, a major loss of water takes place which is termed as “Desiccation” that may lead to

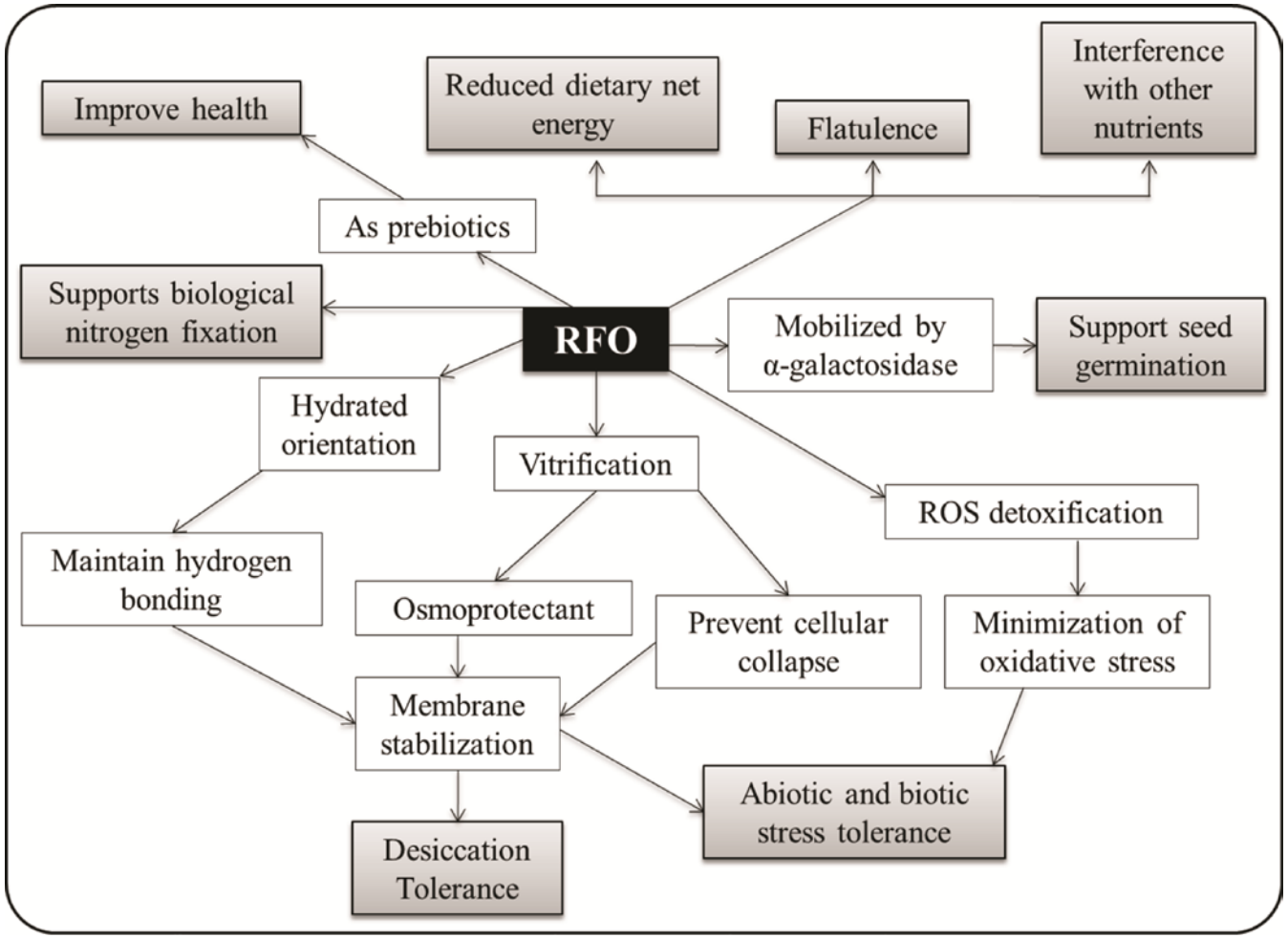


Figure 2.5 Importance of RFO in plants and humans.

membrane damage and death of embryo (Corbineau et al. 2000; Halperin and Koster 2006). Therefore, cells must be protected against potentially lethal changes occurred due to desiccation. Tolerance against desiccation can be achieved by the accumulation of certain non-reducing sugars like sucrose and RFO (Koster and Leopold 1988). Many reports suggested the role of RFO in desiccation tolerance (Blackman et al. 1992; Corbineau et al. 2000; Pukacka et al. 2009; Angelovici et al. 2010) and the first mechanism by which they provide protection is water replacement. The hydroxyl groups of RFO are capable of replacing water molecules and maintaining the hydrophilic interactions with in the cell that is necessary for stabilizing native macromolecules (like protein) and membrane structure during dehydration process (Koster 1991; Pukacka et al. 2009).

Second mechanism for RFO's role in desiccation tolerance is "Vitrification" or formation of glass within the cell. This is the state of a cell solution having very high viscosity due to loss of water. At this state cell solution has the properties like a plastic solid. It is responsible for ensuring stability (by preventing the reactions required diffusion), preventing cellular collapse (by filling the blank spaces within the biomolecules) and maintaining hydrogen bonding within the cell (Koster and Leopold 1988; Koster 1991; Martínez-Villaluenga et al. 2008; Angelovici et al. 2010). It has been reported that late embryogenesis abundant (LEA) proteins and small heat shock proteins (sHSP) along with RFO are responsible for the glassy state (Pukacka et al. 2009). Increased biosynthesis of these oligosaccharides restricts the synthesis of monosaccharides, resulting in decreased respiration rate (site of reactive oxygen species formation). Low monosaccharide content also prevents the protein destruction by inhibiting Maillard's reaction (Martínez-Villaluenga et al. 2008; Pukacka et al. 2009).

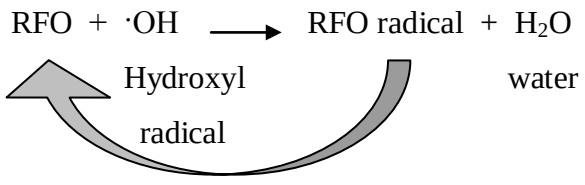
Alpha-galactosides along with sucrose have also been associated with seed storability. Horbowicz and Obendorf (1994) found storability half-viability periods >10 years when sucrose to oligosaccharide ratio was <1.0, while this period was <10 years in case of ratio >1.0.

A positive relationship between desiccation tolerance and RFO accumulation has been reported (Toldi et al. 2009) in many crop species like beans (Bailly et al. 2001), soybean (Koster and Leopold 1988; Blackman et al. 1992), pea, corn (Koster and Leopold 1988; Chen and Burris 1990), *Brassica campestris* (Leprince et al. 1990) and wheat (Black et al. 1999). Wu et al. (2009) showed the importance of raffinose accumulation in desiccation tolerance of the transgenic rice (*Oryza sativa* L.) lines overexpressing *OsWRKY11* (a transcription factor with the WRKY domain and has been reported to be induced by heat shock and drought

stresses in seedlings of rice; Ulker and Somssich 2004). But there are some reports that don't support this hypothesis. Still et al. (1994) and Bochicchio et al. (1994) observed no relationship of oligosaccharides to desiccation tolerance while working with wild rice (*Zizania palustris*) and maize, respectively. Later on, Black et al. (1999) concluded that dehydrins might participate in desiccation tolerance but not by their interaction with raffinose. Therefore, there is need of more resolution on the participation of RFO in desiccation tolerance.

2.4.2 Abiotic and biotic stress tolerance

Plants are sessile and therefore have to cope with varying environmental conditions like high and low temperature, drought, salinity etc. Along with these abiotic factors biotic agents like bacteria, virus, fungi, and insects also affect plant growth and development (Xia et al. 2009). Both biotic and abiotic stresses accumulate harmful and highly reactive forms of molecular oxygen within the plant cell. Such forms of oxygen are known as reactive oxygen species (ROS). These ROS in higher concentrations are capable of damaging proteins, lipids, nucleic acids and other biomolecules irreversibly (Scandalios 2005). Carbohydrates including RFO and sugar alcohols also contribute in protecting cells from oxidative damage and maintaining redox homeostasis (Nishizawa-Yokoi et al. 2008; Keunen et al. 2013). RFO might have the capability to scavenge ROS. During this detoxification process, RFO are proposed to convert in their oxidized radical forms that are further regenerated by reacting with other antioxidants like asorbic acid (ASC) or flavonoids (Van den Ende and Valluru 2009). The following might be the reaction for RFO antioxidant activity (as described for other sugars by Morelli et al. 2003; Bolouri-Moghaddam et al. 2010):



Raffinose as well as galactinol plays important role against oxidative stress in plants (Morsy et al. 2007; Nishizawa et al. 2008; Keunen et al. 2013) and seeds (Buitink et al. 2000; Bailly et al. 2001; Lehner et al. 2006). Liu et al. (2007) found an enhanced tolerance to abiotic stress in Arabidopsis over-expressing *OsUGE-1* (UDP-glucose 4-epimerase from *Oryza sativa*) and associated it with the increased level of raffinose. The overexpression of

galactinol synthase (*GS1*, *GS2* and *GS4*) and raffinose synthase (*RafS2*) in transgenic Arabidopsis increased the concentrations of galactinol and raffinose leading to increased tolerance to methyl-viologen treatment and salinity or chilling stress (Nishizawa et al. 2008). This tolerance is the result of improved ROS scavenging capacity due to accumulation of galactinol and raffinose. Further, these transgenic plants exhibited significantly lower lipid peroxidation and higher PSII activities along with increased level of other antioxidants (Nishizawa et al. 2008; Bolouri-Moghaddam et al. 2010).

RFO are considered as soluble sugars providing tolerance against different environment stresses. Gilbert et al. (1997) discussed the changes in carbohydrate metabolism in source and sink tissues in response to salinity stress in coleus (*Coleus blumei* Benth.) plant. They reported that during stress conditions more sucrose was exported from source that was used to synthesize high-DP RFO in sink tissues by galactan:galactan galactosyltransferase. Taji et al. (2002) confirmed the role of raffinose and galactinol as osmoprotectant. They found an enhanced level of both compounds in transgenic plants overexpressing *GS2* correlated with improved drought tolerance in Arabidopsis. Panikulangara et al. (2004) mentioned *GS1* as a novel heat shock factor (HSF) target gene that accumulated raffinose providing tolerance against heat stress in HSF3 (AtHsfA1b)-overexpressing Arabidopsis plants.

Imanishi et al. (1998) examined the seasonal changes in freezing tolerance, water content and soluble sugar composition of shoot apices of *Lonicera caerulea* L. var. *emphyllocaryx* Nakai. Raffinose and stachyose accumulated rapidly from September to November, while the levels of total soluble sugars and sucrose gradually increased from June to September suggesting their involvement in increasing the freezing tolerance. Pond et al. (2002) showed an increased tolerance to fast desiccation and improved germination of *Picea glauca* somatic embryos that experienced cold treatment before desiccation. Similar result was later reported by Konrádová et al. (2003) in mature cotyledonary somatic embryos of *Picea abies* along with induced RFO accumulation. They found that RFO accumulation induced by cold treatment was substantially responsible for the tolerance. Klotke et al. (2004) reported the accumulation of raffinose in leaves of most of the freezing tolerant plants of *Arabidopsis thaliana*. Wang et al. (2006) revealed the potential basis of low temperature germination advantage of large seeds in *Kraschenninnikovia lanata* (Chenopodiaceae) that was positively correlated with higher concentrations of glucose, raffinose and sucrose. Peters and Keller (2009) demonstrated a positive correlation of frost tolerance in excised *Ajuga reptans* leaves with long-chain RFO accumulation.

In 2004, Zuther et al. experimented on two types of *A. thaliana*, one constitutively over-

expressing a galactinol synthase (*GS*) gene from cucumber and a mutant carrying a knockout of endogenous raffinose synthase (*RS*) gene. They didn't find any change in cold acclimation ability of both RS mutant or the GS overexpressing lines and concluded that raffinose was not essential for basic freezing tolerance or for cold acclimation of *A. thaliana*. However, most of the reports support the role of RFO in freezing tolerance.

2.4.3 Photoassimilate translocation and seed germination

In plants, translocation of photoassimilate from source to sink tissues is necessary for growth and development (McCaskill and Turgeon 2007). Like sucrose, RFO may be used for phloem translocation and storage carbons. According to a review by Gamalei (1989), RFO transporting plants are of type 1, *i.e.* have high numbers of plasmodesmata at the interface of intermediary cell and mesophyll cells/sieve tube. Intermediary cells are the specialized companion cells connected to the bundle sheath, not to phloem parenchyma cells, by branched plasmodesmata (Turgeon 1996). Sucrose-transporting type 2 plants are characterized by low frequencies of plasmodesmata at both the interfaces. Phloem loading of sucrose is achieved by an apoplastic route using proton symport, while in type 1 plants RFO are loaded by symplastic route named as "polymer trap" mechanism (Hannah et al. 2006). Presence of raffinose (small amount) in some apoplasmic loaders has also been reported. Most of the Type 1 plants come under Cucurbitaceae, Verbenaceae, Lamiaceae, Oleaceae and Scrophulariaceae families and are characterized for the formation of RFO in intermediary cells (Turgeon et al. 1993; Sprenger and Keller 2000; Haritatos et al. 2000; Dinant and Lemoine 2010). Galactinol is synthesized from *myo*-inositol and UDP-galactose either in the cytosol of the intermediary cells or in the mesophyll compartment of certain plants. In intermediary cells, galactinol donates its galactosyl unit to sucrose forming raffinose. Turgeon (1996) proposed the above mentioned "polymer trap" mechanism for phloem loading of RFO that explained the higher size exclusion limit of plasmodesmata connections from intermediary cells to sieve tubes. As a result, RFO can only diffuse to the sieve element preventing backward diffusion to mesophyll cells. Formation of RFO in intermediary cells was also supported by Haritatos et al. (2000). They reported the expression specificity (in intermediary or companion cells) of galactinol synthase (*GS*) promoter from cucurbits in source leaves of Arabidopsis and tobacco. Sprenger and Keller (2000) suggested the role of two distinct galactinol synthase genes, *GS1* (expressed in mesophyll cells) and *GS2* (expressed in intermediary cells) in synthesis of storage and transport RFO, respectively. McCaskill and Turgeon (2007) suppressed two galactinol synthase genes in *Verbascum*

phoeniceum (Family: Scrophulariaceae) and resulted in a negatively affected (impaired growth, leaf chlorosis, and necrosis and curling of leaf margins) translocation of photoassimilates confirming the role of RFO for phloem transport in such species.

RFO get accumulated in storage organs (like tubers, seeds) of most of the plants mainly in legumes (Peterbauer et al. 2002) by phloem loading and transport (Turgeon 1996; Sprenger and Keller 2000). During early stages of seed germination, they are rapidly mobilized by *α*-galactosidases (*α*-D-galactoside galactohydrolase, E.C.3.2.1.22) and provide readily available energy and carbon (Zhao et al. 2006). Alpha-galactosidase cleaves the terminal non-reducing α(1→6) linked galactose residues of α-galactosides (Anisha et al. 2011). Blöchl et al. (2008) suggested a model for RFO breakdown in pea seeds. Two types of *α*-galactosidases were reported in this model - Acidic [found in protein storage vacuoles (PSVs)] and Alkaline (active in cytosol; Gao and Schaffer 1999). Acidic *α*-galactosidase (activated by the pH shift during seed imbibition) was proposed to be responsible for the breakdown of RFO in PSV during early stages of seed development and this was considered as the main energy sources for seed metabolism. The resulted increasing sucrose concentration during transition phase, from germination to plant growth, was attributed to induce the expression of alkaline *α*-galactosidase resulting in mobilization of remaining RFO.

de Ruiz and Bressani (1990) analyzed the germinating seeds of *Amaranthus hypochondriacus, A. cruentus,* and *A.* caudate at 0, 24, 48, and 72 h. The raffinose and stachyose concentrations decreased quickly during the first 24 h of germination and almost disappeared after 48 h of the process. Blöchl et al. (2007) applied a specific α-galactosidase inhibitor (1-deoxygalactonojirimycin) to germinating pea seeds and reported drastically dropped germination rate by about 70 % providing clear evidence for RFO's role during early stages of seed germination. Later, Dierking and Bilyeu (2009) compared seed germination potential for wild and low RFO type soybean. Contrastingly, they didn't find any significant difference in germination between normal and low RFO soybean seeds when imbibed or germinated in water. Later, Lahuta and Goszczyńska (2009) and Zalewski et al. (2010) reported delayed seed germination in winter vetch and *Lupinus luteus,* respectively due to inhibited breakdown of RFO. In brief, it might be concluded that RFO are not a common essential source of metabolizable energy for early seed germination events (Lahuta et al. 2005).

2.4.4 RFO and agronomic practices

Legumes are important part of crop rotation as they are capable of fixing atmosph-

eric nitrogen into the soil with the help of nitrogen fixing bacteria. These bacteria are found in legume root nodules in a symbiotic relationship (Larrainzar 2009). RFO are proposed to support the growth and survival of symbiotic nitrogen fixing bacteria (*e.g. Rhizobium meliloti*) in the rhizosphere of germinating seed/young plant of legumes (Gage 1998). This area of research needs more scientific attention to explore role of RFO during establishment of symbiotic relationship and nitrogen fixation (Gage 1998).

2.5 Impact of RFO on human health

Alpha-galactosides of sucrose, namely raffinose, stachyose and verbascose, are widely distributed in higher plants, especially in leguminous seeds (Table 2.9). Humans and monogastric animals are unable to digest RFO because their intestinal mucosa lack a hydrolytic enzyme α-galactosidase and RFO themselves are unable to pass through the intestinal wall (Reddy et al. 1984; Kumar et al. 2010). Therefore, RFO escape digestion and absorption in the small intestine (Saunders and Wiggins 1981). The microflora of large intestine then metabolize RFO and produce large amounts of carbon dioxide, hydrogen, and small quantities of methane and short chain fatty acids, and the pH is lowered (Krause et al. 1994; Naczk et al. 1997). These deleterious gases of bacterial origin make almost 3/4 of the flatulence causing unwanted symptoms of abdominal pain, eructation, bloating stomach, and gut cramps in organisms lacking α-galactosidase (Swennen et al. 2006; Kurbel et al. 2006). Increase in fermentable carbohydrates in lower part of digestive tract may cause the disturbance in the existing microbial balance, causing diarrhoea (Veldman et al. 1993). But, removal of these compounds from beans could not reduce the flatulence problem completely and hence involvement of indigestible polysaccharides was also associated with intestinal gas production (Reddy et al. 1984).

Presence of RFO in diet can reduce the available dietary energy and interferes with the digestion of other nutrients (Martínez-Villaluenga et al. 2008). Coon et al. (1990) compared the diets having 5.3 % and 1 % of α-galactosides on a dry weight basis and found a 20 % decrease in true metabolizable energy in diets containing higher amount of α-galactosides. RFO reduced true metabolizable energy (Leske et al. 1993) and protein utilization (measured by protein efficiency ratio in chicken diets; Leske et al. 1995) available from soy protein. van Barneveld (1999) showed that the extraction of these oligosaccharides significantly improved the digestion of all amino acids increasing the overall nutritional value of lupin diet (Glencross et al. 2003). According to Wagner et al. (1976), levels of raffinose and stachyose greater than 6.7 % could lead to the osmotic pressure imbalance with their small losses before

the completion of hydrolysis and fermentation by intestinal microflora. This osmotic pressure imbalance could lead to the reduction in absorption capacity of small intestine (Wiggins 1984).

On the other hand, RFO are also considered as selective “fertilizer” for the colonic microflora (Roberfroid 2002). Hence, they are considered as prebiotics. A prebiotic is defined as a non-digestible food ingredient that selectively stimulates the growth and activity of bacteria in colon with beneficial consequences for host health (Gibson and Roberfroid 1995; Scholz-Ahrens et al. 2001). Both, *in vitro* (Hayakawa et al. 1990; Saito et al. 1992; Durand et al. 1992; Bouhnik et al. 1997) and *in vivo* (Hayakawa et al. 1990; Andrieux and Szylit 1992; Rowland and Tanaka 1993) studies confirmed the importance of α-galactosides as prebiotics (Gibson and Fuller 2000). RFO are hydrolyzed by intestinal bacteria into mono- or di-saccharides which can be further metabolized to short chain fatty acids (SCFAs), e.g. acetate, propionate, and butyrate resulting in low pH of intestine (Topping 1996). The prebiotic effects of α-galactosides can be studied under following heads:

2.5.1 Influemnce on intestinal microbiota

The end-products of α-galactosides fermentation acidify the colon environment that is beneficial for the development of bacteria such as lactobacilli including bifidobacteria and detrimental to the growth of potential pathogenic species (Swennen et al. 2006). Alpha-galactosides are attributed for the increase in growth rate of *Bifidobacterium* sp. that constitute up to 25 % of total cultivable gut flora (Mitsuoka 1992; Trojanova et al. 2006). Gulewicz et al. (2002) analyzed the effect of diet having 15 mg of lupin and pea *α*-galactosides per 100 g of body weight on Wistar rats and they found significantly increased level of fecal bifidobacteria, while fecal and total coliforms were decreased. Lower caecal pH is believed to prevent growth of pH sensitive pathogens, such as *E. coli* and *Salmonella* (Matteuzzi et al. 2004). Mitsuoka (1992) associated these oligosaccharides to suppress the activities of harmful enzymes like azoreductase activity by 39.9 %, β-glucuronidase by 37.7 %, and β -glucosidase by 31.5 %. Antimicrobial compounds other than organic acids, such as bacteriocins (bifidocin B and Bifidin) and bacteriocin-like inhibitory substance (BLIS; Cheikhyoussef et al. 2010) are reported from *Biofidobactrium sp.* These compounds suppressed the growth of harmful intestinal pathogens like *Clostridium*, *Salmonella*, *Candida*, *E. coli*, *Listeria*, *Enterococcus*, and *Pediococcus* (Touŕe et al. 2003). All these evidences suggest the importance of α-galactosides in maintaining intestinal health by stimulating growth of bifidobacteria.

2.5.2 Improved mineral bioavailability

Alpha-galactosides have been found to stimulate absorption and retention of several minerals, particularly calcium, magnesium and iron (Grizard and Barthomeuf 1999; Scholz-Ahrens et al. 2001; Mitamura et al. 2004; Suzuki and Hara 2004; Swennen et al. 2006). The following mechanisms are proposed for improved mineral bioavailability (Chonan et al. 1995; Cliffe et al. 2005; Swennen et al. 2006; Scholz-Ahrens et al. 2007):

i. Increased solubilization of mineral salts due to lower pH of large intestine.
ii. Degradation of mineral complex with phytic acid.
iii. Enlargement of absorption surface by promoting proliferation of enterocytes that maintains the functional integrity of intestinal epithelial layer. Ishizuka et al. (2009) found a prominent symbiotic effect of exogenous *B. breve* and raffinose on epithelial proliferation in small intestine but not in large intestine.
iv. Another mechanism for improved calcium absorption, involves the activation of calmodulin-dependent myosin light-chain kinase that induces condensation of actin microfilaments resulting in opening of tight junction (Lindmark et al. 1998). This may lead to the enhancement of paracellular calcium transport through intestinal epithelial layer (Mineo et al. 2001).

2.5.3 Protection from diseases

Alpha-galactosides are investigated for their anti-allergic functions against diseases such as atopic dermatitis, allergic rhinitis and asthma. Nagura et al. (2002) reported novel effect of raffinose on suppression of immunoglobulin (Ig) E production by suppressing Th2-type (T-helper cell type 2) immune responses against oral antigen. Swennen et al. (2006) proposed three ways by which these sugars affect immune system: (1) by lactic acid bacteria (can penetrate the intestinal epithelial cells resulting in activation of the gut-associated lymphoid tissue); (2) by butyrate (reduces the requirements of epithelial cells for glutamine, thereby sparing it for cells of the immune system). (3) by other SCFAs (immune-modulatory and anti-inflammatory properties; Kelly-Quagliana et al. 2003). They are also reported to serve against cancer either by producing SCFAs or by stimulating growth of *Biofidobactrium sp.* (Grizard and Barthomeuf 1999; Trojanova et al. 2006). Among SCFAs, butyrate (Scheppach and Weiler 2004) and propionate (Nurmi et al. 2005) are important having chemo-protective and anti-inflammatory effects against colon cancer (Swennen et al. 2006). Kim et al. (2010) studied the anti-inflammatory and anti-proliferative effects of *B. lactis* that might be useful for cancer prevention strategies.

2.5.4 Other aspects of RFO importance

These sugars have received a considerable attention for their hypocholesterolemic (decrease in blood cholesterol level) and hypotriglyceridemic (decrease in blood lipid level) effects (Swannen et al. 2006; Martínez-Villaluenga et al. 2008). Tortuero et al. 1997 reported increase in caecal lactobacilli concentration after raffinose ingestion associated with anti-cholesterolemic action.

Belcourt and Labuza (2007) found significantly softer texture in cookies with added raffinose (5 % w/w). The galactose moiety of the raffinose is proposed to disrupt the crystallization pattern of sucrose that significantly decreased the quantity of recrystallized sucrose.

2.6 Determination of sugar concentration

Analytical estimation is the first step of all RFO related studies. It is also helpful in selecting genotypes with high and low RFO concentration that can be utilized to understand RFO biosynthesis, identify key regulating biosynthetic step and study natural variation together with impact of genotype, environment and their interaction on RFO concentration. RFO represent a class of soluble and non-reducing oligosaccharides sugars. The analytical methods to determine sugars can be categorized into four main groups: (1) chemical, (2) physical, (3) enzymatic, and (4) chromatographic method (http://people.umass.edu/~mcclemen/581Carbohydrates.html; Vinjamoori et al. 2004; Brummer and Cui 2005; Moresco et al. 2008; Raessler et al. 2010). Chemical methods can be subdivided into titration, gravimetric and colorimetric approaches. Lane-Eynon method is a titration based approach to determine total amount of reducing sugars. In this method, sugar solution is added slowly to boiling solution of copper sulfate solution with methylene blue indicator. When the end point of the reaction is achieved, solution color changes from blue to white. The amount of sugar solution required to reach end point is employed to calculate concentration of reducing sugar in the sample on the basis of calibration curve. This method cannot estimate the composition of reducing sugars and direct concentration of non-reducing sugars. Method reported by Munson and Walker follows the gravimetric principles in which reducing sugars are oxidized by heating with excess of copper sulfate and alkaline tartrate under carefully controlled conditions. The resulted copper oxide precipitate was determined by filtration, drying and weighing. Although this method has the same drawbacks as Lane-Eynon method yet it is comparatively more accurate and precise. Colorimetric approach includes phenol-sulfuric acid and anthrone based methods that determine the concentration of

total sugars in the sample. Sugars react with anthrone (with sulfuric acid) or phenol (with sulfuric acid) and produce blue-green or yellow-orange color having absorption maxima at 625 and 490 (480) nm, respectively (Brummer and Cui 2005). Phenol-sulfuric acid method is the most widely used approach to determine total sugars in aqueous solutions (Albalasmeh et al. 2013).

Physical methods to determine sugars utilize polarimetry, refractive index, potentiometry etc. methodologies (http://people.umass.edu/~mcclemen/581Carbo hydrates.html; Moresco et al. 2008). All the above mentioned methods are unable to predict the composition of either total or reducing/non-reducing sugars. Therefore, concentration and composition of RFO cannot be determined. To determine total RFO concentration, enzymatic method includes hydrolysis of RFO and sucrose in to glucose by α-galactosidase and invertase. Thereafter, absorbance of glucose concentration can be measured using spectrophotometer. This approach was adopted by Megazyme (Megazyme International Ireland Ltd., Wicklow, Ireland) and a kit was developed to determine concentration of total RFO.

To perform compositional study for RFO and other soluble sugars, chromatographic techniques have been described as reliable and efficient approach. Among different chromatographic methods reported, high performance liquid chromatography with refractive index detector (HPLC-RI) and high performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD) are widely used approaches. Jones et al. (1999) reported a TLC (thin layer chromatography) based method for qualitative estimation of individual RFO concentration. This method can be utilized to screen a large number of genotypes in a population and selected genotypes can be used for further study. However, TLC is not capable of quantifying individual RFO concentration; hence HPLC-RI or HPAEC-PAD methods should be utilized.

Sánchez-Mata et al. (1998) developed a HPLC with differential refractometer detector based method using Waters µBondapack/carbohydrate column and acetonitrile-water (80:20. v/v) as mobile phase with a flow rate of 0.9 mL/min. Using this method, they reported the concentration of ribose, fructose, glucose, galactose, sucrose, maltose, raffinose and stachyose in seeds of lentils (*Lens esculenta* L.), dry peas (*Pisum sativum* L.), white kidney beans (*Phaseolus vulgaris* L.), pinto beans (*Phaseolus vulgaris* L.) and chickpeas (*Cicer arietinum* L.). However, they could not report the verbascose concentration in these legumes. In 2008, Xiaoli et al. reported a HPLC-RI based method using acetonitrile-water (75:25, v/v) as mobile phase with 1.0 mL/min of flow rate. This method is able to determine verbascose concentration in chickpea seeds. Simultaneously, Bansleben et al. (2008) optimized a

HPAEC-PAD based analytical method using CarboPac PA10 column and 170 mmol/L sodium hydroxide as mobile phase with a flow rate of 1 mL/min for 22.5 min. They validated this method on seeds of *Lupinus albus* and *L. angustifolius* and estimated the concentration of sucrose, raffinose, stachyose and verbascose.

Comparison between HPLC-RI and HPAEC-PAD based approaches must be performed to select the more accurate, precise and rapid method. To solve the puzzle, Frias et al. (1994) compared these two methods by quantifying concentration of fructose, sucrose, raffinose and stachyose in lentil (*Lens culinaris*) seeds. For HPLC-RI, Waters µBondapac/Carbohydrate column was adopted using acetonitrilel:water (75:25, v/v) as mobile phase with a flow rate of 2.0 mL/min whereas in HPAEC-PAD, they employed CarboPac PA100 column using 145 mM sodium hydroxide as mobile phase with 1mL/min of flow rate. They found both the methods reliable but HPAEC-PAD showed higher detection sensitivity thus can be utilized to study RFO biosynthesis at different stages of seed development or in different parts of a seed.

2.7 Effect of genotype by environment interaction on chickpea and RFO concentration

The phenomenon related to significantly different performance of a genotype across environments, is termed as "Genotype by Environment Interaction (G×E)" which is an essential component of plant breeding programs. This interaction can be divided into: 1) crossover interaction reflects change in genotype ranking in diverse environments, whereas 2) non-crossover interaction represents unchanged ranking due to homogeneity either in environment or genetic background (Kang 2002). A significant G×E effect supports the need of multi-location/-environment trials to select genotypes with broad or specific adaptation capacity for various economically important traits like grain yield, biotic/abiotic stress tolerance etc. Consequently, it complicates the selection of superior genotypes in crop improvement programs introducing the concept of "genotype stability" (Kandus 2010). Genotypic stability refers to the consistent performance of a genotype in diverse environments. A significant G×E also points out trait's quantitative nature, *i.e.* phenotype governed by many genes. Hence, contribution of these genes may differ in diverse environments (Kang 2002; Yadav et al. 2010). The pooled/combined analysis of variance (ANOVA) is the widely used method for determining G×E (Kandus 2010).

In chickpea, G×E has been studied for various important traits like seed yield, seed weight, plant height, seed constituents (protein, starch and amylose), disease incidence (Ascochyta blight and Fusarium wilt) etc. as summarized in Table 2.11.

Table 2.11 Effect of genotype (G), environment (E) and G×E on different traits in chickpea

Reference	Characters studied	Results	Experiment		
			Season	Genotype	Location
Singh et al. (1983)	Seed protein	G*, L**, G×L ns	2	47 and 25 desi/15 kabuli	4/3
Khan et al. (1988)	Seed yield	G**, E**, G×E**	1	14	6
Malhotra et al. (1997)	Seed yield	G**, S ns, G×S*	3	24	1
Rubio et al. (1998)	Yield	L***, G ns, G×L**	2	5 pairs	5
	Seed/plant	L***, G ns, G×L**			
	Yield/plant	L***, G***, G×L ns			
	Seed weight	L***, G***, G×L***		5 pairs	4
	Expressiveness of double pod character	L***, G***, G×L ns			
Sirohi et al. (2001)	Days to flowering	G*, E*, G×E*	5	25	-
	Days to maturity				
	Plant height				
	Pod/plant				
	100-seed weight				
	Seed yield/plant				
Arshad et al. (2003)	Grain yield	G**, E**, G×E**	1	25	12
Tekeoğlu et al. (2004)	Ascochyta blight	L**, Y ns, G**, G×L**, G×Y**	2	221 RILs	2

*, ** and *** significant at $P \leq 0.05$, 0.01 and 0.001; ns=Non Significant; G = Genotype, L = Location; E = Environment; Y = Year; S = Season

Table 2.11 contd. Effect of genotype (G), environment (E) and G×E on different traits in chickpea

Reference	Characters studied	Results	Experiment		
			Season	Genotype	Location
Bakhsh et al. (2006)	Primary branches	G×L*			
	Pod/plant	G×L**	1	20	3
	Grain yield	G×L**			
Atta et al. (2009)	Seed yield	G**, E**, G×E*	3	6	4
Frimpong et al. (2009)	Seed yield	G**, E**, G×E**			
	Seed weight	G**, E**, G×E**			
	Protein (Desi/Kabuli)	G**, E**, G×E**/ G*,E**, G×E ns	2	7 desi 9 kabuli	11
	Starch (Desi/Kabuli)	G**, E**, G×E*/ G*,E**, G×E**			
	Amylose (Desi/Kabuli)	G*, E**, G×E**/ G**,E**, G×E**			
ALwawi et al. (2010)	Seed yield /plant	G**, L**, S**, G×L ns, G×E ns, E×L ns, G×E×L ns			
	Days to maturity	G**, L**, S**, G×L**, G×E ns, E×L ns, G×E×L*	2	7	2
	Protein	G**, L**, S**, G×L**, G×E ns, E×L ns, G×E×L ns			
Dehghani et al. (2010)	Grain yield	G**, E**, G×E**	3	17	6
Ebadi segherloo et al. (2010)	Grain yield	G**, L**, G×L**	3	17	6

*, ** and *** significant at $P \leq 0.05$, 0.01 and 0.001; ns=Non Significant; G = Genotype, L = Location; E = Environment; S = Season

Table 2.11 contd. Effect of genotype (G), environment (E) and G×E on different traits in chickpea

Reference	Characters studied	Results	Experiment		
			Season	Genotype	Location
Yadav et al. (2010)	Plant height	G**, E**, G×E**			
	Number of branches/plant	G**, E**, G×E**			
	Days to flowering	G**, E**, G×E**			
	Days to maturity	G**, E**, G×E ns			
	Number of pods/plant	G**, E**, G×E**			
	Number of seeds/pod	G**, E**, G×E**	2	108	2
	100-seed weight	G**, E**, G×E**			
	Biologocal yield/plant	G**, E**, G×E**			
	Seed yield/plant	G**, E**, G×E**			
	Harvest index	G**, E**, G×E**			
	Grain yield	G**, E**, G×E**			
Farshadfar et al. (2011)	Grain yield	G**, L**, G×L**	2	17	5
Sharma et al. (2012)	Fusarium wilt incidence	G***, E***, G×E***	3	27	9
Farshadfar et al. (2013)	Grain yield	G×E**	4 (2 envrironments)	20	1

** and *** significant at $P \leq 0.01$ and 0.001, respectively; ns=Non Significant; G = Genotype, L = Location; E = Environment;

2.7.1 G×E influences RFO concentration in seeds

Effect of G×E on seed RFO concentration, has been reported in some crops like peanut (*Arachis hypogaea* L.; Pattee et al. 2000), soybean (*Glycine max* L. Merr.; Cicek et al. 2006; Jaureguy et al. 2011), sugar beet (*Beta vulgaris* L.; Hoffmann et al. 2009) and lentil (*Lens culinaris* Medikus subsp. Culinaris; Tahir et al. 2011). Most of the studies showed significant effect of G×E on seed RFO concentration (Table 2.12) as RFO act as antioxidants during stress tolerance. Therefore, environmental conditions affect RFO level, *i.e.* more adverse conditions may result in higher RFO concentration.

2.7.2 Heritability

The genotype by environment interaction also affects the heritability of a trait. Heritability (H^2) describes the variation in phenotype by the genotypic factor (Wray and Visscher, 2008). Statistically, H^2 is presented as ratio between genetic (V_G) and phenotypic variance (V_P):

$$H^2 = V_G / V_P$$

V_G is the sum of variation due to additive gene effect (V_A), dominance (V_D) and epistasis (V_I):

$V_G = V_A + V_D + V_I$; where V_A represents variation due to the inheritance of a particular allele. V_D shows the variation due to interaction of alleles present at specific locus whereas V_I is the result of interaction between alleles present at different loci (Byers, 2008).

Similarly, V_P is the total phenotypic variation including V_G and V_E (environmental variance). V_E can be categorized into three groups:
$V_E = V_{Eg} + V_{G\times E} + V_{Es}$; where V_{Eg}, $V_{G\times E}$ and V_{Es} stand for general environmental variance, genotype by environment interaction variance and specific environmental variance, respectively (Byers, 2008).

Based on genetic factor involvement, H^2 is mainly divided into two categories; 1) broad sense heritability includes variation due to total genetic variance, whereas 2) narrow sense heritability focuses on variation due to additive gene effect (V_A) (Piepho and Möhring, 2007; Wray and Visscher, 2008).

To calculate broad sense heritability (h^2) for multilocation trial, Singh et al. (1993) reported the following formulae based on ANOVA table:

$$h^2 = \sigma^2_G / (\sigma^2_G + \sigma^2_I + \sigma^2_e)$$

$$\sigma^2_G = (M_G - M_I)/ (bL),$$

$$\sigma^2_I = (M_I - M_e)/ b, \text{ and}$$

Table 2.12 Effect of genotype (G), environment (E) and G×E on RFO concentration in different crops

References	Crop	Seed Components	Results
Pattee et al. (2000)	Peanut (*Arachis hypogaea* L.)	Inositol	G ns, L*, G×L**
		Glucose	G**, L ns, G×L^
		Fructose	G**, L ns, G×L*
		Sucrose	G**, L ns, G×L**
		Raffinose	G*, L**, G×L**
		Stachyose	G**, L**, G×L**
Mebrahtu and Mohamed (2006)	Soybean [*Glycine max* (L.) Merr.]	Glucose	G ns, Y**, G×Y ns
		Fructose	G*, Y**, G×Y**
		Sucrose	G**, Y**, G×Y**
		Raffinose	G**, Y*, G×Y*
		Stachyose	G ns, Y**, G×Y**
Cicek et al. (2006)	Soybean [*Glycine max* (L.) Merr.]	Sucrose	G***, L**, G×L^
		Raffinose	G***, L***, G×L^
		Stachyose	G***, L***, G×L ns
Hoffmann et al. (2009)	Sugar beet (*Beta vulgaris* L.)	Raffinose	G***, E***, G×E***
Kumar et al. (2010)	Soybean [*Glycine max* (L.) Merr.]	Sucrose	L***, G×L***
		Raffinose	L ns, G×L*
		Stachyose	L ns, G×L*

^, *, ** and *** significant at $P \leq 0.10$, 0.05, 0.01 and 0.001, respectively; ns=Non Significant; G = Genotype, L = Location; E = Environment; Y = Year

Table 2.12 contd. Effect of genotype (G), environment (E) and G×E on RFO concentration in different crops

References	Crop	Seed Components	Results
Jaureguy et al. (2011)	Soybean [*Glycine max* (L.) Merr.]	Sucrose	G***, Y*, L ns, Y×L ns, G×L***
		Raffinose	G***, Y***, L***, Y×L***, G×L***
		Stachyose	G***, Y ns, L***, Y×L***, G×L***
Tahir et al. (2011)	Lentil (*Lens culinaris* Medikus subsp. culinaris)	Glucose	G**, E**, G×E**
		Sucrose	
		Total RFO	
		Raffinose	
		Stachyose	
		Verbascose	
Edmé and Glaz (2013)	Sugarcane (*Saccharum* spp. hybrids)	Sucrose	G***, E***, G×E ns

** and *** significant at $P \leq 0.01$ and 0.001, respectively; ns=Non Significant; G = Genotype, L = Location; E = Environment; Y = Year

$$\sigma^2 e = M_e$$

In these equations, M_G, M_I and M_e are mean sum of squares (MSS) from ANOVA for genotype (G), genotype by environment interaction (G×E) and error, while b and L represent number of replication and environments used in the study, respectively.

Heritability varies between 0 and 1. Broad sense heritability can be described as low (<0.3), medium (0.3 – 0.6) and high (≥0.6) as reported by Ayele (2011). Heritability plays an important role during genotype selection in various plant breeding approaches.

2.8 RFO biosynthesis during seed development

On the basis of results reported by Singh and Jambunathan (1982), Saravitz et al. (1987), Castillo et al. (1990), Black et al. (1996), Frias et al. (1996), Bailly et al. (2001), Peterbauer et al. (2001), Karner et al. (2004), Lahuta et al. (2005), Saldivar et al. (2011) and Zhawar et al. (2011), following general conclusions can be summarized –

1. *Myo*-inositol, fructose, glucose, galactose and sucrose were predominant during early stages of seed development. Decrease in their concentration during later stages of seed development, corresponded to biosynthesis of galactinol followed by RFO.
2. The onset of RFO biosynthesis was also coincident with loss of water from seed indicating their role during seed desiccation.
3. The (raffinose + stachyose)/sucrose ratio was increased during seed filling and showed a value of 1 when all the seeds became tolerant to drying. At this stage, seeds also showed high catalase and glutathione reductase activity whereas superoxide dismutase and ascorbate peroxidase activity was found low.
4. Galactinol dependent RFO biosynthetic enzymes showed the highest activity at pH 7.
5. Galactinol-independent activity to synthesize verbascose was detected in pea seeds that showed activity at pH 7.
6. Transcriptional and post transcriptional regulations were proposed for RFO biosynthesis.
7. There is controversy regarding the key step in regulating final concentration of RFO in seeds. Some reports described galactinol synthase as the key enzyme. However, some favored the concentration of initial substrates like *myo*-inositol and sucrose together with other feed-back loops in controlling RFO concentration rather than galactinol synthase alone.

2.9 Approaches to reduce RFO concentration

2.9.1 Processing methods

Different processing methods like de-hulling, cooking (boiling, autoclaving and microwave cooking), soaking, germination, gamma irradiation, α-galactosidase treatment, ultrasound, hydrostatic pressure and thermal dehydration have been reported to reduce RFO concentration significantly in seeds of chickpea (El-Adawy 2002; Alajaji &and El-Adawy 2006; Han and Baik 2006; Aguilera et al. 2009) and other crops like green gram (*Phaseolus areus*; Rao and Vakil 1983), cow pea (*Vigna unguiculata*; Wang et al. 1997; Onyenekwe et al. 2000), broad bean (*Vicia faba* L.; Al-Kaisey 2003), Black Gram (*Vigna mungo* L.; Girigowda et al. 2005), lentils (*Lens culinaris*; Han and Baik 2006), pea (*Pisum sativum* L.; Han and Baik 2006), mung bean (*Vigna radiata* L.; Anisha and Prema 2008; Tajoddin et al. 2010), Horse gram (*Dolichos biflorus*; Anisha and Prema 2008), red gram (*Cajanus cajan* L.; Devindra et al. 2011) and soybean (*Glycine max* L.; Dixit et al. 2011). However, such physical and mechanical treatments also reduce concentration of protein, B-vitamins, minerals and amino acids in processed seeds/flour (Wang et al. 1997; El-Adawy 2002; Alajali and El-Adawy 2006).

2.9.2 Up-regulation of α-galactosidase

Alpha-galactosidase is a well-known enzyme for RFO break down by hydrolyzing α(1→6) linkage (Blöchl et al. 2008). Using this characteristic together with transformation approach, Polowick et al. (2009) developed transgenic pea lines overexpressing α-galactosidase from coffee (*Coffea arabica* L.). These transgenic lines showed up to 40 % reduction in raffinose and stachyose concentration without affecting seed germination rate (96 %).

2.9.3 Down-regulation of key biosynthetic enzyme

Galactinol synthase (GS) is considered as the first committed and key regulating step of RFO biosynthesis influencing carbon partitioning between sucrose and RFO (Peterbauer et al. 2001; Nishizawa et al. 2008). Recently, Bock et al. (2009) down-regulated the expression of galactinol synthase in canola (*Brassica napus* L.) using antisense approach. Consequently, they observed a decrease in galactinol and stachyose concentration in transgenic canola seeds.

2.9.4 Effect of substrate accumulation

Some reports suggest substrates concentrations as regulating factor of RFO biosyn-

-thesis rather than GS alone. Karner et al. (2004) could not find any significant relationship between GS activity and RFO accumulation in seven pea genotypes varying for RFO concentration. However, they observed a strong positive correlation of *myo*-inositol and sucrose concentration with RFO concentration. Lahuta et al. (2005) on the basis of their feeding experiment reported that ratio of D-pinitol and *myo*-inositol could decide the level of RFO biosynthesis in developing tiny vetch [*Vicia hirsute* (L.) S. F. Gray] seeds. Hence, it can be hypothesized that if *myo*-inositol quantitatively dominates D-pinitol/D-chiro-inositol, RFO will be accumulated at higher concentration and in reverse condition, galactosyl cyclitols will synthesize in higher quantity. In support to the hypothesis, Lahuta et al. (2010) found an increase in concentration of galactosyl cyclitols along with decreased RFO level while feeding with D-pinitol and D-chiro-inositol. However, feeding experiments with *myo*-inositol and sucrose (25 and 50 mM) also stimulated biosynthesis of galactosyl cyclitols instead of RFO. At 100 mM concentration, sucrose reduced galactosyl cyclitols level without showing any effect on RFO. Therefore, a common biosynthetic pathway has been proposed for RFO and galactosyl cyclitols.

2.10 Research hypothesis

On the basis of previous reports, following research hypotheses can be proposed:

1. Chickpea genotypes show natural variation for seed RFO concentration.
2. Activity of RFO biosynthetic enzymes determines the concentration and type of RFO in chickpea seeds.

3. METHODS

3.1 Moisture and dry matter content

To estimate moisture content, seed was weighed together with pre-dried glass vial. The vial was kept at 80 °C till constant weight. Thereafter, tube weight was measured again to calculate seed moisture by using following formulae:

$$\text{Seed moisture (\%)} = \frac{(\text{Weight of tube and fresh seed} - \text{Weight of tube and dried seed}) \times 100}{\text{Weight of tube and fresh seed}}$$

Moisture content (%) was deducted from one hundred to calculate dry matter content of chickpea seeds. Three seeds per genotype were used for the estimation.

3.2 Grinding of seed material

Chickpea seeds were ground into a fine meal using a UDY cyclone mill (Udy Corporation, Fort Collins, CO, USA) to pass through a 0.5 mm sieve. The resulted seed meal was further used to determine total RFO and to extract total soluble sugars including RFO.

3.3 Determination of total RFO concentration

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) provided in a commercial assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland). The assay was performed in following steps:

3.3.1 Enzyme inactivation and sugar extraction

Chickpea seed meal was weighed (500 ± 5 mg) in a glass tube and 5 mL of ethanol (95 % v/v) was added. The tube was incubated in a water bath at 84 - 88 °C for 5 min to inactivate endogenous enzymes. The tube content was transferred to a 50 mL volumetric flask and final volume was adjusted by using sodium acetate buffer (50 mM, pH 4.5; Buffer 1). The sample was allowed to extract over 15 min and thereafter mixed thoroughly. A 5 mL volume of the solution was transferred to another glass tube and mixed vigorously with 2 mL of chloroform. The tube was centrifuged at 1,000 ×*g* for 10 min. The resulted upper aqueous phase (Solution A) was used for further analysis.

3.3.2 Assay for glucose, sucrose and RFO

Before the start of the assay, invertase and α-galactosidase solutions were prepared and stored at -20 °C (see compositions below). In addition, GOPOD reagent was prepared by diluting GOPOD reagent buffer [potassium phosphate buffer (1M, pH 7.4), *p*-hydroxybenzoic acid (0.22 M) and sodium azide (0.02 % (w/w)] in 1 L of nano pure water and dissolving GOPOD reagent enzymes [Glucose oxidase (>12000 U) plus peroxidase (>650 U) and 4-aminoantipyrine (80 mg)] in it. Thereafter, following 5 reactions were prepared:

- 0.4 mL of Buffer 1 (Reagent Blank)
- 0.1 mL of D-glucose standard (supplied with kit) + 0.3 mL of Buffer 1 (Control)
- 0.2 mL of Solution A + 0.2 mL of Buffer 1
- 0.2 mL of Solution A + 0.2 mL of invertase (100 U/12 mL of Buffer 1)
- 0.2 mL of Solution A + 0.2 mL of α-galactosidase + invertase (24 mL of Buffer 1 having 1000 U of α-galactosidase and 200 U of invertase)

All the reactions were incubated at 50 °C for 20 min. Then, 3.0 mL of GOPOD reagent was added to all the reactions. The tubes were again incubated at 50 °C for 20 min. This reaction produced a red colored quinoneimine whose concentration was determined at A_{510} nm using a spectrophotometer.

Glucose, sucrose and total RFO were calculated as:

- D-Glucose, mmoles/100 g = $\Delta A \times F \times 250 \times 200 \times 1/1000$
- Sucrose, mmoles/100 g = $(\Delta B - \Delta A) \times F \times 250 \times 200 \times 1/1000$
- Raffinose family oligosaccharides, mmoles/100 g = $(\Delta C - \Delta B) \times F \times 250 \times 200 \times 1/1000$

In these equations, ΔA = GOPOD absorbance for D-glucose, ΔB = GOPOD absorbance for D-glucose + sucrose, ΔC = GOPOD absorbance for D-glucose + sucrose + Galactosyl-sucrose oligosaccharides. 'F' is a factor to convert absorbance into µmoles of glucose and calculated as:

F = 0.556 (µmoles of glucose)/GOPOD absorbance for 0.556 µmoles of glucose.

The glucose standard provided with the kit has 0.556 µmoles in 100 µL of its solution. Hence, reaction of 100 µL of this glucose with GOPOD reagent gives absorbance for 0.556 µmoles of glucose. Number 250 = conversion of 50 mL of extract to 0.5 g of sample, 200 = conversion from 0.5 to 100 g of sample, 1/1000 = conversion from µmoles to

mmoles. Total RFO concentration was calculated on molar basis as one mole of each oligosaccharide contains one mole of D-glucose.

3.4 Determination of soluble sugars concentration

3.4.1 Extraction of soluble sugars

Soluble sugars were extracted using methods described by Frias et al. (1994) and Sánchez-Mata et al. (1998) with some modifications (Tahir et al. 2011). In brief, 500 mg of chickpea seed meal was weighed in a 15 mL disposable plastic tube and 10 mL of 80 % (v/v) ethanol was added. The tube was thoroughly stirred on a vortex mixer and incubated in a shaking water bath at 60 °C for 45 min with intermittent mixing at 15 min interval. Thereafter, the slurry was centrifuged at 12,100 $\times g$ for 10 min and supernatant was collected in a separate tube. The pellet was used to re-extract soluble sugars as described above. Finally, supernatants from three sequential extractions were pooled and used for purification of soluble sugars as described in the next step.

3.4.2 Purification of soluble sugars

The collected supernatant was filtered through a C18 (Honeywell Burdick & Jackson, Muskegon, MI, USA) cartridge using a vacuum manifold to remove proteins and lipids present in the soluble sugar extract. Cartridge was first prewashed with 5 mL of 99 % (v/v) methanol followed by 5 mL of distilled water and 3 mL of sample extract, respectively. After washing, 3 mL of sample extract was passed through the column and purified extract was collected. A 1.6 mL aliquot of this filtered extract was used for vacuum drying using a Speedvac® Concentrator and universal vacuum system (Thermo Savant, Holbrook, NY, USA).

3.4.3 Sample preparation for HPLC and HPAEC

For HPLC-RI and HPAEC-PAD, vacuum dried samples were dissolved in 0.5 and 1.0 mL of nano pure water, respectively. The sugar solutions were vigorously mixed and centrifuged at 10,000 $\times g$ for 10 min. The supernatant was filtered through a 0.2 µm filter (Phenex-NY 4 mm Syringe Filters, Phenomenex, Torrance, CA, USA) using 1 mL syringe with tuberculin slip tip (Thermo Medical Co., Somerset, NJ, USA). For HPLC-RI, 150 µL of filtrate was directly used to analyze RFO whereas for HPAEC-PAD, 125 µL of filtrate was first diluted to final volume of 500 µL using nano pure water and the diluted sample was used to determine the concentration of individual RFO members.

3.4.4 Instrumentation and chromatographic conditions

Liquid chromatography analysis was performed on a Waters HPLC-RI system (Waters Co., Mississauga, ON, Canada). A LC 60F pump equipped with a Waters 600 system controller was used to deliver mobile phase into guard and analytical columns. Sample injection was performed in a Waters 717 plus autosampler with 10 µL injection volume/sample. The soluble sugars were separated by Rezex RSO Oligosaccharide F analytical column (200 × 10 mm) preceded by Rezex RSO Oligosaccharide F guard column (60 × 10 mm) and a Carbo-Ag security guard cartridge (4 × 3 mm) (Phenomenex, Torrance, CA, USA). The separated sugars were eluted using nano pure water as a mobile phase with a flow rate of 0.1 mL/min. The eluted sugars were detected by Waters 2414 refractive index detector along with data acquisition software (Quick Start, Empower 1154 Chromatography software, Waters Co., Mississauga, ON, Canada). The total run time was 160 min/sample with a 30 min washing interval between two sample runs.

Anion exchange chromatography was carried out on Ion Chromatography System 5000 [ICS 5000 consisting of autosampler, single gradient pump (Model SP-5) and electrochemical detection cell with disposable working gold electrode and Ag/AgCl reference electrode, Thermo Fisher Scientific, Stevens Point, WI, USA]. For sample integration, cleaning and reactivation of the detector, gold standard PAD waveform was used at four different potentials for a total time of 500 milliseconds (msec). In this particular type of waveform, four working potentials (E) were used: +0.1 V for 400 msec (E1), -2.0 V for 20 msec (E2), +0.6 V for 10 msec (E3) and -0.1 V for 70 msec (E4). The complete assembly was controlled by Chromeleon 7.0 software (Dionex Canada Ltd., Oakville, ON, Canada) installed on a Dell Optiplex 780 desktop. Two different strategies using CarboPac PA200 (3 × 250 mm) and CarboPac PA100 (4 × 250 mm) analytical columns (Thermo Fisher Scientific, Stevens Point, WI, USA) were followed to find the best separation among soluble sugars. Both analytical columns were used at 30 °C along with CarboPac PA200 (3 × 50 mm) and CarboPac PA100 (4 × 50 mm) guard columns, respectively. Gradient concentration approaches followed for both columns are summarized in Table 3.1.

Chromatography system, analytical and guard columns along with disposable gold electrode, glass vials for sample injection and sodium hydroxide solution (50 % w/w; used for mobile phase preparation) were purchased from Thermo Scientific (Bannockburn, IL, USA).

3.4.5 Preparation of calibration curve

Table 3.1 Gradient method conditions used for CarboPac PA200 and PA100 to separate soluble sugars and raffinose family oligosaccharides

CarboPac PA200				CarboPac PA100			
Time (min)	Flow (mL/min)	Solvent A*	Solvent B1*	Time (min)	Flow (mL/min)	Solvent A	Solvent B2*
0	0.5	90 %	10 %	0	1.0	90 %	10 %
15	0.5	20 %	80 %	25	1.0	0 %	100 %
15	0.5	90 %	10 %	25	1.0	90 %	10 %
25	0.5	90 %	10 %	35	1.0	90 %	10 %

*Solvent A: Degassed nano pure water, Solvent B1: 100 mM NaOH and Solvent B2: 200 mM NaOH

To prepare calibration curves for *myo*-inositol, galactinol, glucose, fructose, sucrose, raffinose, stachyose and verbascose, serial dilutions were prepared and their readings were analyzed using HPAEC-PAD based approach with CarboPac PA100 analytical and guard column (Figure 3.1).

3.5 Enzyme activity assays

3.5.1 Crude protein extraction

Total cellular protein from frozen chickpea seeds was extracted by following the method as described (Hitz et al. 2002). Seeds were taken out from frozen pods, weighed (about 200 mg) and ground in a pestle and mortar. Ten volumes (2 mL) of extraction buffer [50 mM HEPES-NaOH buffer, pH 7.0 containing 5 mM of 2-mercaptoethanol] was added and thoroughly mixed with ground seeds. On thawing, the cellular homogenate was transferred in to polypropylene tube and centrifuged at 10,000*g* for 10 min. The supernatant was transferred in to a 15 mL disposable tube and desalted using Sephadex G-25 (pre-equilibrated with extraction buffer). The filtrate was collected for protein estimation and enzyme activity assays. A dye binding assay (Bradford method) was used to determine protein concentration in the samples.

3.5.1.1 Dye binding assay to determine protein concentration

The Bradfpord assay (Bradford 1976) is one of the widely used methods of protein determination. It is a colorimetric approach to analyse protein concentration in the sample. Bovine serum albumin (BSA) was used as a standard to prepare calibration curve. Thereafter, reactions were prepared as summarized in Table 3.2. The absorbance was read at 595 nm wavelength. Standard curve was prepared using absorbance for different concentrations of BSA. The resulted coefficient of determination was employed to calculate protein concentration in sample.

3.5.2 Enzyme activity assays

For enzyme activity assays, methods reported by Peterbauer et al. (2001) and Hitz et al. (2002) were followed with some modifications. The compositions and conditions of reactions for different RFO biosynthetic enzymes have been summarized (Table 3.3). For GS activity assay, substrate mix (25 mM HEPES-NaOH pH 7.0, 5 mM $MnCl_2$, 20 mM *myo*-inositol, 10 mM UDP-galactose, 10 mM DTT) along with 10 µg of crude cellular protein was incubated at 25 °C for 10 min. For RS, STS and VS, reaction mix was composed of 30 µg

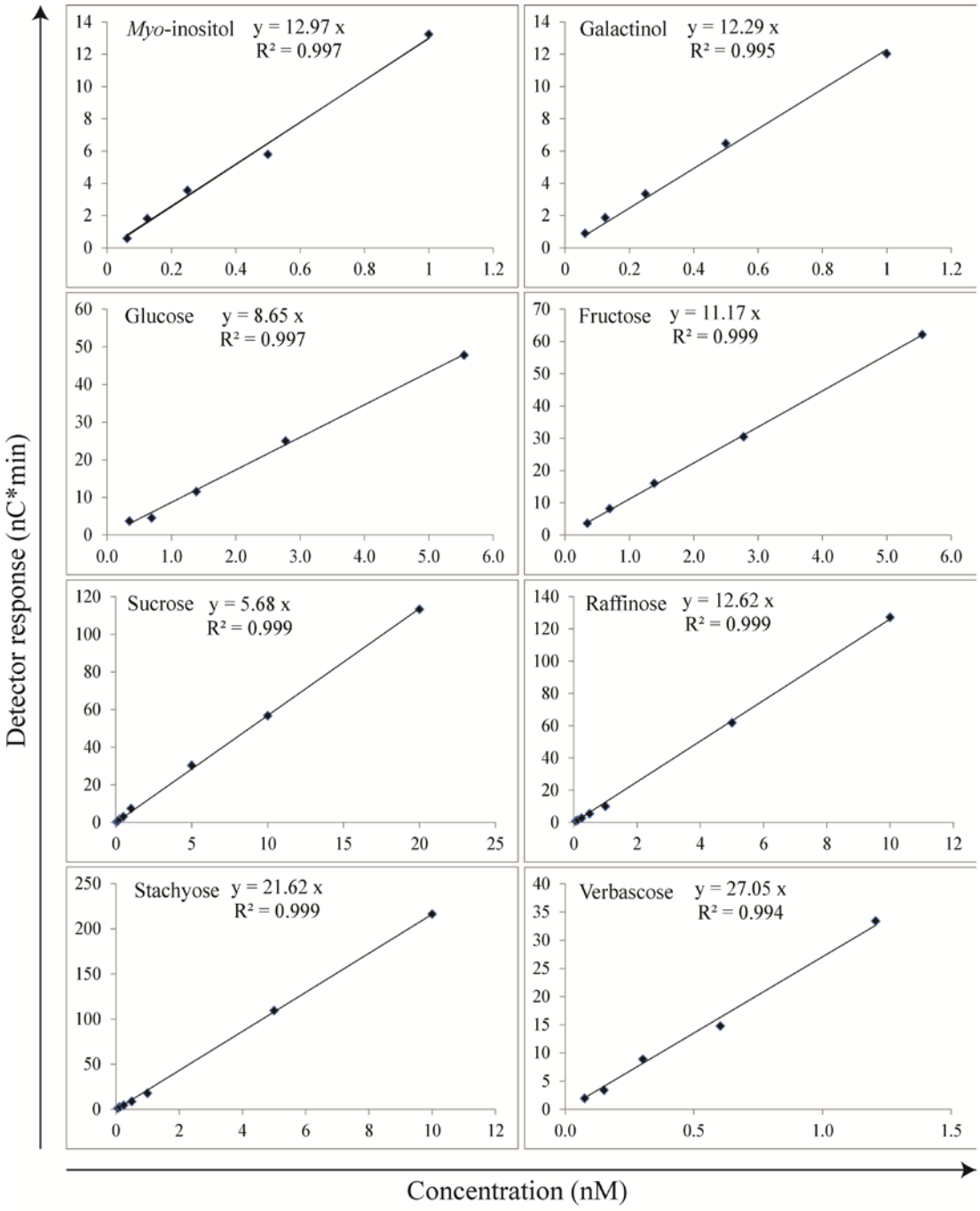


Figure 3.1 Calibration curves for *myo*-inositol, galactinol, glucose, fructose, sucrose, raffinose, stachyose and verbascose.

Table 3.2 Composition of reaction for protein estimation

BSA (µL of stock*)	Nano pure water (µL)	1x Bradford dye (µL)	Remark
0	100	900	Blank
20	80	900	Standard curve
40	60	900	
60	40	900	
80	20	900	
100	0	900	
10 µL of sample	90	900	Sample

* stock solution = 1 µg/10 µL

Table 3.3 Composition of an optimum enzyme activity reaction mixture for different RFO biosynthetic enzymes along with specific conditions

Factors	**Enzymes***				
	GS	**RS**	**STS**	**VS**	**Non-galactinol**
Crude protein (µg)	10	30	30	40	40
Other compounds					
HEPES-NaOH buffer (mM)	25	25	25	25	25
DTT	10	10	10	10	10
$MnCl_2$ (mM; Cofactor for GS)	5	-	-	-	-
Substrate conc. (mM)					
Myo-inositol	20	-	-	-	-
UDP-galactose	10	-	-	-	-
Galactinol	-	10	10	10	-
Sucrose	-	40	-	-	-
Raffinose	-	-	20	-	20
Stachyose	-	-	-	20	-
Nano pure water	accordingly				
Volume (µL)	90	50	50	50	50
pH / Temperature	7.0 / 25 °C				
Time	10 min	1 h	1 h	1 h	1 h

*GS = Galactinol Synthase; RS = Raffinose Synthase; STS = Stachyose Synthase; VS = Verbascose Synthase; GGT = Galactan:galactan galactosyl transferase

(40 µg for VS) of crude cellular protein, 25 mM HEPES-NaOH buffer (pH 7.0), 10 mM galactinol, 10 mM DTT and sucrose (40 mM for RS)/raffinose (20 mM for STS)/stachyose (20 mM for VS). The reaction mix was incubated at 25 °C for 60 min. To determine non-galactinol activity synthesizing RFO, substrate mix [25 mM HEPES-NaOH buffer (pH 7.0), 10 mM DTT and 20 mM of raffinose/stachyose] together with 40 µg of crude protein was incubated at 25 °C for 60 min. Besides enzyme reactions, two controls were also prepared for each enzyme assay: positive (contained only crude protein) and negative (substrates only). The substrates and crude cellular protein concentrations in controls were same as in enzyme activity assay reactions. After incubation, reactions were stopped by adding 50 µL of 95 % (v/v) ethanol and boiling for 1 min to denature proteins. The resulted mixture was centrifuged at 12,000*g* for 10 min. The supernatant was treated by Dowex AG-1×8, H^+ resin and mixture was shaken for 30 min. After resin treatment, enzyme assay mixture was centrifuged at 12,000 ×*g* for 10 min and supernatant was vacuum dried using Speedvac® Concentrator and universal vacuum system (Thermo Savant, Holbrook, NY, USA). The vacuum dried samples were dissolved in 500 µL of nano pure water, vigorously mixed and centrifuged at 10,000 ×*g* for 10 min. 125 µL of supernatant was diluted to a final volume of 500 µL using nano pure water. The diluted sample (injection volume = 10 µL) was used to determine the concentration of individual RFO members as described above.

4. A RELIABLE AND RAPID METHOD FOR SOLUBLE SUGARS AND RFO ANALYSIS IN CHICKPEA USING HPAEC-PAD AND ITS COMPARISON WITH HPLC-RI

4.1 Study 1*

In this study, a modified HPAEC-PAD (High performance anion exchange chromatography with pulsed ampreometric detector) based analytical method was optimized to determine soluble sugars concentration in chickpea seeds.

*Gangola, M. P., Jaiswal, S., Khedikar, Y. P. and Chibbar, R. N. 2014. A reliable and rapid method for soluble sugars and RFO analysis in chickpea using HPAEC–PAD and its comparison with HPLC–RI. Food Chem. 154: 127-133.

4.2 Abstract

A high performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) was optimized to separate with precision, accuracy and high reproducibility soluble sugars including oligosaccharides present in pulse meal samples. The optimized method within 20 min separated *myo*-inositol, galactinol, glucose, fructose, sucrose, raffinose, stachyose and verbascose in chickpea seed meal extracts. Gradient method of eluting solvent (sodium hydroxide) resulted in higher sensitivity and rapid detection compared to similar analytical methods. Peaks asymmetry equivalent to one and resolution value ≥1.5 support column's precision and accuracy for quantitative determinations of soluble sugars in complex mixtures. Intermediate precision determined as relative standard deviation (1.8 - 3.5 %) for different soluble sugars confirms reproducibility of the optimized method. The developed method has superior sensitivity to detect even scarcely present verbascose in chickpea. It also quantifies *myo*-inositol and galactinol making it suitable both for RFO related genotype screening and biosynthetic studies.

4.3 Introduction

Carbon storage and translocation is an important phenomenon in plants to sustain their growth and development. Raffinose family oligosaccharides (RFO) or α-galactosides constitute a group of soluble, non-reducing carbohydrates used to transport and store carbon in plant families like Cucurbitaceae, Leguminosae, Lamiaceae, Oleaceae and Scrophulariaceae (Sprenger and Keller 2000). RFO are non-structural carbohydrates characterized by the presence of α (1→6) linkage between the galactosyl and sucrose residues (Tapernoux-Luthi et al. 2004). RFO are ubiquitous in plant kingdom and only second to sucrose in concentration among soluble sugars (Frias et al. 1999). Raffinose is the first member of this family followed by stachyose and verbascose.

RFO mainly accumulate in seeds during their later stages of development (Peterbauer et al. 2001) and play important physiological roles in plants, such as inducing desiccation tolerance, seed longevity (Koster 1991), detoxification of reactive oxygen species (Bolouri-Moghaddam et al. 2010) and tolerance against biotic and abiotic stresses (Liu et al. 2007; Cho et al. 2010). However, human and mono-gastric animals cannot digest RFO and escape small intestinal digestion and absorption due to lack of α-galactosidase enzyme required for the hydrolysis of α (1→6) glycosidic linkages (Saunders and Wiggins 1981). However, microflora of large intestine metabolizes RFO and produce substantial amounts of carbon dioxide, hydrogen, and small quantities of methane. Therefore, consumption of food with

high RFO in humans causes stomach discomfort, flatulence and diarrhea (Veldman et al. 1993). It also leads to nutrient deficiency in animal feed due to decreased intestinal absorption (Wiggins 1984) and hence reduced availability of metabolizable energy (Coon et al. 1990). In human diet RFO stimulate growth of some therapeutic microorganisms such as *Bifidobacterium* spp. (a lactic acid bacteria) in large intestine and are considered as prebiotics (Peterbauer and Richter 2001; Trojanová et al. 2006). In pulse crops, α-galactosides contribute up to 9.5 % of total dry matter that reduces their acceptability in human diet, particularly in western countries (Martínez-Villaluenga et al. 2008; Alonso et al. 2010). In chickpea, stachyose (0.18 − 2.38 g/100 g) is reported as one of the major soluble sugars (Gangola et al. 2013). Therefore, to reduce the negative effects of pulses in human diet and increase its consumption, seed RFO concentration needs to be reduced without affecting their beneficial attributes in humans and plants. To achieve this objective a rapid, precise and accurate method is needed to determine the concentration of three RFO members, raffinose, stachyose and verbascose in small samples of pulse seeds.

Chromatographic separation followed by visual detection [thin layer chromatography (TLC); Jones et al. 1999] and high performance liquid chromatography (HPLC) with refractive index (RI) or pulse amperometeric detector (PAD) are the most commonly used methods to detect and quantify RFO members (Table 4.1). To precisely and accurately determine RFO members' concentration in chickpea seed meal samples, we compared different methods reported in the literature (Table 4.1). High performance anion exchange chromatography (HPAEC) with CarboPac PA100 column and PAD (HPAEC-PAD) was optimized using gradient of sodium hydroxide as eluent. The optimized method was assessed for its accuracy, precision and reproducibility in separating soluble sugars of complex chickpea seed meal samples prepared from several genotypes.

4.4 Materials and methods

4.4.1 Sugar standards

Standards of *myo*-inositol, galactinol, glucose, fructose raffinose, stachyose and verbascose were purchased from Sigma-Aldrich (Oakville, ON, Canada), while sucrose was from EMD Chemicals (Mississauga, ON, Canada).

4.4.2 Plant Material

Seeds from 17 genotypes (ICCV 2, ICC 4951, ICC 4918, ICC 4958, ICC 1882, ICC 283, ICC 8261, ICC 506-EB, ICC 16382, ICC 995, ICC 5912, ICC 6263, ICC 1431,

Table 4.1 Comparison of different methods reported in the literature to determine soluble sugars concentration

Properties	Type of chromatography					
	HPLC				HPAEC-PAD	
Seeds used	*Lens culinaris*[a]	*Cicer arietinum*[b]	*Cicer arietinum*[c]	*Lens culinaris*[d]	*Lens culinaris*[a]	*Lupinus* sp.[e]
Detector	RI[f]	Differential Refractometer	RI[f]	RI[f]	PAD[f]	PAD[f]
Mobile phase (isocratic elution)	Acetonitrile : water (75:25)	Acetonitrile : water (80:20)	Acetonitrile : water (75:25)	CaN_2EDTA (0.0001 M)	145 mM NaOH	170 mM NaOH
Flow rate (mL/min)	2.0	0.9	1.0	0.5	1.0	1.0
Retention time (min)						
Sucrose	5.0	10.2	8.5*	NA	7.9	8.45
Raffinose	9.0	18.8	13.5*	6.7	15.1	14.02
Stachyose	16.5	38.6	24.0*	6.1	17.0	16.17
Verbascose	25.0*	NA	27.5*	5.7	24.5*	20.95
LOD/LOQ[g] (unit)	(µg/mL)	NA	NA	NA	(ng/mL)	(µg/mL)
Sucrose	450/NA				138/NA	1.39/4.80
Raffinose	170/NA				16/NA	0.59/2.07
Stachyose	1380/NA				43/NA	0.64/2.25
Verbascose	NA/NA				NA/NA	0.62/2.19

[a] Frias et al. (1994); [b] Sánchez-Mata et al. (1998); [c] Xioli et al. (2008); [d] Tahir et al. (2011); [e] Bansleben et al. (2008)

[f] RI and PAD stand for refractive index and pulsed amperometric detector, respectively.

[g] LOD/LOQ is the level of detection/quantification.

* not mentioned in the manuscript but estimated from the chromatogram shown.

NA = not available in the publication.

ICCV 93954, ICCV 05530, ICC 4991 and ICCV 04516) of chickpea (*Cicer arietinum* L.) were used to validate the proposed method. Seeds were ground into a fine meal using a UDY cyclone mill (Udy Corporation, Fort Collins, CO, USA) to pass through a 0.5 mm sieve. The seed meal was used to extract and measure total soluble sugars including RFO.

4.4.3 Estimation of total RFO

Total RFO concentration in chickpea seed meal (500 ± 5 mg) was determined by stepwise hydrolysis of complex RFO into D-galactose, D-fructose and D-glucose molecules using α-galactosidase (from *Aspergillus niger*) and invertase (from yeast), using raffinose/sucrose/glucose assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). The resulting D-glucose was estimated using glucose oxidase/peroxidase reagent (GOPOD) that produced a red colored quinoneimine whose concentration was determined at $A_{510\ nm}$ using a spectrophotometer. This method determined concentration of different members of raffinose family as a group. Glucose, sucrose and total RFO were calculated as:

D-Glucose, mmoles/100 g = ΔA x F x 250 x 200 x 1/1000

Sucrose, mmoles/100 g = (ΔB-ΔA) x F x 250 x 200 x 1/1000

Total RFO, mmoles/100 g = (ΔC-ΔB) x F x 250 x 200 x 1/1000

In these equations, ΔA = GOPOD absorbance for D-glucose, ΔB = GOPOD absorbance for D-glucose + sucrose, ΔC = GOPOD absorbance for D-glucose + sucrose + Galactosyl-sucrose oligosaccharides. "F" is a factor to convert absorbance into µmoles of glucose and calculated as:

F = 0.556 (µmoles of glucose)/GOPOD absorbance for 0.556 µmoles of glucose.

The glucose standard provided with the kit has 0.556 µmoles in 100 µL of its solution. Hence, reaction of 100 µL of this glucose with GOPOD reagent gives absorbance for 0.556 µmoles of glucose. Number 250 = conversion of 50 mL of extract to 0.5 g of sample, 200 = conversion from 0.5 to 100 g of sample, 1/1000 = conversion from µmoles to mmoles. Total RFO concentration was calculated on molar basis as one mole of each oligosaccharide contains one mole of D-glucose.

4.4.4 Extraction of soluble sugars

Soluble sugars were extracted using methods described by Frias et al. (1994) and Sánchez-Mata et al. (1998) with some modifications (Tahir et al. 2011). In brief, 500 mg of chickpea seed meal was weighed in a 15 mL disposable plastic tube and 10 mL of 80 % (v/v) ethanol was added. The tube was thoroughly stirred on a vortex mixer and incubated in a

shaking water bath at 60 °C for 45 min with intermittent mixing at 15 min interval. Thereafter, the slurry was centrifuged at 12,100 × *g* for 10 min and supernatant was collected in a separate tube. The pellet was used to re-extract soluble sugars as described above. Finally, supernatants from three sequential extractions were pooled and used for purification of soluble sugars as described in the next step.

4.4.5 Purification of soluble sugars

The supernatant collected from the above described protocol was filtered through a prewashed C18 (Honeywell Burdick & Jackson, Muskegon, MI, USA) cartridge using a vacuum manifold to remove proteins and lipids present in the soluble sugar extract. Cartridge was first washed with 5 mL of 99 % (v/v) methanol followed by 5 mL of distilled water and 3 mL of sample extract, respectively. After washing, 3 mL of sample extract was passed through the column and purified extract was collected. A 1.6 mL aliquot of this extract was used for vacuum drying using Speedvac® Concentrator and universal vacuum system (Thermo Savant, Holbrook, NY, USA).

4.4.6 Sample preparation for HPLC and HPAEC

For HPLC-RI and HPAEC-PAD, vacuum dried samples were dissolved in 0.5 and 1.0 mL of nano pure water, respectively. The sugar solutions were vigorously mixed and centrifuged at 10,000 × *g* for 10 min. The supernatant was filtered through a 0.2 µm filter (Phenex-NY 4 mm Syringe Filters, Phenomenex, Torrance, CA, USA) using 1 mL syringe with tuberculin slip tip (Thermo Medical Co., Somerset, NJ, USA). For HPLC-RI, 150 µL of filtrate was directly used to analyze RFO whereas for HPAEC-PAD, 125 µL of filtrate was first diluted to final volume of 500 µL using nano pure water and the diluted sample was used to determine the concentration of individual RFO members.

4.4.7 Instrumentation and chromatographic conditions

Liquid chromatography analysis was performed on a Waters HPLC-RI system (Waters Co., Mississauga, ON, Canada). A LC 60F pump equipped with a Waters 600 system controller was used to deliver mobile phase into guard and analytical column. Sample injection was performed in a Waters 717 plus autosampler with 10 µL injection volume/sample. The soluble sugars were separated by Rezex RSO Oligosaccharide F analytical column (200 x 10 mm) preceded by Rezex RSO Oligosaccharide F guard column (60 x 10 mm) and a Carbo-Ag security guard cartridge (4 x 3 mm) (Phenomenex, Torrance,

CA, USA). The separated sugars were eluted using nano pure water as a mobile phase with a flow rate of 0.1 mL/min. The eluted sugars were detected by Waters 2414 refractive index detector along with data acquisition software (Quick Start, Empower 1154 Chromatography software, Waters Co., Mississauga, ON, Canada). The total run time was 160 min/sample with a 30 min washing interval between two sample runs.

Anion exchange chromatography was carried out on Ion Chromatography System 5000 [ICS 5000 consisting of autosampler, single gradient pump (Model SP-5) and electrochemical detection cell with disposable working gold electrode and Ag/AgCl reference electrode, Dionex Canada Ltd., Oakville, ON, Canada]. For sample integration, cleaning and reactivation of the detector, gold standard PAD waveform was used at four different potentials for a total time of 500 milliseconds (msec). In this particular type of waveform, four working potentials (E) were used: +0.1 V for 400 msec (E1), -2.0 V for 20 msec (E2), +0.6 V for 10 msec (E3) and -0.1 V for 70 msec (E4). The complete assembly was controlled by Chromeleon 7.0 software (Dionex Canada Ltd., Oakville, ON, Canada) installed on a Dell Optiplex 780 desktop. Two different strategies using CarboPac PA200 (3 x 250 mm) and CarboPac PA100 (4 x 250 mm) analytical columns (Thermo Fisher Scientific, Stevens Point, WI, USA) were followed to find the best separation among soluble sugars. Both analytical columns were used at 30 °C along with CarboPac PA200 (3 x 50 mm) and CarboPac PA100 (4 x 50 mm) guard columns, respectively. Elutant concentration gradient methods used for both the columns are summarized in Table 3.1. Solvent bottles were continuously supplied with helium gas to prevent bicarbonate contamination. Chromatography system, analytical and guard columns along with disposable gold electrode, glass vials for sample injection and sodium hydroxide solution (50 % w/w; used for mobile phase preparation) were purchased from Thermo Scientific (Bannockburn, IL, USA).

4.4.8 Assessment of analytical method

The performance of optimized method was evaluated by calculating the coefficient of determination (R^2), level of detection (LOD) and level of quantification (LOQ). To interpret accuracy and precision, recovery per cent, repeatability and intermediate precision was calculated. The suitability of chromatography column was assessed by determining peak resolution, peak asymmetry and plate number. As a first step, calibration curves using five concentrations [62.5, 125, 250, 500 and 1000 ng/ injection volume (10 µL)] for each sugar was prepared to develop a regression equation and calculate R^2. Further, on the basis of calibration curves, LOD and LOQ scores were calculated using formulae as per ICH

harmonized tripartite guidelines (2005; http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf).

To estimate recovery per cent, repeatability and intermediate precision of various soluble sugars, known concentrations (0.05, 0.03 and 0.01 mg/mL) of standards were analyzed on the same day (to calculate repeatability) and on different days (to evaluate intermediate precision). Repeatability and intermediate precision were expressed in terms of per cent relative standard deviation. Recovery per cent (R%) for different analytes was calculated using R% = [(observed concentration × 100) / actual concentration]. Peak resolution (*R*) and asymmetry (*A*) were measured by using guidelines by European Pharmacopeia. In addition, relative per cent of different sugars in chickpea samples were also calculated as relative% = (peak area of sugar × 100) / total area under identified and unidentified peaks.

4.4.9 Statistical analysis

Data with three replicates was analyzed to calculate analysis of variance (ANOVA) and correlation coefficient using MINITAB 14.0 statistical software (Minitab Inc., State College, PA, USA). General linear model was applied to calculate ANOVA and resulted MSS (mean sum of squares) for genotype and replication were utilized to confirm applicability and reproducibility of the method.

4.5 Results and Discussion

4.5.1 Comparison and selection of suitable method

Three methods were compared to separate and quantify different soluble sugars in a mixture of standard solutions (sucrose, raffinose, stachyose and verbascose) and sugar extract of chickpea seed meals (Table 4.2). The first method was based on separation of sugars by size exclusion chromatography (SEC) and detection using a RI detector (HPLC-RI). Chromatogram resulted from SEC showed a good separation among raffinose, stachyose and verbascose in standards' mixture having retention time of 86.7, 73.9 and 62.8 min, respectively (Figure 4.1a). However, this method was not suitable for chickpea seed meal samples as peaks were not well separated (Figure 4.1b). The other disadvantage of HPLC-RI was the long run time/sample (160 min run time + 30 min washing time). This method also suffered with high back pressure problem due to blockage of guard column resulting in frequent change of security guard cartridge after every ~50 samples. Second method using HPAEC-PAD with CarboPac PA200 column was able to separate and quantify soluble sugars within 6 min of total run time (25 min) in both standard mix (Figure 4.1c) and chickpea

Table 4.2 Comparison of HPLC-RI and HPAEC-PAD methods to separate soluble sugars and raffinose family oligosaccharides in chickpea seed meal extracts

Properties	HPLC-RI (Waters)	HPAEC-PAD (Dionex ICS 5000)	
Chromatography type	Size exclusion	Ion exchange	Ion exchange
Column	Rezex RSO Oligosaccharide F	CarboPac PA200	CarboPac PA100
Elution	Isocratic	Gradient	Gradient
Mobile phase	Water	10 – 80 mM NaOH	20 – 200 mM NaOH
Flow rate (mL/min)	0.1	0.5	1.0
Run time (min)	160	25	35
Separation	Good	Good	Good
Retention time (min)			
Raffinose	86.7	4.8	16.1
Stachyose	73.9	5.1	17.0
Verbascose	62.8	6.0	19.5
Reproducibility	Good	Poor	Good
Back pressure problem	Yes	No	No

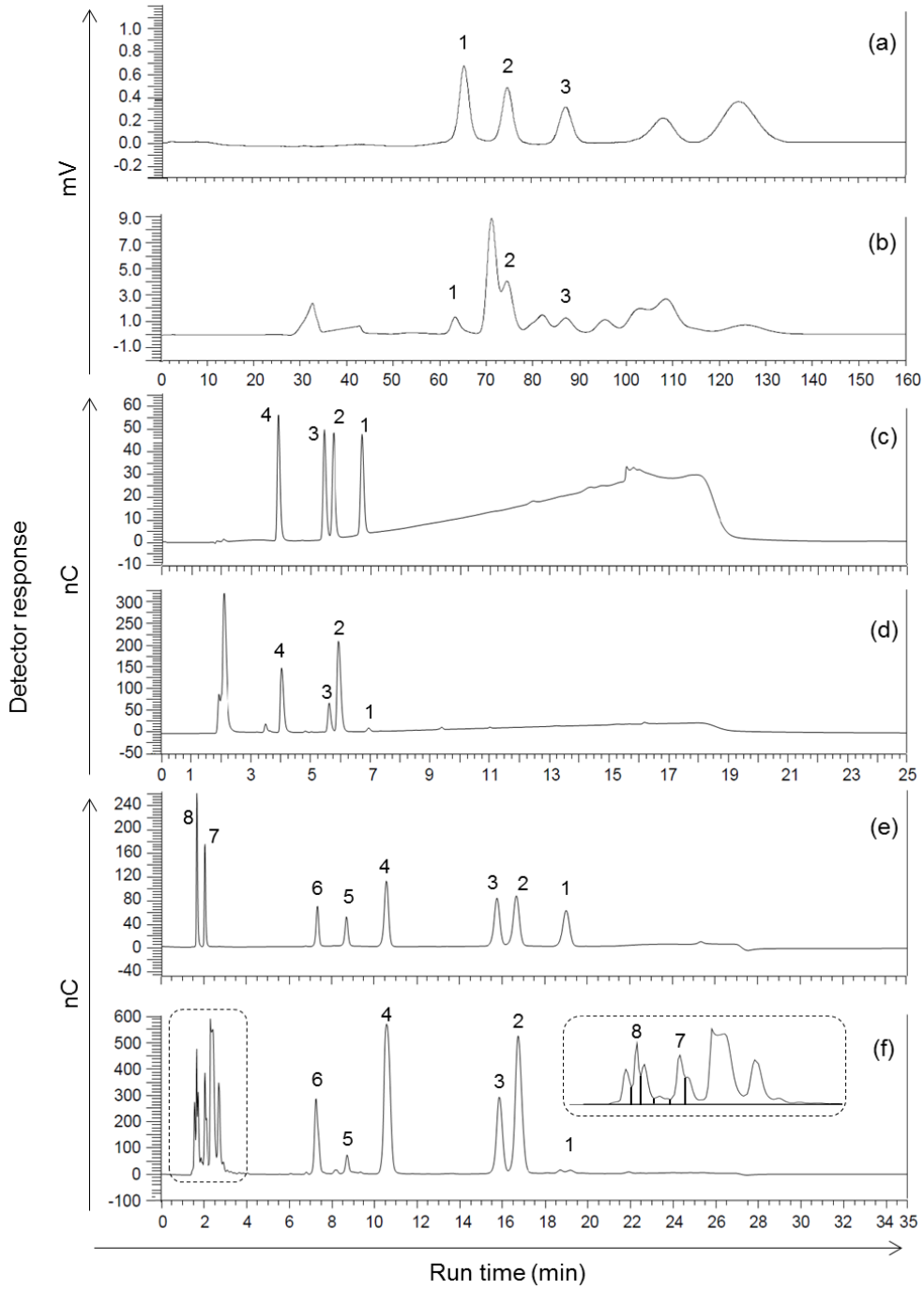


Figure 4.1 Comparison of three chromatographic methods to determine soluble sugars in sugar standards and chickpea seed meal extracts.

Chromatograms of different sugars (1-verbascose, 2-stachyose, 3-raffinose, 4-sucrose, 5-fructose, 6-glucose, 7-galactinol, 8-*myo*-inositol and unlabeled peaks are unknown) resulted from, (a) & (b) HPLC-RI, (c) & (d) HPAEC-PAD with CarboPac PA200 column and (e) & (f) HPAEC-PAD with CarboPac PA100 column where, (a), (c) & (e) represent pattern for standards and (b), (d) & (f) show separation of sugars from chickpea seed meal extracts.

sample (Figure 4.1d). Sucrose, raffinose, stachyose and verbascose were detected at 4.1, 4.8, 5.1 and 6.0 min, respectively. During assessment, shift in peak retention time was observed suggesting poor reproducibility of this method thus causing imprecision in quantifying different sugars. Compared to CarboPac PA1 and CarboPac PA100, CarboPac PA200 is composed of smaller substrate (5.5 µm) and latex beads (43 nm) beads which allows the use of a wide range of eluent concentration to separate small sugars with improved resolution (DIONEX application update 150). However, it is difficult to speculate the specific reason for inefficient performance of CarboPAC PA200 in this study; it could be due to potential difference arising due to finer changes in the column matrix.

Third method employing HPAEC-PAD with CarboPac PA100 column had a slightly longer run time (35 min) compared to 25 min with CarboPac PA200 column, but achieved good separation of soluble sugars both in standard mix (Figure 4.1e) and chickpea seed meal samples (Figure 4.1f). The optimized method separated glucose (7.4 min), fructose (8.8 min), sucrose (10.8 min), raffinose (16.1 min), stachyose (17.0 min) and verbascose (19.5 min) using a total runtime of 35 min (complete separation in < 20 min). In addition, this method can also quantify *myo*-inositol (retention time 1.7 min) and galactinol (retention time 2.0 min) concurrently. Galactinol (peak 7) and *myo*-inositol (peak 8) can be separated in standard but in plant extract we suspect other stereoisomers of cyclohexanehexol eluting in the same region as *myo*-inositol, resulting in its poor separation. Modifications in the run time and solvent concentration were tried but no success in resolving this issue was achieved. The optimized method was further assessed regarding sensitivity, accuracy and precision.

4.5.2 Assessment of optimized analytical method

The optimized gradient method was examined for its applicability to estimate soluble sugars including glucose, fructose, sucrose, raffinose, stachyose and verbascose. At first, calibration curves for these sugars were prepared along with coefficients of determination (R^2) and regression line equations which were utilized to quantify sugars in a sample. R^2 value (>0.99) for all sugars confirmed the linearity of calibration curves. LOD and LOQ were observed in a range of 3 – 48 and 9 – 144 ng/mL, respectively. To the best of our knowledge, these scores represent lowest concentrations of detection reported to-date. Recovery per cent was observed to vary from 95.8% to 103.2 %. Repeatability and intermediate precision was expressed in terms of relative standard deviation per cent (RSD%). The optimized method showed a variation from 1.1 % to 2.1 % and 1.8 % to 2.1 %, for repeatability and intermediate precision, respectively. Peaks for all the standards were

found to be ideal as asymmetry was ~1 supporting column precision to determine quantitative results. The peak resolution value that was >1.5 in all cases indicated complete separation among all sugars in standard mix and chickpea seed meal. It also proved the column's utility to separate sugars from complex mixtures. In addition, relative per cent of different identified and unidentified peaks was also calculated (Table 4.3). The area under identified peaks was about 64.5 % while unidentified peaks shared about 35.5 % of total peak area.

4.5.3 Comparison with other reported methods

Many reports are available to separate RFO using various chromatography techniques. Frias et al. (1994) confirmed the higher sensitivity of HPAEC-PAD while comparing it with HPLC-RI using lentil (*Lens culinaris*) seeds. The sugar separations were performed on CarboPac PA100 column (4 x 250 mm) using an isocratic method with 145 mM NaOH as mobile phase at a flow rate of 1 mL/min. This method did not show good separation specifically between raffinose and stachyose in chickpea seed meal sample. Bansleben et al. (2008) also reported a CarboPac PA10 column based HPAEC-PAD method using 170 mM NaOH as mobile phase at 1 mL/min flow rate. In both the reports, the resulting retention times of sugars are comparable to that of proposed method. However, using a gradient of eluting solvent could be responsible for higher sensitivity of detection achieved by the optimized method. Introduction of gold standard PAD waveform may be another reason for higher detection sensitivity of the optimized method.

Sánchez-Mata et al. (1998) developed a modified HPLC method to determine soluble sugars' concentration in chickpea seed meal. However, in this method the chromatographic separation of raffinose and stachyose at 18.8 and 38.6 min, respectively, was much slower compared to the method optimized in this report. Later, Xioli et al. (2008) separated chickpea seed meal soluble sugars on Sugar-D column (4.6 x 250 mm, Nacalai Tesque Inc., Japan) in which acetonitrile–water (75:25; v/v) was used as mobile phase with a flow rate of 1.0 mL/min. The sugar separation was good, but the retention times were higher (8.5, 13.5, 24.0 and 27.5 min for sucrose, raffinose, stachyose and verbascose, respectively) compared to the optimized method in this study. A major advantage of the method developed in this study is the ability to detect and determine verbascose concentration which is present in very low concentration in chickpea seeds. Besides this, *myo*- inositol and galactinol concentration were also determined using proposed gradient method that will assist in exploring the RFO biosynthesis by examining substrate and product level in the same sample (Figures 4.1e and 4.1f). *Myo*-inositol is a substrate for galactinol formation, the first committed step in RFO

Table 4.3 Assessment of analytical method regarding linearity, sensitivity, accuracy, precision and column evaluation

Component	t_R[a] (min)	R^2[a]	LOD[b] (µM)	LOQ[b] (µM)	Recovery (%)	Repeatability (RSD%)[c]	*IP*[b] (RSD%)	Peak Asymmetry[d]	Peak Resolution[d, e]	Number of plates	Relative%
Glucose	7.4	0.996	0.266	0.799	103.23 ± 1.2	1.8	2.9	1.0 (1.1)	0.0 (0.0)	16476	4.49 ± 0.96
Fructose	8.8	0.992	0.139	0.416	97.3 ± 2.8	2.1	3.5	1.0 (0.9)	5.4 (5.1)	17366	0.32 ± 0.06
Sucrose	10.8	0.997	0.099	0.298	97.2 ± 1.4	1.1	2.4	0.9 (1.0)	10.3 (11.1)	14102	19.29 ± 2.38
Raffinose	16.1	0.999	0.008	0.025	96.0 ± 3.8	1.6	1.8	1.0 (1.0)	22.8 (24.4)	17537	10.19 ± 0.69
Stachyose	17.0	0.998	0.023	0.066	95.8 ± 2.0	1.8	2.3	0.9 (1.1)	24.1 (24.8)	17604	22.39 ± 2.25
Verbascose	19.5	0.994	0.004	0.011	100.0 ± 2.0	2.0	2.3	0.9 (1.0)	28.3 (24.7)	18922	0.92 ± 0.11
n[f]		3	3	3	3 x 3	3 x 3	3 x 3	3	3	3	10

[a] t_R represents retention time while R^2 indicates coefficient of determination.

[b] LOD and LOQ stand for level of detection and quantification whereas *IP* is for intermediate precision.

[c] RSD is used as acronym for Relative Standard Deviation.

[d] Values outside and inside the brackets are calculated from standards and chickpea samples, respectively.

[e] For peak resolution, glucose was considered as reference peak.

[f] n represents the number of replications.

biosynthesis, whereas galactinol can be used as a substrate for the biosynthesis of all the members of raffinose family oligosaccharides (Martínez-Villaluenga et al. 2008).

4.5.4 Validation of optimized method with chickpea seed meal extracts

To validate the utility of method, soluble sugars extracted from seventeen chickpea cultivars varying in total RFO concentration (3.30 to 4.91 mmol/100 g chickpea seed meal) were analyzed using the optimized method (Table 4.4). HPAEC-PAD analysis revealed the presence of stachyose (1.88 – 2.83 mmol/100 g chickpea seed meal) as major RFO in chickpea seeds followed by raffinose (1.22 – 1.87 mmoles/100 g chickpea seed meal) and verbascose (0.06 – 0.14 mmol/100 g chickpea seed meal). A strong positive correlation was found between total RFO and individual concentration of raffinose (r = 0.87), stachyose (r = 0.91) and verbascose (r = 0.88) significant at $P \leq 0.001$ confirming the utility of the analytical method for screening chickpea germplasm for variation in soluble sugars including RFO constituents. Sucrose was found as major soluble sugar in chickpea seeds showing variation from 4.51 to 7.90 mmol/100 g of chickpea seed meal. In most of the genotypes, total RFO concentration was found a little higher than the sum of raffinose, stachyose and verbascose that might be due to indirect estimation of total RFO using enzyme hydrolysis or presence of some unidentified member of this family in chickpea.

Glucose and fructose were detected in a range from 0.62 to 1.68 and 0.03 to 0.09 mmol/100 g of chickpea seed meal, respectively. *Myo*-inositol and galactinol were also determined varying from 0.32 to 0.83 and 0.31 to 0.69 mmol/100 g of chickpea seed meal, respectively. The results for soluble sugars are in agreement with previous reports by Sánchez-Mata et al. (1999) and Xioli et al. (2008) describing sucrose as major soluble sugar and stachyose as major RFO in chickpea seed meal. All the components of chickpea seeds determined using the proposed method showed significant difference among genotypes whereas difference between replications was non-significant (Table 4.4), which further emphasizes reproducibility of the method.

4.6 Conclusion

HPAEC coupled with pulsed amperometric detection (HPAEC-PAD) has been optimized to detect a wide range of soluble sugars extracted from chickpea seed meal. Present study showed the higher sensitivity and shorter run time of HPAEC-PAD compared to HPLC-RI for analyzing the members of raffinose family oligosaccharides and other soluble sugars. The described method is able to separate glucose, fructose, sucrose, raffinose, stachyose,

Table 4.4 Concentrations of *myo*-inositol, galactinol and soluble sugars in seed meal extract in seventeen chickpea genotypes

Genotypes	Concentration (mmoles/100 g of chickpea seed meal on fresh weight basis) ± Standard Deviation								
	myo-inositol	Galactinol	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Verbascose	Total RFO
ICCV 2	0.59 ± 0.19	0.60 ± 0.03	1.57 ± 0.05	0.04 ± 0.01	6.33 ± 0.28	1.87 ± 0.14	2.60 ± 0.10	0.11 ± 0.02	4.91 ± 0.14
ICC 8261	0.39 ± 0.17	0.69 ± 0.03	1.28 ± 0.15	0.04 ± 0.01	4.81 ± 0.35	1.82 ± 0.14	2.72 ± 0.08	0.14 ± 0.01	4.84 ± 0.12
ICC 4951	0.37 ± 0.05	0.49 ± 0.13	1.24 ± 0.26	0.03 ± 0.01	5.41 ± 0.20	1.63 ± 0.07	2.83 ± 0.06	0.12 ± 0.01	4.70 ± 0.12
ICC 4918	0.57 ± 0.18	0.49 ± 0.10	1.07 ± 0.21	0.04 ± 0.01	4.86 ± 0.22	1.55 ± 0.14	2.44 ± 0.09	0.11 ± 0.02	4.48 ± 0.21
ICC 16382	0.61 ± 0.09	0.60 ± 0.09	0.62 ± 0.14	0.06 ± 0.02	4.67 ± 0.11	1.52 ± 0.08	2.57 ± 0.07	0.12 ± 0.02	4.48 ± 0.22
ICC 283	0.58 ± 0.14	0.47 ± 0.10	0.98 ± 0.26	0.06 ± 0.02	4.81 ± 0.35	1.36 ± 0.08	2.54 ± 0.04	0.10 ± 0.02	4.17 ± 0.11
ICC 5912	0.61 ± 0.07	0.60 ± 0.07	1.32 ± 0.21	0.03 ± 0.01	7.90 ± 0.47	1.54 ± 0.03	2.31 ± 0.08	0.11 ± 0.02	4.08 ± 0.15
ICC 506-EB	0.80 ± 0.12	0.61 ± 0.11	1.54 ± 0.18	0.05 ± 0.01	4.58 ± 0.34	1.46 ± 0.15	2.48 ± 0.08	0.10 ± 0.03	4.05 ± 0.09
ICC 4958	0.61 ± 0.08	0.60 ± 0.09	1.68 ± 0.06	0.05 ± 0.01	6.69 ± 0.20	1.35 ± 0.15	2.21 ± 0.05	0.09 ± 0.01	4.00 ± 0.17
ICC 1882	0.71 ± 0.17	0.48 ± 0.10	1.36 ± 0.04	0.03 ± 0.01	6.25 ± 0.38	1.27 ± 0.06	2.36 ± 0.10	0.09 ± 0.03	3.97 ± 0.22
ICCV 04516	0.32 ± 0.06	0.48 ± 0.12	1.34 ± 0.17	0.05 ± 0.01	5.22 ± 0.43	1.53 ± 0.10	1.95 ± 0.17	0.06 ± 0.01	3.82 ± 0.09
ICC 1431	0.35 ± 0.18	0.38 ± 0.05	1.28 ± 0.24	0.06 ± 0.02	5.72 ± 0.56	1.46 ± 0.12	2.13 ± 0.19	0.09 ± 0.01	3.82 ± 0.21
ICC V93954	0.65 ± 0.18	0.31 ± 0.03	1.55 ± 0.07	0.03 ± 0.00	5.18 ± 0.40	1.43 ± 0.16	2.01 ± 0.23	0.09 ± 0.03	3.61 ± 0.20
ICC 6263	0.36 ± 0.19	0.42 ± 0.08	1.32 ± 0.06	0.03 ± 0.00	5.82 ± 0.41	1.27 ± 0.21	2.08 ± 0.02	0.09 ± 0.02	3.55 ± 0.18
ICC V05530	0.39 ± 0.08	0.44 ± 0.02	1.39 ± 0.23	0.09 ± 0.01	4.64 ± 0.65	1.22 ± 0.08	1.96 ± 0.08	0.08 ± 0.01	3.46 ± 0.10
ICC 4991	0.83 ± 0.15	0.61 ± 0.11	1.25 ± 0.35	0.04 ± 0.02	4.51 ± 0.31	1.23 ± 0.03	2.08 ± 0.07	0.08 ± 0.02	3.31 ± 0.06
ICC 995	0.61 ± 0.16	0.41 ± 0.09	1.15 ± 0.18	0.02 ± 0.01	5.51 ± 0.14	1.27 ± 0.16	1.88 ± 0.18	0.07 ± 0.02	3.30 ± 0.16
MSSG	1.6×10^{-3}*	2.9×10^{-3}*	4.0×10^{-3}*	1.3×10^{-5}*	0.20***	0.03***	0.07***	4.9×10^{-4}*	0.52***
MSSR	1.7×10^{-4}ns	1.3×10^{-3}ns	5.0×10^{-6}ns	3.0×10^{-7}ns	0.01ns	0.01ns	1.1×10^{-3}ns	2.2×10^{-6}ns	0.02ns

MSSG and MSSR represent mean sum of squares for genotypes and replications whereas *** and * show significance at $P \leq 0.001$ and 0.05 level, respectively. ns stands for non-significant difference.

and verbascose in a 35 min (complete separation in <20 min) of total run time and also to identify genotypes with varying RFO concentrations. It thus provides a platform for studies related to association mapping, correlation analysis and food quality improvement. The separation and precise determination of *myo*-inositol and galactinol in the same extract makes it very valuable to study RFO biosynthesis in seeds.

5. 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

5.1 Study 2*

In this study, natural variation for RFO concentration among 171 chickpea germplasms was studied. In addition, Shannon-weaver diversity index, effect of genotype, environment and their interaction on RFO concentration and heritability were also analyzed.

*Gangola, M. P., Khedikar, Y. P., Gaur, P. M., Båga, M. and Chibbar, R. N. 2013. 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. J. Agric. Food Chem. 61: 4943−4952.

5.2 Abstract

To develop genetic improvement strategies to modulate raffinose family oligosaccharides (RFO) concentration in chickpea (*Cicer arietinum* L.) seeds, we analyzed RFO and their precursor concentration in 171 chickpea genotypes from diverse geographical origins. The genotypes were grown in replicated trials over two years in field (Patancheru, India) and Greenhouse (Saskatoon, Canada). Analysis of variance revealed significant impact of genotype, environment and their interaction on RFO concentration in chickpea seeds. Total RFO concentration ranged from 1.58 to 5.31 and 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 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 use of multi-location trials based approach in chickpea seed quality improvement programs.

5.3 Introduction

Chickpea (*Cicer arietinum* L.) is the second most important pulse crop after dry beans cultivated over 11.98 million hectare area with a total production of 1.09 million tonnes around the world during 2010 (FAO STAT 2010; Upadhyaya et al. 2011). Chickpea is broadly classified into two clusters, (a) Kabuli type (white flower and large, cream-colored seeds) is usually grown in temperate regions, whereas (b) desi type (purple flower and small, dark, angular seeds) is mainly produced in semi-arid tropical regions of the world (Cobos et al. 2007; Jukanti et al. 2012). Chickpea seeds make an important nutritional contribution to the population of developing countries as they are excellent source of carbohydrate (40 - 59 %), protein (13.5 - 31.7 %), vitamins and minerals. In addition, chickpea seed constituents like PUFA (polyunsaturated fatty acid), saturated fatty acid (<1 %) and dietary fibers (about 10 %) have been associated with several beneficial health-promoting properties (Veenstra et al. 2010). Hence, chickpea is considered as part of a health promoting diet (Yust et al. 2012). However, presence of some anti-nutritional factors like raffinose family oligosaccharides (RFO) or α-galactosides reduce chickpea's acceptability in food products particularly in western countries (Alonso et al. 2010). 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 % (Martínez-Villaluenga et al. 2008). Raffinose is the first member of this family followed by stachyose and verbascose (Sprenger and Keller 2000). Some alternative RFO like lychnose and manninotriose have

been recently reported from Caryophyllacean (Vanhaecke et al. 2010) and Lamiaceae (Dos Santos et al. 2013) plants, respectively but their presence in chickpea seeds has not yet been reported. RFO represent a class of soluble but non-reducing and non-structural oligosaccharides having α (1→6) linkage between sucrose and galactosyl subunit (Tapernoux-Luthi et al. 2004). Therefore, these sugars are indigestible in human and monogastric animals as they lack α-galactosidase a hydrolyzing enzyme responsible for RFO breakdown (Reddy et al. 1984; Kumar et al. 2010). Consequently, RFO escape digestion and absorption in 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 (Saunders et al. 1981; Naczk et al. 1997; Swennen et al. 2006). 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 source in early stages of germination, translocation of photo-assimilates and abiotic stress tolerance (Turgeon 1996; Martínez-Villaluenga et al. 2008; Blöchl et al. 2008; Pukacka et al. 2009;). 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 (Gage et al. 1998). Therefore, to increase the acceptability of chickpea in human and animal diet, 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 gamma radiation exposure can be used to reduce RFO in legume seeds (Girigowda et al. 2005; Alajali et al. 2006; Han and Baik 2006). Exposure to such mechanical and chemical treatments can reduce the nutritional quality of seeds. Therefore, 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 concentration in chickpea seeds. Both genotype and environment affect accumulation of RFO concentration in chickpea seeds.

5.4 Materials and methods

5.4.1 Plant material and growing conditions

A set of 171 chickpea genotypes (116 desi and 55 kabuli type was selected from genebank of ICRISAT (International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India) based on geographic origin. These genotypes represented eight different geographic regions including chickpea's center of origin and center of diversity (Table 5.1). These genotypes were grown in field as well as in greenhouse conditions in two biological

Table 5.1 Geographical origin of chickpea genotypes used in the study

Region	No. of Genotypes	
	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. Southwest Asia	13	11
8. Sub Saharan Africa	11	5
Total	116	55

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, mean daily minimum and maximum temperature was 15.0 and 31.1 °C, respectively. The average bright sunshine hours were 8.9 with approximately 352.1 μM/m^2/sec of solar radiation. The daily mean minimum and maximum temperatures during 2009-2010 were 16.2 and 30.0 °C, respectively along with average 8.1 h of bright sunshine and approximately 333.4 μM/m^2/sec of solar radiation. These genotypes were also grown in 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 GH, the mean daily minimum and maximum temperatures were 18 and 23 °C with 18 h photoperiod and 385 μM/m^2/sec of photosynthetically active radiation.

5.4.2 Determination of Total RFO concentration

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 colored quinoneimine whose concentration was determined at $A_{510\ nm}$ using 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 molar basis as one mole of each oligosaccharide contains one mole of D-glucose.

5.4.3 HPAEC-PAD analysis of chickpea seeds soluble sugars

Soluble sugars from chickpea seed meal (500 ± 5 mg) were extracted as described by Frias et al. (1994) and Sánchez-Mata et al. (1998) with some modifications (Tahir et al. 2011). For quantification of each member of 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 disposable gold electrode, Ag/AgCl reference electrode and CarboPac PA100 (4 x 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.

5.4.4 Data and statistical analysis

Box plot analysis was employed to represent variation among geographical regions for selected seed constituents (Figures 5.1 and 5.2). Shannon-Weaver diversity index (SDI) was calculated to analyze the diversity present in each geographical region (Tables 5.2 and 5.3). For both SDI and box plot analysis, pooled data from all three growing environments was used.

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

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

Where, n represents total number of phenotypic classes and P_i is the proportion of total number of entries in the i^{th} class. Phenotypic classes were prepared by using MINITAB 14 statistical software.

5.5 Results and discussion

5.5.1 Diversity pattern among geographical regions

On the basis of their origin, desi and kabuli genotypes were grouped into seven geographical regions. In desi genotypes, South Asian region showed highest diversity index (0.33 - 0.87) for all the selected seed constituents, as this region has maximum representation (68 genotypes contributing about 59 % to total desi genotypes) in the germplasm collection (Figure 5.1). Consequently, South Asian genotypes showed the highest variation in seed constituents and it ranged from 0.01 - 0.10, 0.03 - 0.31, 0.03 -0.42, 0.01 - 0.05, 0.60 - 2.93, 0.09 - 1.19, 0.18 - 2.36 and 0.01 - 0.13 g/100 g for *myo*-inositol, galactinol, glucose, fructose, sucrose, raffinose, stachyose and verbascose with an average value 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 5.1). Southwest Asia is one of chickpea's primary centers of origin whereas Sub Saharan Africa contained genotypes from Ethiopia considered as secondary center of genetic diversity for chickpea. Therefore, second highest SDI for all the traits were expressed by genotypes either from Southwest Asia or Sub Saharan Africa. SDI ranged from 0.29 - 0.76, 0.13 - 0.68, 0.15 - 0.68, 0.27 - 0.68 and 0.23 - 0.51 for Southwest Asia, Sub Saharan Africa, North Africa, Europe and

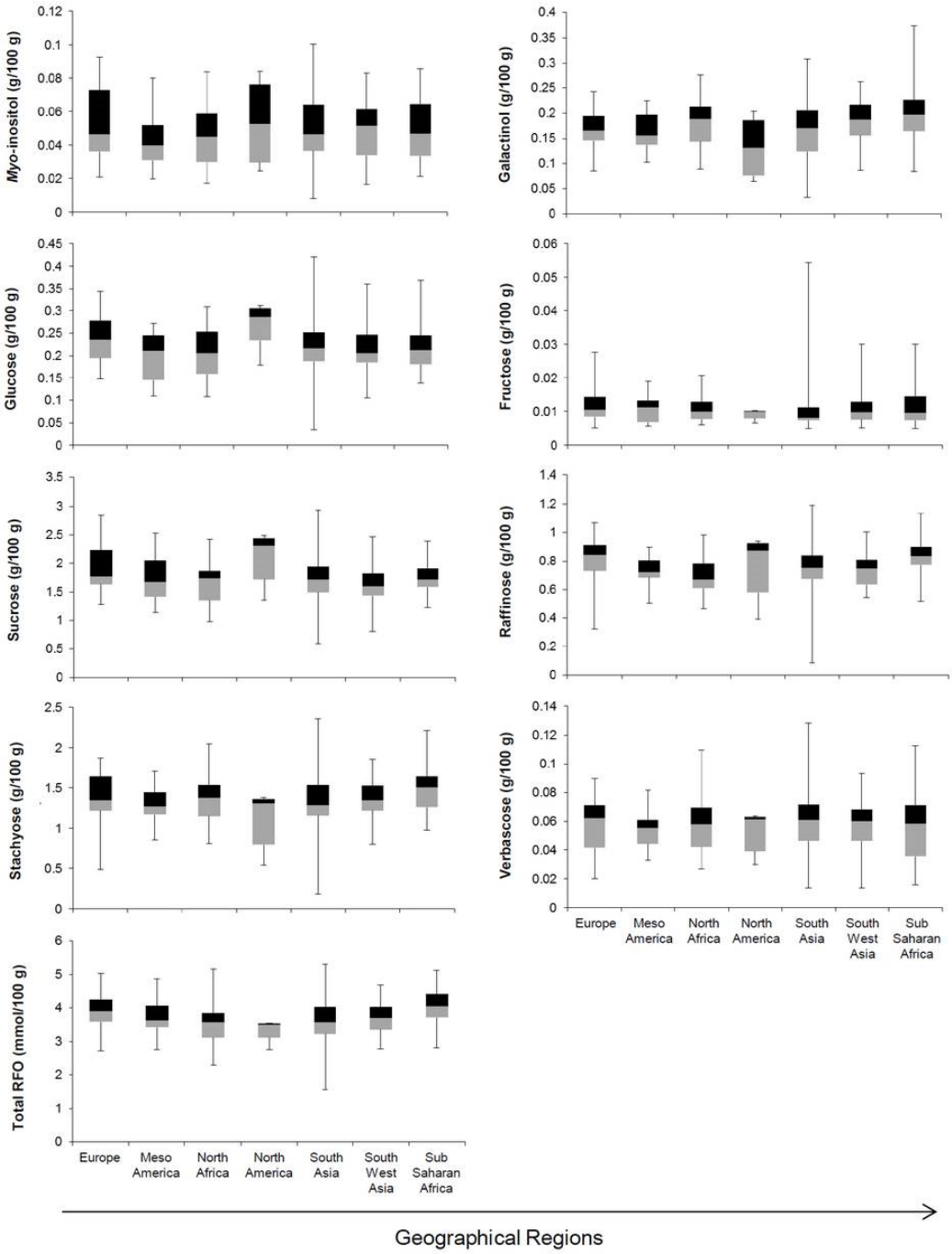


Figure 5.1 Box plot analysis for desi genotypes in different geographical regions.

Figure shows variation for selected chickpea seed constituents (g/100 g of chickpea seed meal on fresh weight basis) using pooled data from different growing environments. Upper and lower limits represent the lowest and highest concentration. Black and grey boxes indicate third and second quartile whereas middle line shows the median of the dataset.

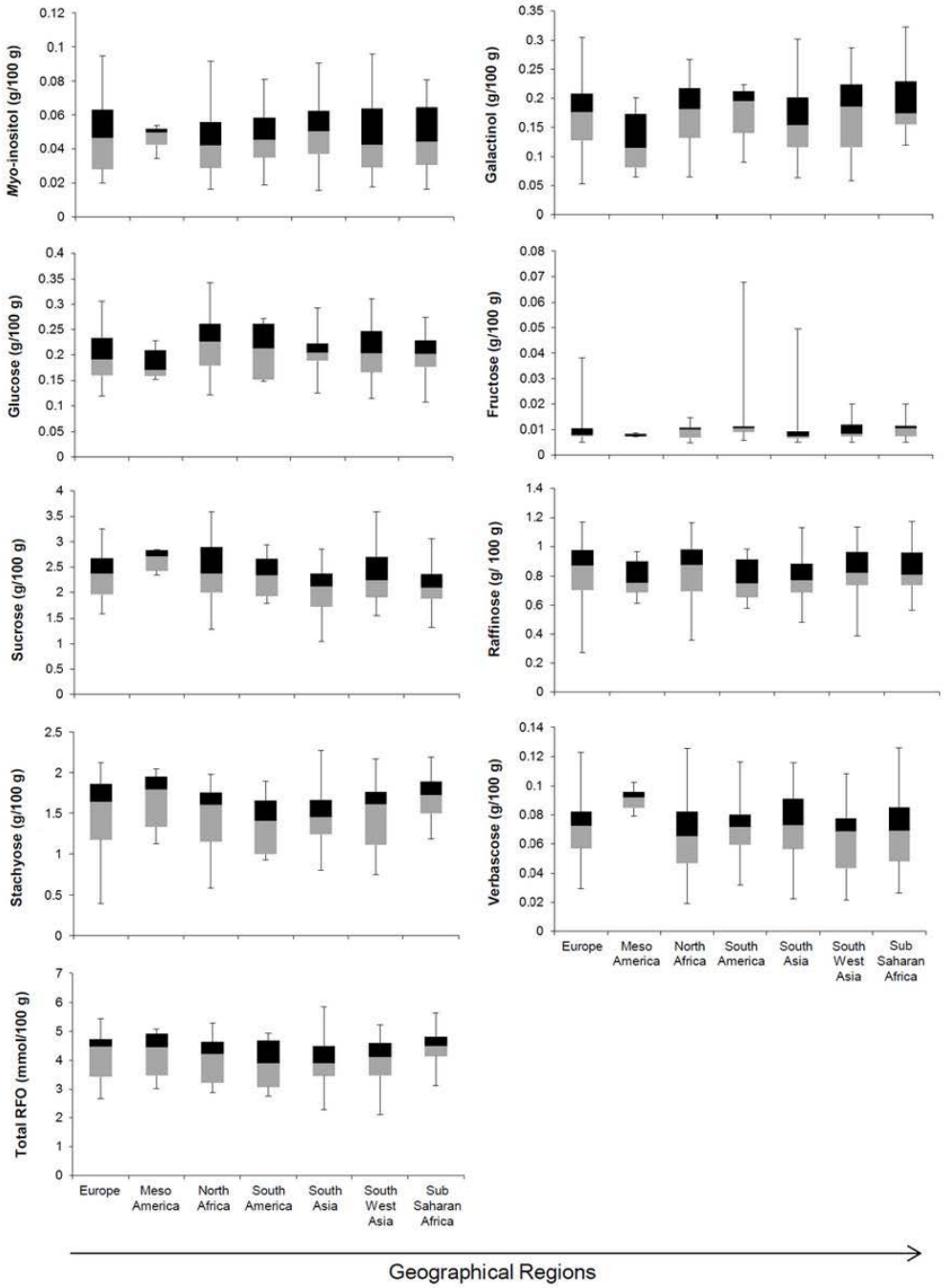


Figure 5.2 Box plot analysis for kabuli genotypes in different geographical regions.

Figure shows variation for selected chickpea seed constituents (g/100 g of chickpea seed meal on fresh weight basis) using pooled data from different growing environments. Upper and lower limits represent the lowest and highest concentration. Black and grey boxes indicate third and second quartile whereas middle line shows the median of the dataset.

Table 5.2 Shannon-Weaver diversity index (SDI) of selected chickpea seed constituents in different geographical regions for desi genotypes

Seed constituents	SDI as per geographical region					
	Europe	Meso America	North Africa	South Asia	Southwest 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

Table 5.3 Shannon-Weaver diversity index (SDI) of selected chickpea seed constituents in different geographical regions for kabuli genotypes

Seed constituents	SDI as per geographical region					
	Europe	South America	North Africa	South Asia	Southwest 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

Meso-America, respectively. This germplasm collection represented no desi genotype from South America whereas only one and four from North America.

In kabuli genotypes, South Asian region showed highest SDI 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 - 0.05, 0.48 - 1.13, 0.80 - 2.28, 0.02 - 0.12 and 2.27 - 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 5.2). Highest SDI for *myo*-inositol (0.88) and sucrose (0.77) was observed for North African genotypes with concentrations ranging from 0.02 - 0.09 and 1.29 - 3.59 g/100 g with a mean value of 0.05 and 2.41 g/100 g of chickpea seed meal, respectively. Galactinol concentration ranged from 0.05 - 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 highest SDI of 0.89 among all geographical regions. However, highest SDI for glucose (0.75) was calculated for Southwest Asian genotypes with concentrations ranging from 0.11 - 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 collection sharing about 32.7% of total kabuli genotypes followed by genotypes from Southwest 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.

5.5.2 Influence of genotype and environment on seed constituents concentration

Analysis of variance (ANOVA) showed significant effect ($P \leq 0.001$) of genotype (G) and growing environment (E) on concentration of *myo*-inositol, galactinol, glucose, fructose, sucrose, raffinose, stachyose, verbascose and total RFO in both desi and kabuli genotypes (Table 5.4). The interaction between genotype and growing environment (G×E) also exhibited significant effect ($P \leq 0.001$) on these seed constituents (Table 5.4). These results concur with the conclusions of Kumar et al. (2010) showing significant effect ($P \leq 0.05$) of genotype × location on sucrose, raffinose and stachyose concentration in seven soybean genotypes. Recently, Tahir et al. (2011) reported significant ($P \leq 0.0001$) effect of cultivar, environment and their interaction on glucose, sucrose and RFO concentration in lentil seeds.

5.5.3 Variation for selected seed constituents in desi and kabuli genotypes

Table 5.4 Analysis of variance and heritability of chickpea selected seed constituents

	Seed constituents	Mean sum of squares				Heritability (h^2)
		Genotype (G)	Environment (E)	Replication	G × E	
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

*** significant at $P \leq 0.001$; ns = non-significant

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. (2000), El-Adawy (2002), Aguilera et al. (2009) and Berrios et al. (2010) also reported stachyose as a major RFO in chickpea seeds. In desi type (Figure 5.3), 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 field 2009 and 2010 it was 4.09 and 3.66 mmol/100 g, respectively. Similar pattern of total RFO was observed in kabuli type (Figure 5.4) showing lower concentration (2.11 - 4.56 mmol/100 g) in GH grown genotypes than that in field-grown 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 - 0.95 g/100 g), stachyose (0.43 - 1.86 g/100 g) and verbascose (0.01 -0.11 g/100 g) had a mean value of 0.68, 1.15 and 0.05 g/100 g, respectively (Figure 5.3). Genotypes grown in field during 2009 had average value of 0.85, 1.57 and 0.07 g/100 g for raffinose, stachyose and verbascose with a range of 0.09 - 1.10, 0.18 - 2.36 and 0.02 - 0.11 g/100 g, respectively whereas, genotypes grown in field during 2010 showed variation from 0.40 to 1.19, 0.78 to 1.99 and 0.01 to 0.13 g/100 g for raffinose, stachyose and verbascose with mean value of 0.75, 1.35 and 0.06 g/100 g, respectively (Figure 5.3). 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 a mean value of 0.66, 1.12, and 0.05 g/100 g, respectively (Figure 5.4). Kabuli genotypes grown in 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 - 1.17, 1.31 - 2.38 and 0.05 - 0.13 g/100 g, respectively. However, genotypes grown in field during 2010 ranged from 0.58 to 1.08, 1.06 to 2.17 and 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 5.4). 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 (Martínez-Villaluenga et al. 2008; Krasensky and Jonak 2012). RFO act as reactive oxygen

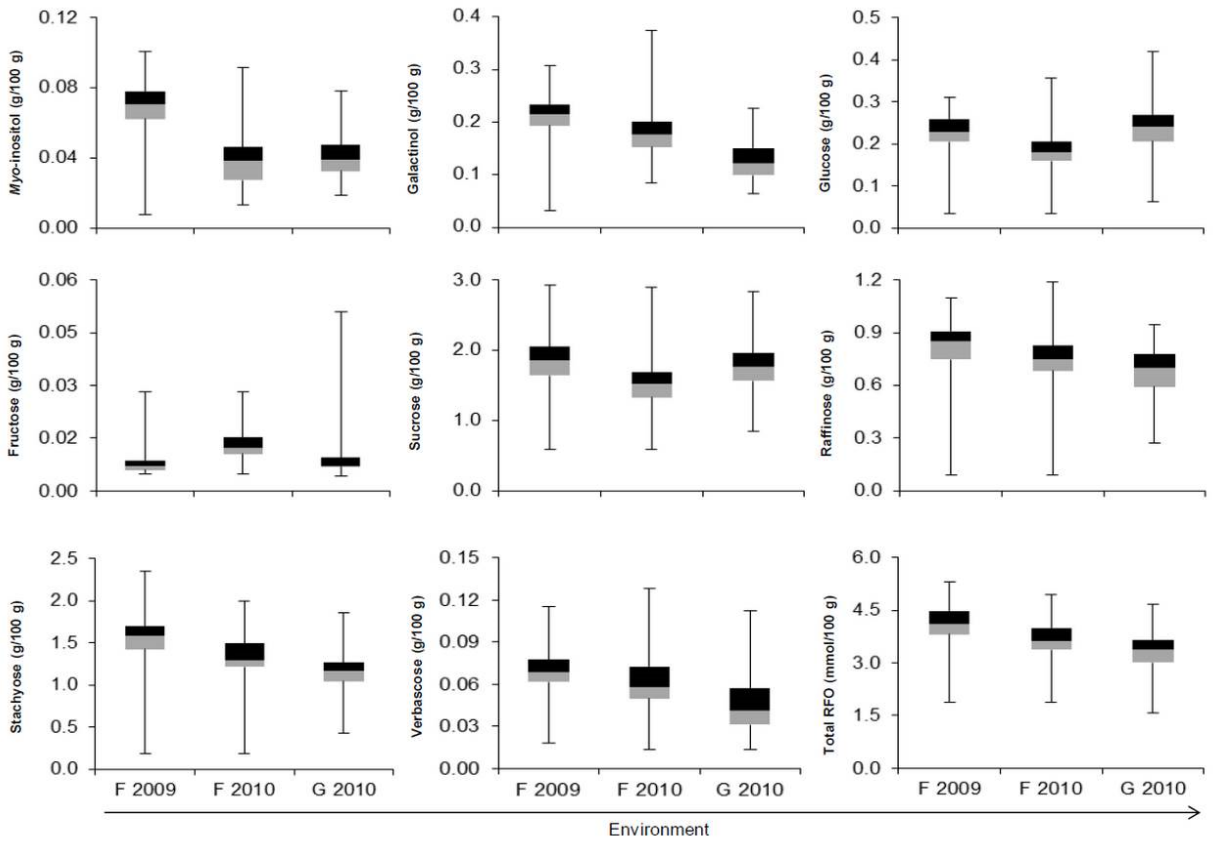


Figure 5.3 Box plot analysis for selected chickpea seed constituents of desi genotypes in different growing environments.

Genotypes grown in field during 2008 - 2009 and 2009 - 2010 are represented as F 2009 and F2010, respectively whereas G 2010 represents greenhouse genotypes grown in 2010. Upper and lower limits represent the lowest and highest concentration (g/100 g of chickpea seed meal on fresh weight basis). Black and grey boxes indicate third and second quartile whereas middle line shows the median of the dataset.

species scavengers, signaling molecules and osmo-protectants thus providing protection against oxidative, freezing, salinity and drought stress (Taji et al. 2002; Peters et al. 2007; Guy et al. 2008; Nishizawa et al. 2008; Van den Ende and Valluru 2009; Bolouri-Moghaddam et al. 2010).

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 - 2.93 g/100 g and 0.81 - 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, 1.33 to 3.59 and 1.07 to 2.94 g/100 g in kabuli genotypes grown in 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 universal molecule to transport carbon and a substrate for raffinose biosynthesis (Peterbauer and Richter 2001; Shiratake 2007; Kuhn and Grof 2010). Sosulski et al. (1982) estimated sucrose concentration in hull free chickpea seeds with mean value of 2.69 g/100 g that was about 32 % of total sugars. Later, Xiaoli et al. (2008) reported the concentration of sucrose, raffinose, stachyose and verbascose in seeds of 19 chickpea cultivars varied from 1.80 to 5.22, 0.46 to 0.92, 1.60 to 3.10 and 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 Sánchez-Mata et al. (1999), Frias et al. (2000), Alajaji and El-Adawy (2006), Aguilera et al. (2009) and Berrios et al. (2010) concluding varying range of mean values for sucrose, raffinose and stachyose from 0.79 to 3.53, 0.32 to 1.45 and 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 5.3), *myo*-inositol and galactinol ranged from 0.01 to 0.10 and 0.03 to 0.37 g/100 g with a mean value 0.05 and 0.17 g/100 g, respectively. Similarly, *myo*-inositol in kabuli type (Figure 5.4) varied from 0.02 to 0.10 g/100 g but with 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 and 0.11 to 0.34 g/100 g for glucose concentration with an average of 0.22 and 0.10 g/100 g, respectively. Whereas, fructose concentration varied from 0.001 to 0.03 and 0.003 to 0.07 g/100 g in desi and kabuli genotypes with a mean value of 0.01 and 0.006 g/100 g, respectively (Figures 5.3 and 5.4). Sosulski et al. (1982) and Jukanti et al. (2012) also reported low concentration of galactinol in chickpea seeds with a mean value 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

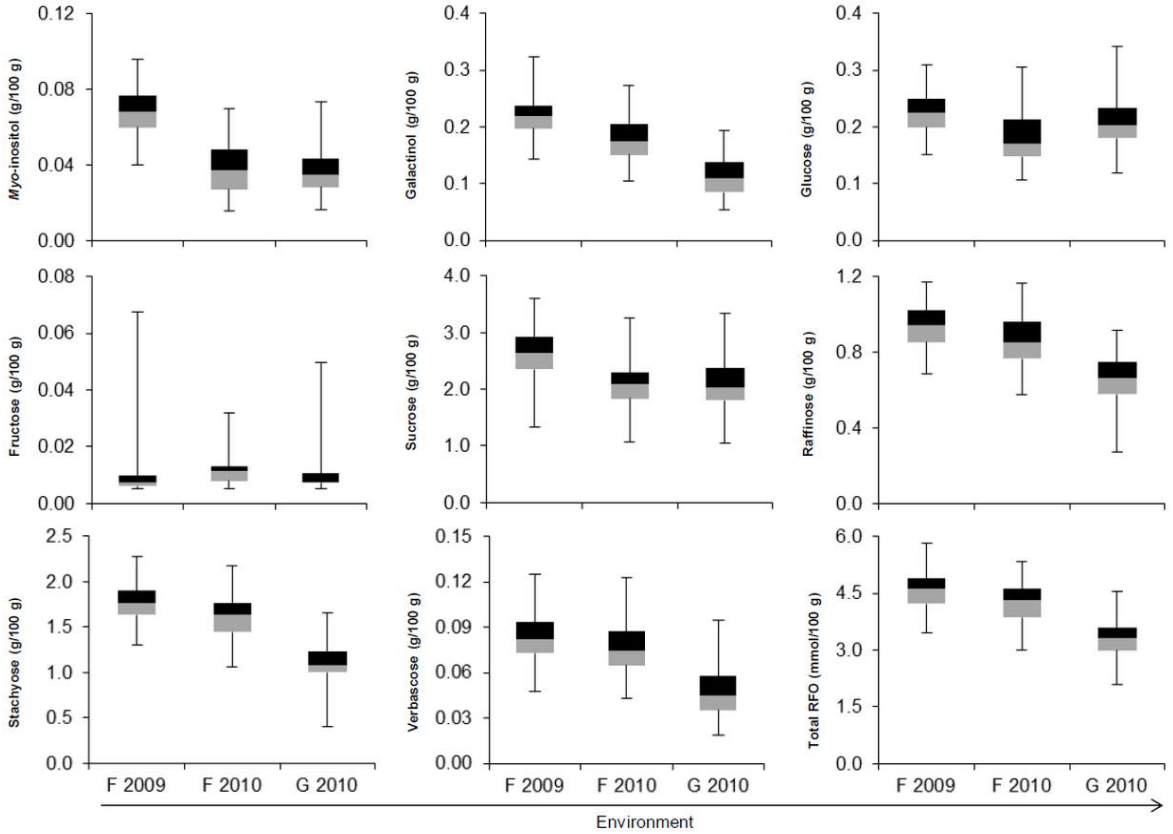


Figure 5.4 Box plot analysis for selected chickpea seed constituents of kabuli genotypes in different growing environments.

Genotypes grown in field during 2008 - 2009 and 2009 - 2010 are represented as F 2009 and F2010, respectively whereas G 2010 represents greenhouse genotypes grown in 2010. Upper and lower limits represent the lowest and highest concentration (g/100 g of chickpea seed meal on fresh weight basis). Black and grey boxes indicate third and second quartile whereas middle line shows the median of the dataset.

matter) and fructose (0.1 - 0.3 % of dry matter) in chickpea seeds reported earlier (Aguilera et al. 2009; Berrios et al. 2010).

5.5.4 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 5.5). Raffinose, stachyose and verbascose were collectively determined during total RFO assay; hence resulted correlation confirmed the accuracy and precision of 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 correlation 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 a significant positive correlation with raffinose and stachyose concentration in soybean seeds (Hartwig et al. 1997; Cicek et al. 2006).

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. Further, 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. Like in case of *Brassica napus* (Bock et al. 2009), antisense technology was used to down-regulate galactinol synthase that resulted into substantial reduction in galactinol and stachyose concentration in mature transgenic seeds. Such transgenic approaches can also be followed in chickpea to develop varieties with reduced RFO concentration.

Table 5.5 Correlation among chickpea selected seed constituents in desi and kabuli genotypes

	***Myo*-inositol**	**Galactinol**	**Glucose**	**Fructose**	**Sucrose**	**Raffinose**	**Stachyose**	**Verbascose**
Desi								
Galactinol	0.64***							
Glucose	0.39***	0.00 ns						
Fructose	-0.03 ns	0.07 ns	0.01 ns					
Sucrose	0.36***	0.03 ns	0.56***	-0.07 ns				
Raffinose	0.40***	0.39***	0.12**	0.07 ns	0.15***			
Stachyose	0.50***	0.53***	-0.01ns	0.07 ns	0.09*	0.78***		
Verbascose	0.49***	0.40***	-0.03ns	0.08 ns	0.18***	0.50***	0.64***	
Total RFO	0.46***	0.47***	-0.01ns	0.04 ns	0.08*	0.85***	0.91***	0.60***
Kabuli								
Galactinol	0.68***							
Glucose	0.47***	0.12*						
Fructose	0.04 ns	0.15**	-0.01 ns					
Sucrose	0.33***	0.23***	0.39***	-0.08 ns				
Raffinose	0.42***	0.55***	0.11 ns	0.05 ns	0.41***			
Stachyose	0.44***	0.64***	0.01 ns	0.07 ns	0.35***	0.89***		
Verbascose	0.47***	0.49***	0.09 ns	0.05 ns	0.41***	0.66***	0.72***	
Total RFO	0.44***	0.62***	0.01 ns	0.06 ns	0.33***	0.89***	0.92***	0.69***

***, ** and * are significant at $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$, respectively; ns = non-significant

5.5.5 Heritability of important chickpea seed constituents

Significant impact of environment and genotype × environment on the performance of a particular genotype suggests complex genetic regulation of traits (McPhee 2002; Cicek et al. 2006). Broad sense heritability (h^2) was estimated on the basis of the pooled ANOVA of genotypes grown in field and greenhouse environments (Table 5.4). Ayele (2011) 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 range reported for sucrose (0.43 - 0.87), raffinose (0.42 - 0.56) and stachyose (0.30 - 0.74) in soybean seeds (Cicek et al. 2006; Mebrahtu and Mohamed 2006; Jaureguy 2011). McPhee et al. (2002) also estimated narrow sense heritability for sucrose, raffinose and stachyose in common bean seeds with a value of 0.22, 0.54 and 0.44, respectively.

5.6 Conclusion

Present study revealed significant impact of genotype (G), environment (E) and G×E on concentration of raffinose family oligosaccharides suggesting their complex genetic regulation in chickpea seeds. Sucrose and stachyose were identified as predominant soluble sugar and RFO in chickpea seeds. A significant positive correlation was observed between substrate and product concentration in RFO biosynthetic pathway. Among all the genotypes screened, some were identified 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 field as well as GH growing 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. Moderate heritability of RFO trait suggested the use of multi-location trials based approach while using germplasms for chickpea improvement programs.

6. Deciphering raffinose family oligosaccharides biosynthesis during chickpea (*Cicer arietinum* L.) seed development

6.1 Study 3*

In this study, RFO accumulation and corresponding biosynthetic enzymes activities were determined to understand RFO biosynthesis during seed development in desi and kabuli type chickpeas.

*Gangola, M. P., Jaiswal, S., Kannan, U., Båga, M. and Chibbar, R. N. 2014. Deciphering raffinose family oligosaccharides biosynthesis during chickpea (*Cicer arietinum* L.) seed development (to be submitted).

6.2 Abstract

Concentration of soluble sugars and activities of raffinose family oligosaccharides (RFO) biosynthetic enzymes were studied during chickpea seed development of two released varieties: CDC Vanguard (desi type) and CDC Frontier (kabuli type). In both genotypes, sucrose (1.7 – 2.0 g/100 g) was the major soluble sugar while stachyose (0.9 – 1.1 g/100 g) was predominant among RFO in mature seeds. During 18 – 38 DAF (days after flowering), seed moisture was decreased by 90 %. Increased RFO accumulation during later stages of seed development provided desiccation tolerance to maturing seeds. The initial substrates *myo*-inositol and sucrose were observed throughout seed development with maximum accumulation of 0.50 – 0.57 and 9.94 – 11.17 g/100 g at 18 – 20 and 20 – 22 DAF supporting the biosynthesis of galactinol and raffinose, respectively. Galactinol, the universal galactosyl donor showed the highest concentration at 30 DAF that was later utilized for increased RFO accumulation till 36 DAF. RFO biosynthetic enzymes activities were observed 2 – 6 days prior to first detection of corresponding RFO product and the highest enzymes activities were determined 2 – 4 days prior to maximum RFO accumulation. However, the highest activity of GS (galactinol synthase) was observed at 36 DAF that did not correspond with galactinol accumulation suggesting galactinol biosynthesis in higher amounts even after 30 DAF but it was utilized in RFO biosynthesis. A galactinol independent pathway was also found operative in chickpea seeds. These results suggested substrate concentration and GS activity as possible factors regulating seed RFO concentration in chickpea.

6.3 Introduction

Chickpea (*Cicer arietinum* L.) is one of the potential crops able to feed world's growing population as it is an inexpensive but excellent source of protein, carbohydrate, polyunsaturated fatty acids (PUFA), dietary fibre, vitamins and minerals (Jukanti et al. 2012). However, worldwide acceptability of chickpea in human diet (particularly in western countries) is constrained due to the presence of anti-nutritional factors like raffinose family oligosaccharides (RFO) (Alonso et al. 2010). RFO represent a class of non-structural sucrosyl-galactosides characterized by the presence of α(1→6) linkage between sucrose and galactosyl moiety (Tapernoux-Luthi et al. 2004). Raffinose is the first member of this family followed by stachyose and verbascose (Sprenger and Keller 2000). Higher consumption of food with RFO causes flatulence, diarrhea and stomach discomfort in the human and mono-gastric animal as they lack α-galactosidase, a hydrolyzing enzyme responsible for RFO breakdown (Kumar et al. 2010; Tahir et al. 2011). However, RFO are also considered as

prebiotics in the human diet (Martínez-Villaluenga et al. 2008). In plants, RFO participate in important physiological mechanisms like seed desiccation and germination, translocation of photo-assimilates and abiotic stress tolerance (Turgeon et al. 1993; Nishizawa et al. 2008; Martínez-Villaluenga et al. 2008). Therefore, chickpea seed RFO concentration needs to be reduced to increase acceptability of chickpea in the human diet. In chickpea seeds, RFO ranged from 1.58 to 5.31 mmol/100 g and 2.11 to 5.83 mmol/100 g in desi (dark-colored, angular seeds) and kabuli (cream or beige-colored, smooth surface seeds) types, respectively (Gangola et al. 2013). Consequently, understanding RFO biosynthesis in chickpea seeds is a prerequisite to develop strategy to reduce RFO concentration in chickpea seeds.

RFO biosynthesis is initiated by galactinol synthase (GS; EC 2.4.1.123), which catalyzes the transfer of galactosyl unit from UDP-D-galactose to *myo*-inositol producing galactinol, the first committed step in RFO biosynthesis. Galactinol synthesis regulates carbon partitioning between sucrose and RFO (Nishizawa et al. 2008). The RFO biosynthetic process is further extended by raffinose synthase (RS; EC 2.4.1.82; Peterbauer and Richter 2001), catalyzing reversible transfer of galactosyl residue from donor galactinol to sucrose synthesizing raffinose with the release of inositol. For stachyose and higher homologues biosynthesis, two pathways have been reported: 1) galactinol dependent and, 2) galactinol independent (Peterbauer et al. 2001). In galactinol dependent pathway, galactinol donates galactosyl moiety to raffinose and stachyose yielding stachyose and verbascose in reactions catalyzed by stachyose synthase (STS; EC 2.4.1.67) and verbascose synthase (VS; EC 2.4.1.x), respectively. However, existence of VS in plants has yet to be confirmed (Lahuta 2006). Verbascose synthase activity has been observed in purified stachyose synthase from pea seeds (Peterbauer et al. 2002), while stachyose synthase from adzuki bean seeds was devoid of verbascose synthase activity (Peterbauer and Richter 1998). Therefore, a new galactinol-independent pathway has been proposed for the biosynthesis of higher members of RFO (Bachmann et al. 1994; Haab and Keller 2002). In this pathway, an already present RFO molecule transfers its terminal galatosyl residue to another RFO molecule synthesizing a higher member of raffinose family. Galactan:galactan galactosyltransferase (GGT) plays central role in this pathway (Haab and Keller 2002; Tapernoux-Luthi et al. 2004). GGT is considered as non-galactinol enzyme because existence of GGT in chickpea seeds has not been reported yet.

To analyse RFO biosynthesis in chickpea seeds, RFO accumulation and corresponding biosynthetic enzyme activity during seed development were studied. RFO biosynthetic enzymes activities were detected from 14 – 22 days after flowering (DAF) till maturity. RFO

biosynthetic enzymes activities were observed 2 – 6 days prior to first corresponding RFO accumulation.

6.4 Material and methods

6.4.1 Plant material

Breeder seeds of chickpea (*Cicer arietinum* L.) released varieties CDC Vanguard (desi type) and CDC Frontier (Kabuli type) were procured from Saskatchewan Pulse Growers (Saskatoon). Seeds were grown during March – July, 2011 in agricultural greenhouse (University of Saskatchewan; 52° 07' N latitude, 106° 38' W longitude and 481.5 m altitude, Saskatoon, SK, Canada) with 18 h photoperiod, ~345 µM/m^2/sec of integrated PAR (photo-synthetically active radiation) and mean temperature of 18 °C (night) to 23 °C (day). Fully opened flowers were tagged and pods were collected from six days after flowering (DAF) till maturity (46 DAF) at two days interval. Pods after collection were instantly frozen in liquid N_2 and stored at -80 °C till used in experiments. To determine moisture content (% on fresh weight basis), seeds were kept in an oven at 80 °C till there was no change in weight and were weighed to calculate average dry seed weight at different developmental stages.

Frozen seeds of chickpea (20 g till 12 DAF and 5 g for later stages) were ground using pestle and mortar. The resulted flour was lyophilized and used to determine concentration of total RFO and soluble sugars.

6.4.2 Determination of total RFO concentration

Total RFO concentration in lyophilized chickpea seed meal (500 ± 5 mg) was determined by stepwise hydrolysis of complex RFO into D-galactose, D-fructose and D-glucose molecules using α-galactosidase (from *Aspergillus niger*) and invertase (from yeast), using raffinose/sucrose/glucose assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). The resulting D-glucose was determined using glucose oxidase/peroxidase reagent (GOPOD) that produced a red colored quinoneimine whose concentration was determined at $A_{510\ nm}$ using a spectrophotometer. This method determined concentration of Glucose, sucrose and total RFO as described (Gangola et al. 2013).

6.4.3 Determination of soluble sugars concentration

Soluble sugars (*myo*-inositol, galactinol, sucrose, raffinose, stachyose and verbascose) were extracted and their concentrations (g/100 g chickpea seed meal on dry weight basis) were determined using a modified HPAEC-PAD (High Performance Anion

Exchange Chromatography with Pulsed Amperometric Detector) based method as reported (Gangola et al. 2014).

6.4.4 *In vitro* activity assays of RFO biosynthetic enzymes

Enzyme activity assays for RFO biosynthetic enzymes were performed as described in section 3.5 of the thesis.

6.5 Results

6.5.1 Water content and dry weight of seed at different developmental stages

Chickpea seeds contained about 95 % of water at 6 DAF in both desi and kabuli type. Thereafter, a gradual decrease in seed moisture was observed till 18 DAF followed by a rapid decrease till 38 DAF *i.e.* about 75 % reduction in seed moisture was observed from 18 DAF to 38 DAF in both chickpea types (Figure 6.1). The rate of moisture reduction was highest during 34 – 38 DAF in desi (22.1 to 51.9 %) and kabuli (31.0 to 43.3 %) types. After 38 DAF, no significant decrease in seed moisture was observed. Mature chickpea seeds contained about 6.3 ± 0.3 and 7.2 ± 0.3 % water in desi and kabuli type, respectively. Decrease in water concentration was accompanied by dry matter accumulation in seed. Initially, seed dry matter was similar in both desi and kabuli type. However, seed dry weight increased rapidly from 20 DAF till 34 DAF (299.1 ± 11.5 mg) in desi type and up to 36 DAF (431.2 ± 28.5 mg) in kabuli type. After the stage of highest dry weight accumulation (34 – 36 DAF), reduction in seed dry weight was detected followed by a gradual increase till maturity (46 DAF). The average dry weight of mature seed of CDC Vanguard (desi type) and CDC Frontier (kabuli type) were 269.6 ± 17.4 and 371.2 ± 26.2 mg/seed.

6.5.2 Accumulation of RFO and corresponding biosynthetic enzymes activities during seed development

In mature seeds of CDC Vanguard (desi type; 3.1 ± 0.1 mmol/100 g), total RFO concentration was higher compared to CDC Frontier (kabuli type; 2.9 ± 0.1 mmol/100 g) (Figure 6.2). Total RFO were first detected at 28 DAF (0.21 ± 0.05 and 0.22 ± 0.04 mmol/100 g) in both genotypes (desi and kabuli type) with the peak accumulation at 36 DAF (2.5 ± 0.1 and 2.1 ± 0.1 mmol/100 g).

The first committed step of RFO biosynthesis is formation of galactinol that utilizes *myo*-inositol as one of the substrates (other is UDP galactose). *Myo*-inositol was detected throughout seed development (6 DAF till maturity) with its highest concentration at 18 and

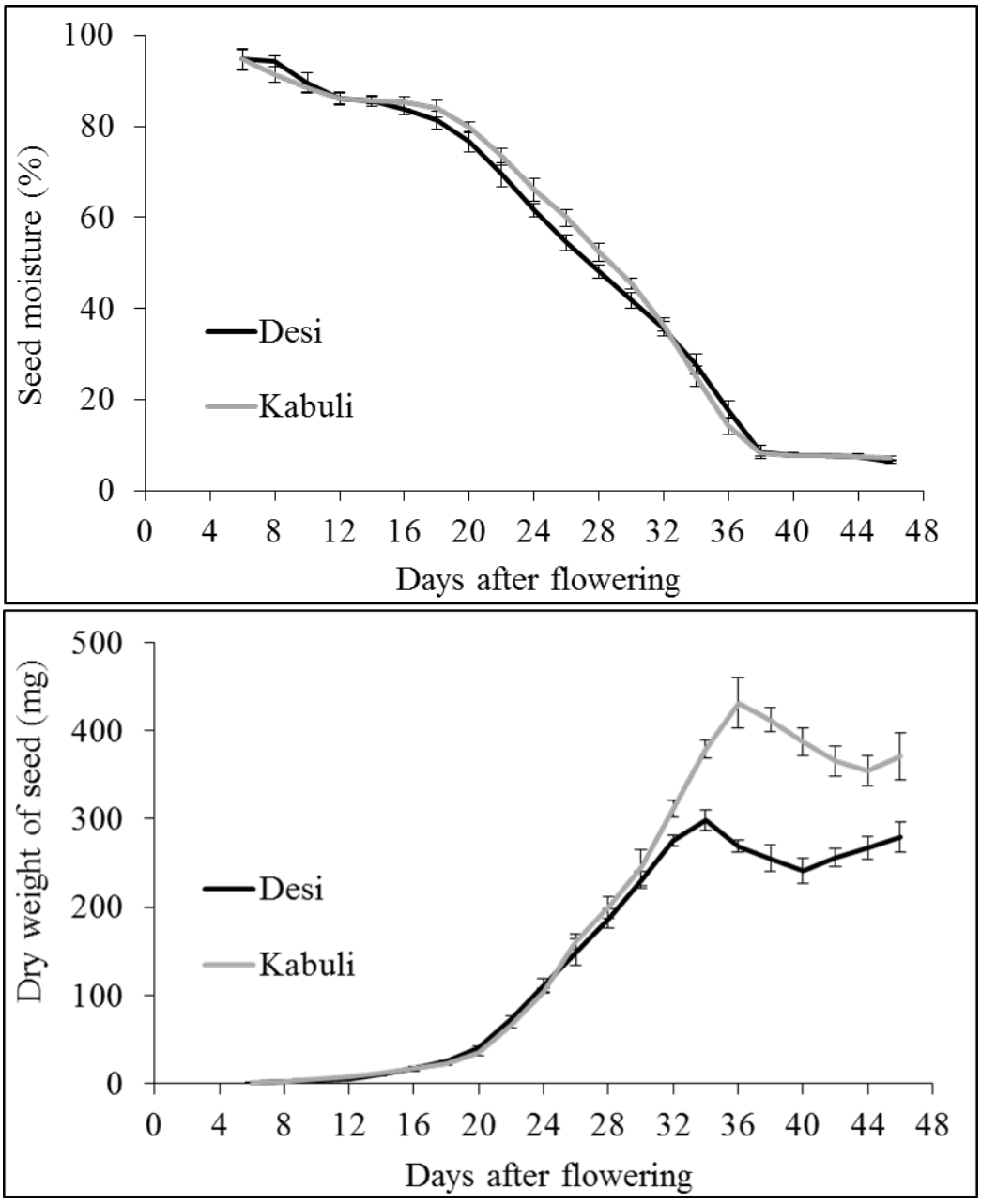


Figure 6.1 Moisture content and dry matter of chickpea seeds (desi and kabuli type) during seed development.

Data represent the mean ± SD from three independent replicates. Moisture content is shown in % on fresh weight basis.

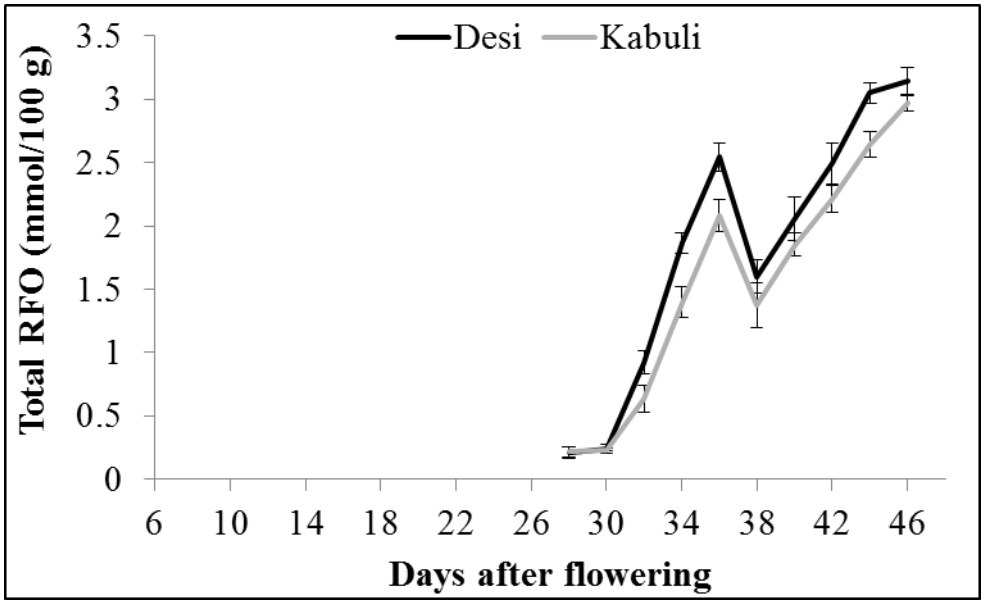


Figure 6.2 Accumulation of total RFO during chickpea seed development.

Data represent the mean (mmol/100 g of chickpea seed meal on dry weight basis) ± SD from three independent replicates.

20 DAF in desi (0.57 ± 0.01 g/100 g) and kabuli (0.50 ± 0.02 g/100 g) type, respectively.

Thereafter, a rapid decrease in *myo*-inositol concentration was observed corresponding to increased accumulation of galactinol after 22 DAF in both desi and kabuli genotypes (Figure 6.3). Mature seeds of desi and kabuli types contained about 0.05 ± 0.01 g/100 g of *myo*-inositol. Galactinol was first detected at 20 and 18 DAF in desi (0.02 ± 0.01 g/100 g) and kabuli type (0.02 ± 0.002 g/100 g), respectively and showed highest accumulation at 30 DAF in both desi (0.90 ± 0.07 g/100 g) and kabuli (0.67 ± 0.04 g/100 g) type chickpea. Thereafter, a sudden decrease in galactinol concentration was observed (Figure 6.4). In desi and kabuli type, mature seeds contained 0.12 ± 0.02 and 0.13 ± 0.004 g/100 g galactinol. GS catalyzes the first step of RFO biosynthesis and also showed the highest enzymatic activity among all the RFO biosynthetic enzymes. The first GS activity was detected at 14 DAF (8.6 ± 2.1 and 13.7 ± 2.6 pkat/mg protein) that increased till 36 DAF (854.7 ± 47.3 and 687.3 ± 51.1 pkat/mg protein) in both desi and kabuli type. Mature seeds of desi (91.1 ± 8.0 pkat mg^{-1} protein) and kabuli (67.6 ± 16.5 pkat/mg protein) types also showed GS activity (Figure 6.4). Raffinose biosynthesis is a galactinol dependent step. The other substrate required for raffinose synthesis is sucrose. Like *myo*-inositol, sucrose was also present from 6 DAF (6.3 ± 0.2 and 5.9 ± 0.6 g/100 g) till maturity (1.7 ± 0.1 and 2.0 ± 0.1 g/100 g) during chickpea seed development in both desi and kabuli type (Figure 2). The highest concentration of sucrose was found at 20 (11.17 ± 0.19 g/100 g)/22 (9.94 ± 0.09 g/100 g) DAF followed by a decrease in concentration from 22/24 DAF in desi/kabuli type that resulted in raffinose accumulation (0.02 ± 0.004/0.03 ± 0.006 g/100 g). Raffinose accumulated rapidly from 30/32 DAF till 36 DAF (0.60 ± 0.01 and 0.42 ± 0.01 g/100 g) in both desi and kabuli types (Figure 6.5). Thereafter, a decrease in raffinose concentration was observed (36 to 38 DAF) followed by gradual increase till maturity. In mature seeds of desi and kabuli types, raffinose concentration was 0.6 ± 0.01 and 0.5 ± 0.02 g/100 g, respectively. RS activity was first observed at 18 DAF in both desi (2.8 ± 0.6 pkat/mg protein) and kabuli (2.5 ± 0.1 pkat/mg protein) type. Maximum RS activity of 47.6 ± 4.8 and 36.7 ± 3.5 pkat/mg protein was observed at 32 DAF for desi and kabuli types, respectively (Figure 6.5). Mature seeds of desi and kabuli types had residual RS activity of 5.7 ± 0.5 and 4.3 ± 0.3 pkat/mg protein, respectively (Figure 6.5).

In the desi type, accumulation of stachyose and verbascose was initiated at 22 (0.01 ± 0.001 g/100 g) and 24 (0.01 ± 0.002 g/100 g) DAF, respectively. Where as in kabuli type, stachyose and verbascose accumulation was delayed with first detection at 26 (0.01 ± 0.003) and 28 (0.003 ± 0.001 g/100 g) DAF, respectively. Seeds at 36 DAF had the highest

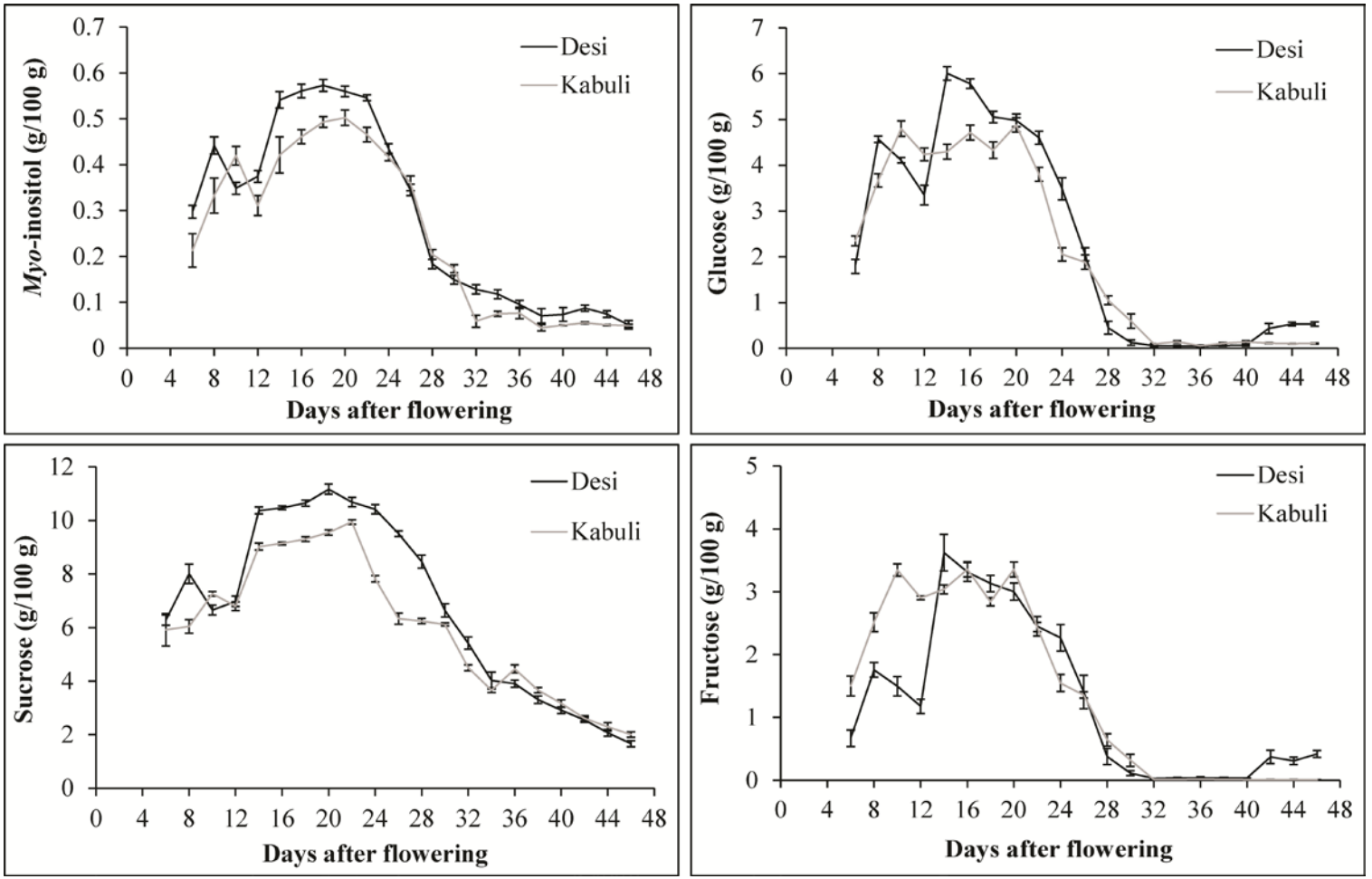


Figure 6.3 Accumulation of initial substrates of RFO biosynthesis during chickpea seed development (desi and kabuli type).

Data represent the mean (g/100 g of chickpea seed meal on dry weight basis) ± SD from three independent replicates.

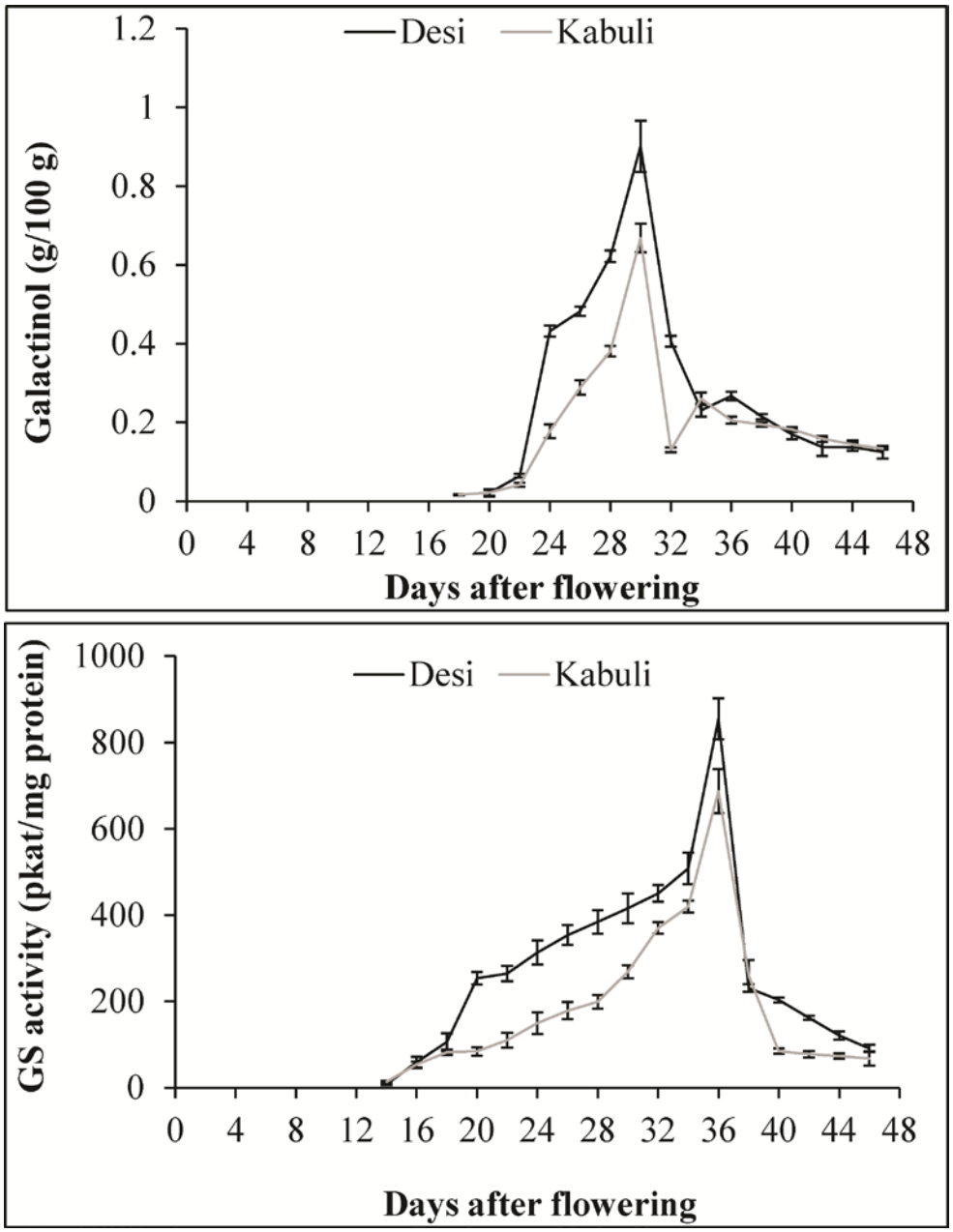


Figure 6.4 Accumulation of galactinol and GS (Galactinol Synthase) activity during chickpea (desi and kabuli type) seed development.

Data represent the mean ± SD from three independent replicates. Galactinol was determined as g/100 g of chickpea seed meal on dry weight basis.

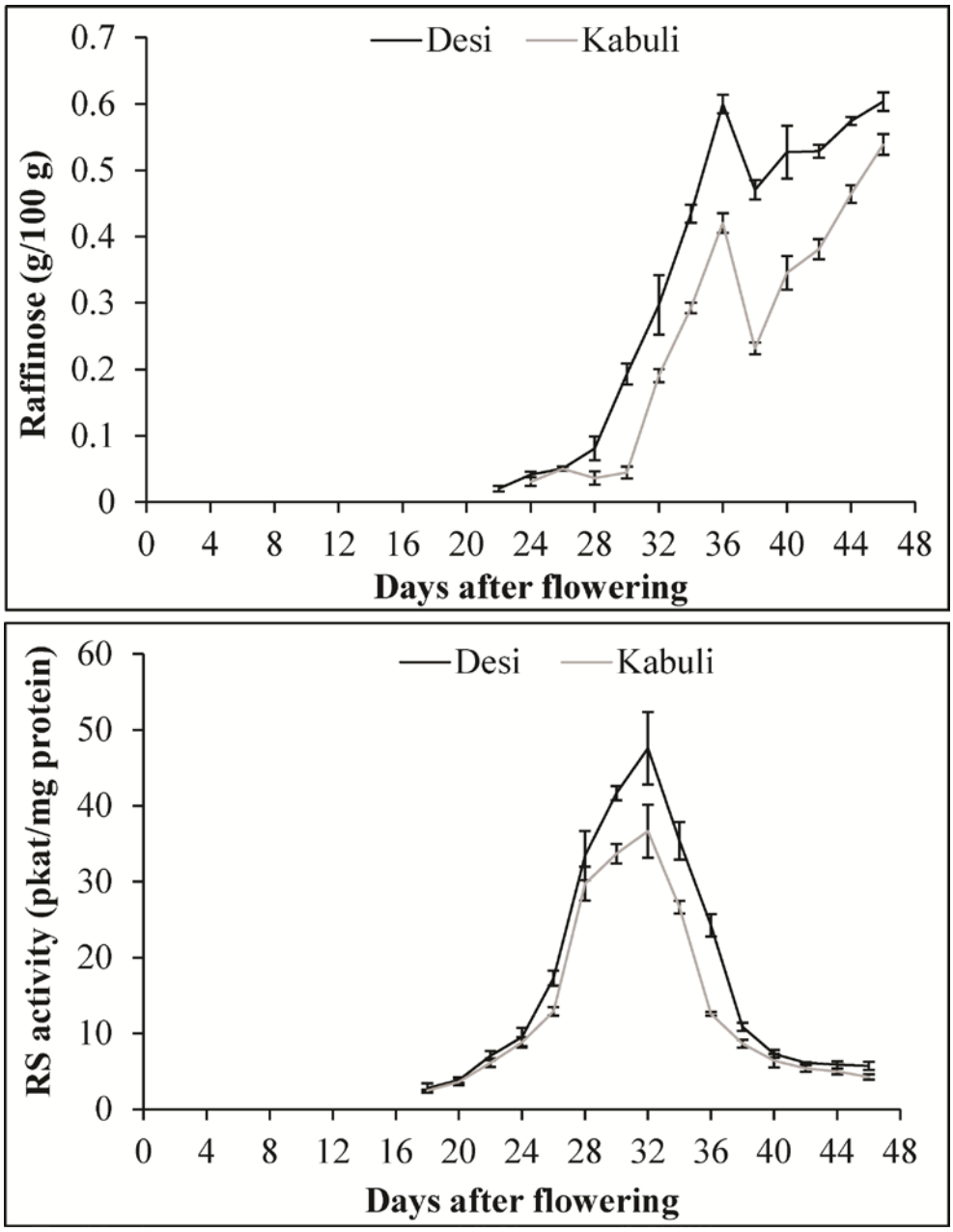


Figure 6.5 Accumulation of raffinose and RS (Raffinose Synthase) activity during chickpea (desi and kabuli type) seed development.

Data represent the mean ± SD from three independent replicates. Raffinose was determined as g/100 g of chickpea seed meal on dry weight basis.

concentration of stachyose (1.18 ± 0.13 and 0.78 ± 0.02 g/100 g) and verbascose (0.03 ± 0.003 and 0.05 ± 0.009 g/100 g) in both desi and kabuli type. A decrease in stachyose and verbascose concentration was observed at 38 DAF followed by a gradual increase till maturity (Figure 6.6 and 6.7). At seed maturity, stachyose and verbascose concentrations were 1.1 ± 0.06/0.9 ± 0.01 and 0.03 ± 0.003/0.04 ± 0.002 g/100 g in desi/kabuli type, respectively. STS activity was first detected at 20 DAF (4.7 ± 0.4 and 2.1 ± 0.2 pkat/mg protein) whereas maximum activity was observed at 32 DAF (57.3 ± 1.5 and 49.1 ± 0.9 pkat/mg protein) in chickpea seeds (desi and kabuli type; Figure 6.6). VS activity was observed starting from 22 DAF (1.7 ± 0.2 and 1.3 ± 0.2 pkat/mg protein) till maturity with maximum activity at 34 DAF (10.5±0.3 and 10.0±0.6 pkat/mg protein) in chickpea seeds (desi and kabuli type; Figure 6.7). STS and VS activity was also detected in mature seeds of desi (6.4 ± 0.6 and 2.2 ± 0.2 pkat/mg protein) and kabuli (4.1 ± 0.3 and 2.4 ± 0.2 pkat/mg protein) type. Non-galactinol enzyme activities synthesizing stachyose (using raffinose as substrate) and verbascose (using stachyose as substrate) were first observed at 22 DAF (0.7 ± 0.2/0.9 ± 0.04 and 0.4 ± 0.1/1.2 ± 0.1 pkat/mg protein) using raffinose/stachyose that reached to a maxima of 6.1 ± 0.3/6.7 ± 0.3 and 4.6 ± 0.2/4.8 ± 0.8 pkat/mg protein at 34 DAF in desi and kabuli type, respectively. Mature seeds of desi and kabuli type also showed non-galatinol enzyme activity synthesizing stachyose (2.9 ± 0.1 and 3.2 ± 0.2 pkat/mg protein) and verbascose (2.5 ± 0.2 and 2.8 ± 0.1 pkat/mg protein) (Figure 6.8).

Monosaccharides glucose and fructose were also detected during chickpea seed development in both desi and kabuli types (Figure 6.3). Glucose concentration was 1.8 ± 0.2/2.4 ± 0.1 and 0.5 ± 0.04/0.1 ± 0.01 g/100 g at 6 DAF and maturity in desi/kabuli type, respectively. Fructose concentration was 0.7 ± 0.1/1.5 ± 0.2 and 0.4 ± 0.05/0.01 ± 0.001 g/100 g at 6 DAF and maturity in desi/kabuli type, respectively. In desi type, the highest concentration of glucose (6.00 ± 0.15 g/100 g) and fructose (3.62 ± 0.29 g/100 g) was observed at 14 DAF where as in kabuli type, it was at 20 (4.88 ± 0.16 g/100 g) and 16 (3.35 ± 0.12 g/100 g) DAF, respectively (Figure 6.3).

In mature chickpea seeds (desi/kabuli type), sucrose (1.67/2.00 g/100 g) was the major soluble sugar whereas stachyose (1.13/0.94 g/100 g) was predominant among RFO followed by raffinose (0.60/0.54 g/100 g) and verbascose (0.03/0.04 g/100 g).

6.6 Discussion

6.6.1 RFO accumulation provides desiccation tolerance to developing chickpea seeds

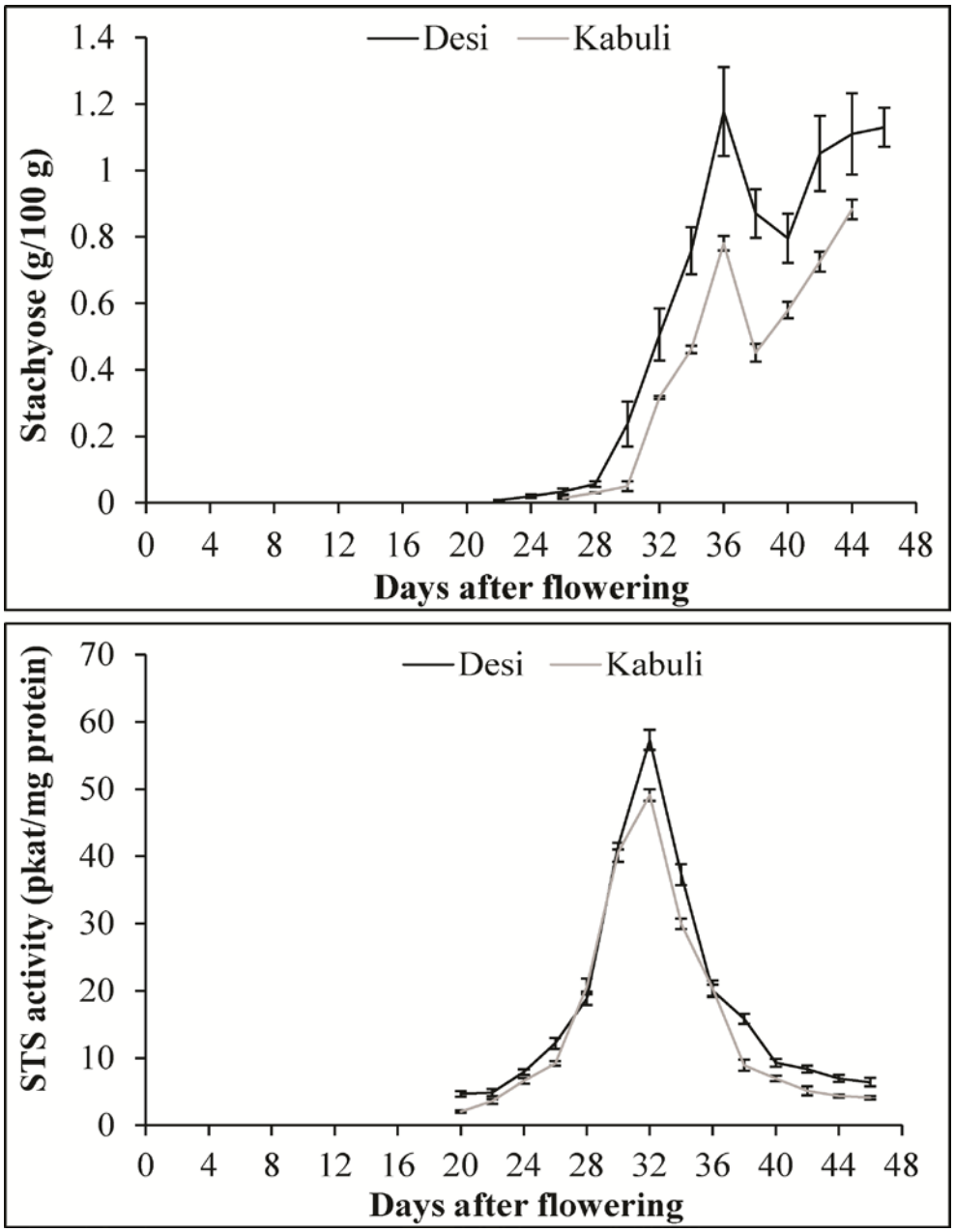


Figure 6.6 Accumulation of stachyose and STS (Stachyose Synthase) activity during chickpea (desi and kabuli type) seed development.

Data represent the mean ± SD from three independent replicates. Stachyose was determined as g/100 g of chickpea seed meal on dry weight basis.

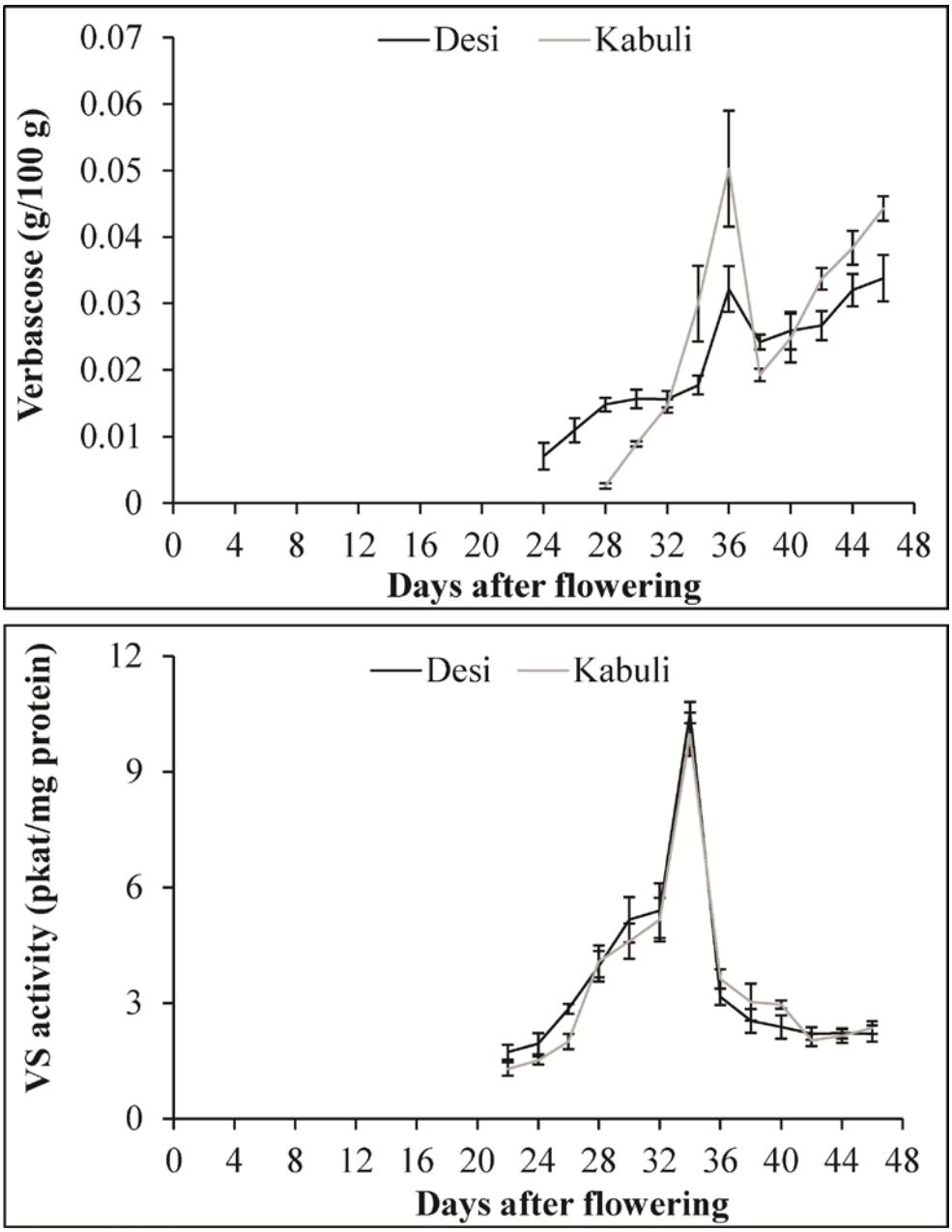


Figure 6.7 Accumulation of verbascose and VS (Verbascose Synthase) activity during chickpea (desi and kabuli type) seed development.

Data represent the mean ± SD from three independent replicates. Verbascose was determined as g/100 g of chickpea seed meal on dry weight basis.

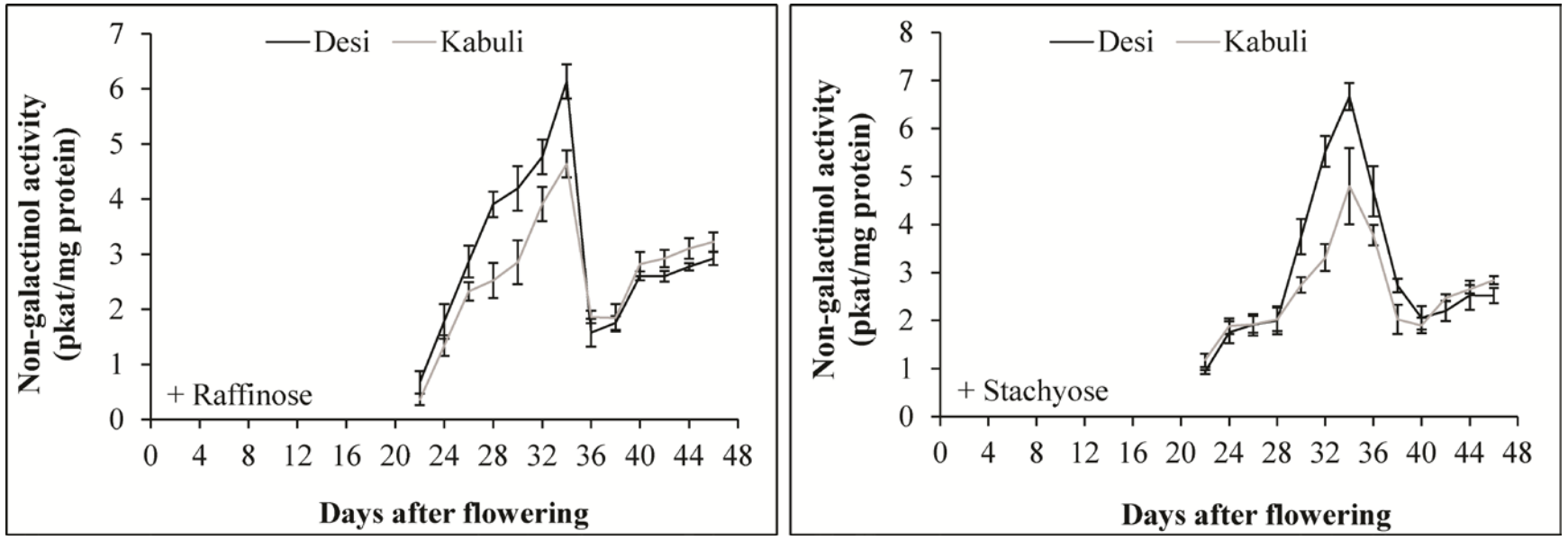


Figure 6.8 Non-galactinol enzymatic activity of RFO biosynthesis during chickpea (desi and kabuli type) seed development.

Raffinose and stachyose were used as substrates. Data represent the mean ± SD from three independent replicates.

In chickpea seeds, RFO biosynthesis is triggered by the loss of moisture and simultaneous accumulation of dry matter in chickpea seeds. The total reduction in seed moisture during early (6 – 18 DAF) and late (38 DAF till maturity) stages of seed development were 14.1 and 26.1 % for desi type whereas 11.5 and 13.5 % for kabuli type, respectively. During 18 – 38 DAF, about 75 % reduction in seed moisture was observed in both genotypes (Figure 6.1). To minimize the negative effects of rapid water loss, chickpea seeds accumulated RFO after 18 DAF till 36 DAF. Higher rate of reduction in seed moisture also corresponded to the increased rate of RFO accumulation during 34 – 36 DAF in both desi and kabuli type (Figures 6.1 and 6.2). RFO provide tolerance against rapid water loss during seed maturity (seed desiccation) either by replacing water (Koster 1991; Pukacka et al. 2009) or by the process of vitrification (Koster 1991; Martínez-Villaluenga et al. 2008; Angelovici et al. 2010) to maintain hydrophilic and hydrophobic interactions required to stabilize native macromolecules and membrane structure (Blackman et al. 1992; Corbineau et al. 2000; Pukacka et al. 2009; Angelovici et al. 2010). Consequently, loss of seed moisture induced RFO accumulation providing desiccation tolerance in maturing chickpea seeds.

6.6.2 Initial substrates concentrations influence seed RFO concentration

Accumulation of total RFO concurred with the biosynthesis of individual member of raffinose family (Figure 6.2). Galactinol is the first compound committed for RFO biosynthesis (Paterbauer et al. 2001). It is a universal galactosyl unit donor during RFO biosynthesis except in galactinol-independent pathway (Martínez-Villaluenga et al. 2008). Therefore, a decline in galactinol concentration after 30 DAF supported the increased biosynthesis of raffinose, stachyose and verbascose till 36 DAF. During chickpea seed development, raffinose was detected immediately after galactinol, followed by stachyose and verbascose. CDC Vanguard (desi type) had higher seed RFO concentration compared to CDC Frontier (kabuli type). Although in a previous study (Gangola et al. 2013), kabuli types (2.11 to 5.83 mmol/100 g) showed higher total seed RFO concentration compared to desi types (1.58 to 5.31 mmol/100 g) but total RFO concentration in mature seeds CDC Vanguard and CDC Frontier are within the range. Higher seed RFO concentration also coincided with higher concentration of substrates concentrations in desi type compared to kabuli type. These results suggest that higher members of raffinose family require a lower RFO member/substrate for their biosynthesis. Therefore, RFO biosynthesis might be regulated by initial substrates concentration like *myo*-inositol, galactinol or sucrose. Consequently, higher concentration of substrates supports the increased biosynthesis of products (RFO) which are

translocated in to vacuole for continued RFO biosynthesis in cytosol. These results concur with the earlier observations suggesting reversible nature of RFO biosynthetic reactions regulated by mass action ratio and equilibrium constant (Peterbauer et al. 2001). Karner et al. (2004) by substrate feeding experiment also suggested the regulation of RFO concentration by the initial substrates concentration (*myo*-inositol and sucrose) rather than GS activity alone. In a recent study using 171 chickpea genotypes, a significant positive correlation was observed between seed RFO and substrate concentration (Gangola et al. 2013).

6.6.3 Galactinol independent RFO biosynthetic pathway is operative in chickpea seeds

Chickpea seeds also showed a non-galactinol enzyme activity synthesizing verbascose as well as stachyose. Enzymes catalyzing galactinol dependent RFO biosynthesis showed higher activity compared to non-galactinol enzyme thus suggesting the predominance of galactinol dependent RFO biosynthesis in seeds of both desi and kabuli types (Figures 6.4 – 6.8). The optimum pH for non-galactinol enzyme activity was 7.0 which concurred with results of Peterbauer et al. (2001) but in contrast to Bachmann et al. (1994) who demonstrated that acidic pH (4.5 – 5.0) was optimum for non-galactinol activity in leaves of *Ajuga reptans* (Tapernoux-Lüthi et al. 2004). The enzyme with non-galactinol RFO biosynthetic activity was suggested as multifunctional STS in *P. sativum* (Peterbauer et al. 2002) and a separate GGT in *A. reptans* (Bachmann et al. 1994; Haab and Keller 2002; Tapernoux-Lüthi et al. 2004). Optimum pH of 7.0 suggests non-galactinol enzyme in chickpea seeds to be a multifunctional STS. However, GGT has not been characterized from any other plant species except *A. reptans* (Tapernoux-Lüthi et al. 2004).

6.6.4 GS activity – another factor influencing seed RFO concentration

The accumulation of RFO was in good agreement with their corresponding biosynthetic enzymes activities. The highest biosynthetic enzymes (RS, STS, VS and non-galactinol) activities were detected at 2 – 4 days prior to the accumulation of raffinose, stachyose and verbascose. However, GS showed maximum enzyme activity after six days of highest galactinol accumulation. This might be due to continuous utilization of galactinol in RFO biosynthesis even after 30 DAF. Galactinol was synthesized in higher amount even after 30 DAF and utilized in RFO biosynthesis thus could not accumulate in seeds. Almost similar pattern for GS activity and galactinol accumulation was reported in seeds of *Pisum sativum* (in one out of two genotypes; Peterbauer et al. 2001) and *Brassica napus* (Li et al. 2011). GS

was identified as a biosynthetic enzyme with the highest enzymatic activity showing its importance in RFO biosynthesis. The interval between initial and maximum GS activity was 22 days which is the longest time period compared to RS (14 days), STS (12 days), VS (12 days) and non-galactinol enzyme (12 days). These results suggest that GS is the most active RFO biosynthetic enzyme during seed development and a potential target to reduce seed RFO concentration in chickpea.

6.7 Conclusion and future prospects

The present study revealed the presence of both galactinol –dependent and –independent pathways of RFO biosynthesis in chickpea seeds. Galactinol-independent pathway confirmed its capability to synthesize higher members of the raffinose family (stachyose and verbascose). RFO mostly accumulated during later stages of seed development providing desiccation tolerance to the maturing chickpea seeds. Sucrose and stachyose were predominant soluble sugar and RFO, respectively. GS was identified as the most active RFO biosynthetic enzyme followed by STS, RS and VS/non-galactinol. The results suggest two possible factors regulating RFO biosynthesis in chickpea seeds: (1) substrate concentration, and (2) GS activity. To further decipher the RFO biosynthesis in chickpea seeds, comparative study with contrasting RFO genotypes could be targeted in future.

7. CHICKPEA GENOTYPES WITH CONTRASTING RFO CONCENTRATIONS SUGGEST GALACTINOL SYNTHASE AS A POTENTIAL TARGET TO REDUCE RFO CONCENTRATION IN CHICKPEA (*Cicer arietinum* L.) SEEDS

7.1 Study 4*

In this study, chickpea genotypes with contrasting RFO concentrations were studied to identify key regulating step of RFO biosynthesis. Consequently, galactinol synthase was proposed as a potential target to reduce seed RFO concentration in chickpea.

*Gangola, M. P., Jaiswal, S., Kannan, U., Gaur, P. M., Båga, M. and Chibbar, R. N. 2014. Chickpea genotypes with contrasting RFO concentrations suggest galactinol synthase as a potential target to reduce RFO concentration in chickpea (*Cicer arietinum* L.) seeds (to be submitted).

7.2 Abstract

To understand the regulation of RFO (Raffinose Family Oligosaccharides) biosynthesis in chickpea (*Cicer arietinum* L.) seeds, RFO accumulation and corresponding biosynthetic enzymes activities were determined during seed development of desi and kabuli genotypes with contrasting RFO concentrations. In mature chickpea seeds, sucrose concentration was positively correlated with seed weight and size significant at $P < 0.05$. RFO concentration in mature chickpea seeds was found as a facilitator rather than the regulating step of seed germination. In mature seeds, raffinose concentration ranged from 0.38 to 0.68 and 0.75 to 0.99 g/100 g whereas stachyose concentration varied from 0.79 to 1.26 and 1.70 to 1.87 g/100 g indicating significant difference between low and high RFO genotypes, respectively. During seed development, rapid decrease in seed moisture after 22 – 26 DAF (days after flowering) stimulated biosynthesis of RFO required to provide desiccation tolerance. Chickpea genotypes with high RFO concentration accumulated higher concentration of *myo*-inositol and sucrose during early seed developmental stages suggesting initial substrates concentrations may influence RFO concentration in mature seeds. High RFO genotypes showed about two to three times higher activity for all RFO biosynthetic enzymes (galactinol synthase, raffinose synthase, stachyose synthase, verbascose synthase and non-galactinol enzyme) compared to those with low RFO concentration. RFO biosynthetic enzymes activities corresponded with the accumulation of individual RFO during chickpea seed development. Galactinol is the first committed step in RFO biosynthesis. Therefore, down-regulation of GS activity could reduce RFO concentration in chickpea seeds.

7.3 Introduction

Chickpea (*Cicer arietinum* L.) is the second most important pulse crop cultivated over 12.3 million hectare of harvested area with total production of 11.6 million tonnes during 2012 (FAO STAT 2012). Chickpea is broadly classified into two categories, (a) desi type (pink flower and small, angular, dark colored seeds), and (b) kabuli type (white flower and large, cream/beige colored, smooth surfaced seeds) (Cobos et al. 2007; Agarwal 2012). Chickpea is an excellent source of carbohydrate, protein, dietary fibers, polyunsaturated fatty acid, minerals and vitamins (Jukanti et al. 2012). However, presence of anti-nutrients like raffinose family oligosaccharides (RFO) restricts worldwide acceptability of chickpea (Alonso et al. 2010). RFO are soluble, non-structural and non-reducing oligosaccharides that are needed in physiological processes like seed desiccation tolerance, seed germination (Blöchl et al. 2008; Pukacka et al. 2009), photosynthate translocation (Turgeon 1996) and

stress tolerance (Nishizawa et al. 2008). However in human diet, food rich in RFO causes stomach discomfort, flatulence and diarrhoea in human and mono-gastric animals as they lack α-galactosidase, a hydrolyzing enzyme needed for RFO breakdown (Naczk et al. 1997; Swennen et al. 2006; Kumar et al. 2010). RFO when consumed in lower concentration are considered as prebiotic supporting growth of beneficial intestinal microflora (Martínez-Villaluenga et al. 2008). Therefore, to develop strategy to reduce seed RFO concentration in chickpea, key steps in RFO biosynthesis need to be characterized.

RFO biosynthesis begins in cytosol with the formation of galactinol from *myo*-inosito and UDP-galactose in a reaction catalyzed by galactinol synthase (GS; EC 2.4.1.123) (Nishizawa et al. 2008). In the next step, galactinol donates its galactosyl unit to sucrose in the presence of raffinose synthase (RS, EC 2.4.1.82) yielding raffinose, the first RFO member (Sprenger and Keller 2000). Raffinose and stachyose together with galactinol synthesize stachyose and verbascose in two separate reactions catalyzed by stachyose synthase (STS, EC 2.4.1.67) and verbascose synthase (VS, EC 2.4.1.x), respectively (Peterbauer et al. 2001). In a galactinol independent pathway, already present RFO react with each other to synthesize higher members of RFO. Galactan:galactan Galactosyl Transferase (GGT) is the sole enzyme catalysing the reaction (Bachmann et al. 1994, Haab and Keller 2002). From here onwards, GGT will be referred as non-galactinol enzyme as its existence has yet to be proven in chickpea. In previous studies with soybean (*Glycine max* L., Saravitz et al. 1987; Castillo et al. 1990; Saldivar et al. 2011), bean (*Phaseolus vulgaris*, Castillo et al. 1990; Bailly 2001), pea (*Pisum sativum* L., Frias et al. 1996; Peterbauer et al. 2001), faba bean (*Vicia faba*, Frias et al. 1996), yellow pea lupin (*Lupinus luteus* L., Frias et al. 1996) and *Vicia* spp. (Lahuta et al. 2005), RFO mainly accumulated during later stages of seed development. However, it is still unclear – which biochemical step should be targeted to reduce seed RFO concentration in chickpea. Besides this, role of RFO during seed germination is also not well-understood. RFO metabolism supports early stages of seed germination by providing carbon and energy (Blöchl et al. 2008). In pea, inhibition of RFO metabolism significantly delayed the seed germination (Blöchl et al. 2007). However in wild type soybean, inhibition of RFO metabolism by 1-deoxygalactonojirimycin delayed seed germination but low RFO genotypes did show significant delay in germination (Dierking and Bilyeu 2009). Therefore, it is important to study the effect of RFO concentration and metabolism on chickpea seed germination. Imino sugar 1-deoxygalactonojirimycin (DGJ) is an inhibitor of acidic and alkaline α-galactosidase thus can inhibit the RFO metabolism during seed germination (Blöchl et al. 2007). Comparison of seed germination among chickpea genotypes with

contrasting RFO concentration and *in vitro* treatment with DGJ would be helpful in investigating whether RFO concentration or metabolism is critical for chickpea seed germination (Blöchl et al. 2007; Dierking and Bilyeu 2009).

Chickpea genotypes with contrasting RFO concentration were analysed for seed weight and size, germination and RFO biosynthesis. Sucrose concentration showed a significant positive (r = 0.728, $P \leq 0.05$) correlation with chickpea seed size and weight. Chickpea genotypes with contrasting RFO concentration did not show significant difference in seed germination. However, inhibition of RFO mobilization significantly delayed germination in DGJ treated seeds. Chickpea genotypes with high RFO concentration showed significantly higher accumulation of initial substrates (*myo*-inositol and sucrose) as well as higher RFO biosynthetic enzymes activities compared to low RFO genotypes.

7.4 Material and methods

7.4.1 Plant material and chemicals

Desi (ICC 1163, ICC 1471, ICC 9562, ICCV 07115, ICCV 07116 and ICCV 07117) and kabuli (ICC 5270, ICC 10674, ICC 16216, ICC 16528, ICCV 3 and ICCV 91302) chickpea genotypes with high and low RFO concentration (high RFO genotypes are underlined), were selected from a germplasm collection procured from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, Patancheru, India) (Figure 7.1, Gangola et al. 2013). Seeds were grown during May – August, 2013 in University of Saskatchewan agricultural greenhouse (52° 07′ N latit-ude, 106° 38′ W longitude and 481.5 m altitude, Saskatoon, SK, Canada) with 18 h photoperiod, ~325 µM/m^2/sec of integrated PAR (photo-synthetically active radiation) and mean temperature of 17 °C (night) to 23 °C (day). For germination test, two released chickpea varieties, CDC Vanguard (desi type) and CDC Frontier (kabuli type) were used as control. Fully opened flowers were tagged and pods were collected from 10 days after flowering (DAF) till 50 DAF at four days interval for all the genotypes. Pods were collected and immediately frozen in liquid N_2 and stored at -80 °C till needed. To determine moisture (fresh weight basis), seeds were kept at 80°C till constant weight was observed. *M*yo-inositol, DGJ, galactinol, UDP-galactose, raffinose, stachyose and verbascose were purchased from Sigma-Aldrich (Oakville, ON, Canada), while sucrose was from EMD Chemicals (Mississauga, ON, Canada).

7.4.2 Seed germination test

Germination test was performed using all desi and kabuli genotypes including two

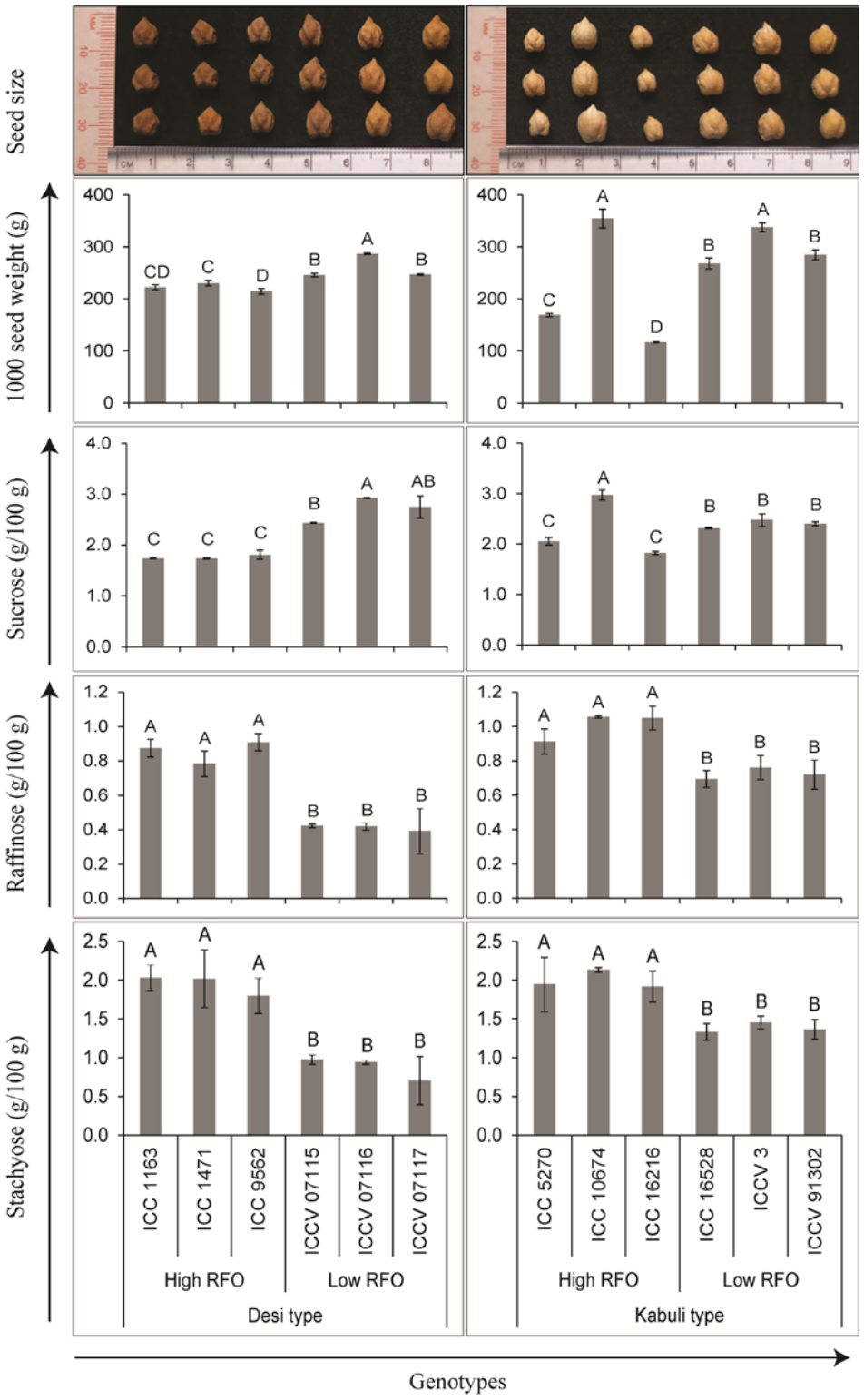


Figure 7.1 Chickpea genotypes showing variation for seed size, seed weight and concentrations of sucrose, raffinose and stachyose.

Mean values from three independent replicates ± SD are shown. The unit of concentration is g/100 g of chickpea seed meal on fresh weight basis.

control genotypes. Germination percentage was calculated as described (Blöchl et al. 2007, Dierking and Bilyeu 2009). In brief, seeds (25 seeds/genotype in three replications) were imbibed in water for 16 h at 25 °C in dark and transferred to petri-plates lined with wetted filter papers. Petri-plates were placed at 25 °C in dark for seed germination. Germinated seeds were counted at 12 h interval till ≥95 % seed germination was achieved. The piercing of seed coat by radicle was considered as germination stage or zero hour after germination (HAG).

Seeds (25 seeds/genotype in three replications) of CDC Vanguard were exposed to six treatments to analyse germination rate: water (control), 50 mM DGJ (Sigma, Oakville, ON, Canada), 25 mM sucrose (Sigma, Oakville, ON, Canada), 50 mM galactose (Sigma, Oakville, ON, Canada), 50 mM DGJ + 25 mM sucrose and 50 mM DGJ + 50 mM galactose. Seeds were imbibed and placed for germination following the same method as mentioned earlier. Seeds were collected at 8 and 16 h after soaking (HAS) and zero, 12 and 24 HAG to determine soluble sugars' concentration.

7.4.3 Determination of soluble sugars concentration

To determine concentration (g/100 g of chickpea seeds meal on dry weight basis) of soluble sugars (*myo*-inositol, galactinol, sucrose, raffinose, stachyose and verbascose), a modified HPAEC-PAD (High Performance Anion Exchange Chromatography with Pulsed Amperometric Detector) based method was utilized (Gangola et al. 2014).

7.4.4 *In vitro* assays for RFO biosynthetic enzymes activities

Enzyme activity assays for RFO biosynthetic enzymes were performed as described in section 3.5 of the thesis.

7.4.5 Statistical analysis

Pairwise comparisons using Tukey's method and correlation analysis were performed on MINITAB 14.0 statistical software (Minitab Inc., State College, PA, USA).

7.5 Results

7.5.1 Concentration of soluble sugars in selected chickpea genotypes

Pairwise comparison revealed significant difference in RFO concentration among desi and kabuli genotypes, therefore high and low RFO genotypes (desi and kabuli typoe) were grouped separately (Figure 7.1). In high RFO desi genotypes, stachyose/raffinose varied

from 1.73 to 1.87/0.75 to 0.90 g/100 g, while it ranged from 0.79 to 0.82/0.38 to 0.43 g/100 g among low RFO genotypes. In kabuli types, stachyose concentration varied from 1.70 to 1.85 and 1.19 to 1.26 g/100 g in high and low RFO genotypes, respectively whereas raffinose concentration varied from 0.89 to 0.99 and 0.59 to 0.68 g/100 g. Verbascose concentration did not vary significantly among high and low RFO genotypes in both desi and kabuli type chickpeas. Sucrose concentration varied from 1.74 to 1.81 and 2.44 to 2.92 g/100 g among low and high RFO desi genotypes, respectively. Kabuli high and low RFO genotypes also followed the same pattern for sucrose concentration that varied 1.82 to 2.97 and 2.32 to 2.48 g/100 g, respectively. The higher variation in sucrose concentration among kabuli genotypes compared to desi types is attributed to exceptionally high sucrose level in seeds of ICC 10674 (high RFO kabuli genotype).

7.5.2 Seed weight and size variation among contrasting RFO genotypes

Among desi types, ICCV 07116 (286.7 ± 2.3 g) showed the highest thousand seed weight while it was the lowest for ICC 9562 (214.2 ± 5.6 g). In kabuli types, the highest and lowest 1000 seed weight was observed for ICC 10674 (354.2 ± 17.9 g) and ICC 16216 (116.5 ± 1.1 g), respectively. Low RFO genotypes (245.7 to 286.7 and 268.1 to 337.5 g for desi and kabuli genotypes, respectively) showed significantly higher thousand seed weight than genotypes with high RFO concentration (214.2 to 229.9 and 116.5 to 169.0 g for desi and kabuli genotypes, respectively) reflecting larger seed size of low RFO genotypes (Figure 7.1). However, ICC 10674 is the exception, *i.e.* this genotype is one of the high RFO kabuli genotypes showing significantly higher seed weight and larger seed size. Correlation analysis revealed a negative but insignificant (r = -0.219 and -0.362, $P \geq 0.05$ for raffinose and stachyose) association between raffinose/stachyose concentration and seed weight/size in chickpea seeds. However, sucrose concentration showed significant positive correlation (r = 0.728, $P \leq 0.05$) with chickpea seed weight and size.

7.5.3 Seed germination test

All desi and kabuli genotypes showed about 95 % germination at 84 h after imbibition (HAI). However, kabuli type chickpeas germinated faster than that of desi types. For >50 % germination, kabuli types took 24 HAI whereas desi types required 36 HAI. The germination in high and low RFO genotypes were compared at two time points: 1) when most of the genotypes- (24 HAI in desi type and 12 HAI in kabuli type), and 2) control- (36 and 24 HAI in desi and kabuli type, respectively), -showed ≥50 % germination. ICCV 07117

(74.7 % at 24 HAI and 89.3 % at 36 HAI) and ICC 16216 (96 % at 12 HAI and 100 % at 24 HAI) showed the highest rate of germination among desi and kabuli genotypes, respectively (Figure 7.2). At both time points, no significant difference in germination was observed that could be directly associated with high and low RFO concentrations in desi and kabuli chickpea types (Figure 7.2).

To study if RFO are utilized during seed germination, seeds of CDC Vanguard were treated with water, DGJ, sucrose, galactose, DGJ + sucrose and DGJ + galactose. The germination in different treatments were compared at 24, 36, 48 and 60 HAI at which germination rate was observed very high compared to other time points (Figure 7.3). No germination was observed in DGJ treated seeds till 24 HAI. Thereafter, effect of DGJ on seed germination was gradually reduced causing insignificant difference in germination at 96 HAI (Figure 7.3). Water and sucrose treated seeds showed ≥50 % germination at 36 HAI whereas galactose treated seeds aceived similar germination percentage at 48 HAI. In DGJ treated seeds, ≥50 % germination was observed at 72 HAI. However, seeds treated with DGJ + sucrose and DGJ + galactose required 48 and 60 HAI for >50 % germination, respectively (Figure 7.3). At 84 HAI, all the treatments exhibited >95 % germination except DGJ (85.3 %) and DGJ + galactose (92.0 %) whereas no significant difference in germination was observed at 96 HAI. RFO concentration did not change significantly during germination in DGJ treated seeds (Figure 7.4). In other treatments (water, sucrose and galactose), RFO were completely utilized during germination that simultaneously increased the concentration of basic molecules like *myo*-inositol, glucose, fructose and sucrose (Figure 7.4).

7.5.4 Moisture and dry matter conetent in developing chickpea seeds

At 10 DAF, desi genotypes contained 82.0 to 87.8 and 88.9 to 92.3 % moisture in seeds of high and low RFO genotypes where as in kabuli types, seed moisture ranged from 86.9 to 91.1 and 87.2 to 89.9 % in high and low RFO genotypes, respectively. Seed moisture gradually decreased till about 38 DAF in both chickpea types *i.e.* 30.3 – 39.5 % reduction in seed moisture was observed (Figure 7.5). The rate of seed moisture reduction was higher after 38 DAF. Chickpea genotypes showed about 41.7 to 56.2 % reduction in seed moisture from 38 DAF till maturity. Chickpea seeds moisture decreased continuously with the progression of seed development that corresponded with the increased accumulation of dry matter. The seed moisture concentration decreased from 82.0 - 92.3 % at 10 DAF to 4.7 - 7.1 % at maturity (Figure 7.5). After 38 DAF, rapid decrease in seed moisture was observed that corresponded with the accumulation of RFO in seeds. Dry matter in chickpea seeds increased

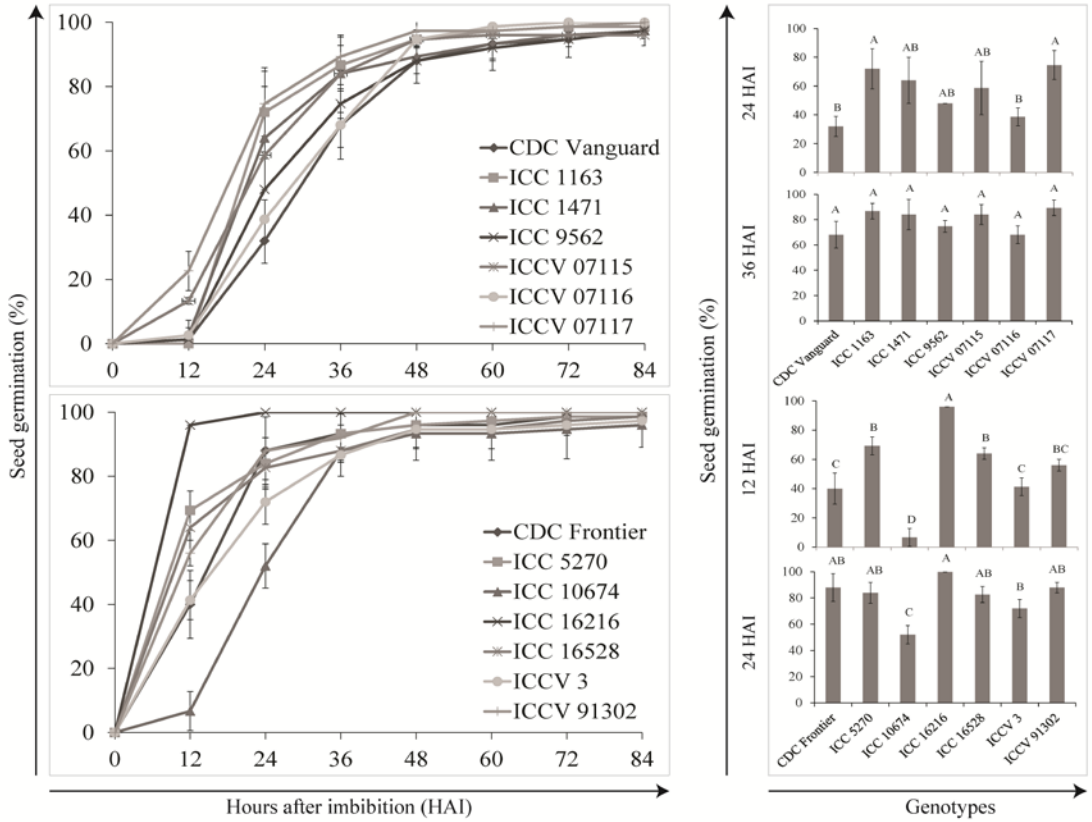


Figure 7.2 Germination percentage of chickpea genotypes with contrasting RFO concentration.

CDC Vanguard (desi type) and CDC Frontier (kabuli type) were used as control. Mean values from three independent replicates ± SD are shown.

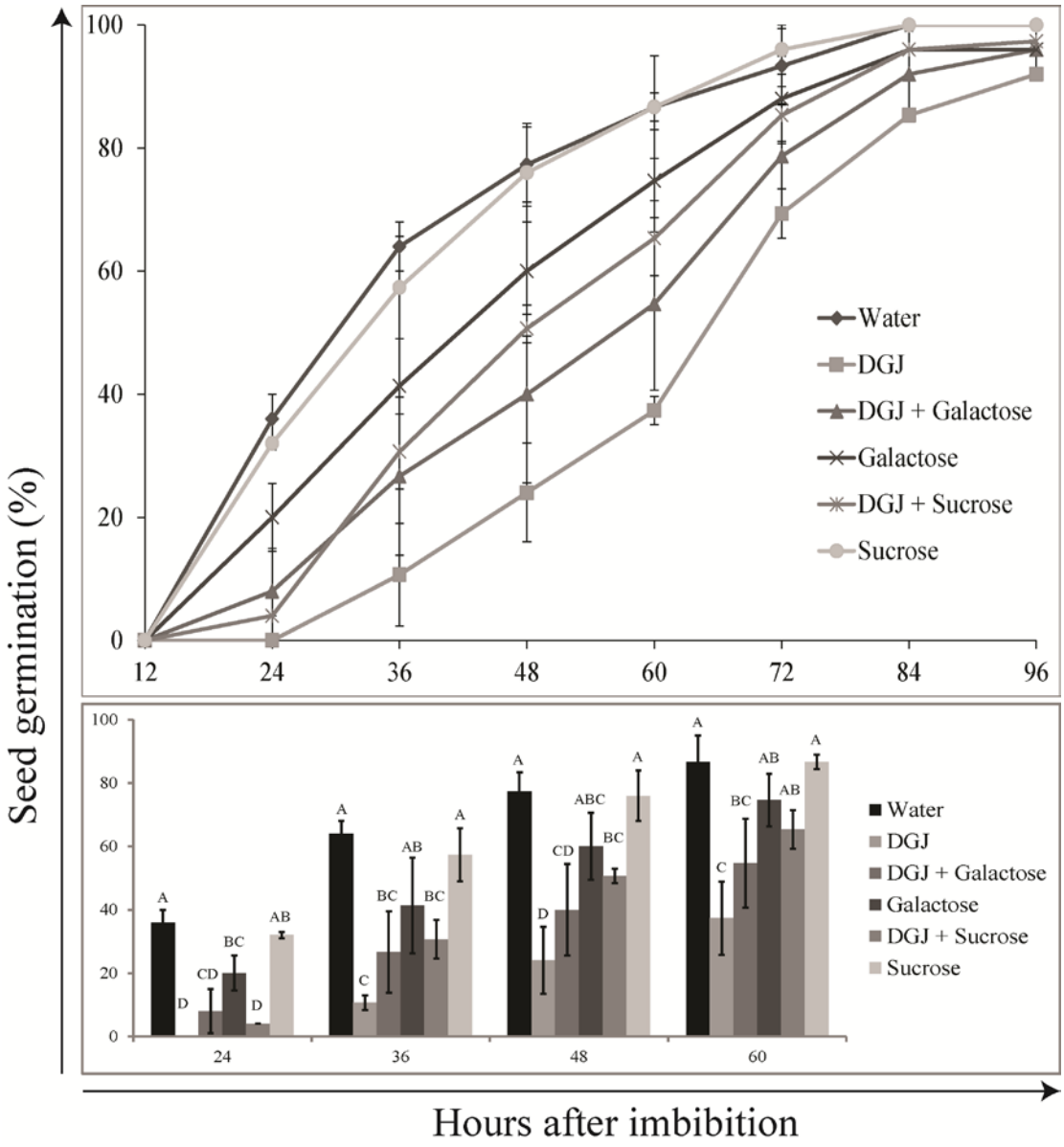


Figure 7.3 Inhibitory effect of DGJ on chickpea seed germination.

Different treatments used in the study were: water (control), 50 mM DGJ, 50 mM DGJ + 50mM galactose, 50 mM galactose, 50 mM DGJ + 25mM sucrose and 25 mM sucrose (DGJ inhibitor of acidic and alkaline α-galactosidase, stands for 1-deoxygalactonojirimycin). Mean values from three independent replicates ± SD are shown.

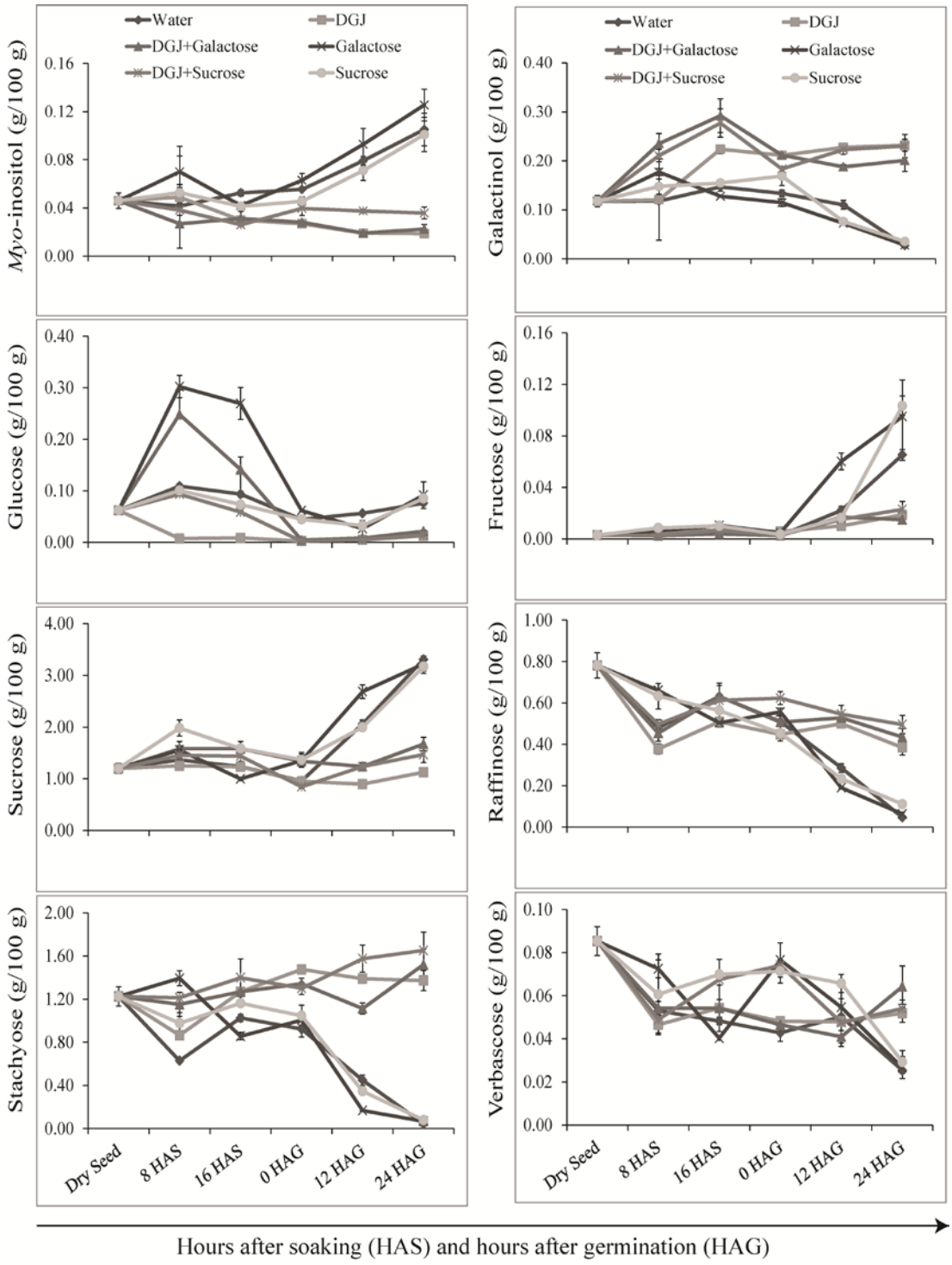


Figure 7.4 Concentration of different compounds regarding RFO biosynthesis during germination test of CDC Vanguard using different treatments.

Traetments were: water (control), 50 mM DGJ, 50 mM DGJ + 50mM galactose, 50 mM galactose, 50 mM DGJ + 25mM sucrose and 25 mM sucrose (DGJ stands for 1-deoxygalactonojirimycin). Mean values from three independent replications ± SD are shown. The unit of concentration is g/100 g of chickpea seed meal on dry weight basis.

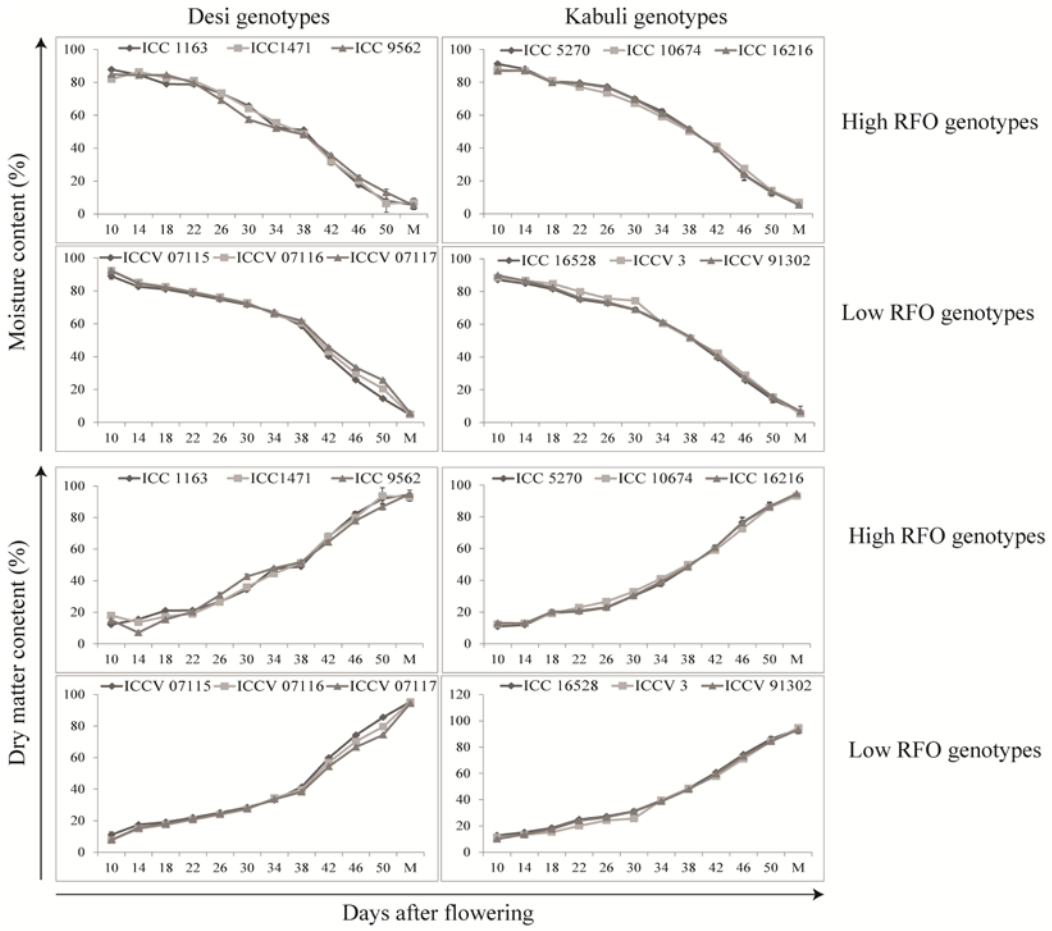


Figure 7.5 Moisture loss and accumulation of dry matter during seed development of contrasting RFO genotypes.

Mean values from three independent replicates ± SD are shown.

from 7.7 - 18.0 % at 10 DAF to 92.9 - 95.3 % at maturity (Figure 7.5). Therefore, in contrast to moisture, seed dry matter increased rapidly after 38 DAF. Seed dry matter increased about 41.7 to 56.2 % from 38 DAF till maturity. The change in seed dry matter and moisture was almost similar in all the genotypes disrespect to chickpea type (desi or kabuli) and high or low RFO genotypes.

7.5.5 RFO accumulation and corresponding biosynthetic enzymes activities during chickpea seed development

The initial substrates of RFO biosynthesis *myo*-inositol and sucrose were detected throughout seed development process. In desi genotypes, *myo*-inositol concentration in seeds was 0.48 to 0.83 g/100 g at 10 DAF that reduced to 0.03 to 0.07 g/100 g in mature seeds. *Myo*-inositol concentration in seeds of kabuli genotypes ranged from 0.44 to 1.15 and 0.03 to 0.06 g/100 g at 10 DAF and in mature seeds, respectively. Sucrose concentration varied 7.7 to 10.7/1.1 to 2.8 and 6.1 to 15.9/1.6 to 3.7 g/100 g at 10 DAF/in mature seeds of desi and kabuli types, respectively. During early stages, these molecules were present in higher concentration which was utilized for RFO biosynthesis thus reduced their concentration gradually during seed development (Figures 7.6 and 7.7). Primary substrates *myo*-inositol and sucrose showed significant differences ($P \leq 0.05$) in concentrations among high and low RFO chickpea genotypes. In high RFO genotypes, maximum *myo*-inositol concentration ranged 0.73 to 0.83 and 0.97 to 1.15 g/100 g at 10 DAF in desi and kabuli types, respectively whereas low RFO genotypes showed variation from 0.48 to 0.53 and 0.50 to 0.67 g/100 g at 10 and 14 DAF, respectively. In desi genotypes, sucrose concentration showed its peak accumulation at 18 - 22 DAF and varied from 13.2 to 14.1/16.9 to 17.9 g/100 g in low/high RFO genotypes. In kabuli genotypes, maximum sucrose concentration was observed at 14 - 18 DAF and ranged 12.1 to 12.4/14.3 to 19.8 g/100 g in low/high RFO genotypes. Monosaccharides like glucose and fructose were also present throughout the seed development. Glucose concentration was 2.6 to 8.4/0.2 to 0.3 g/100 g in desi genotypes whereas varied from 1.9 to 6.5/0.2 to 0.3 g/100 g in kabuli genotypes at 6 DAF/in mature seeds. In desi genotypes, fructose concentration ranged 1.7 to 5.1/0.005 to 0.008 g/100 g while in kabuli genotypes varied 1.1 to 3.9/0.006 to 0.01 g/100 g at 6 DAF/in mature seeds.

RFO biosynthesis started with the detection of galactinol at 14 – 22 DAF (Figures 7.6 and 7.7). In desi genotypes, galactinol was first detected at 14 – 18 (0.02 to 0.15 g/100 g) and 18 – 22 (0.09 to 0.11 g/100 g) DAF, whereas in kabuli genotypes at 18 (0.14 to 0.16 g/100 g) and 14 – 22 (0.03 to 0.09 g/100 g) DAF in high and low RFO genotypes, respectively.

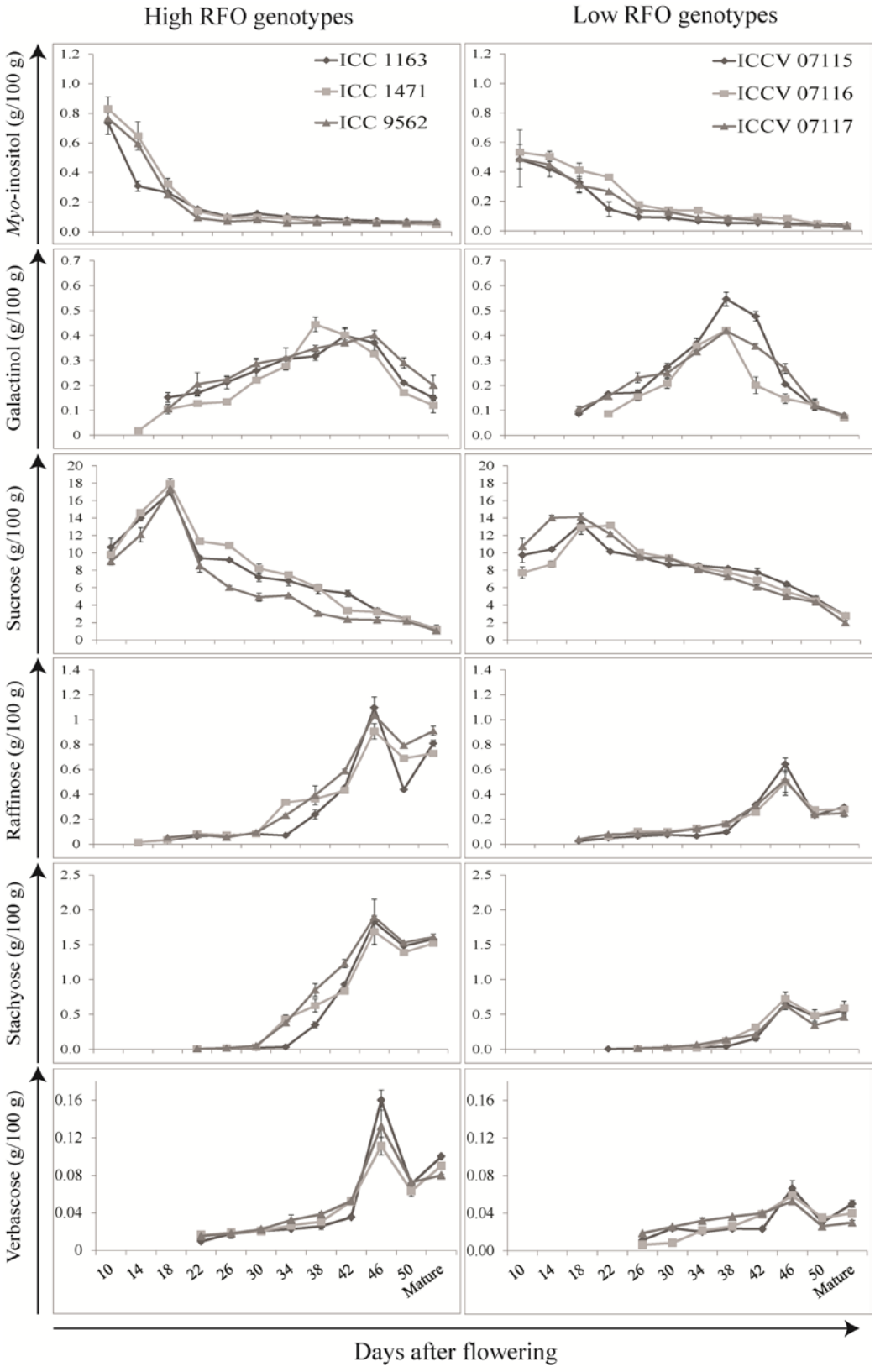


Figure 7.6 Concentration of different compounds regarding RFO biosynthesis during seed development of contrasting RFO genotypes (desi type).

Mean values from three independent replicates ± SD are shown. The unit of concentration is g/100 g of chickpea seed meal on dry weight basis.

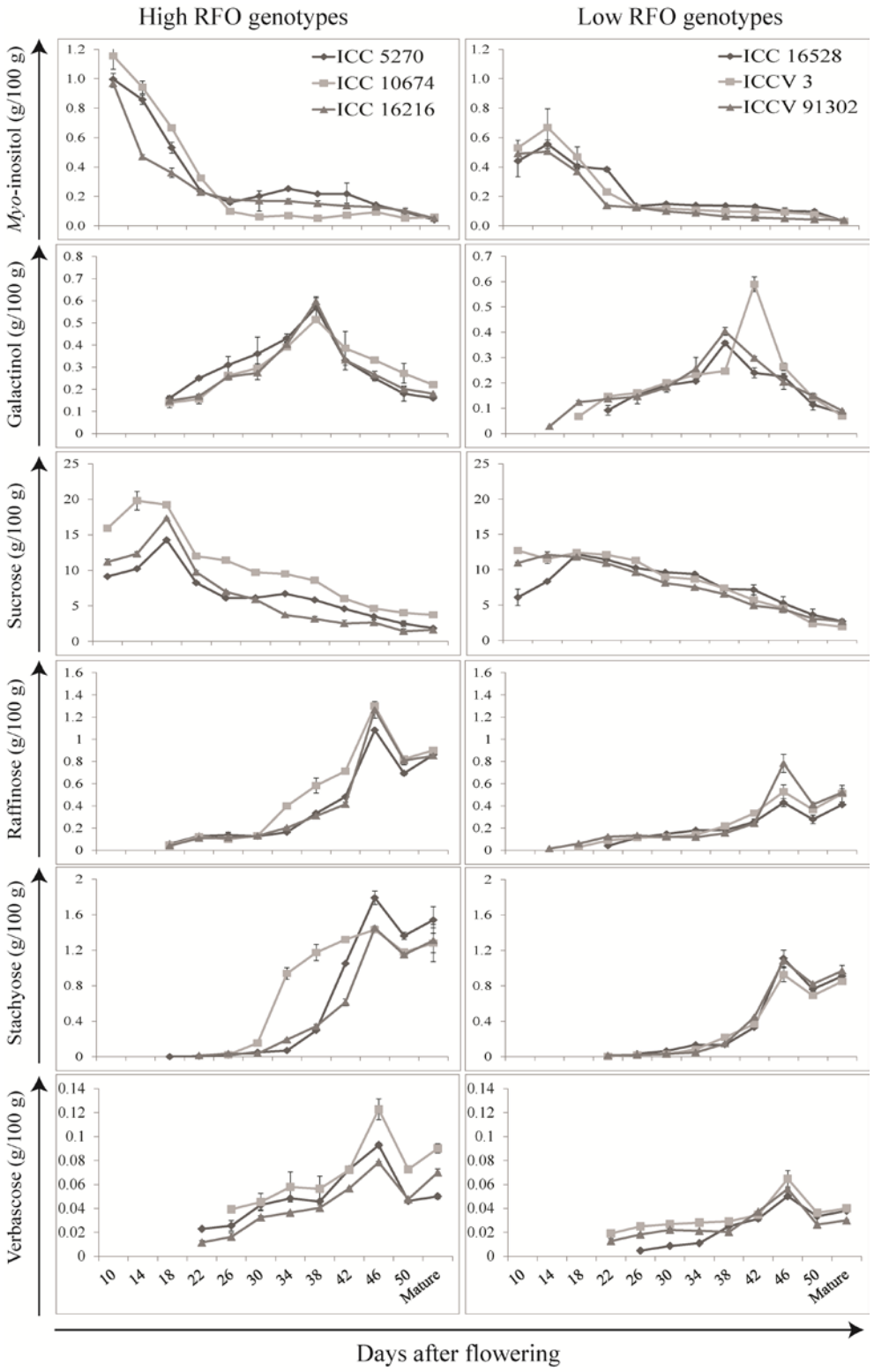


Figure 7.7 Concentration of different compounds regarding RFO biosynthesis at different developmental stages during seed development of contrasting RFO genotypes (kabuli type).

Mean values from three independent replicates ± SD are shown. The unit of concentration is g/100 g of chickpea seed meal on dry weight basis.

Among desi and kabuli types, maximum accumulation of galactinol was observed at 38 – 42 DAF in both high (0.44 to 0.46 and 0.52 to 0.60 g/100 g) and low (0.53 to 0.56 and 0.46 to 0.69 g/100 g) RFO genotypes. Thereafter, galactinol concentration decreased till seed maturity. In mature seeds of desi and kabuli types, galactinol concentration ranged 0.12 to 0.20/0.07 to 0.08 and 0.16 to 0.22/0.07 to 0.09 g/100 g in high and low RFO genotypes.

In both desi and kabuli types, maximum GS activity was observed at 42 and 46 DAF in low and high RFO genotypes, respectively. Among desi types, maximum GS activity ranged from 1211.6 to 1346.9 and 544.1 to 615.4 pkat/mg protein in high and low RFO genotypes, respectively. Maximum GS activity in kabuli types ranged from 1376.2 to 1490.8 and 639.6 to 670.4 pkat/mg protein in high and low RFO genotypes, respectively (Figure 7.8). Mature seeds of desi and kabuli types showed GS activity varying from 544.8 to 783.4/315.7 to 408.3 and 505.0 to 1046.0/247.4 to 391.1 pkat/mg protein in high/low RFO genotypes, respectively. Decrease in galactinol concentration after 38 - 42 DAF in desi and kabuli genotypes supported the biosynthesis of raffinose and other higher members of the family.

In desi genotypes with high RFO concentration, raffinose was first detected at 14 – 18 DAF with concentrations of 0.01 – 0.05 g/100 g whereas in low RFO genotypes at 18 – 22 DAF with concentration ranging from 0.03 to 0.06 g/100 g (Figures 7.6 and 7.7). Among kabuli types, high and low RFO genotypes showed first accumulation of raffinose at 18 and 14 – 22 DAF with varying concentration of 0.04 – 0.06 and 0.01 – 0.04 g/100 g, respectively. The highest accumulation of raffinose was observed at 46 DAF in high/low RFO genotypes of desi and kabuli type with concentrations of 0.91 to 1.10/0.50 to 0.64 and 1.08 to 1.30/0.43 to 0.78 g/100 g, respectively. Among mature seeds of desi and kabuli types, raffinose concentration was 0.73 to 0.91/0.25 to 0.30 and 0.41 to 0.52/0.85 to 0.90 g/100 g in high/low RFO genotypes, respectively. Accumulation of raffinose in chickpea seeds was association with RS activity. In desi and kabuli type, the highest RS activity was found at 42 DAF in low/high RFO genotypes of desi (18.3 – 25.5/43.5 – 57.8 pkat/mg protein) and kabuli (20.0 – 27.7/52.3 – 64.9 pkat/mg protein) type (Figure 7.8). Mature seeds also expressed RS activity ranged from 24.2 to 24.9/9.2 to 11.4 and 11.2 to 20.1/7.8 to 11.4 pkat/mg protein in high/low RFO genotypes of desi and kabuli types, respectively.

Stachyose accumulation was first observed at 18 - 26 DAF which is after the initiation of raffinose biosynthesis as raffinose acts as a substrate for stachyose biosynthesis. Thereafter, raffinose and stachyose were synthesised simultaneously during seed development. Stachyose was first detected at 22 and 22 – 26 DAF with concentrations ranging from 0.01 to 0.02 and 0.005 to 0.01 g/100 g in high and low RFO genotypes of desi

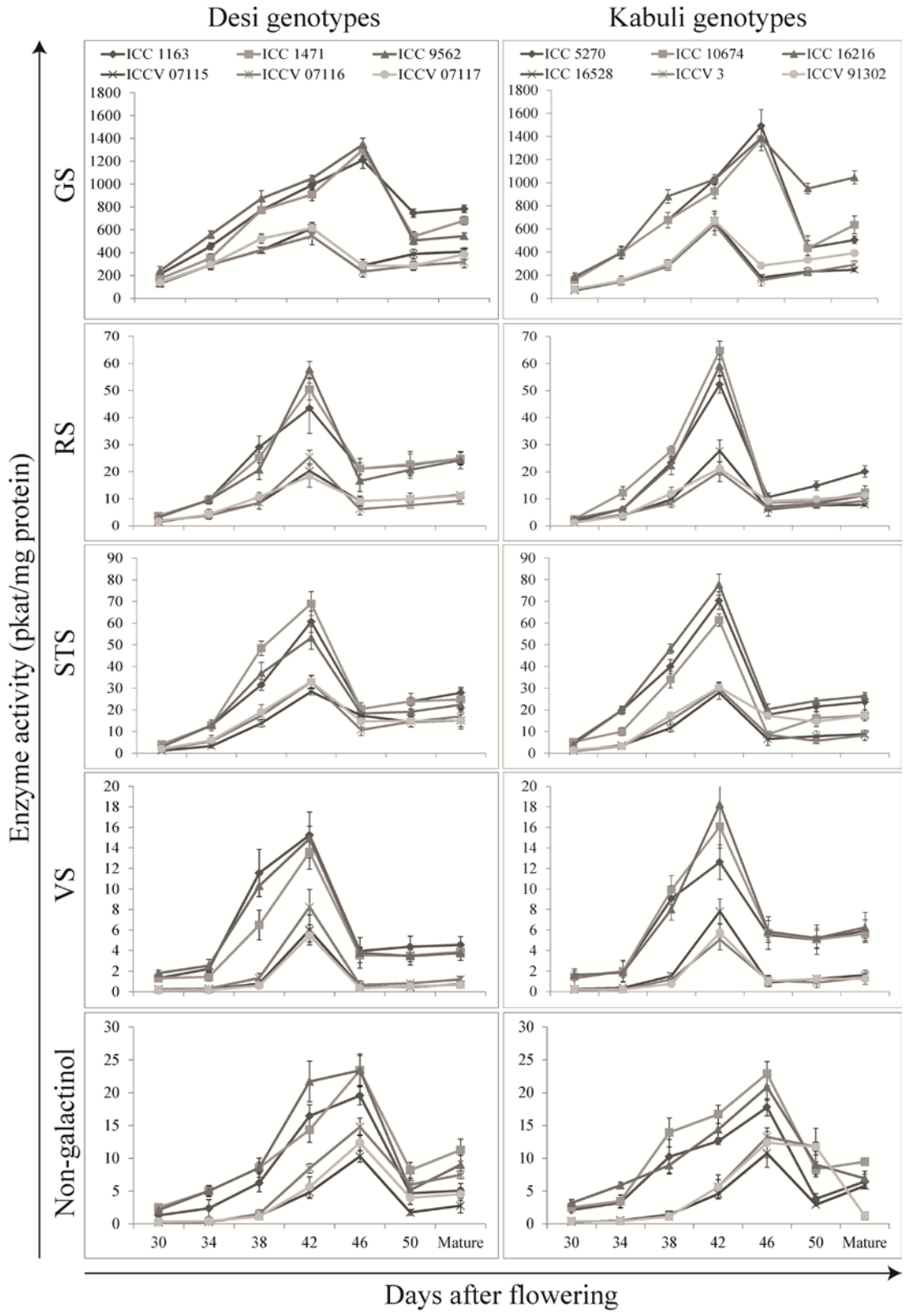


Figure 7.8 Enzymatic activities of RFO biosynthetic enzymes during seed development of contrasting RFO genotypes.

Mean values from three independent replicates ± SD are shown.

type, respectively (Figures 7.6 and 7.7). Among kabuli types, stachyose accumulation occurred between 18 to 26 and 22 to 26 DAF with varying concentration from 0.001 to 0.02 and 0.003 to 0.03 g/100 g in high and low RFO genotypes, respectively. Stachyose showed the highest concentration at 46 DAF in desi and kabuli types that ranged from 1.69 to 1.90/0.63 to 0.73 and 1.43 to 1.79/0.93 to 1.11 g/100 g among high/low RFO genotypes, respectively. In mature seeds, stachyose concentration varied from 1.52 to 1.61/0.46 to 0.59 and 1.28 to 1.54/0.85 to 0.97 g/100 g in high/low RFO genotypes of desi and kabuli types, respectively.

Stachyose biosynthesis is catalyzed by STS that showed comparatively higher enzymatic activity in high RFO genotypes (desi/kabuli type) (53.2 to 69.0/61.4 to 77.7 pkat/mg protein) than in low RFO genotypes (28.4 to 33.0/28.0 to 30.3 pkat/mg protein) at 42 DAF (Figure 7.8). Mature seeds also showed STS activity that ranged from 22.4 to 27.9/15.1 to 17.0 and 17.5 to 26.3/8.3 to 17.4 pkat/mg protein in high/low RFO genotypes of desi and kabuli types, respectively.

Verbascose is the RFO with least concentration in chickpea seeds and detected after the onset of stachyose biosynthesis. All desi and kabuli genotypes at 46 DAF showed the highest verbascose concentration varying from 0.11 to 0.16/0.05 to 0.07 and 0.08 to 0.12/0.05 to 0.06 g/100 g among high/low RFO genotypes, respectively (Figures 7.6 and 7.7). Verbascose synthesizing enzyme showed the highest activity of 13.6 to 15.2/5.5 to 8.2 and 12.6 to 18.3/5.1 to 7.8 pkat/mg protein at 42 DAF for high/low RFO genotypes of desi and kabuli type, respectively (Figure 7.8).

Chickpea seeds also exhibited enzymatic activity for non-galactinol biosynthesis of RFO. Desi and kabuli genotypes showed maximum activity of 19.6 to 23.4/10.3 to 14.8 and 17.8 to 22.9/10.7 to 13.3 pkat/mg protein at 46 DAF for non-galactinol RFO biosynthesis in high/low RFO genotypes (Figure 7.8). Mature seeds also showed non-galactinol RFO biosynthetic activity in the range of 5.1 to 11.3/2.8 to 7.5 and 6.5 to 9.5/1.2 to 5.8 pkat/mg protein in high/low RFO genotypes of desi and kabuli types, respectively.

7.6 Discussion

7.6.1 Concentration of soluble sugars influencing seed size and weight

In all selected chickpea genotypes seeds, sucrose was identified as the major soluble sugar followed by RFO. Stachyose was the predominant member of RFO followed by raffinose whereas verbascose was present in very low concentrations in mature chickpea seeds (Alajaji and El-Adawy 2006; Martínez-Villaluenga et al. 2008; Gangola et al. 2013).

Sucrose showed a negative correlation with raffinose (r = -0.48, $P \geq 0.05$) and stachyose (r = -0.66, $P \leq 0.05$) as it acts as a substrate for the biosynthesis of raffinose which is further utilized to synthesize stachyose. Therefore, reduced level of sucrose reflects the higher accumulation of RFO (Figures 7.6 and 7.7). Sucrose in the phloem sap is also considered as an important determinant of seed growth by modulating the mitotic activity in embryo (Munier-Jolain and Salon 2003) thus explains its significant positive correlation with seed weight and size. Sucrose synthase activity in developing cotyledons was also correlated with chickpea seed size (Turner et al. 2009). This correlation also describes the exceptionally higher seed weight and size of ICC 10674 among kabuli genotypes with high RFO concentrations. Sucrose concentration in other high RFO kabuli genotypes is significantly lower than ICC 10674 and low RFO genotypes resulting in significantly reduced seed weight and size. Cicek et al. (2006) also reported a significant ($P \leq 0.001$) positive correlation of sucrose with seed yield (r = 0.39/0.35) and seed size (r = 0.39/0.56) in two recombinant inbred populations of soybean [*Glycine max* (L.) Merr.]. RFO biosynthesis utilizes sucrose as substrate thus affects its concentration indirectly. Consequently, RFO concentration is in negative but insignificant correlation with seed weight and size.

7.6.2 RFO mobilization facilitates chickpea seed germination

In chickpea, RFO concentration did not demonstrate any significant association with seed germination ability thus suggesting that reduced RFO chickpea genotypes have no adverse effect on agricultural production. However, it contradicts the participation of RFO during early seed germination process (Blöchl et al. 2007; Rosnoblet et al. 2007; Blöchl et al. 2008). DGJ an imino sugar and analog of terminal galactose acts as a reversible competitive inhibitor of α-galactosidase (Khanna et al. 2010), declined the rate of germination in chickpea seeds. The inhibitory effect of DGJ was compensated by sucrose and galactose suggesting galactose and sucrose are needed for seed germination. RFO are mobilized by α-galactosidase during germination providing carbon and energy to the growing seedling (Zhao et al. 2006). Sucrose (through sucrose transporters, Li et al. 2012) and galactose (through hexokinase signalling, Jang et al. 1997; Frommer et al. 2003) may also be needed for sugar signaling influencing seed germination. Determination of soluble sugars confirmed the obstruction of RFO mobilization in DGJ treated seeds that resulted in delayed seed germination. In absence of RFO mobilization, other carbon sources might be utilized to support seed germination thus compensating inhibitory effect of RFO immobilization. Therefore, chickpea genotypes with contrasting RFO concentration showed no significant difference in seed germination after 96

HAI. The results from both germination experiments (comparing contrasting RFO genotypes and DGJ treatment) suggest that RFO mobilization may be a facilitator but RFO concentration is not the regulatory factor to determine chickpea
seed germination capacity.

7.6.3 Initial substrate concentration and RFO biosynthetic enzymes activities influence seed RFO concentration

Significant difference in *myo*-inositol and galactinol concentration between high and low RFO genotypes suggests that initial substrates concentrations influence the seed RFO concentration as reported in pea (*Pisumm sativum* L., Peterbauer et al. 2001; Karner et al. 2004). *Myo*-inositol and sucrose concentration did not show any effect on galactinol synthesis as no significant difference in galactinol concentration was observed between low and high RFO genotypes. It might be due to simultaneous utilization of galactinol in RFO biosynthesis. These results also confirmed the reversible nature of RFO biosynthetic reactions regulated by mass action ratio and equilibrium constant (Peterbauer et al. 2001). Therefore, increased accumulation of substrates supports the higher accumulation of RFO. Later on the basis of substrates feeding experiments, Karner et al. (2004) suggested the regulation of RFO concentration by the initial substrates level (*myo*-inositol and sucrose) rather than GS activity alone.

Accumulation of different members of raffinose family (raffinose, stachyose and verbascose) is supported by the corresponding biosynthetic enzymes activities. RFO biosynthetic enzymes expressed their maximum activities about four days prior to RFO accumulation. GS showed the highest enzymatic activity (42 - 46 DAF) even after the highest accumulation of galactinol (38 - 42 DAF) thus suggesting that active utilization of galactinol after about 38 DAF in RFO biosynthesis outstripped the accumulation of galactinol in developing seeds. The highest GS activity after maximum galactinol accumulation was also observed during seed development in *Pisum sativum* (Peterbauer et al. 2001) and *Brassica napus* (Li et al. 2011). In addition, Galactinol has been designated as universal galactosyl unit donor in RFO biosynthesis (Peterbauer et al. 2001). In galactinol independent pathway, galactinol participates indirectly by synthesizing raffinose and stachyose that are further utilized to synthesize stachyose and verbascose, respectively. Therefore, GS showed the highest enzymatic activity followed by STS, RS, non-galactinol and VS. Hence, GS may be considered as a determinant of RFO concentration in chickpea seeds. A galactinol independent pathway was also found operative in chickpea seeds and showed its pH optimum

at 7.0. Similarly, neutral pH was observed optimum for non-galactinol RFO biosynthesis in *P. sativum* (Peterbauer et al. 2001). They described STS as a multifunctional enzyme participating in galactinol –dependent as well as –independent pathway of RFO biosynthesis. However, Bachmann et al. (1994) reported GGT a separate enzyme for galactinol independent RFO biosynthesis pathway, from leaves of *Ajuga reptans* and found its pH optimum at 4.5 - 5.0 (Tapernoux-Lüthi et al. 2004). In summary, initial substrate concentration and regulating RFO biosynthetic enzymes activities could be the most potential candidates to reduce RFO concentration in chickpea seeds.

7.6.4 GS – an appropriate strategy to reduce seed RFO concentration in chickpea

Among RFO biosynthetic enzymes, GS was found as the potential target to reduce seed RFO concentration in chickpea. The six main characteristics supporting GS candidature over other biosynthetic enzymes are: (1) galactinol is the universal substrate to synthesize every member of raffinose family. The galactinol independent pathway is also operative in chickpea seeds but showed very low enzymatic activity and the availability of substrates (raffinose and stachyose) depends of galactinol availability. (2) Galactinol connects two biosynthetic pathways including *myo*-inositol and UDP-galactose therefore considered as first committed step in RFO biosynthesis (McCaskill and Turgeon 2007). (3) GS showed 2-3 times higher activity in high RFO genotypes compared to low RFO genotypes. (4) GS showed about 20-30 times higher enzymatic activity than that of other biosynthetic enzymes. (5) During seed developmental stages, GS is the biosynthetic enzyme showing first activity and also the last with maximum activity depicting its highest involvement in RFO biosynthesis. (6) In previous study (Gangola et al. 2013), galactinol followed the same pattern of variation as total RFO, raffinose and stachyose in field and greenhouse growing conditions. Therefore, GS should be targeted to reduce RFO concentration in chickpea seeds.

7.7 Conclusion and future prospects

The present study compared RFO biosynthesis in contrasting chickpea genotypes. It also revealed the significant positive correlation of seed size/weight to sucrose concentration. Seed RFO concentration was described as a facilitator of germination rather than regulating factor in chickpea. RFO mainly accumulated during later stages of seed development thus providing desiccation tolerance to maturing seed. Sucrose and stachyose were designated as predominant soluble sugar and RFO in chickpea seeds, respectively. By comparing RFO biosynthesis in contrasting RFO genotypes, substrate concentration and biosynthetic

enzymes' activities were described as determinants of seed RFO concentration in chickpea. GS was identified as most direct and potent target to reduce RFO concentration in chickpea seeds. Purification and characterization of GS from chickpea seeds would be helpful to understand the structure and kinetics of this enzyme that might be utilized to further study its regulation with in plant cell. It might also be employed to develop chickpea varieties with reduced seed RFO concentration that will lead to increased acceptability of chickpea worldwide.

8. GENERAL DISCUSSION

Chickpea (*Cicer arietinum* L.) is the second most important pulse crop in terms of production during 2012 (FAO STAT 2012). Chickpea seeds are excellent source of carbohydrates (including dietary fibres), proteins, polyunsaturated fatty acids, minerals and vitamins. It is also with in the economical accessibility of the people (Jukanti et al. 2012). However, presence of anti-nutritional factors reduces the worldwide acceptability of chickpea seeds especially in western countries. Among different antinbutritional factors present in chickpea (phytate, saponins etc.), RFO are one of the crucial factors causing flatulence thus deter people to consume more chickpea (Martínez-Villaluenga et al. 2008). Therefore, it is more important to focus on RFO rather than other anti-nutrients to make chickpea more acceptable as food and feed globally. RFO is a group of soluble, non-reducing and non-structural oligosaccharides that are indigestible to humans and monogastric animals but participate in some important physiological mechanisms in plants (Martínez-Villaluenga et al. 2008). Besides this, RFO are also designated as prebiotic in human diet as they support the growth of some beneficial bacteria in intestine. Therefore, RFO concentration needs to be reduced without affecting their role in plants and positive impact on human health. Therefore, this thesis targeted to identify the key regulating step of RFO biosynthesis in chickpea seeds. The project was initiated based on two hypotheses: (1) chickpea genotypes show natural variation for seed RFO concentration, and (2) activity of RFO biosynthetic enzymes determines the concentration and type of RFO in chickpea seeds. The first hypothesis of the study was supported by the results obtained during the second study (Chapter 5) revealing the natural variation for seed RFO concentration among chickpea genotypes. Total RFO concentration ranged from 1.58 to 5.31 and 2.11 to 5.83 mmol/100 g in desi and kabuli genotypes, respectively. Consequently, concentration of individual members of raffinose family *viz.* raffinose (0.09 – 1.19 g/100 g), stachyose (0.18 − 2.38 g/100 g) and verbascose (0.01 – 0.13 g/100 g) also showed variation among chickpea genotypes. Second hypothesis of the research was evidenced by third (Chapter 6) and fourth (Chapter 7) studies. Specific activity of RFO biosynthetic enzymes was observed 2 – 6 days prior to corresponding RFO accumulation. The other important conclusions of the thesis have been discussed below.

8.1 A modified HPAEC-PAD based gradient method determines soluble sugars concentration in chickpea seeds with higher accuracy and precision

The first objective of the thesis focused on the optimization of a rapid, precise and

accurate chromatographic method to determine soluble sugars (including RFO) in chickpea seeds. Among analytical methods available to determine sugars, chromatographic methods can distinguish individual sugar in a mixture and also estimate their concentration. The use of other chemical, physical or enzymatic methods is limited because of their ability to estimate either total sugars or a particular group of sugars collectively thus cannot be used for compositional studies. For sugar composition analysis specially regarding RFO, high performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD) is the most widely used technique. This technique utilizes the weak acidic properties of sugars at high pH/strong alkaline solutions (Cataldi et al. 2000; Rohrer et al. 2013). Therefore, classical silica based columns cannot be employed in this approach as silica is unstable at high pH. Alternatively, polymer based anion exchange columns have been developed that are stable even at high pH (Technical note 20, Dionex, Thermo Scientific; http://www.dionex.com/en-us/webdocs/5023-TN20_LPN032857-04.pdf). Amperometric detection allows the use of different waveforms required for sample integration and electrode cleaning. Separation in HPAEC can be explained by two factors: (1) acidity of the molecule, and (2) capacity factor (Lee 1996). In HPAEC, sugars are separated on the basis of their acidity. Less acidic compounds are retained for less time. Accordingly, alditols showed comparatively lower retention time than their parent sugars. The capacity factor reflects the number of carbon atoms in a sugar molecule. In a series of sugars, retention time increases with increase in number of carbon atoms (Lee 1996).

A HPAEC-PAD based analytical method using CarboPac PA100 analytical column was optimized to determine a wide range of compounds in legume seeds *viz.* *myo*-inositol, galactinol, glucose, fructose, sucrose, raffinose, stachyose and verbascose. A gradient (10 to 100 %) of 200 mM sodium hydroxide was used as mobile phase with a flow rate of 1 mL/min. Gradient elution efficiently resolved the compounds specially raffinose and stachyose. Gradient approach also resulted in narrower chromatographic peak and shorter run time thus increased the separation power (Chandrul et al. 2010; Ukić et al. 2013). The optimized method demonstrated higher accuracy and precision compared to HPLC-RI (High Pressure Liquid Chromatography with Refractive Index; Frias et al. 1994) and methods reported previously (Sánchez-Mata et al. 1998; Xiaoli et al. 2008; Bansleben et al. 2008; Tahir et al. 2011). The detection and quantification (LOD and LOQ) of very low concentrations reflected the higher sensitivity of the method whereas values for peak asymmetry (equivalent to 1) and resolution (≥1.5) confirmed the suitability of the method and column to determine soluble sugars. The analytical method also showed higher accuracy and

precision in terms of coefficient of determination, recovery percentage, repeatability and intermediate precision. The method separated different compounds in 35 min of total run time (retention time) including *myo*-inositol (1.7 min), galactinol (2.0 min), glucose (7.4 min), fructose (8.8 min), sucrose (10.8 min), raffinose (16.1 min), stachyose (17.0 min) and verbascose (19.5 min). The method was validated on seventeen chickpea genotypes having varying concentrations of total RFO (3.30 – 4.91 mmol/100 g), raffinose (1.22 – 1.87 mmol/100 g), stachyose (1.88 – 2.83 mmol/100 g) and verbascose (0.06 – 0.14 mmol/100 g). A strong positive correlation was observed between total RFO and individual concentration of raffinose (r = 0.87), stachyose (r = 0.91) and verbascose (r = 0.88) significant at $P \leq 0.001$ confirming the utility of the analytical method to screen chickpea germplasms for variation in soluble sugars including RFO constituents.

8.2 Accmulation of RFO in chickpea seeds is influenced by genotype and environment

The optimized analytical approach was utilized to study natural variation for seed RFO concentration in a collection of 171 chickpea germplasms procured from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, Patancheru, India) (Appendix 8.1 and 8.2). These genotypes were grown in field and greenhouse conditions. Analysis of variance revealed a significant impact ($P \leq 0.001$) of genotype (G), environment (E), and their interaction (G×E) on seed RFO concentration in chickpea. Kumar et al. (2010) and Tahir et al. (2011) also reported significant impact of G, E and G×E on RFO concentration in soybean [*Glycine max* (L.) Merr.] and lentil (*Lens culinaris* Medikus subsp. culinaris), respectively. The range of seed RFO concentration in desi and kabuli genotypes did not differ significantly. Total RFO concentration ranged from 1.58 to 5.31 and 2.11 to 5.83 mmol/100 g in desi and kabuli genotypes, respectively. Sucrose (0.60 − 3.59 g/100 g) was predominant among soluble sugars in chickpea seeds. Stachyose (0.18 − 2.38 g/100 g) was distinguished as the major RFO followed by raffinose (0.09 – 1.19 g/100 g) and verbascose (0.01 – 0.13 g/100 g). Genotypes grown in greenhouse conditions (controlled growing environment with less temperature variation, longer photoperiod, and higher photosynthetically active radiation) accumulated lower concentration of RFO in their seeds thus supporting RFO participation during stress tolerance (Martínez-Villaluenga et al. 2008; Krasensky et al. 2012). RFO have been described as reactive oxygen species scavengers, signaling molecules and osmo-protectants, therefore provide protection against abiotic and biotic stresses (Taji et al. 2002; Peters et al. 2007; Guy et al. 2008; Nishizawa et al. 2008; Van den Ende et al. 2009; Bolouri-Moghaddam et al. 2010). A significant positive correlation was observed between

substrate and product concentration regarding RFO biosynthesis. Due to the significant impact of E and G×E on RFO concentration, raffinose, stachyose, and verbascose showed moderate broad sense heritability (0.25 − 0.56) suggesting the quantitative characteristic of RFO trait in chickpea seeds. Desi (ICC 1163, ICC 1471, ICC 9562, ICCV 07115, ICCV 07116 and ICCV 07117) and kabuli (ICC 5270, ICC 10674, ICC 16216, ICC 16528, ICCV 3 and ICCV 91302) chickpea genotypes were identified with high and low RFO concentrations (high RFO genotypes are underlined). These results show the natural variation for seed RFO concentration among chickpea genotypes, thus proving the first hypothesis.

8.3 Initial substrates and RFO biosynthetic enzymes inflence seed RFO concentration in chickpea

To identify the key regulating step of RFO biosynthesis, RFO accumulation and corresponding biosynthetic enzymes activities were determined during chickpea seed development of two released varieties: CDC Vanguard (desi type) and CDC Frontier (kabuli type). In both genotypes, sucrose (1.7 – 2.0 g/100 g) was identified as major soluble sugar while stachyose (0.9 – 1.1 g/100 g) was predominant among RFO in mature seeds. The initial substrate *myo*-inositol showed maximum accumulation of 0.50 – 0.57 g/100 g at 18 – 20 DAF that decreased afterwards supporting galactinol biosynthesis. Galactinol acts as universal galatosyl unit donor for RFO biosynthesis. The maximum accumulation of galactinol was observed at 30 DAF that later utilized for increased RFO biosynthesis till 36 DAF. Raffinose was detected immediately after galactinol, followed by stachyose and verbascose. These results suggest that higher members of raffinose family require backbone of immediate lower member for their biosynthesis. Therefore, RFO biosynthesis might be regulated by substrates concentration like *myo*-inositol, galactinol or sucrose. These results confirmed the reversible nature of RFO biosynthetic reactions regulated by mass action ratio and equilibrium constant as proposed by Peterbauer et al. (2001). Therefore, higher level of substrates supports the increased biosynthesis of products (RFO) which are translocated in to vacuole to allow further RFO biosynthesis in cytosol (Peterbauer et al. (2001). Later on the basis of substrate feeding experiments, Karner et al. (2004) suggested the regulation of RFO concentration by the initial substrates level (*myo*-inositol and sucrose) rather than GS alone. The highest RFO biosynthetic enzymes activities were determined 2 - 4 days prior to maximum accumulation of raffinose, stachyose and verbascose. However, maximum GS activity was observed at 36 DAF that did not concur with galactinol accumulation indicating galactinol biosynthesis in higher amount even after 30 DAF but directed towards RFO

biosynthesis thus could not accumulate in seeds. GS was identified as a biosynthetic enzyme with the highest enzymatic activity indicating its importance in RFO biosynthesis. The interval between initial detection and maximum GS activity was 22 days which is the longest time period compared to other RFO biosynthetic enzymes.

A galactinol independent pathway was also found operative in chickpea seeds synthesizing both stachyose and verbascose. The non-galactinol enzyme showed pH optimum at 7.0 that concurred with results of Peterbauer et al. (2001) but in contrast to Bachmann et al. (1994). The enzyme showing non-galactinol RFO biosynthetic activity might be a multifunctional STS as reported in *P. sativum* (Peterbauer et al. 2002) which also showed maximum activity at pH 7.0. These results suggested substrate concentration and GS activity as possible factors regulating RFO concentration in chickpea seeds.

In low and high RFO genotypes, concentration of raffinose/stachyose showed significant ($P \leq 0.001$) difference in pairwise comparison and ranged 0.38 - 0.68/0.79 - 1.26 and 0.75 - 0.99/1.70 - 1.87 g 100 g^{-1}, respectively. The pairwise comparisons revealed significantly ($P \leq 0.05$) higher accumulation of *myo*-inositol and sucrose during seed development in high RFO genotypes supporting initial substrates as regulating factor of seed RFO concentration. High RFO genotypes expressed about 2 - 3 times higher activity of all RFO biosynthetic enzymes in comparison to those with low RFO concentration. The enzyme activity data corresponded with the accumulation of raffinose, stachyose and verbascose during chickpea seed development. Consequently, RFO biosynthetic enzymes may be another regulating factor of seed RFO concentration. The results show that the RFO biosynthetic enzyme activities influence RFO type and concentration in chickpea seeds, thus proving the second hypothesis.

8.4 GS – a potential target to reduce seed RFO concentration in chickpea

Targeting RFO biosynthetic enzymes is the direct approach to reduce RFO concentration in chickpea seeds. In literature, four main approaches have been demonstrated to reduce RFO concentration in seeds: (1) various processing treatments, (2) overexpressing RFO degrading enzyme, (3) down-regulating enzymes present in the upstream of RFO biosynthesis (substrate concentration), and (4) down-regulating RFO biosynthetic enzymes. In first approach, different processing methods like de-hulling, cooking (boiling, autoclaving and microwave cooking), soaking, germination, gamma irradiation, α-galactosidase treatment, ultrasound, hydrostatic pressure and thermal dehydration have been reported to reduce RFO concentration significantly in seeds of chickpea (El-Adawy 2002, Alajaji and El-Adawy 2006, Han and Baik 2006, Aguilera et al. 2009). However, such physical and mechanical

treatments also reduce concentration of protein, B-vitamins, minerals and amino acids in processed seeds/flour (Wang et al. 1997, El-Adawy 2002, Alajali and El-Adawy 2006). Such mechanical methods also increase the processing cost from consumer point of view. In second method, α-galactosidase is a well-known enzyme for RFO break down by hydrolyzing α(1→6) linkage (Blöchl et al. 2008). Substrate specificity at different biochemical conditions is the main problem in using α-galactosidase to reduce RFO level. Singh and Kayastha (2012) purified α-galactosidase from white chickpea and characterized it to have highest activity for raffinose and stachyose at pH 5.0 and 6.0, respectively. RFO biosynthesis mainly occurs in cytoplasm (alkaline) and they are stored in protein storage vacuole (alkaline). Therefore, modulating α-galactosidase activity may not affect RFO concentration in chickpea seeds. In third alternative, enzymes in the upstream of RFO biosynthesis *viz.* MIPS (*Myo*-inositol phosphate synthase) is reported to affect *myo*-inositol biosynthesis leading to regulate RFO biosynthesis. *Myo*-inositol on the other hand, participates in various physiochemical mechanisms including phosphorus storage, signal transduction, stress protection, hormonal homeostasis and cell wall biosynthesis (Abid et al. 2009). Sucrose (the other initial substrate of RFO biosynthesis) and its biosynthetic enzyme sucrose synthase also participate in various important physiological processes in plants (Abid et al. 2009). Hence, it would be complicated to down-regulate MIPS in chickpea.

RFO participates in some physiological processes in plants like photoassimilate translocation (also supported by sucrose, Turgeon 1996, Sprenger and Keller 2000), stress tolerance (Nishizawa-Yokoi et al. 2008, Bolouri-Moghaddam et al. 2010, Keunen et al. 2013), seed desiccation (Blackman et al. 1992, Corbineau et al. 2000, Martínez-Villaluenga et al. 2008, Pukacka et al. 2009, Angelovici et al. 2010) and germination (Blöchl et al. 2008) but their concentration can be reduced as evidenced by the contrasting RFO used in the study. The low RFO genotypes did not show any significant difference while growing in field as well as greenhouse conditions (Gangola et al. 2013). Among RFO biosynthetic enzymes, GS was found as the potential target to reduce seed RFO concentration in chickpea. The six main characteristics supporting GS candidature over other biosynthetic enzymes are: (1) galactinol is the universal substrate to synthesize every member of raffinose family. The galactinol independent pathway is also operative in chickpea seeds but showed very low enzymatic activity and the availability of substrates (raffinose and stachyose) depends of galactinol availability. (2) Galactinol connects two biosynthetic pathways including myo-inositol and UDP-galactose therefore considered as first committed step in RFO biosynthesis (McCaskill and Turgeon 2007). (3) GS showed 2 – 3 fold higher activity in high RFO genotypes

compared to low RFO genotypes. (4) GS showed about 20 – 30 times higher enzymatic activity than that of other biosynthetic enzymes. (5) During seed developmental stages, GS is the biosynthetic enzyme showing first activity and also the last with maximum activity depicting its highest involvement in RFO biosynthesis. (6) In previous study (Gangola et al. 2013), galactinol followed the same pattern of variation as total RFO, raffinose and stachyose during field as well as greenhouse growing conditions. Therefore, GS should be targeted to reduce RFO concentration in chickpea seeds.

On the basis of the reports available till date, different approaches to reduce chickpea seed RFO concentration like mechanical processing treatments (El-Adawy 2002, Alajaji and El-Adawy 2006, Han and Baik 2006, Aguilera et al. 2009), overexpression of α-galactosidase (Polowick et al. 2009), decrease substrate (*myo*-inositol and sucrose) concentration and reduced biosynthetic enzymes activity/gene expression (Bock et al. 2009) have been compared. Among all these approaches, targeting RFO biosynthetic enzymes is a direct startegy to reduce seed RFO concentration and does not have limitations like others. On the basis of the following results obtained during the present research work, galactinol synthase (GS) was proposed as a potential target among all RFO biosynthetic enzymes to reduce seed RFO concentration in chickpea: (1) 20 – 30 times higher activity compared to other RFO biosynthetic enzymes, (2) 2 – 3 times more enzymatic activity in high RFO genotypes compared to low RFO genotypes, and (3) highest involvement in RFO biosynthesis during chickpea seed development.

8.5 Future prospects

Present work on RFO biosynthesis in chickpea seeds can be extended in following directions:

- Chickpea genome has been sequenced recently. The sequence information can be utilized to synthesize probe for transcriptional analysis of RFO biosynthetic genes. The transcriptional and enzyme activity data can be combined to predict the probable cause of high and low RFO concentrations in contrasting genotypes. It might be at transcriptional or posttranslational level.
- Presence of GGT in chickpea seeds is still not validated. Hence, STS should be identified and characterized to confirm whether it is a multifunctional enzyme as in pea seeds.
- DNA sequence information can also be utilized to detect allelic variations of RFO

biosynthetic genes and their correlation with seed RFO concentration.

- GS was proposed as a potential target to reduce seed RFO concentration in chickpea. Therefore, effect of GS on other seed characteristics (RFO concentration, germination capacity, seed weight/size and yield) would be studied.

8.6 Conclusions and novel findings

The conclusions of the study have proven both the hypotheses of the research project. The novel findings of the thesis can be summarized as following points:

- The optimized HPAEC-PAD based method is a rapid and reliable approach to determine soluble sugars in legume seeds with higher sensitivity, accuracy and precision compared to previous reported methods.
- G, E and G×E showed significant impact on chickpea seed RFO concentration; therefore, broad sense heritability for RFO traits ranged from 0.25 to 0.56.
- Chickpea seed RFO concentration was affected by varying growth parameters as they participate in stress tolerance. Consequently, greenhouse grown genotypes accumulated lower concentrations of RFO compared to their field grown counterparts.
- Sucrose was identified as major soluble sugars while stachyose was predominant constituent of RFO followed by raffinose and verbascose.
- A significant positive correlation was observed between seed sucrose concentration and seed weight/size.
- Seed RFO concentration was showed as a facilitator of seed germination rather than critical regulating factor.
- RFO accumulated during later stages of chickpea seed development thus provided desiccation tolerance to maturing seeds.
- Specific activity of RFO biosynthetic enzymes was observed 2 – 6 days prior to corresponding RFO accumulation.
- Both galactinol dependent and independent pathways of RFO biosynthesis were operative in chickpea seeds.
- *Myo*-inositol and sucrose showed a significant difference in accumulation between low and high RFO genotypes.
- All biosynthetic enzymes (GS, RS, STS, VS and non-galactinol enzyme) showed about 2 – 3 times higher activity in high RFO genotypes compared to low RFO genotypes.

- GS showed the highest enzymatic activity (20 – 30 times higher than that of other biosynthetic enzymes) followed by STS and RS whereas VS and non-galactinol enzymes showed the least enzyme activities.
- Initial substrates concentrations and biosynthetic enzymes activities were identified as regulating elements of RFO biosynthesis in chickpea seeds.
- Galactinol synthase was proposed as a potential target to reduce seed RFO concentration in chickpea.

LITERATURE CITED

Abbo, S., Berger, J. and Turner, N. C. 2003. Evolution of cultivated chickpea: four bottlenecks limit diversity and constrain adaptation. Funct. Plant Biol. 30: 1081-1087.

Abbo, S., Molina, C., Jungmann, R., Grusak, M. A., Berkovitch, Z., Reifen, R., Kahl, G., Winter, P. and Reifen, R. 2005. Quantitative trait loci governing carotenoid concentration and weight in seeds of chickpea (*Cicer arietinum* L.). Theor. Appl. Genet. 111: 185–195.

Abid, G., Silue, S., Muhovski, Y., Jacquemin, J. M., Toussaint, A. and Baudoin, J. P. 2009. Role of *myo*-inositol phosphate synthase and sucrose synthase genes in plant seed development. Gene 439: 1-10.

Agarwal, G., Jhanwar, S., Priya, P., Singh, V. K., Saxena, M. S., Parida, S. K., Garg, R., Tyagi, A. K. and Jain, M. 2012. Comparative analysis of kabuli chickpea transcriptome with desi and wild chickpea provides a rich resource for development of functional markers. PLoS ONE 7: e52443.

Aguilera, Y., Martin-Cabrejas, M. A., Benitez, V., Molla, E., Lopez-Andreu, F. J. and Esteban, R. M. 2009. Changes in carbohydrate fraction during dehydration process of common legumes. J. Food Compos. Anal. 22: 678-683.

Alajaji, S. A. and El-Adawy, T. A. 2006. Nutritional composition of chickpea (*Cicer arietinum* L.) as affected by microwave cooking and other traditional cooking methods. J. Food Compos. Anal. 19: 806-812.

Albalasmeh, A. A., Berhe, A. A. and Ghezzehei, T. A. 2013. A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry. Carbohydr. Polym. 97: 253-261.

Alexandrov, N., Brover, V., Freidin, S., Troukhan, M., Tatarinova, T., Zhang, H., Swaller, T., Lu, Y., Bouck, J., Flavell, R. and Feldmann, K. 2009. Insights into corn genes derived from large-scale cDNA sequencing. Plant Mol. Biol. 69: 179-194.

Al-Kaisey, M. T., Alwan, A. K. H., Mohammad, M. H. and Saeed, A. H. 2003. Effect of gamma irradiation on antinutritional factors in broad bean. Radiat. Phys. Chem. 67: 493-496.

Alonso, B. O., Rovir, R. F., Vegas, C. A. and Pedrosa, M. M. 2010. The role of pulses in the present-day diet. Act. Diet. 14: 72-76.

ALwawi, H., Moulla, M. and Choumane, W. 2010. Genotype-environment interaction and genetic parameters in chickpea (*Cicer arietinum* L.) landraces. J. Agric. Sci. 2: 154-161.

Andersen, K. E., Bjergegaard, C., Moller, P., Sorensen, J. C. and Sorensen, H. 2005. Compositional variations for α-galactosides in different species of leguminosae, brassicaceae, and barley: a chemotaxonomic study based on chemometrics and high-performance capillary electrophoresis. J. Agric. Food Chem. 53: 5809-5817.

Andrieux, C. and Szylit, O. 1992. Effects of galacto-oligosaccharides (TOS) on bacterial enzyme activities and metabolite production in rats associated with a human faecal flora. Proc. Nutr. Soc. 51, 7A (abs.).

Angelovici, R., Galili, G., Fernie, A. R. and Fait, A. 2010. Seed desiccation: a bridge between

maturation and germination. Trends Plant Sci. 15: 211-218.

Anisha, G. S. and Prema, P. 2008. Reduction of non-digestible oligosaccharides in horse gram and green gram flours using crude α-galactosidase from *Streptomyces griseoloalbus*. Food Chem. 106: 1175-1179.

Anisha, G. S., John, R. P. and Prema, P. 2011. Substrate specificities and mechanism of action of multiple α-galactosidases from *Streptomyces griseoloalbus*. Food Chem. 124: 349-353.

Arshad, M., Bakhsh, A., Haqqani, A. M. and Bashir, M. 2003. Genotype - environment interaction for grain yield in chickpea (*Cicer arietinum* L.). Pak. J. Bot. 35: 181-186.

Asp, N. 1995. Classification and methodology of food carbohydrates as related to nutritional effects. Am. J. Clin. Nutr. 61: 930S-937S.

Atta, B. M., Shah, T. M., Abbas, G. and Haq, M. A. 2009. Genotype x environment interaction for seed yield in kabuli chickpea (*Cicer arietinum* L.) genotypes developed through mutation breeding. Pak. J. Bot. 41: 1883-1890.

Ayele, A. G. 2011. Heritability and genetic advance in recombinant inbred lines for drought tolerance and other related traits in sorghum (*Sorghum bicolor*). C. J. Agric. Sci. 5: 1-9.

Bachmann, M. and Keller, F. 1995. Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. (inter- and intracellular compartmentation). Plant Physiol. 109: 991-998.

Bachmann, M., Matile, P. and Keller, F. 1994. Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. (Cold acclimation, translocation, and sink to source transition: discovery of chain elongation enzyme). Plant Physiol. 105: 1335-1345.

Bailly, C., Audigier, C., Ladonne, F., Wagner, M. H., Coste, F., Corbineau, F. and Come, D. 2001. Changes in oligosaccharide content and antioxidant enzyme activities in developing bean seeds as related to acquisition of drying tolerance and seed quality. J Exp Bot. 52: 701-708.

Bakhsh, A., Arshad, M. and Haqqani, A. M. 2006. Effect of genotype x environment interaction on relationship between grain yield and its components in chickpea [*Cicer arietinum* (L.)]. Pak. J. Bot. 38: 683-690.

Bansleben, D., Schellenberg, I. and Wolff, A. C. 2008. Highly automated and fast determination of raffinose family oligosaccharides in *Lupinus* seeds using pressurized liquid extraction and high-performance anion-exchange chromatography with pulsed amperometric detection. J. Sci. Food Agric. 88: 1949-1953.

Belcourt, L. A. and Labuza, T. P. 2007. Effect of raffinose on sucrose recrystallization and textural changes in soft cookies. J. Food Sci. 72: C065-071.

Bernabe, M., Fenwick, R., Frias, J., Jimenez-Barbero, J., Price, K., Valverde, S. and Vidal-Valverde, C. 1993. Determination, by NMR spectroscopy, of the structure of ciceritol, a pseudotrisaccharide isolated from lentils. J. Agric. Food Chem. 41: 870-872.

Black, M., Corbineau, F., Gee, H. and Come, D. 1999. Water content, raffinose, and dehydrins in the induction of desiccation tolerance in immature wheat embryos. Plant Physiol. 120: 463-472.

Black, M., Corbineau, F., Grzesik, M., Guy, P. and Come, D. 1996. Carbohydrate metabolism in the developing and maturing wheat embryo in relation to its desiccation

tolerance. J. Exp. Bot. 47: 161-169.
Blackman, S. A., Obendorf, R. L. and Leopold, A. C. 1992. Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. Plant Physiol. 100: 225-230.
Blöchl, A., Peterbauer, T. and Richter, A. 2007. Inhibition of raffinose oligosaccharide breakdown delays germination of pea seeds. J. Plant Physiol. 164: 1093-1096.
Blöchl, A., Peterbauer, T., Hofmann, J. and Richter, A. 2008. Enzymatic breakdown of raffinose oligosaccharides in pea seeds. Planta 228: 99-110.
Bochicchio, A., Rizzi, E., Balconi, C., Vernieri, P. and Vazzana, C. 1994. Sucrose and raffinose contents and acquisition of desiccation tolerance in immature maize embryos. Seed Sci. Res. 4: 123.
Bock, C., Ray, H. and Georges, F. 2009. Down-regulation of galactinol synthesis in oilseed *Brassica napus* leads to significant reduction of antinutritional oligosaccharides. Botany 87: 597-603.
Bolouri-Moghaddam, M., Le Roy, K., Xiang, L., Rolland, F. and Van, D. E. 2010. Sugar signalling and antioxidant network connections in plant cells. FEBS J. 277: 2022-2037.
Bouhnik, Y., Flourie, B., D'Agay-Abensour, L., Pochart, P., Gramet, G., Durand, M. and Rambaud, J. C. 1997. Administration of transgalacto-oligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. J. Nutr. 127: 444-448.
Boye, J., Zare, F. and Pletch, A. 2010. Pulse proteins: processing, characterization, functional properties and applications in food and feed. Food Res. Int. 43: 414-431.
Bradford, M. M. 1976. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.
Brown, A. H. D. 1989a. The case for core collections. In: The use of plant genetic resources. A. H. D. Brown, O. H. Frankel, D. R. Marshall, and J. T. Williams (eds.). Cambridge University Press, Cambridge. pp. 136-156.
Brown, A. H. D. 1989b. Core collections: a practical approach to genetic resources management. Genome 31: 818-824.
Brummer, Y. and Cui, S. W. 2005. Understanding Carbohydrate Analysis. In: Food carbohydrates: Chemistry, physical properties and applications. S. W. Cui (eds.). CRC Press, Taylor & Francis Group (USA).
Buitink, J., Hemminga, M. A. and Hoekstra, F. A. 2000. Is there a role for oligosaccharides in seed longevity? An assessment of intracellular glass stability. Plant Physiol. 122: 1217-1224.
Byers, D. 2008. Components of phenotypic variance. Nature Education 1(1).
Cabrera, C., Lloris, F., Gimenez, R., Olalla, M. and Lopez, M. C. 2003. Mineral content in legumes and nuts: contribution to the Spanish dietary intake. Sci. Total Environ. 308: 1-14.
Campos-Vega, R., Loarca-Piña, G. and Oomah, B. D. 2010. Minor components of pulses and their potential impact on human health. Food Res. Int. 43: 461-482.
Castillo, E. M., de Lumen, B. O., Reyes, P. S. and de Lumen, H. 1990. Raffinose synthase and galactinol synthase in developing seeds and leaves of legumes. J. Agric. Food Chem. 38: 351-355.
Cataldi, T. R. I., Campa, C. and De Benedetto, G. E. 2000. Carbohydrate analysis by high-

performance anion-exchange chromatography with pulsed amperometric detection: The potential is still growing. Fresenius J. Anal. Chem. 368: 739-758.

Champ, M. M. J. 2002. Non-nutrient bioactive substances of pulses. Br. J. Nutr. 88: S307–S319.

Chandrul, K. K. and Srivastava, B. 2010. A process of method development: A chromatographic approach. J. Chem. Pharm. Res. 2: 519-545.

Chavan, J. K., Kadam, S. S. and Salunkhe, D. K. 1986. Biochemistry and technology of chickpea (*Cicer arietinum* L.) seeds. Crit. Rev. Food Sci. Nutr. 25: 107-157.

Cheikhyoussef, A., Cheikhyoussef, N., Chen, H., Zhao, J., Tang, J., Zhang, H. and Chen, W. 2010. Bifidin I – A new bacteriocin produced by *Bifidobacterium infantis* BCRC 14602: purification and partial amino acid sequence. Food Control 21: 746-753.

Chen, Y. and Burris, J. S. 1990. Role of carbohydrates in desiccation tolerance and membrane behavior in maturing maize seed. Crop Sci. 30: 971–975.

Chibbar, R. N., Ambigaipalan, P. and Hoover, R. 2010. Molecular diversity in pulse seed starch and complex carbohydrates and its role in human nutrition and health. Cereal Chem. 87: 342–352.

Cho, S. M., Kang, E. Y., Kim, M. S., Yoo, S. J., Im, Y. J., Kim, Y. C., Yang, K. Y., Kim, K. Y., Kim, K. S., Choi, Y. S. and Cho, B. H. 2010. Jasmonate-dependent expression of a galactinol synthase gene is involved in priming of systemic fungal resistance in *Arabidopsis Thaliana*. Botany 88: 452-461.

Chonan, O., Matsumoto, K. and Watanuki, M. 1995. Effect of galactooligosaccharides on calcium absorption and preventing bone loss in ovariectomized rats. Biosci. Biotechnol. Biochem. 59: 236-239.

Cicek, M. S., Chen, P., Saghai Maroof, M. A. and Buss, G. R. 2006. Interrelationships among agronomic and seed quality traits in an interspecific soybean recombinant inbred population. Crop Sci. 46: 1253-1259.

Ciftci, H., Ozkaya, A., Cevrimli, S. and Bakoglu, A. 2010. Levels of Fat-Soluble Vitamins in Some Foods. Asian J. Chem. 22: 1251-1256.

Cliffe, L. J., Humphreys, N. E., Lane, T. E., Potten, C. S., Booth, C. and Grencis, R. K. 2005. Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. Science 308: 1463-1465.

Cobos, M. J., Rubio, J., Fernandez-Romero, M. D., Garza, R., Moreno, M. T., Millan, T. and Gil, J. 2007. Genetic analysis of seed size, yield and days to flowering in a chickpea recombinant inbred line population derived from a Kabuli × Desi cross. Ann. Appl. Biol. 151: 33–42.

Coon, C. N., Leske, K. L., Akavanichan, O. and Cheng, T. K. 1990. Effect of oligosaccharide-free soybean meal on true metabolizable energy and fiber digestion in adult roosters. Poult. Sci. 69: 787-793.

Corbineau, F., Picard, M. A., Fougereux, J., Ladonne, F. and Côme, D. 2000. Effects of dehydration conditions on desiccation tolerance of developing pea seeds as related to oligosaccharide content and cell membrane properties. Seed Sci. Res. 10: 329-339.

Cummings, J. H. and Stephen, A. M. 2007. Carbohydrate terminology and classification. Eur. J. Clin. Nutr. 61: S5-S18.

Cunningham, S. M., Nadeau, P., Castonguay, Y., Laberge, S. and Volenec, J. J. 2003.

Raffinose and stachyose accumulation, galactinol synthase expression, and winter injury of contrasting alfalfa germplasms. Crop Sci. 43: 562-570.

Dalgetty, D. D. and Baik, B. K. 2003. Isolation and characterization of cotyledon fibers from peas, lentils, and chickpeas. Cereal Chem. 80: 310-315.

de Ruiz', A. S. C. and Bressani, R. 1990. Effect of germination on the chemical composition and nutritive value of Amaranth grain. Cereal Chem. 67: 519-522.

Dehghani, H., Sabaghpour, S. H. and Ebadi, A. 2010. Study of genotype × environment interaction for chickpea yield in Iran. Agron. J. 102: 1-8.

Devindra, S., Rao, J. S., Krishnaswamya, P. and Bhaskarb, V. 2011. Reduction of α-galactoside content in red gram (*Cajanus cajan* L.) upon germination followed by heat treatment. J. Sci. Food Agric. 91: 1829-1835.

Dhawan, K., Malhotra, S., Dahiya, B. S. and Singh, D. 1991. Seed protein fractions and amino acid composition in gram (*Cicer arietinum*). Plant Foods Hum. Nutr. 41: 225-232.

Dierking, E. C. and Bilyeu, K. D. 2008. Association of a soybean raffinose synthase gene with low raffinose and stachyose seed phenotype. Plant Genome 1: 135-145.

Dierking, E. C. and Bilyeu, K. D. 2009. Raffinose and stachyose metabolism are not required for efficient soybean seed germination. J. Plant Physiol. 166: 1329-1335.

Dilis, V. and Trichopoulou, A. 2009. Nutritional and health properties of pulses. Mediterr. J. Nutr. Metab. 1: 149-157.

Dinant, S. and Lemoine, R. 2010. The phloem pathway: New issues and old debates. C. R. Biol. 333: 307-319.

Dixit, A. K., Kumar, V., Rani, A., Manjaya, J. G. and Bhatnagar, D. 2011. Effect of gamma irradiation on lipoxygenases, trypsin inhibitor, raffinose family oligosaccharides and nutritional factors of different seed coat colored soybean (*Glycine max* L.). Radiat. Phys. Chem. 80: 597-603.

dos Santos, T. B., Budzinski, I. G. F., Marur, C. J., Petkowicz, C. L. O., Pereira, L. F. P. and Vieira, L. G. E. 2011. Expression of three galactinol synthase isoforms in *Coffea arabica* L. and accumulation of raffinose and stachyose in response to abiotic stresses. Plant Physiol. Biochem. 49: 441-448.

Downie, B., Gurusinghe, S., Dahal, P., Thacker, R. R., Snyder, J. C., Nonogaki, H., Yim, K., Fukanaga, K., Alvarado, V. and Bradford, K. J. 2003. Expression of a galactinol synthase gene in tomato seeds is up-regulated before maturation desiccation and again after imbibition whenever radicle protrusion is prevented. Plant Physiol. 131: 1347-1359.

Durand, M., Cordelet, C., Hannequart, G. and Beaumatin, P. 1992. *In vitro* fermentation of a galacto-oligosaccharide by human bacteria in continuous culture. Proc. Nutr. Soc. 51: 6A (abs.).

Ebadi segherloo, A., Sabaghpour, S. H., Dehghani, H. and Kamrani, M. 2010. Screening of superior chickpea genotypes for various environments of Iran using genotype plus genotype × environment (GGE) biplot analysis. J. Plant Breed. Crop Sci. 2: 286-292.

Edmé, S. J. and Glaz, B. S. 2013. Field response of sugarcane genotypes to freeze stress with genotype x environment effects on quality traits. J. Crop Improv. 27: 1-30.

Egert, A., Keller, F. and Peters, S. 2013. Abiotic stress-induced accumulation of raffinose in

Arabidopsis leaves is mediated by a single raffinose synthase (*RS5*, At5g40390). BMC Plant Biol. 13: 218.

El-Adawy, T. A. 2002. Nutritional composition and antinutritional factors of chickpeas (*Cicer arietinum* L.) undergoing different cooking methods and germination. Plant Foods Hum. Nutr. 57: 83-97.

FAO STAT. 2010. http://faostat.fao.org/site/567/DesktopDefault.aspx (accessed May 19, 2010).

FAO STAT. 2012. http://faostat.fao.org/site/567/DesktopDefault.aspx (accessed April 30, 2014).

Farshadfar, E., Rashidi, M., Jowkar, M. M. and Zali, H. 2013. GGE Biplot analysis of genotype × environment interaction in chickpea genotypes. Eur. J. Exp. Biol. 3: 417-423.

Farshadfar, E., Zali, H. and Mohammadi, R. 2011. Evaluation of phenotypic stability in chickpea genotypes using GGE-biplot. Ann. Biol. Res. 2: 282-292.

Frankel, O. H. 1984. Genetic perspectives of germplasm conservation. In: Genetic manipulation: impact on Man and society. W. K. Arber, K. Llimensee, W. J. Peacock, and P. Starlinger (eds.). Cambridge University Press, Cambridge. pp. 161 - 170.

Frankel, O. H. and Brown, A. H. D. 1984. Current plant genetic resources-a critical appraisal. In: Genetics: new frontiers. Vol. 4. Oxford & IBH Publishing Co., New Delhi. pp. 1-11.

Frias, J., Bakhsh, A., Jones, D. A., Arthur, A. E., Vidal-Valverde, C., Rhodes, M. J. C. and Hedley, C. L. 1999. Genetic analysis of the raffinose oligosaccharide pathway in lentil seeds. J. Exp. Bot. 50: 469-476.

Frias, J., Hedley, C. L., Price, K. R., Fenwick, G. R. and Vidal-Valverde, C. 1994. Improved methods of oligosaccharide analysis for genetic studies of legume seeds. J. Liq. Chromatogr. Relat. Technol. 17: 2469-2483.

Frias, J., Vidal-Valverde, C., Kozlowska, H., Gorecki, R., Honke, J. and Hedley, C. L. 1996. Evolution of soluble carbohydrates during the development of pea, faba bean and lupin seeds. Z. Lebensm. Unters. Forsch. 203: 27-32.

Frias, J., Vidal-Valverde, C., Sotomayor, C., Diaz-Pollan, C. and Urbano, G. 2000. Influence of processing on available carbohydrate content and antinutritional factors of chickpeas. Eur. Food Res. Technol. 210: 340-345.

Frimpong, A., Sinha, A., Taran, B., Warkentin, T. D., Gossen, B. D. and Chibbar, R. N. 2009. Genotype and growing environment influence chickpea (*Cicer arietinum* L.) seed composition. J. Sci. Food Agric. 89: 2052-2063.

Frommer, W. B., Schulze, W. X. and Lalonde, S. 2003. Hexokinase, Jack-of-all-trades. Science 300: 261-263.

Gage, D. J. and Long, S. R. 1998. α-galactoside uptake in Rhizobium meliloti: isolation and characterization of *agpA*, a gene encoding a periplasmic binding protein required for melibiose and raffinose utilization. J. Bacteriol. 180: 5739-5748.

Gamalei, Y. 1989. Structure and function of leaf minor veins in trees and herbs. Trees 3: 96-110.

Gangola M. P., Jaiswal, S., Khedikar, Y. P. and Chibbar, R. N. 2014. A reliable and rapid method for soluble sugars and RFO analysis in chickpea using HPAEC-PAD and its comparison with HPLC-RI. Food chem. 154: 127-133.

Gangola, M. P., Khedikar, Y. P., Gaur, P. M., Båga, M. and Chibbar, R. N. 2013. 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. J. Agric. Food Chem. 61: 4943-4952.

Gao, Z. and Schaffer, A. A. 1999. A novel alkaline α-galactosidase from melon fruit with a substrate preference for raffinose. Plant Physiol. 119: 979-988.

Gaudreault, P. and Webb, J. A. 1981. Stachyose synthesis in leaves of *Cucurbita pepo*. Phytochem. 20: 2629-2633.

Gaur, P. M., Srinivasan, S., Suresh, K., Deepika, S. R., and Rao, B. V. 2012. Inheritance of natural seed-coat cracking in chickpea. J. Hered. 103: 898-902.

Gibson, G. R. and Fuller, R. 2000. Aspects of *in vitro* and *in vivo* research approaches directed toward identifying probiotics and prebiotics for human use. J. Nutr. 130: 391-391.

Gibson, G. R. and Roberfroid, M. B. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125: 1401-1412.

Gilbert, G. A., Wilson, C. and Madore, M. A. 1997. Root-zone salinity alters raffinose oligosaccharide metabolism and transport in *Coleus*. Plant Physiol. 115: 1267-1276.

Girigowda, K., Prashanth, S. J. and Mulimani, V. H. 2005. Oligosaccharins of black gram (*Vigna mungo* L.) as affected by processing methods. Plant Foods Hum. Nutr. 60: 173-180.

Glencross, B. D., Boujard, T. and Kaushik, S. J. 2003. Influence of oligosaccharides on the digestibility of lupin meals when fed to rainbow trout, *Oncorhynchus mykiss*. Aquaculture 219: 703-713.

Grant, T. N., Dami, I. E., Ji, T., Scurlock, D. and Streeter, J. 2009. Variation in leaf and bud soluble sugar concentration among vitis genotypes grown under two temperature regimes. Can. J. Plant Sci.89: 961-968.

Grizard, D. and Barthomeuf, C. 1999. Non-digestible oligosaccharides used as prebiotic agents: mode of production and beneficial effects on animal and human health. Reprod Nutr Dev. 39: 563-588.

Gulewicz, P., Szymaniec, S., Bubak, B., Frias, J., Vidal-Valverde, C., Trojanowska, K. and Gulewicz, K. 2002. Biological activity of α-galactoside preparations from *Lupinus angustifolius* L. and *Pisum sativum* L. seeds. J. Agric. Food Chem. 50: 384-389.

Gupta, R., and Dhillon, S. 1993. Characterization of seed storage proteins of lentil (*Lens culinaris* M.). Ann. Biol. 9: 71-78.

Haab, C. I. and Keller, F. 2002. Purification and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan:galactan galactosyltransferase (GGT), from *Ajuga reptans* leaves. Physiol. Plant.114: 361-371.

Halperin, S. J. and Koster, K. L. 2006. Sugar effects on membrane damage during desiccation of pea embryo protoplasts. J. Exp. Bot. 57: 2303-2311.

Han, I. H. and Baik, B. 2006. Oligosaccharide content and composition of legumes and their reduction by soaking, cooking, ultrasound, and high hydrostatic pressure. Cereal Chem. 83: 428-433.

Hannah, M. A., Zuther, E., Buchel, K. and Heyer, A. G. 2006. Transport and metabolism of raffinose family oligosaccharides in transgenic potato. J. Exp. Bot. 57: 3801-3811.

Hannan, R., Açikgöz, N. and Robertson, L. D. 2001. Chickpeas (*Cicer* L.). In: N. Maxted and S. J. Bennett (eds.). Plant Genetic Resources of Legumes in the Mediterranean. Springer Netherlands. pp. 115-124.

Haritatos, E., Ayre, B. G. and Turgeon, R. 2000. Identification of phloem involved in assimilate loading in leaves by the activity of the galactinol synthase promoter. Plant Physiol. 123: 929-938.

Hayakawa, K., Mizutani, J., Wada, K., Masai, T., Yoshihara, I. and Mitsuoka, T. 1990. Effects of soybean oligosaccharides on human faecal flora. Microb. Ecol. Health Dis. 3: 293-303.

Hitz, W. D., Carlson, T. J., Kerr, P. S. and Sebastian, S. A. 2002. Biochemical and molecular characterization of a mutation that confers a decreased raffinosaccharide and phytic acid phenotype on soybean seeds. Plant Physiol. 128: 650-660.

Hoch, G., Peterbauer, T. and Richter, A. 1999. Purification and characterization of stachyose synthase from lentil (*Lens culinaris*) seeds: galactopinitol and stachyose synthesis. Arch. Biochem. Biophys. 366: 75-81.

Hoffmann, C. M., Huijbregts, T., van Swaaij, N. and Jansen, R. 2009. Impact of different environments in Europe on yield and quality of sugar beet genotypes. Eur. J. Agron. 30: 17-26.

Holthaus, U. and Schmitz, K. 1991. Stachyose synthesis in mature leaves of *Cucumis melo*. Purification and characterization of stachyose synthase (EC 2.4.1.67). Planta 184: 525-531.

Horbowicz, M. and Obendorf, R. L. 1994. Seed desiccation tolerance and storability: Dependence on flatulence-producing oligosaccharides and cyclitols—review and survey. Seed Sci. Res. 4: 385.

Huber, J. L. A., Mason Pharr, D. and Huber, S. C. 1990. Partial purification and characterization of stachyose syynthase in leaves of *Cucumis sativus* and *Cucumis melo*: utilization of a rapid assay for *myo*-inositol. Plant Sci. 69: 179-188.

Huynh, B., Palmer, L., Mather, D. E., Wallwork, H., Graham, R. D., Welch, R. M. and Stangoulis, J. C. R. 2008. Genotypic variation in wheat grain fructan content revealed by a simplified HPLC method. J. Cereal Sci. 48: 369-378.

Ibáñez, M. V., Rincón, F., Amaro, M. and Martínez, B. 1998. Intrinsic variability of mneral composition of chickpea (*Cicer arietinum* L.). Food Chem. 63: 55-60.

ICRISAT. 2013. (http://www.icrisat.org/what-we-do/crops/ChickPea/Project1/pfirst. asp). Accessed on May 18, 2013.

Imanishi, H. T., Suzuki, T., Masuda, K. and Harada, T. 1998. Accumulation of raffinose and stachyose in shoot apices of *Lonicera caerulea* L. during cold acclimation. Sci. Hort. 72: 255-263.

Iqbal, A., Khalil, I. A., Ateeq, N. and Khan, M. S. 2006. Nutritional quality of important food legumes. Food Chem. 97: 331-335.

Ishizuka, S., Iwama, A., Dinoto, A., Suksomcheep, A., Maeta, K., Kasai, T., Hara, H. and Yokota, A. 2009. Synbiotic promotion of epithelial proliferation by orally ingested encapsulated *Bifidobacterium breve* and raffinose in the small intestine of rats. Mol. Nutr. Food Res. 53: S62-S67.

Jang, J. C., León, P., Zhou, L. and Sheen, J. 1997. Hexokinase as a sugar sensor in higher

plants. Plant Cell 9: 5-19.

Jaureguy, L. M., Chen, P. and Scaboo, A. M. 2011. Heritability and correlations among food-grade traits in soybean. Plant Breed.13: 647-652.

Joersbo, M., Guldager Pedersen, S., Nielsen, J. E., Marcussen, J. and Brunstedt, J. 1999. Isolation and expression of two cDNA clones encoding UDP-galactose epimerase expressed in developing seeds of the endospermous legume guar. Plant Sci. 142: 147-154.

Jones D. A., DuPont, M. S., Ambrose, M. J., Frias, J. and Hedley, C. L. 1999. The discovery of compositional variation for the raffinose family of oligosaccharides in pea seeds. Seed Sci. Res. 9: 305-310.

Jukanti, A. K., Gaur, P. M., Gowda, C. L. L. and Chibbar, R. N. 2012. Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. Br. J. Nutr. 108: S11−S26.

Kandus, M., Almorza, D., Ronceros, R. B. and Salerno, J. C. 2010. Statistical models for evaluating the genotype-environment interaction in maize (*Zea mays* L.). Int. J. Exp. Bot.79: 39-46.

Kang, M. S. 2002. Genotype-environment interaction: progress and prospects. In: M. S. Kang (eds.). Quantitative Genetics, Genomics and Plant Breeding. CAB International. pp. 221-243.

Karner, U., Peterbauer, T., Raboy, V., Jones, D. A., Hedley, C. L. and Richter, A. 2004. *Myo*-Inositol and sucrose concentrations affect the accumulation of raffinose family oligosaccharides in seeds. J. Exp. Bot.55: 1981-1987.

Keller, F. 1992. Galactinol synthase is an extravacuolar enzyme in tubers of japanese artichoke (*Stachys sieboldii*). Plant Physiol. 99: 1251-1253.

Kelly-Quagliana, K. A., Nelson, P. D. and Buddington, R. K. 2003. Dietary oligofructose and inulin modulate immune functions in mice. Nutr. Res. 23: 257-267.

Kerem, Z., Lev-Yadun, S., Gopher, A., Weinberg, P. and Abbo, S. 2007. Chickpea domestication in the Neolithic Levant through the nutritional perspective. J. Archaeol. Sci. 34: 1289-1293.

Keunen, E., Peshev, D., Vangronsveld, J., Van den Ende, W. and Cuypers, A. 2013. Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. Plant Cell Environ. 36: 1242-1255.

Khan, I. A., Malik, B. A. and Bashir, M. 1988. Investigation of genotype × environment interaction for seed yield in chickpea (*Cicer arietinum* L.). Pak. J. Bot. 20: 201-204.

Khan, M. A., Ahtar, N., Ullah, I. and Jaffery, S. 1995. Nutritional evaluation of desi and kabuli chickpeas and their products commonly consumed in Pakistan. Int. J. Food Sci. Nutr. 46: 215-223.

Khanna, R., Soska, R., Lun, Y., Feng, J., Frascella, M., Young, B., Brignol, N., Pellegrino, L., Sitaraman, S. A., Desnick, R. J., Benjamin, E. R., Lockhart, D. J. and Valenzano, K. J. 2010. The pharmacological chaperone 1-deoxygalactonojirimycin reduces tissue globotriaosylceramide levels in a mouse model of fabry disease. Mol. Ther. 18: 23-33.

Kim, S. W., Kim, H. M., Yang, K. M., Kim, S., Kim, S., An, M. J., Park, J. J., Lee, S. K., Kim, T. I., Kim, W. H. and Cheon, J. H. 2010. *Bifidobacterium lactis* inhibits NF-kB in intestinal epithelial cells and prevents acute colitis and colitis-associated colon cancer in mice. Inflamm. Bowel Dis. 16: 1514-1525.

Klotke, J., Kopka, J., Gatzke, N. and Heyer, A. G. 2004. Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation - evidence for a role of raffinose in cold acclimation. Plant, Cell Environ.27: 1395-1404.

Konrádová, H., Gricová, M. and Lipavská, H. 2003. Cold-induced accumulation of raffinose family oligosaccharides in somatic embryos of Norway spruce (*Picea abies*). In Vitro Cell. Dev. Biol. Plant 39: 425-427; 427.

Koster, K. L. 1991. Glass formation and desiccation tolerance in seeds. Plant Physiol. 96: 302-304.

Koster, K. L. and Leopold, A. C. 1988. Sugars and desiccation tolerance in seeds. Plant Physiol. 88: 829-832.

Krause, D. O., Easter, R. A. and Mackie, R. I. 1994. Fermentation of stachyose and raffinose by hind-gut bacteria of the weanling pig. Lett. Appl. Microbiol. 18: 349-352.

Kumar, V., Rani, A., Goyal, L., Dixit, A. K., Manjaya, J. G., Dev, J. and Swamy, M. 2010. Sucrose and raffinose family oligosaccharides (RFOs) in soybean seeds as influenced by genotype and growing location. J. Agric. Food Chem. 58: 5081-5085.

Kurbel, S., Kurbel, B. and Včev, A. 2006. Intestinal gases and flatulence: possible causes of occurrence. Med. Hypotheses 67: 235-239.

Ladizinsky, G. and Adler, A. 1976. Genetic relationships among the annual species of *Cicer* L. Theor. Appl. Genet.48: 197-203.

Lahuta, L. B. 2006. Biosynthesis of raffinose family oligosaccharides and galactosyl pinitols in developing and maturing seeds of winter vetch (*Vicia villosa* Roth.). Acta Soc. Bot. Pol. 75: 219-227.

Lahuta, L. B. and Goszczyńska, J. 2009. Inhibition of raffinose family oligosaccharides and galactosyl pinitols breakdown delays germination of winter vetch (*Vicia villosa* Roth.) seeds. Acta Soc. Bot. Pol. 78: 203–208.

Lahuta, L. B., Górecki, R. J., Gojlo, E. and Horbowicz, M. 2005. Differences in accumulation of soluble α-galactosides during seed maturation of several *Vicia* species. Acta Physiol. Plant. 27: 163-171.

Lahuta, L. B., Goszczyńska, J., Horbowicz, M., Hołdyński, C. and Górecki, R. J. 2010. Cyclitols affect accumulation of α-d-galactosides in developing *Vicia* seeds. Acta Physiol. Plant. 32: 933-944.

Larrainzar, E., Wienkoop, S., Scherling, C., Kempa, S., Ladrera, R., Arrese-Igor, C., Weckwerth, W. and González, E. M. 2009. Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in *Medicago truncatula* under drought and recovery. Mol. Plant Microbe Interact. 22: 1565-1576.

Lee, Y. C. 1996. Carbohydrate analyses with high-performance anion-exchange chromatography. J. Chromatogr. A 720: 137-149.

Lehle, L. and Tanner, W. 1973. The function of myo-inositol in the biosynthesis of raffinose. Eur. J. Biochem. 38: 103-110.

Lehner, A., Bailly, C., Flechel, B., Poels, P., Côme, D. and Corbineau, F. 2006. Changes in wheat seed germination ability, soluble carbohydrate and antioxidant enzyme activities in the embryo during the desiccation phase of maturation. J. Cereal Sci. 43: 175-182.

Leprince, O., Bronchart, R. and Deltour, R. 1990. Changes in starch and soluble sugars in

relation to the acquisition of desiccation tolerance during maturation of *Brassica campestris* seed. Plant, Cell Environ.13: 539-546.

Leske, K. L., Jevne, C. J. and Coon, C. N. 1993. Effect of oligosaccharide additions on nitrogen-corrected metabolizable energy of soy protein concentrate. Poult. Sci. 72: 664-668.

Leske, K. L., Zhang, B. and Coon, C. N. 1995. The use of low alpha-galactoside protein products as a protein source in chicken diets. Anim. Feed Sci. Tech. 54: 275-286.

Leterme, P. and Muñoz, L. C. 2002. Factors influencing pulse consumption in Latin America. Br. J. Nutr. 88: 251-254.

Li, S., Li, T., Kim, W., Kitaoka, M., Yoshida, S., Nakajima, M. and Kobayashi, H. 2007. Characterization of raffinose synthase from rice (*Oryza sativa* L. var. Nipponbare). Biotechnol. Lett. 29: 635-640.

Li, X., Zhuo, J., Jing, Y., Liu, X. and Wang, X. 2011. Expression of a galactinol synthase gene is positively associated with desiccation tolerance of *Brassica napus* seeds during development. J. Plant Physiol. 168: 1761-1770.

Li, Y., Li, L. L., Fan, R. C., Peng, C. C., Sun, H. L., Zhu, S. Y., Wang, X. F., Zhang, L. Y. and Zhang D. P. 2012. Arabidopsis sucrose transporter SUT4 interacts with cytochrome b5-2 to regulate seed germination in response to sucrose and glucose. Mol. Plant. 5: 1029-1041.

Lindmark, T., Kimura, Y. and Artursson, P. 1998. Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in caco-2 Cells. J. Pharmacol. Exp. Ther. 284: 362-369.

Liu, H., Dai, X., Xu, Y. and Chong, K. 2007. Over-expression of OsUGE-1 altered raffinose level and tolerance to abiotic stress but not morphology in Arabidopsis. J. Plant Physiol. 164: 1384-1390.

Liu, J. J., Odegard, W. and de Lumen, B. O. 1995. Galactinol synthase from kidney bean cotyledon and zucchini leaf (purification and n-terminal sequences). Plant Physiol. 109: 505-511.

Ma, J. M., Horbowicz, M. and Obendorf, R. L. 2005. Cyclitol galactosides in embryos of buckwheat stem-leaf-seed explants fed D-chiro-inositol, *myo*- inositol or D-pinitol. Seed Sci. Res. 15: 329-338.

Malhotra, R. S., Singh, K. B. and Saxena, M. C. 1997. Effect of irrigation on winter- sown chickpea in a Mediterranean environment. J. Agron. Crop Sci. 178: 237-243.

Martín-Cabrejas, M. A., Díaz, M. F., Aguilera, Y., Benítez, V., Mollá, E. and Esteban, R. M. 2008. Influence of germination on the soluble carbohydrates and dietary fibre fractions in non-conventional legumes. Food Chem. 107: 1045-1052.

Martínez-Villaluenga, C., Frias, J. and Vidal-Valverde, C. 2008. Alpha-galactosides: antinutritional factors or functional ingredients? Crit. Rev. Food Sci. Nutr. 48: 301-316.

Matteuzzi, D., Swennen, E., Rossi, M., Hartman, T. and Lebet, V. 2004. Prebiotic effects of a wheat germ preparation in human healthy subjects. Food Microbiol. 21: 119-124.

McCaskill, A. and Turgeon, R. 2007. Phloem loading in *Verbascum phoeniceum* L. depends on the synthesis of raffinose-family oligosaccharides. Proc. Natl. Acad. Sci. USA 104: 19619-19624.

Mebrahtu, T. and Mohamed, A. 2006. Genetic variation for green pod yield and quality

among vegetable soybean genotypes. J. Crop Improv. 16: 113-130.
Mineo, H., Hara, H., Kikuchi, H., Sakurai, H. and Tomita, F. 2001. Various indigestible saccharides enhance net calcium transport from the epithelium of the small and large intestine of rats *in vitro*. J. Nutr. 131: 3243-3246.
Mitamura, R., Hara, H. and Aoyama, Y. 2004. Ingestion of raffinose promotes calcium absorption in the large intestine of rats. Biosci., Biotechnol., Biochem. 68: 384-389.
Mitsuoka, T. 1992. Intestinal flora and aging. Nutr. Rev. 50: 438-446.
Mittal, R., Nagi, H. P. S., Sharma, P. and Sharma, S. 2012. Effect of processing on chemical composition and antinutritional factors in chickpea flour. J. Food Sci. Eng. 2: 180-186.
Morelli, R., Russo-Volpe, S., Bruno, N. and Lo Scalzo, R. 2003. Fenton-dependent damage to carbohydrates: free radical scavenging activity of some simple sugars. J. Agric. Food Chem. 51: 7418-7425.
Moresco, H., Sansón, P. and Seoane, G. 2008. Simple potentiometric determination of reducing sugars. J. Chem. Edu. 85: 1091-1093.
Morsy, M. R., Jouve, L., Hausman, J., Hoffmann, L. and Stewart, J. M. 2007. Alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting in chilling tolerance. J. Plant Physiol. 164: 157-167.
Munier-Jolain, N. and Salon, C. 2003. Can sucrose content in the phloem sap reaching field pea seeds (*Pisum sativum* L.) be an accurate indicator of seed growth potential? J. Exp. Bot. 54: 2457-2465.
Naczk, M., Amarowicz, R. and Shahidi, F. 1997. Alpha-Galactosides of sucrose in foods: composition, flatulence-causing effects, and removal. ACS Symposium Series 662: 127–151.
Nagura, T., Hachimur, S., Hashiguchi, M., Ueda, Y., Kanno, T., Kikuchi, H., Sayama, K. and Kaminogawa, S. 2002. Suppressive effect of dietary raffinose on T-helper 2 cell-mediated immunity. Br. J. Nutr. 88: 421-426.
Nishizawa, A., Yabuta, Y. and Shigeoka, S. 2008. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiol. 147: 1251-1263.
Nishizawa-Yokoi, A., Yabuta, Y. and Shigeoka, S. 2008. The contribution of carbohydrates including raffinose family oligosaccharides and sugar alcohols to protection of plant cells from oxidative damage. Plant Signaling Behav. 3: 1016-1018.
Nurmi, J. T., Puolakkainen, P. A. and Rautonen, N. E. 2005. *Bifidobacterium Lactis* sp. 420 up-regulates cyclooxygenase (Cox)-1 and down-regulates Cox-2 gene expression in a Caco-2 cell culture model. Nutr. Cancer. 51: 83-92.
Obendorf, R. L., Odorcic, S., Ueda, T., Coseo, M. P. and Vassallo, E. 2004. Soybean galactinol synthase forms fagopyritol B1 but not galactopinitols: substrate feeding of isolated embryos and heterologous expression. Seed Sci. Res. 14: 321-333.
Obendorf, R. L., Zimmerman, A. D., Zhang, Q., Castillo, A., Kosina, S. M., Bryant, E. G., Sensenig, E. M., Wu, J. and Schnebly, S. R. 2009. Accumulation of soluble carbohydrates during seed development and maturation of low-raffinose, low-stachyose soybean. Crop Sci. 49: 329-341.
Onyenekwe, P. C., Njoku, G. C. and Ameh, D. A. 2000. Effect of cowpea (*Vigna ilwguiculata*) processing methods on flatus causing oligosaccharides. Nutr. Res. 20: 349-358.

Panikulangara, T. J., Eggers-Schumacher, G., Wunderlich, M., Stransky, H. and Schöffl, F. 2004. Galactinol synthase1. A novel heat shock factor target gene responsible for heat-induced synthesis of raffinose family oligosaccharides in arabidopsis. Plant Physiol. 136: 3148-3158.

Pattee, H. E., Isleib, T. G., Giesbrecht, F. G. and McFeeters, R. F. 2000. Investigations into Genotypic Variations of Peanut Carbohydrates. J. Agric. Food Chem. 48: 750-756.

Peterbauer, T. and Richter, A. 1998. Galactosylononitol and stachyose synthesis in seeds of adzuki bean: purification and characterization of stachyose synthase. Plant Physiol. 117: 165-172.

Peterbauer, T. and Richter, A. 2001. Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. Seed Sci. Res. 11: 185-197.

Peterbauer, T., Lahuta, L. B., Blöchl, A., Mucha, J., Jones, D. A., Hedley, C. L., Gòrecki, R. J. and Richter, A. 2001. Analysis of the raffinose family oligosaccharide pathway in pea seeds with contrasting carbohydrate composition. Plant Physiol. 127: 1764-1772.

Peterbauer, T., Mach, L., Mucha, J. and Richter, A. 2002a. Functional expression of a cDNA encoding pea (*Pisum sativum* L.) raffinose synthase, partial purification of the enzyme from maturing seeds, and steady-state kinetic analysis of raffinose synthesis. Planta 215: 839-846.

Peterbauer, T., Mucha, J., Mach, L. and Richter, A. 2002b. Chain elongation of raffinose in pea seeds. Isolation, characterization, and molecular cloning of a multifunctional enzyme catalyzing the synthesis of stachyose and verbascose. J. Biol. Chem. 277: 194-200.

Peterbauer, T., Mucha, J., Mayer, U., Popp, M., Glossl, J. and Richter, A. 1999. Stachyose synthasis in seeds of adzuki bean (*Vigna angularis*): molecular cloning and functional expression of stachyose synthase. Plant J. 20: 509-518.

Peters, S. and Keller, F. 2009. Frost tolerance in excised leaves of the common bugle (*Ajuga reptans* L.) correlates positively with the concentrations of raffinose family oligosaccharides (RFOs). Plant Cell Environ.32: 1099-1107.

Peters, S., Mundree, S. G., Thomson, J. A., Farrant, J. M. and Keller, F. 2007. Protection mechanisms in the resurrection plant *Xerophyta viscosa* (Baker): Both sucrose and raffinose family oligosaccharides (RFOs) accumulate in leaves in response to water deficit. J. Exp. Bot. 58: 1947-1956.

Philippe, R. N., Ralph, S. G., Mansfield, S. D. and Bohlmann, J. 2010. Transcriptome profiles of hybrid poplar (*Populus trichocarpa* X *deltoides*) reveal rapid changes in undamaged, systemic sink leaves after simulated feeding by forest tent caterpillar (*Malacosoma disstria*). New Phytol. 188: 787-802.

Piepho, H. P. and Möhring, J. 2007. Computing heritability and selection response from unbalanced Plant Breed.trials. Genetics 177: 1881-1888.

Polowick, P. L., Baliski, D. S., Bock, C., Ray, H. and Georges, F. 2009. Over-expression of α-galactosidase in pea seeds to reduce raffinose oligosaccharide content. Botany 87: 526-532.

Pond, S. E., Patrick, v. A. and Bonga, J. M. 2002. Improving tolerance of somatic embryos of *Picea glauca* to flash desiccation with a cold treatment (desiccation after cold acclimation). In Vitro Cell. Dev. Biol. Plant 38: 334-341.

Pukacka, S., Ratajczak, E. and Kalemba, E. 2009. Non-reducing sugar levels in beech (*Fagus sylvatica*) seeds as related to withstanding desiccation and storage. J. Plant Physiol. 166: 1381-1390.

Quemener, B. and Brillouet, J. 1983. Ciceritol, a pinitol digalactoside form seeds of chickpea, lentil and white lupin. Phytochemistry 22: 1745-1751.

Raessler, M., Wissuwa, B., Breul, A., Unger, W. and Grimm, T. 2010. Chromatographic analysis of major non-structural carbohydrates in several wood species – an analytical approach for higher accuracy of data. Anal. Methods 2: 532–538.

Rajesh, P. N., O'Bleness, M., Roe, B. A. and Muehlbauer, F. J. 2008. Analysis of genome organization, composition and microsynteny using 500 kb BAC sequences in chickpea. Theor. Appl. Genet. 117: 449-458.

Rao, V. S. and Vakil, U. K. 1983. Effects of gamma-irradiation on flatulence-causing oligosaccharides in green gram (*Phaseolus areus*). J. Food Sci. 48: 1791-1795.

Redden, R. J. and Berger, J. D. 2007. History and origin of chickpea. In: S.S. Yadav, R. Redden, W. Chen and B. Sharma (eds.). Chickpea Breeding and Management. CAB International. pp. 1-13.

Reddy, N. R. and Salunkhe, D. K. 1980. Changes in oligosaccharides during germination and cooking of black gram and fermentation of black gram/rice blend. Cereal Chem. 57: 356-360.

Reddy, N. R., Pierson, M. D., Sathe, S. K. and Salunkhe, D. K. 1984. Chemical, nutritional and physiological aspects of dry bean carbohydrates—A review. Food Chem. 13: 25-68.

Rincón, F., Martínez, B. and Ibáñez, M. V. 1998. Proximate composition and antinutritive substances in chickpea (*Cicer arietinum* L.) as affected by the biotype factor. J. Sci. Food Agric. 78: 382-388.

Roberfroid, M. 2002. Functional food concept and its application to prebiotics. Dig. Liver Dis. 34: S105-S110.

Rohrer, J. S., Basumallick, L. and Hurum, D. 2013. High-performance anion-exchange chromatography with pulsed amperometric detection for carbohydrate analysis of glycoproteins. Biochem. (Moscow) 78: 697-709.

Rosnoblet, C., Aubry, C., Leprince, O., Vu, B. L., Rogniaux, H. and Buitink J. 2007. The regulatory gamma subunit SNF4b of the sucrose non-fermenting-related kinase complex is involved in longevity and stachyose accumulation during maturation of *Medicago truncatula* seeds. Plant J. 51: 47-59.

Rowland, I. R. and Tanaka, R. 1993. The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human faecal microflora. J. Appl. Bacteriol. 74: 667–674.

Roy, F., Boye, J. I. and Simpson, B. K. 2010. Bioactive proteins and peptides in pulse crops: pea, chickpea and lentil. Food Res. Int.43: 432-442.

Rubio, J., Moreno, M. T., Cubero, J. I. and Gil, J. 1998. Effect of the gene for double pod in chickpea on yield, yield components and stability of yield. Plant Breed.117: 585-587.

Saini, H. S. and Knights, E. J. 1984. Chemical constitution of starch and oligosaccharide components of 'desi' and 'kabuli' chickpea (*Cicer arietinum*) seed types. J. Agric. Food Chem. 32: 940-944.

Saito, Y., Takano, T. and Rowland, I. 1992. Effects of soybean oligosaccharidles on the human gut microflora in *in vitro* culture. Microb. Ecol. Health Dis. 5: 105-110.

Saldivar, X., Wanga, Y. J., Chen, P. and Hou, A. 2011. Changes in chemical composition during soybean seed development. Food Chem. 124: 1369–1375.

Sánchez-Mata, M. C., Penuela-Teruel, M. J., Cámara Hurtado, M., Díez-Marques, C. and Torija-Isasa, M. E. 1998. Determination of mono-, di-, and oligosaccharide in legumes by high-performance liquid chromatography using an amino-bonded silica column. J. Agric. Food Chem. 46: 3648-3652.

Sánchez-Mata, M. C., Cámara Hurtado, M. and Díez-Marqués, C. 1999. Effect of domestic processes and water hardness on soluble sugars content of chickpeas (*Cicer arietinum* L.). Food Chem. 65: 331-338.

Saravitz, D. M., Pharr, D. M. and Carter, T. E. Jr. 1987. Galactinol synthase activity and soluble sugars in developing seeds of four soybean genotypes. Plant Physiol. 83: 185-189.

Saunders, D. R. and Wiggins, H. S. 1981. Conservation of mannitol, lactulose, and raffinose by the human colon. Am. J. Physiol. Gastrointest. Liver Physiol. 241: G397-G402.

Scandalios, J. G. 2005. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz. J. Med. Biol. Res. 38: 995-1014.

Scheppach, W. and Weiler, F. 2004. The butyrate story: old wine in new bottles? Curr. Opin. Clin. Nutr. Metab. Care *7*: 563-567.

Scholz-Ahrens, K. E., Ade, P., Marten, B., Weber, P., Timm, W., Avarsigmail, Y., Glüer, C. and Schrezenmeir, J. 2007. Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. J. Nutr. 137: 838S-846S.

Scholz-Ahrens, K. E., Schaafsma, G., Van den Heuvel, E. G. and Schrezenmeir, J. 2001. Effects of prebiotics on mineral metabolism. Am. J. Clin. Nutr. 73: 459S-464S.

Sharma, M., Kiran Babu, T., Gaur, P. M., Ghosh, R., Rameshwar, T., Chaudhary, R. G., Upadhyay, J. P., Gupta, O., Saxena, D. R., Kaur, L., Dubey, S. C., Anandani, V. P., Harer, P. N., Rathore, A. and Pande, S. 2012. Identification and multi-environment validation of resistance to *Fusarium oxysporum* f. sp. *ciceris* in chickpea. Field Crops Res. 135: 82-88.

Singh, N. and Kayastha, A. M. 2012. Purification and characterization of α-galactosidase from white chickpea (*Cicer arietinum*). J. Agric. Food Chem. 60: 3253-3259.

Singh, F. and Diwakar, B. 1995. Chickpea botany and production practices. Skill Development Series no. 16. ICRISAT - Training and Fellowships Program.

Singh, K. B. 1997. Chickpea (*Cicer arietinum* L.). Field Crop Res. 53: 161-170.

Singh, M., Ceccarelli, S. and Hamblin, J. 1993. Estimation of heritability from varietal trials data. Theor. Appl. Genet. 86: 437-441.

Singh, U. and Jambunathan, R. 1982. Changes in starch, oligosaccharides and soluble sugars in developing pod wall and seed of chickpea. Phytochemistry 21: 297-299.

Singh, U., Kumar, J.and Gowda, C. L. L. 1983. The protein content of chickpea (*Cicer arietinum* L.) grown at different locations. Plant Foods Hum. Nutr. 32: 79-184.

Sirohi, A., Singh, A., Panwar, K.S. and Chauhan, K.C. 2001. Genotype-environment interactions and phenotypic stability in gram (*Cicer arietinum*). Indian J. Agric. Sci. 71: 411-413.

Smith, P. T., Kuo, T. M. and Gerald Crawford, C. 1991. Purification and characterization of galactinol synthase from mature zucchini squash leaves. Plant Physiol. 96: 693-698.

Sosulski, F. W., Elkowicz, L. and Reichert, R. D. 1982. Oligosaccharides in eleven legumes and their air-classified protein and starch fractions. J. Food Sci. 47: 498-

Sprenger, N. and Keller, F. 2000. Allocation of raffinose family oligosaccharides to transport and storage pools in *Ajuga reptans*: the roles of two distinct galactinol synthases. Plant J. 21: 249-258.

Srinivasan, S. and Gaur, P. M. 2012. Genetics and characterization of an open flower mutant in chickpea. J. Hered. 103: 297–302.

Still, D. W., Kovach, D. A. and Bradford, K. J. 1994. Development of desiccation tolerance during embryogenesis in rice (*Oryza sativa*) and wild rice (*Zizania palustris*) (dehydrin expression, abscisic acid content, and sucrose accumulation). Plant Physiol. 104: 431-438.

Sui, X. L., Meng, F. Z., Wang, H. Y., Wei, Y. X., Li, R. F., Wang, Z. Y., Hu, L. P., Wang, S. H. and Zhang, Z. X. 2012. Molecular cloning, characteristics and low temperature response of raffinose synthase gene in *Cucumis sativus* L. J. Plant Physiol. 169: 1883-1891.

Suzuki, T. and Hara, H. 2004. Various nondigestible saccharides open a paracellular calcium transport pathway with the induction of intracellular calcium signaling in human intestinal caco-2 cells. J. Nutr. 134: 1935-1941.

Swennen, K., Courtin, C. M. and Delcour, J. A. 2006. Non-digestible oligosaccharides with prebiotic properties. Crit. Rev. Food Sci. Nutr. 46: 459-471.

Tahir, M., Vandenberg, A. and Chibbar, R. N. 2011. Influence of environment on seed soluble carbohydrates in selected lentil cultivars. J. Food Compos. Anal. 24: 596-602.

Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yamaguchi-Shinozaki, K. and Shinozaki, K. 2002. Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J. 29: 417-426.

Tajoddin, M., Shinde, M. and Junna, L. 2010. Raffinose, stachyose and sucrose contents of mung bean cultivars differing in seed coat color from hyderabad-karnataka region of india: effect of soaking and germination. The Bioscan 5 : 343-346.

Takanashi, K., Shitan, N., Sugiyama, A., Kamimoto, Y., Hamamoto, M., Iwaki, T., Takegawa, K. and Yazaki, K. 2008. Galactinol synthase gene of *Coptis japonica* is involved in berberine tolerance. Biosci. Biotechnol. Biochem. 72: 398-405.

Tapernoux-Luthi, E., Bohm, A. and Keller, F. 2004. Cloning, Functional Expression, and Characterization of the raffinose oligosaccharide chain elongation enzyme, galactan:galactan galactosyltransferase, from common bugle leaves. Plant Physiol. 134: 1377-1387.

Tekeoğlu, M., Işik, M. and Muehlbauer, F. J. 2004. QTL Analysis of ascochyta blight resistance in chickpea. Turk. J. Agric. For. 28: 183-187.

Tekeoğlu, M., Rajesh, P. N. and Muehlbauer, F. J. 2002. Integration of sequence tagged microsatellite sites to the chickpea genetic map. Theor. Appl. Genet. 105: 847-854.

Thavarajah, D. and Thavarajah, P. 2012. Evaluation of chickpea (*Cicer arietinum* L.) micronutrient composition: biofortification opportunities to combat global micronutrie-

-nt malnutrition. Food Res. Int.49: 99-104.

Toldi, O., Tuba, Z. and Scott, P. 2009. Vegetative desiccation tolerance: Is it a goldmine for bioengineering crops? Plant Sci. 176: 187-199.

Topping, D. L. 1996. Short-chain fatty acids produced by intestinal bacteria. Asia Pac. J. Clin. Nutr. 5: 15-19.

Tortuero, F., Fernández, E., Rupérez, P. and Moreno, M. 1997. Raffinose and lactic acid bacteria influence caecal fermentation and serum cholesterol in rats. Nutr. Res. 17: 41-49.

Tosh, S. M. and Yada, S. 2010. Dietary fibres in pulse seeds and fractions: Characterization, functional attributes, and applications. Food Res. Int. 43: 450-460.

Touŕe, R., Kheadr, E., Lacroix, C., Moroni, O. and Fliss, I. 2003. Production of antibacterial substances by bifidobacterial isolates from infant stool active against *Listeria monocytogenes*. J. Appl. Microbiol. 95: 1058-1069.

Trojanová, I., Vlková, E., Rada, V. and Marounek, M. 2006. Different utilization of glucose and raffinose in *Bifidobacterium breve* and *Bifidobacterium animalis*. Folia Microbiol. 51: 320-324.

Turgeon, R. 1996. Phloem loading and plasmodesmata. Trends Plant Sci. 1: 418-423.

Turgeon, R., Beebe, D. U. and Gowan, E. 1993. The intermediary cell: Minor-vein anatomy and raffinose oligosaccharide synthesis in the Scrophulariaceae. Planta 191: 446-456.

Turner, N. C., Furbank, R. T., Berger, J. D., Gremigni, P., Abbo, S. and Leport, L. 2009. Seed size is associated with sucrose synthase activity in developing cotyledons of chickpea. Crop Sci. 49: 621-627.

Ueda, T., Coseo, M. P., Harrell, T. J. and Obendorf, R. L. 2005. A multifunctional galactinol synthase catalyzes the synthesis of fagopyritol A1 and fagopyritol B1 in buckwheat seed. Plant Sci. 168: 681-690.

Ukić, Š., Rogošić, M., Novak, M., Šimović, E., Tišler, V. and and Bolanča, T. 2013. Optimization of IC separation based on isocratic-to-gradient retention modeling in combination with sequential searching or evolutionary algorithm. J. Anal. Methods Chem. 2013: 549729.

Ülker, B. and Somssich, I. E. 2004. WRKY transcription factors: from DNA binding towards biological function. Curr. Opin. Plant Biol. 7: 491-498.

Ulukan, H. 2011. The use of plant genetic resources and biodiversity in classical plant breeding. Acta Agric. Scand. B. 61: 97-104.

Upadhyaya, H. D. and Ortiz, R. 2001. A mini core subset for capturing diversity and promoting utilization of chickpea genetic resources in crop improvement. Theor. Appl. Genet. 102: 1292-1298.

Upadhyaya, H. D., Bramel, P. J. and Singh, S. 2001. Plant genetic resources: development of a chickpea core subset using geographic distribution and quantitative traits. Crop Sci. 41: 206-210

Upadhyaya, H. D., Furman, B. J., Dwivedi, S. L., Udupa, S. M., Gowda, C. L. L., Baum, M., Crouch, J. H., Buhariwalla, H. K. and Singh, S. 2006. Development of a composite collection for mining germplasm possessing allelic variation for beneficial traits in chickpea. Plant Genet. Resour. 4: 13-19.

Upadhyaya, H. D., Thudi, M., Dronavalli, N., Gujaria, N., Singh, S., Sharma, S. and

Varshney, R. K. 2011. Genomic tools and germplasm diversity for chickpea improvement. Plant Genet. Resour. 9: 45-58.

van Barneveld, R. J. 1999. Understanding the nutritional chemistry of lupin (*Lupinus* spp.) seed to improve livestock production efficiency. Nutr. Res. Rev. 12: 203-230.

Van den Ende, W. and Valluru, R. 2009. Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging? J. Exp. Bot.60: 9–18.

Van der Maesen, L. J. G. 1972. *Cicer* L., a monograph of the genus, with special reference to the chickpea (*Cicer arietinum* L.), its ecology and cultivation. Meded. Landbouwhoge school Wageningen 72-10.

Van der Maesen, L. J. G., Maxted, N., . Javadi, F., Coles, S. and Davies, A. M. R. 2007. Taxanomy of the genus *Cicer* revisited. In: S.S. Yadav, R. Redden, W. Chen, B. Sharma (eds.). Chickpea Breeding and Management, CABI International, Wallingford, pp. 14-46.

Van, K., Kim, D. H., Shin, J. H. and Lee, S. H. 2011. Genomics of plant genetic resources: past, present and future. Plant Genet. Resour. 9: 155-158.

Vandecasteele, C., Teulat-Merah, B., Morère-Le Paven, M. C., Leprince, O., Ly Vu, B., Viau, L., Ledroit, L., Pelletier, S., Payet, N., Satour, P., Lebras, C., Gallardo, K., Huguet, T., Limami, A. M., Prosperi, J. M. and Buitink, J. 2011. Quantitative trait loci analysis reveals a correlation between the ratio of sucrose/raffinose family oligosaccharides and seed vigour in *Medicago truncatula*. Plant Cell Environ. 34: 1473-1487.

Varshney, R. K., Song, C., Saxena, R. K., Azam, S., Yu, S., Sharpe, A. G., Cannon, S., Baek, J., Rosen, B. D., Tar'an, B., Millan, T., Zhang, X., Ramsay, L. D., Iwata, A., Wang, Y., Nelson, W., Farmer, A. D., Gaur, P. M., Soderlund, C., Penmetsa, R. V., Xu, C., Bharti, A. K., He, W., Winter, P., Zhao, S., Hane, J. K., Carrasquilla-Garcia, N., Condie, J. A., Upadhyaya, H. D., Luo, M. C., Thudi, M., Gowda, C. L. L., Singh, N. P., Lichtenzveig, J., Gali, K. K., Rubio, J., Nadarajan, N., Dolezel, J., Bansal, K. C., Xu, X., Edwards, D., Zhang, G., Kahl, G., Gil, J., Singh, K. B., Datta, S. D., Jackson, S. A., Wang, J. and Cook, D. R. 2013. Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. Nat. Biotechnol. 31: 240-246.

Veldman, A., Veen, W. A. G., Barug, D. and Van Paridon, P. A. 1993. Effect of α-galactosides and α-galactosidase in feed on ileal piglet digestive physiology. J. Anim. Physiol. Anim. Nutr. 69: 57-65.

Velíšek, J. and Cejpek, K. 2005. Biosynthesis of food constituents: saccharides.1. monosaccharides, oligosaccharides, and related compounds – a review. Czech J. Food Sci. 23: 129-144.

Vidal-Valverde, C., Frias, J., Hernández, A., Martín-Alvarez, P. J., Sierra, I., Rodríguez, C., Blazquez, I. and Vicente, G. 2003. Assessment of nutritional compounds and antinutritional factors in pea (*Pisum sativum*) seeds. J. Sci. Food Agric. 83: 298-306.

Vidal-Valverde, C., Frias, J., Sotomayor, C., Diaz-Pollan, C., Fernandez, M. and Urbano, G. 1998. Nutrients and antinutritional factors in faba beans as affected by processing. Z. Lebensm. Unters. Forsch. 207: 140-145.

Vinjamoori, D. V., Byrum, J. R., Hayes T. and Das, P. K. 2004. Challenges and opportunities in the analysis of raffinose oligosaccharides, pentosans, phytate, and glucosinolates. J.

Anim. Sci. 82: 319-328.

Wagner, J. R., Becker, R., Gumbmann, M. R. and Olson, A. C. 1976. Hydrogen production in the rat following ingestion of raffinose, stachyose and oligosaccharide-free bean residue. J. Nutr. 106: 466-470.

Wang, D., Yao, W., Song, Y., Liu, W. and Wang, Z. 2012. Molecular characterization and expression of three galactinol synthase genes that confer stress tolerance in *Salvia miltiorrhiza*. J. Plant Physiol. 169: 1838-1848.

Wang, N. and Daun, J. K. 2004. Effect of variety and crude protein content on nutrients and certain antinutrients in field (*Pisum sativum*). J. Sci. Food Agric. 84: 1021-1029.

Wang, N. and Daun, J. K. 2004. The chemical composition and nutritive value of Canadian pulses. In: Canadian Grain Commission Report. pp. 19-29.

Wang, N., Hatcher, D. W., Tyler, R. T., Toews, R. and Gawalko, E. J. 2010. Effect of cooking on the composition of beans (*Phaseolus vulgaris* L.) and chickpeas (*Cicer arietinum* L.). Food Res. Int. 43: 589-594.

Wang, N., Lewis, M. J., Brennan, J. G. and Westby, A. 1997. Effect of processing methods on nutrients and anti-nutritional factors in cowpea. Food Chem. 58: 59-68.

Wang, R., Bai, Y., Low, N. H. and Tanino, K. 2006. Seed size variation in cold and freezing tolerance during seed germination of winterfat (*Krascheninnikovia lanata*) (Chenopodiaceae). Can. J. Bot. 84: 49-59.

Wang, Z., Zhu, Y., Wang, L., Liu, X., Liu, Y., Phillips, J. and Deng, X. 2009. A WRKY transcription factor participates in dehydration tolerance in *Boea hygrometrica* by binding to the W-box elements of the galactinol synthase (*BhGolS1*) promoter. Planta 230: 1155-1166; 1166.

Wiggins, H. S. 1984. Nutritional value of sugars and related compounds undigested in the small gut. Proc. Nutr. Soc. 43: 69-75.

Wojciechowski, M.F., Lavin, M. and Sanderson, M. J. 2004. A phylogeny of legumes (Legumenosae) based on analyses of the plastid matK gene resolves many well-supported subclades within the family. Am. J. Bot. 91: 1846-1862.

Wood, J. A. and Grusak, M. A. 2007. Nutritional value of chickpea. In: S. S. Yadav, R. Redden, W. Chen and B. Sharma (eds.) Chickpea breeding and management. CAB International. pp. 101-142.

Wood, J. A., Knights, E. J. and Choct, M. 2011. Morphology of chickpea seeds (*Cicer arietinum* L.): comparison of desi and kabuli types. Int. J. Plant Sci. 172: 632-643.

Wray, N. and Visscher, P. 2008. Estimating trait heritability. Nature Edu. 1(1).

Wu, X., Kishitani, S., Ito, Y. and Toriyama, K. 2009. Accumulation of raffinose in rice seedlings overexpressing OsWRKY11 in relation to desiccation tolerance. Plant Biotechnol. 26: 431-434.

Xia, X., Wang, Y., Zhou, Y., Tao, Y., Mao, W., Shi, K., Asami, T., Chen, Z. and Yu, J. 2009. Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. Plant Physiol. 150: 801-814.

Xiaoli, X., Liyi, Y., Shuang, H., Wei, L., Yi, S., Hao, M., Jusong, Z. and Xiaoxiong, Z. 2008. Determination of oligosaccharide contents in 19 cultivars of chickpea (*Cicer arietinum* L) seeds by high performance liquid chromatography. Food Chem. 111: 215-219.

Yadav, S. S., Verma, A. K., Rizvi, A. H., Singh, D., Kumar, J. and Andrews, M. 2010.

Impact of genotype × environment interactions on the relative performance of diverse groups of chickpea (*Cicer arietinum* L.) cultivars. Arch. Agron. Soil Sci. 56: 49-64.

Zalewski, K., Nitkiewicz, B., Lahuta, L. B., Głowacka, K., Socha, A. and Amarowicz, R. 2010. Effect of jasmonic acid–methyl ester on the composition of carbohydrates and germination of yellow lupine (*Lupinus luteus* L.) seeds. J. Plant Physiol. 167: 967-973.

Zhao, T., Corum III, J. W., Mullen, J., Meeley, R. B., Helentjaris, T., Martin, D. and Downie, B. 2006. An alkaline α-galactosidase transcript is present in maize seeds and cultured embryo cells, and accumulates during stress. Seed Sci. Res. 16: 107-121.

Zhawar, V. K., Kaur, N. and Gupta, A. K. 2011. Phytic acid and raffinose series oligosaccharides metabolism in developing chickpea seeds. Physiol. Mol. Biol. Plants 17: 355-362.

Zhou, T., Zhang, R. and Guo, S. 2012. Molecular cloning and characterization of *GhGolS1*, a novel gene encoding galactinol synthase from cotton (*Gossypium hirsutum*). Plant Mol. Biol. Rep. 30: 699-709.

Zhuo, C., Wang, T., Lu, S., Zhao, Y., Li, X. and Guo, Z. 2013. Cold responsive galactinol synthase gene from *Medicago falcata* (*MfGolS1*) is induced by *myo*-inositol and confers multiple tolerances to abiotic stresses. Physiol. Plant. 149: 67-78.

Zia-Ul-Haq, M., Iqbal, S., Ahmad, S., Imran, M., Niaz, A. and Bhanger, M. I. 2007. Nutritional and compositional study of desi chickpea (*Cicer arietinum* L.) cultivars grown in Punjab. Pakistan. Food Chem. 105: 1357-1363.

Zuther, E., Büchel, K., Hundertmark, M., Stitt, M., Hincha, D. K. and Heyer, A. G. 2004. The role of raffinose in the cold acclimation response of *Arabidopsis thaliana*. FEBS Lett. 576: 169-173.

Appendix 8.1 Concentration (value ± standard deviation) of soluble sugars in desi chickpea accessions [unit of concentration is g/100 g of chickpea seed meal on fresh weight basis except for RFO (mmol/100 g of chickpea seed meal on fresh weight basis)]

Accession Number	*Myo*-inositol	Galactinol	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Verbascose	Total RFO
ICC 4951	0.07 ± 0.02	0.23 ± 0.05	0.25 ± 0.02	0.016 ± 0.013	1.7 ± 0.07	0.83 ± 0.14	1.57 ± 0.27	0.09 ± 0.03	3.94 ± 0.68
ICC 4918	0.06 ± 0.01	0.18 ± 0.01	0.23 ± 0.04	0.019 ± 0.018	1.93 ± 0.45	0.78 ± 0.11	1.42 ± 0.17	0.08 ± 0.01	3.84 ± 0.54
ICC 506-EB	0.06 ± 0.01	0.2 ± 0.03	0.22 ± 0.04	0.011 ± 0.002	1.32 ± 0.22	0.79 ± 0.09	1.56 ± 0.12	0.08 ± 0.01	3.91 ± 0.18
ICC 16382	0.07 ± 0.02	0.19 ± 0.03	0.26 ± 0.06	0.054 ± 0.085	1.85 ± 0.35	0.92 ± 0.11	1.69 ± 0.02	0.1 ± 0.01	4.46 ± 0.18
ICC 995	0.04 ± 0.01	0.16 ± 0.03	0.17 ± 0.06	0.009 ± 0.004	1.88 ± 0.25	0.69 ± 0.14	1.19 ± 0.26	0.06 ± 0.01	3.44 ± 0.59
ICC 1431	0.04 ± 0.01	0.11 ± 0.02	0.21 ± 0.06	0.007 ± 0.003	1.52 ± 0.33	0.83 ± 0.14	1.34 ± 0.11	0.08 ± 0.01	3.82 ± 0.24
ICC 4991	0.05 ± 0.005	0.21 ± 0.02	0.14 ± 0.07	0.012 ± 0.007	1.11 ± 0.34	0.64 ± 0.13	1.26 ± 0.2	0.07 ± 0.01	3.39 ± 0.17
ICCV 04516	0.06 ± 0.01	0.17 ± 0.01	0.16 ± 0.05	0.012 ± 0.007	1.72 ± 0.15	0.93 ± 0.22	1.23 ± 0.22	0.06 ± 0.02	3.94 ± 0.34
ICC 982	0.05 ± 0.02	0.19 ± 0.04	0.23 ± 0.07	0.01 ± 0.002	1.51 ± 0.39	0.74 ± 0.06	1.25 ± 0.02	0.05 ± 0.02	3.42 ± 0.07
ICC 988	0.04 ± 0.01	0.16 ± 0.05	0.23 ± 0.04	0.011 ± 0.003	1.51 ± 0.29	0.73 ± 0.06	1.31 ± 0.17	0.05 ± 0.01	3.7 ± 0.16
ICC 1017	0.05 ± 0.02	0.18 ± 0.05	0.25 ± 0.06	0.011 ± 0.002	1.96 ± 0.49	0.76 ± 0.08	1.31 ± 0.09	0.06 ± 0.03	3.66 ± 0.16
ICC 1025	0.04 ± 0.02	0.17 ± 0.04	0.24 ± 0.05	0.011 ± 0.002	1.57 ± 0.34	0.59 ± 0.05	1.07 ± 0.08	0.05 ± 0.01	3.08 ± 0.17
ICC 1026	0.05 ± 0.02	0.19 ± 0.05	0.2 ± 0.04	0.009 ± 0.003	1.53 ± 0.32	0.68 ± 0.06	1.24 ± 0.06	0.05 ± 0.01	3.34 ± 0.14
ICC 1163	0.05 ± 0.02	0.17 ± 0.04	0.19 ± 0.03	0.009 ± 0.003	1.75 ± 0.13	0.85 ± 0.05	1.88 ± 0.27	0.08 ± 0.04	4.65 ± 0.35
ICC 1471	0.04 ± 0.02	0.16 ± 0.05	0.16 ± 0.03	0.008 ± 0.004	1.61 ± 0.27	0.76 ± 0.07	1.86 ± 0.38	0.09 ± 0.03	4.58 ± 0.54
ICC 2204	0.05 ± 0.02	0.19 ± 0.04	0.2 ± 0.06	0.01 ± 0.002	1.55 ± 0.32	0.65 ± 0.02	1.33 ± 0.12	0.06 ± 0.01	3.36 ± 0.27
ICC 2234	0.04 ± 0.02	0.19 ± 0.04	0.21 ± 0.05	0.01 ± 0.003	1.47 ± 0.27	0.67 ± 0.06	1.33 ± 0.2	0.06 ± 0.02	3.43 ± 0.38
ICC 2935	0.04 ± 0.02	0.16 ± 0.04	0.2 ± 0.06	0.009 ± 0.003	1.27 ± 0.37	0.6 ± 0.02	1.15 ± 0.26	0.05 ± 0.01	3.12 ± 0.27
ICC 3335	0.05 ± 0.02	0.19 ± 0.04	0.2 ± 0.06	0.011 ± 0.002	1.44 ± 0.36	0.73 ± 0.03	1.57 ± 0.06	0.06 ± 0.01	3.84 ± 0.15
ICC 3336	0.05 ± 0.02	0.17 ± 0.03	0.21 ± 0.05	0.011 ± 0.003	1.72 ± 0.3	0.76 ± 0.11	1.56 ± 0.1	0.06 ± 0.02	3.89 ± 0.36
ICC 3429	0.04 ± 0.02	0.21 ± 0.04	0.17 ± 0.05	0.01 ± 0.003	1.59 ± 0.31	0.64 ± 0.02	1.4 ± 0.13	0.07 ± 0.004	3.49 ± 0.21
ICC 3485	0.05 ± 0.02	0.2 ± 0.04	0.24 ± 0.04	0.01 ± 0.002	1.52 ± 0.22	0.79 ± 0.05	1.52 ± 0.12	0.08 ± 0.01	4.04 ± 0.23
ICC 3867	0.05 ± 0.01	0.18 ± 0.03	0.22 ± 0.04	0.012 ± 0.002	1.74 ± 0.23	0.76 ± 0.08	1.38 ± 0.1	0.06 ± 0.01	3.75 ± 0.19
ICC 3935	0.05 ± 0.03	0.21 ± 0.05	0.23 ± 0.06	0.01 ± 0.002	1.73 ± 0.2	0.76 ± 0.07	1.76 ± 0.07	0.07 ± 0.02	4.37 ± 0.25
ICC 4482	0.05 ± 0.02	0.21 ± 0.04	0.17 ± 0.05	0.01 ± 0.003	1.5 ± 0.21	0.61 ± 0.05	1.39 ± 0.05	0.06 ± 0.01	3.45 ± 0.22
ICC 4902	0.05 ± 0.02	0.17 ± 0.02	0.21 ± 0.03	0.011 ± 0.003	1.54 ± 0.24	0.71 ± 0.09	1.34 ± 0.31	0.06 ± 0.01	3.71 ± 0.47
ICC 4933	0.04 ± 0.02	0.2 ± 0.05	0.22 ± 0.03	0.01 ± 0.003	1.51 ± 0.14	0.65 ± 0.08	1.23 ± 0.28	0.04 ± 0.02	3.5 ± 0.42

Accession Number	*Myo*-inositol	Galactinol	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Verbascose	Total RFO
ICC 5186	0.05 ± 0.02	0.16 ± 0.04	0.2 ± 0.03	0.009 ± 0.003	1.38 ± 0.16	0.72 ± 0.06	1.3 ± 0.22	0.05 ± 0.02	3.62 ± 0.34
ICC 5384	0.06 ± 0.03	0.21 ± 0.05	0.23 ± 0.03	0.01 ± 0.002	1.56 ± 0.24	0.72 ± 0.14	1.29 ± 0.28	0.05 ± 0.02	3.64 ± 0.63
ICC 5566	0.04 ± 0.02	0.17 ± 0.04	0.19 ± 0.04	0.014 ± 0.004	1.34 ± 0.11	0.77 ± 0.09	1.38 ± 0.24	0.05 ± 0.02	4.14 ± 0.54
ICC 5794	0.05 ± 0.03	0.18 ± 0.04	0.2 ± 0.03	0.01 ± 0.003	1.58 ± 0.25	0.79 ± 0.15	1.33 ± 0.16	0.05 ± 0.02	3.79 ± 0.49
ICC 5912	0.05 ± 0.02	0.16 ± 0.07	0.23 ± 0.04	0.009 ± 0.003	1.54 ± 0.16	0.84 ± 0.03	1.57 ± 0.39	0.06 ± 0.02	4.32 ± 0.7
ICC 6152	0.04 ± 0.02	0.15 ± 0.05	0.19 ± 0.04	0.009 ± 0.003	1.85 ± 0.41	0.79 ± 0.05	1.26 ± 0.32	0.06 ± 0.02	3.85 ± 0.51
ICC 6293	0.05 ± 0.02	0.15 ± 0.05	0.23 ± 0.06	0.011 ± 0.002	1.78 ± 0.38	0.67 ± 0.1	1.24 ± 0.23	0.06 ± 0.03	3.52 ± 0.5
ICC 7192	0.04 ± 0.03	0.12 ± 0.1	0.25 ± 0.02	0.023 ± 0.007	1.88 ± 0.1	0.78 ± 0.36	1.32 ± 0.63	0.06 ± 0.02	4.04 ± 0.13
ICC 7669	0.06 ± 0.03	0.16 ± 0.03	0.25 ± 0.09	0.013 ± 0.002	2.31 ± 0.52	0.86 ± 0.09	1.59 ± 0.27	0.06 ± 0.02	4.26 ± 0.53
ICC 8166	0.06 ± 0.02	0.18 ± 0.02	0.24 ± 0.05	0.011 ± 0.006	1.6 ± 0.28	0.71 ± 0.12	1.32 ± 0.25	0.05 ± 0.02	3.42 ± 0.62
ICC 8397	0.05 ± 0.02	0.18 ± 0.06	0.29 ± 0.11	0.009 ± 0.002	1.57 ± 0.41	0.85 ± 0.13	1.55 ± 0.31	0.05 ± 0.03	4.08 ± 0.69
ICC 8474	0.06 ± 0.02	0.17 ± 0.02	0.22 ± 0.06	0.01 ± 0.005	2.11 ± 0.49	0.8 ± 0.07	1.38 ± 0.22	0.07 ± 0.02	3.78 ± 0.38
ICC 8943	0.04 ± 0.01	0.14 ± 0.04	0.23 ± 0.05	0.011 ± 0.004	2.04 ± 0.35	0.67 ± 0.16	1.21 ± 0.26	0.05 ± 0.02	3.13 ± 0.65
ICC 9125	0.06 ± 0.01	0.17 ± 0.03	0.2 ± 0.05	0.009 ± 0.005	1.89 ± 0.34	0.8 ± 0.1	1.53 ± 0.24	0.05 ± 0.03	4.1 ± 0.45
ICC 9557	0.04 ± 0.02	0.16 ± 0.04	0.21 ± 0.06	0.01 ± 0.003	2.12 ± 0.36	0.73 ± 0.1	1.34 ± 0.27	0.05 ± 0.01	3.58 ± 0.51
ICC 9562	0.05 ± 0.01	0.19 ± 0.03	0.16 ± 0.05	0.01 ± 0.006	1.88 ± 0.28	0.9 ± 0.05	1.73 ± 0.21	0.08 ± 0.01	4.6 ± 0.38
ICC 9567	0.05 ± 0.02	0.2 ± 0.05	0.24 ± 0.07	0.013 ± 0.006	1.38 ± 0.37	0.58 ± 0.07	1.12 ± 0.28	0.05 ± 0.02	2.95 ± 0.59
ICC 10090	0.05 ± 0.005	0.2 ± 0.02	0.26 ± 0.08	0.02 ± 0.008	2 ± 0.37	0.84 ± 0.03	1.32 ± 0.17	0.05 ± 0.01	3.7 ± 0.26
ICC 10134	0.04 ± 0.01	0.14 ± 0.04	0.23 ± 0.09	0.009 ± 0.002	1.87 ± 0.41	0.6 ± 0.09	1.02 ± 0.35	0.03 ± 0.02	2.86 ± 0.64
ICC 10600	0.06 ± 0.01	0.19 ± 0.04	0.25 ± 0.07	0.011 ± 0.004	1.49 ± 0.33	0.9 ± 0.12	1.58 ± 0.28	0.06 ± 0.01	4.19 ± 0.54
ICC 11886	0.05 ± 0.02	0.2 ± 0.02	0.27 ± 0.08	0.024 ± 0.005	2 ± 0.31	0.92 ± 0.07	1.56 ± 0.26	0.06 ± 0.02	4.23 ± 0.51
ICC 11903	0.06 ± 0.01	0.19 ± 0.03	0.26 ± 0.04	0.011 ± 0.005	2.46 ± 0.18	0.91 ± 0.13	1.63 ± 0.29	0.07 ± 0.01	4.5 ± 0.66
ICC 12123	0.05 ± 0.03	0.21 ± 0.04	0.22 ± 0.06	0.012 ± 0.004	1.91 ± 0.32	0.79 ± 0.04	1.35 ± 0.16	0.07 ± 0.03	3.8 ± 0.22
ICC 12169	0.06 ± 0.02	0.22 ± 0.04	0.25 ± 0.05	0.012 ± 0.005	1.93 ± 0.27	0.69 ± 0.06	1.21 ± 0.18	0.05 ± 0.02	3.34 ± 0.26
ICC 12184	0.05 ± 0.03	0.22 ± 0.02	0.24 ± 0.08	0.008 ± 0.003	1.85 ± 0.43	0.67 ± 0.06	1.25 ± 0.18	0.05 ± 0.02	3.43 ± 0.31
ICC 12289	0.06 ± 0.02	0.2 ± 0.05	0.27 ± 0.07	0.01 ± 0.002	1.84 ± 0.22	0.78 ± 0.07	1.28 ± 0.17	0.04 ± 0.02	3.63 ± 0.31
ICC 12312	0.05 ± 0.01	0.18 ± 0.03	0.21 ± 0.05	0.011 ± 0.005	1.51 ± 0.19	0.68 ± 0.08	1.25 ± 0.25	0.04 ± 0.02	3.37 ± 0.55
ICC 12511	0.06 ± 0.02	0.19 ± 0.04	0.25 ± 0.07	0.011 ± 0.003	1.41 ± 0.38	0.57 ± 0.04	1.17 ± 0.12	0.04 ± 0.02	2.92 ± 0.24
ICC 12554	0.05 ± 0.02	0.18 ± 0.03	0.23 ± 0.05	0.009 ± 0.002	1.81 ± 0.22	0.82 ± 0.11	1.47 ± 0.27	0.05 ± 0.02	3.92 ± 0.61
ICC 12620	0.05 ± 0.02	0.2 ± 0.03	0.24 ± 0.04	0.01 ± 0.004	1.9 ± 0.18	0.78 ± 0.05	1.57 ± 0.24	0.05 ± 0.02	3.99 ± 0.37

Accession Number	*Myo*-inositol	Galactinol	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Verbascose	Total RFO
ICC 12787	0.05 ± 0.01	0.28 ± 0.08	0.23 ± 0.04	0.01 ± 0.003	1.87 ± 0.1	1.02 ± 0.14	1.47 ± 0.11	0.04 ± 0.02	4.38 ± 0.5
ICC 13941	0.05 ± 0.02	0.2 ± 0.03	0.22 ± 0.03	0.011 ± 0.005	1.71 ± 0.17	0.87 ± 0.06	1.47 ± 0.18	0.06 ± 0.02	4.01 ± 0.21
ICC 14176	0.05 ± 0.02	0.18 ± 0.03	0.24 ± 0.03	0.01 ± 0.003	1.7 ± 0.07	0.89 ± 0.09	1.49 ± 0.26	0.05 ± 0.02	4.15 ± 0.42
ICC 14177	0.04 ± 0.02	0.17 ± 0.04	0.23 ± 0.05	0.01 ± 0.002	1.91 ± 0.34	0.81 ± 0.03	1.3 ± 0.08	0.05 ± 0.02	3.82 ± 0.25
ICC 14179	0.05 ± 0.03	0.19 ± 0.03	0.23 ± 0.04	0.012 ± 0.004	1.87 ± 0.22	0.87 ± 0.13	1.43 ± 0.22	0.05 ± 0.02	4.05 ± 0.47
ICC 14183	0.06 ± 0.03	0.15 ± 0.04	0.23 ± 0.05	0.012 ± 0.004	1.71 ± 0.21	0.82 ± 0.11	1.26 ± 0.15	0.05 ± 0.02	3.51 ± 0.44
ICC 14315	0.06 ± 0.01	0.21 ± 0.04	0.22 ± 0.04	0.018 ± 0.007	1.32 ± 0.11	0.82 ± 0.04	1.51 ± 0.2	0.06 ± 0.02	4.01 ± 0.28
ICC 14406	0.05 ± 0.01	0.18 ± 0.04	0.21 ± 0.05	0.011 ± 0.006	1.45 ± 0.07	0.7 ± 0.04	1.4 ± 0.23	0.05 ± 0.02	3.52 ± 0.34
ICC 14456	0.05 ± 0.02	0.19 ± 0.04	0.25 ± 0.05	0.01 ± 0.003	1.44 ± 0.11	0.9 ± 0.03	1.45 ± 0.17	0.06 ± 0.02	4.02 ± 0.22
ICC 14497	0.05 ± 0.02	0.21 ± 0.05	0.22 ± 0.05	0.009 ± 0.002	1.35 ± 0.14	0.89 ± 0.03	1.51 ± 0.17	0.06 ± 0.02	4.07 ± 0.25
ICC 14575	0.06 ± 0.02	0.19 ± 0.06	0.25 ± 0.05	0.013 ± 0.008	1.6 ± 0.34	0.87 ± 0.05	1.49 ± 0.19	0.06 ± 0.02	3.99 ± 0.24
ICC 14592	0.05 ± 0.02	0.16 ± 0.04	0.25 ± 0.04	0.011 ± 0.005	1.57 ± 0.12	0.85 ± 0.07	1.45 ± 0.2	0.06 ± 0.02	3.97 ± 0.42
ICC 14674	0.05 ± 0.02	0.19 ± 0.05	0.19 ± 0.02	0.011 ± 0.007	1.95 ± 0.53	0.55 ± 0.14	1.01 ± 0.27	0.04 ± 0.02	2.65 ± 0.61
ICC 15536	0.05 ± 0.02	0.19 ± 0.06	0.17 ± 0.03	0.011 ± 0.005	1.54 ± 0.27	0.61 ± 0.1	1.18 ± 0.17	0.05 ± 0.01	3.09 ± 0.53
ICC 16141	0.05 ± 0.02	0.18 ± 0.06	0.22 ± 0.03	0.01 ± 0.004	1.63 ± 0.2	0.78 ± 0.12	1.27 ± 0.14	0.06 ± 0.02	3.64 ± 0.38
ICC 16173	0.05 ± 0.02	0.19 ± 0.06	0.21 ± 0.05	0.01 ± 0.005	1.5 ± 0.12	0.74 ± 0.12	1.25 ± 0.18	0.05 ± 0.02	3.5 ± 0.59
ICC 16181	0.06 ± 0.03	0.17 ± 0.06	0.23 ± 0.06	0.01 ± 0.004	1.63 ± 0.2	0.8 ± 0.14	1.29 ± 0.13	0.05 ± 0.02	3.49 ± 0.42
ICC 16219	0.05 ± 0.03	0.18 ± 0.07	0.21 ± 0.04	0.011 ± 0.006	1.65 ± 0.31	0.82 ± 0.13	1.34 ± 0.2	0.05 ± 0.02	3.64 ± 0.49
ICC 16298	0.05 ± 0.02	0.21 ± 0.07	0.19 ± 0.03	0.01 ± 0.006	1.6 ± 0.16	0.75 ± 0.16	1.31 ± 0.25	0.05 ± 0.02	3.71 ± 0.66
ICC 16343	0.04 ± 0.04	0.09 ± 0.09	0.27 ± 0.05	0.009 ± 0.002	2.08 ± 0.5	0.75 ± 0.25	1.09 ± 0.4	0.05 ± 0.02	3.31 ± 0.34
ICC 16436	0.05 ± 0.02	0.18 ± 0.05	0.19 ± 0.02	0.012 ± 0.01	1.59 ± 0.12	0.7 ± 0.06	1.21 ± 0.18	0.05 ± 0.02	3.34 ± 0.44
ICC 16833	0.05 ± 0.01	0.19 ± 0.07	0.18 ± 0.01	0.012 ± 0.005	1.51 ± 0.13	0.83 ± 0.15	1.53 ± 0.38	0.06 ± 0.02	4.03 ± 0.93
ICC 16835	0.04 ± 0.02	0.14 ± 0.04	0.2 ± 0.02	0.011 ± 0.005	1.7 ± 0.17	0.76 ± 0.08	1.35 ± 0.23	0.06 ± 0.02	3.73 ± 0.62
ICC 17083	0.05 ± 0.02	0.16 ± 0.05	0.2 ± 0.02	0.01 ± 0.004	1.35 ± 0.1	0.72 ± 0.08	1.28 ± 0.25	0.04 ± 0.02	3.47 ± 0.56
ICCC 37	0.05 ± 0.02	0.14 ± 0.05	0.22 ± 0.03	0.01 ± 0.002	2.08 ± 0.26	0.79 ± 0.16	1.36 ± 0.33	0.06 ± 0.02	3.72 ± 0.89
ICCL 81248	0.06 ± 0.02	0.21 ± 0.07	0.25 ± 0.03	0.011 ± 0.005	1.83 ± 0.18	0.86 ± 0.12	1.49 ± 0.33	0.06 ± 0.02	4 ± 0.68
ICCL 83149	0.05 ± 0.02	0.15 ± 0.05	0.22 ± 0.03	0.011 ± 0.005	2.3 ± 0.37	0.75 ± 0.13	1.29 ± 0.34	0.06 ± 0.02	3.57 ± 0.71
ICCL 87207	0.05 ± 0.02	0.14 ± 0.05	0.17 ± 0.02	0.009 ± 0.002	1.94 ± 0.22	0.8 ± 0.06	1.33 ± 0.23	0.05 ± 0.01	3.7 ± 0.44
ICCV 88202	0.03 ± 0.03	0.11 ± 0.11	0.19 ± 0.01	0.004 ± 0.001	1.49 ± 0.12	0.68 ± 0.08	1.17 ± 0.28	0.05 ± 0.01	3.45 ± 0.46
ICCV 89314	0.05 ± 0.02	0.18 ± 0.05	0.23 ± 0.02	0.01 ± 0.005	2.08 ± 0.26	0.76 ± 0.13	1.38 ± 0.38	0.06 ± 0.01	3.73 ± 0.7

Accession Number	*Myo*-inositol	Galactinol	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Verbascose	Total RFO
ICCV 90201	0.05 ± 0.02	0.14 ± 0.04	0.24 ± 0.02	0.011 ± 0.003	1.62 ± 0.13	0.67 ± 0.06	1.16 ± 0.09	0.05 ± 0.01	3.34 ± 0.33
ICCV 92809	0.05 ± 0.02	0.17 ± 0.07	0.18 ± 0.03	0.015 ± 0.003	1.42 ± 0.21	0.8 ± 0.05	1.5 ± 0.27	0.07 ± 0.01	4.09 ± 0.55
ICCV 92944	0.06 ± 0.02	0.17 ± 0.05	0.25 ± 0.04	0.01 ± 0.004	1.63 ± 0.16	0.72 ± 0.03	1.43 ± 0.15	0.07 ± 0.03	3.64 ± 0.29
ICCV 93952	0.05 ± 0.02	0.14 ± 0.05	0.23 ± 0.02	0.009 ± 0.002	1.95 ± 0.12	0.74 ± 0.09	1.33 ± 0.26	0.06 ± 0.01	3.55 ± 0.65
ICCV 93954	0.05 ± 0.02	0.14 ± 0.06	0.29 ± 0.06	0.011 ± 0.005	2.09 ± 0.13	0.67 ± 0.2	1.15 ± 0.27	0.05 ± 0.02	3.12 ± 0.7
ICCV 94954	0.04 ± 0.02	0.13 ± 0.05	0.17 ± 0.01	0.008 ± 0.002	1.86 ± 0.36	0.76 ± 0.22	1.44 ± 0.44	0.07 ± 0.03	3.78 ± 1.05
ICCV 96836	0.06 ± 0.03	0.18 ± 0.07	0.25 ± 0.04	0.009 ± 0.005	1.58 ± 0.18	0.62 ± 0.09	1.2 ± 0.2	0.06 ± 0.02	3.16 ± 0.37
ICCX 820065	0.06 ± 0.02	0.16 ± 0.04	0.2 ± 0.02	0.007 ± 0.0002	1.76 ± 0.11	0.87 ± 0.06	1.4 ± 0.27	0.08 ± 0.01	4.01 ± 0.45
ICCV 97105	0.04 ± 0.02	0.13 ± 0.05	0.21 ± 0.02	0.008 ± 0.002	1.68 ± 0.14	0.73 ± 0.12	1.43 ± 0.24	0.06 ± 0.01	3.76 ± 0.41
ICCV 96030	0.06 ± 0.02	0.17 ± 0.04	0.2 ± 0.03	0.008 ± 0.001	1.39 ± 0.05	0.79 ± 0.04	1.39 ± 0.13	0.07 ± 0.02	3.78 ± 0.25
ICCV 07102	0.05 ± 0.02	0.13 ± 0.04	0.25 ± 0.01	0.009 ± 0.002	1.86 ± 0.12	0.74 ± 0.08	1.46 ± 0.23	0.05 ± 0.01	3.83 ± 0.44
ICCV 07104	0.05 ± 0.02	0.17 ± 0.05	0.24 ± 0.02	0.009 ± 0.004	2.05 ± 0.21	0.84 ± 0.09	1.38 ± 0.22	0.06 ± 0.01	3.75 ± 0.51
ICCV 07105	0.05 ± 0.02	0.12 ± 0.04	0.23 ± 0.02	0.008 ± 0.001	1.75 ± 0.13	0.76 ± 0.06	1.26 ± 0.09	0.05 ± 0.01	3.5 ± 0.35
ICCV 07108	0.05 ± 0.02	0.14 ± 0.05	0.23 ± 0.04	0.008 ± 0.002	1.95 ± 0.34	0.81 ± 0.07	1.26 ± 0.06	0.07 ± 0.01	3.61 ± 0.34
ICCV 07109	0.06 ± 0.02	0.16 ± 0.05	0.24 ± 0.02	0.009 ± 0.003	1.93 ± 0.16	0.86 ± 0.07	1.4 ± 0.21	0.07 ± 0.01	3.91 ± 0.57
ICCV 07110	0.05 ± 0.02	0.15 ± 0.04	0.21 ± 0.02	0.009 ± 0.002	1.82 ± 0.21	0.8 ± 0.07	1.38 ± 0.21	0.07 ± 0.01	3.68 ± 0.46
ICCV 07113	0.05 ± 0.02	0.17 ± 0.06	0.21 ± 0.03	0.011 ± 0.005	1.87 ± 0.15	0.64 ± 0.1	1.15 ± 0.27	0.05 ± 0.01	3.07 ± 0.57
ICCV 07115	0.05 ± 0.01	0.16 ± 0.07	0.23 ± 0.04	0.01 ± 0.004	1.94 ± 0.44	0.38 ± 0.06	0.81 ± 0.26	0.05 ± 0.01	2.16 ± 0.37
ICCV 07116	0.05 ± 0.02	0.14 ± 0.07	0.22 ± 0.02	0.011 ± 0.005	2.25 ± 0.53	0.38 ± 0.07	0.81 ± 0.21	0.05 ± 0.01	2.13 ± 0.21
ICCV 07117	0.02 ± 0.03	0.07 ± 0.08	0.16 ± 0.1	0.004 ± 0.002	1.87 ± 1.05	0.3 ± 0.2	0.53 ± 0.36	0.04 ± 0.02	1.9 ± 0.11
ICC 283	0.04 ± 0.02	0.16 ± 0.04	0.17 ± 0.03	0.007 ± 0.001	1.69 ± 0.18	0.76 ± 0.03	1.41 ± 0.15	0.06 ± 0.01	4.05 ± 0.29
ICC 1882	0.05 ± 0.02	0.16 ± 0.05	0.23 ± 0.01	0.008 ± 0.004	1.79 ± 0.18	0.74 ± 0.04	1.33 ± 0.26	0.06 ± 0.01	3.62 ± 0.39
ICC 4958	0.04 ± 0.01	0.14 ± 0.04	0.21 ± 0.03	0.007 ± 0.001	1.82 ± 0.1	0.75 ± 0.07	1.35 ± 0.3	0.07 ± 0.003	3.81 ± 0.5
ICCV 94916-4	0.05 ± 0.01	0.13 ± 0.03	0.23 ± 0.02	0.007 ± 0.002	1.9 ± 0.17	0.81 ± 0.07	1.54 ± 0.25	0.08 ± 0.01	4.12 ± 0.62
ICCV 94916-8	0.05 ± 0.01	0.13 ± 0.04	0.23 ± 0.02	0.007 ± 0.002	1.94 ± 0.24	0.83 ± 0.1	1.44 ± 0.27	0.07 ± 0.01	4.17 ± 0.54
ICCV 98901	0.05 ± 0.01	0.15 ± 0.03	0.22 ± 0.04	0.008 ± 0.003	1.99 ± 0.26	0.67 ± 0.08	1.5 ± 0.4	0.06 ± 0.01	3.63 ± 0.54
ICCV 98902	0.04 ± 0.02	0.14 ± 0.06	0.19 ± 0.02	0.008 ± 0.003	1.67 ± 0.2	0.76 ± 0.08	1.39 ± 0.27	0.06 ± 0.01	3.7 ± 0.45
ICCV 98903	0.04 ± 0.01	0.12 ± 0.03	0.18 ± 0.01	0.007 ± 0.001	1.72 ± 0.12	0.76 ± 0.04	1.34 ± 0.17	0.06 ± 0.005	3.67 ± 0.26
ICCV 98904	0.05 ± 0.02	0.14 ± 0.05	0.19 ± 0.01	0.007 ± 0.002	1.91 ± 0.2	0.81 ± 0.1	1.38 ± 0.19	0.06 ± 0.01	3.96 ± 0.44

Appendix 8.2 Concentration (value ± standard deviation) of soluble sugars in kabuli chickpea accessions [unit of concentration is g/100 g of chickpea seed meal on fresh weight basis except for RFO (mmol/100 g of chickpea seed meal on fresh weight basis)]

Accession Number	*Myo*-inositol	Galactinol	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Verbascose	RFO
ICCV 2	0.06 ± 0.01	0.17 ± 0.04	0.25 ± 0.03	0.021 ± 0.017	2.11 ± 0.37	0.97 ± 0.14	1.63 ± 0.15	0.1 ± 0.01	4.41 ± 0.42
ICC 6263	0.06 ± 0.01	0.15 ± 0.02	0.18 ± 0.05	0.015 ± 0.011	1.77 ± 0.18	0.8 ± 0.22	1.38 ± 0.29	0.09 ± 0.03	3.95 ± 0.79
ICC V05530	0.07 ± 0.01	0.16 ± 0.02	0.18 ± 0.06	0.011 ± 0.005	1.28 ± 0.26	0.71 ± 0.07	1.34 ± 0.15	0.08 ± 0.02	3.84 ± 0.34
ICC 1164	0.05 ± 0.02	0.2 ± 0.07	0.2 ± 0.05	0.011 ± 0.002	1.94 ± 0.59	0.75 ± 0.03	1.75 ± 0.24	0.07 ± 0.03	4.42 ± 0.34
ICC 4861	0.06 ± 0.03	0.18 ± 0.04	0.24 ± 0.06	0.009 ± 0.003	2.17 ± 0.28	0.92 ± 0.15	1.64 ± 0.34	0.07 ± 0.02	4.82 ± 0.5
ICC 4969	0.05 ± 0.02	0.21 ± 0.05	0.21 ± 0.03	0.012 ± 0.001	1.32 ± 0.2	0.67 ± 0.07	1.43 ± 0.14	0.05 ± 0.02	3.7 ± 0.29
ICC 5116	0.05 ± 0.03	0.21 ± 0.05	0.2 ± 0.07	0.011 ± 0.002	2.04 ± 0.36	0.9 ± 0.11	1.75 ± 0.22	0.07 ± 0.02	4.57 ± 0.28
ICC 5270	0.05 ± 0.02	0.19 ± 0.05	0.19 ± 0.02	0.01 ± 0.002	1.75 ± 0.33	0.89 ± 0.07	1.81 ± 0.34	0.06 ± 0.02	4.89 ± 0.46
ICC 6169	0.04 ± 0.02	0.17 ± 0.05	0.21 ± 0.04	0.01 ± 0.003	2.6 ± 0.38	0.81 ± 0.11	1.45 ± 0.34	0.05 ± 0.02	4.14 ± 0.58
ICC 6231	0.04 ± 0.03	0.22 ± 0.08	0.19 ± 0.05	0.01 ± 0.003	2.2 ± 0.47	0.83 ± 0.15	1.58 ± 0.47	0.06 ± 0.03	4.18 ± 0.7
ICC 6283	0.04 ± 0.03	0.16 ± 0.06	0.19 ± 0.04	0.009 ± 0.003	2.04 ± 0.26	0.71 ± 0.07	1.26 ± 0.3	0.06 ± 0.02	3.64 ± 0.53
ICC 6334	0.05 ± 0.03	0.19 ± 0.06	0.24 ± 0.05	0.01 ± 0.003	1.5 ± 0.2	0.68 ± 0.14	1.19 ± 0.26	0.05 ± 0.02	3.3 ± 0.46
ICC 6969	0.05 ± 0.02	0.17 ± 0.06	0.26 ± 0.04	0.01 ± 0.002	1.75 ± 0.16	0.81 ± 0.05	1.28 ± 0.26	0.05 ± 0.02	3.69 ± 0.33
ICC 7241	0.05 ± 0.02	0.16 ± 0.06	0.2 ± 0.06	0.009 ± 0.003	2.71 ± 0.67	0.92 ± 0.08	1.55 ± 0.34	0.06 ± 0.03	4.28 ± 0.54
ICC 7263	0.03 ± 0.03	0.1 ± 0.1	0.26 ± 0.04	0.007 ± 0.001	2.73 ± 0.63	0.82 ± 0.43	1.41 ± 0.78	0.07 ± 0.03	4.15 ± 0.69
ICC 7292	0.04 ± 0.02	0.13 ± 0.04	0.2 ± 0.06	0.01 ± 0.003	2.47 ± 0.52	0.78 ± 0.15	1.45 ± 0.3	0.09 ± 0.03	3.81 ± 0.61
ICC 7294	0.05 ± 0.02	0.17 ± 0.02	0.24 ± 0.08	0.01 ± 0.002	2.63 ± 0.54	0.83 ± 0.14	1.5 ± 0.35	0.06 ± 0.01	4.12 ± 0.67
ICC 7298	0.04 ± 0.01	0.14 ± 0.03	0.21 ± 0.07	0.01 ± 0.003	2.37 ± 0.4	0.85 ± 0.24	1.55 ± 0.29	0.07 ± 0.02	4.03 ± 0.82
ICC 7570	0.04 ± 0.03	0.12 ± 0.1	0.18 ± 0.02	0.005 ± 0.001	2.5 ± 0.11	0.84 ± 0.25	1.39 ± 0.51	0.06 ± 0.01	3.99 ± 0.8
ICC 8273	0.07 ± 0.02	0.23 ± 0.05	0.27 ± 0.05	0.008 ± 0.003	2.63 ± 0.31	0.88 ± 0.19	1.57 ± 0.4	0.08 ± 0.03	4.16 ± 0.9
ICC 8527	0.05 ± 0.01	0.18 ± 0.04	0.24 ± 0.05	0.008 ± 0.002	2.59 ± 0.34	0.89 ± 0.15	1.66 ± 0.3	0.07 ± 0.02	4.21 ± 0.69
ICC 10674	0.05 ± 0.02	0.21 ± 0.02	0.21 ± 0.07	0.008 ± 0.002	2.8 ± 0.56	1 ± 0.1	1.85 ± 0.43	0.08 ± 0.01	4.71 ± 0.69
ICC 11553	0.06 ± 0.02	0.17 ± 0.03	0.24 ± 0.05	0.009 ± 0.002	2.29 ± 0.17	0.99 ± 0.13	1.55 ± 0.41	0.06 ± 0.02	4.3 ± 0.94
ICC 11795	0.05 ± 0.01	0.19 ± 0.04	0.23 ± 0.06	0.02 ± 0.023	2.63 ± 0.26	0.84 ± 0.19	1.49 ± 0.42	0.08 ± 0.02	4.14 ± 1.05
ICC 11901	0.05 ± 0.02	0.2 ± 0.05	0.21 ± 0.04	0.009 ± 0.001	2.23 ± 0.21	0.9 ± 0.21	1.7 ± 0.4	0.06 ± 0.03	4.44 ± 0.97
ICC 12121	0.04 ± 0.02	0.17 ± 0.05	0.17 ± 0.05	0.01 ± 0.005	2.48 ± 0.49	0.87 ± 0.18	1.67 ± 0.34	0.06 ± 0.02	4.36 ± 0.77
ICC 14533	0.05 ± 0.02	0.22 ± 0.06	0.2 ± 0.02	0.01 ± 0.004	1.59 ± 0.15	0.65 ± 0.1	1.21 ± 0.17	0.04 ± 0.02	3.35 ± 0.35
ICC 14913	0.05 ± 0.02	0.2 ± 0.05	0.21 ± 0.03	0.009 ± 0.003	1.88 ± 0.27	0.97 ± 0.08	1.8 ± 0.2	0.07 ± 0.03	4.85 ± 0.51
ICC 15367	0.04 ± 0.02	0.16 ± 0.07	0.18 ± 0.04	0.01 ± 0.003	2.6 ± 0.72	0.88 ± 0.14	1.5 ± 0.29	0.06 ± 0.03	4.08 ± 0.66
ICC 15380	0.03 ± 0.03	0.11 ± 0.1	0.22 ± 0.03	0.006 ± 0.001	2.78 ± 0.46	0.72 ± 0.24	1.19 ± 0.44	0.06 ± 0.01	3.92 ± 0.48
ICC 15388	0.05 ± 0.03	0.2 ± 0.08	0.2 ± 0.06	0.009 ± 0.003	2.84 ± 0.67	0.82 ± 0.18	1.53 ± 0.39	0.08 ± 0.03	4.06 ± 0.85
ICC 15779	0.05 ± 0.03	0.2 ± 0.08	0.22 ± 0.06	0.009 ± 0.003	2.42 ± 0.51	0.93 ± 0.21	1.53 ± 0.36	0.06 ± 0.02	4.17 ± 0.79
ICC 15807	0.04 ± 0.02	0.18 ± 0.06	0.18 ± 0.03	0.01 ± 0.002	2.7 ± 0.62	0.92 ± 0.14	1.58 ± 0.24	0.07 ± 0.02	4.21 ± 0.49

Accession Number	*Myo*-inositol	Galactinol	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Verbascose	RFO
ICC 16216	0.06 ± 0.02	0.23 ± 0.07	0.21 ± 0.02	0.009 ± 0.005	1.77 ± 0.18	0.97 ± 0.13	1.7 ± 0.37	0.08 ± 0.03	4.74 ± 0.79
ICC 16453	0.05 ± 0.02	0.17 ± 0.07	0.19 ± 0.05	0.014 ± 0.006	2.35 ± 0.57	0.78 ± 0.12	1.55 ± 0.41	0.06 ± 0.02	4.18 ± 0.94
ICC 16528	0.04 ± 0.01	0.13 ± 0.05	0.19 ± 0.03	0.009 ± 0.002	1.87 ± 0.35	0.6 ± 0.15	1.19 ± 0.23	0.04 ± 0.02	3.07 ± 0.73
ICC 16626	0.04 ± 0.02	0.16 ± 0.08	0.17 ± 0.04	0.011 ± 0.005	1.99 ± 0.37	0.72 ± 0.15	1.41 ± 0.32	0.06 ± 0.02	3.52 ± 0.71
ICC 16774	0.03 ± 0.01	0.14 ± 0.06	0.15 ± 0.02	0.009 ± 0.002	2.49 ± 0.31	0.81 ± 0.18	1.59 ± 0.41	0.07 ± 0.02	4.23 ± 1.13
ICC 16820	0.04 ± 0.02	0.15 ± 0.06	0.17 ± 0.04	0.011 ± 0.005	2.44 ± 0.42	0.75 ± 0.16	1.34 ± 0.33	0.07 ± 0.02	3.66 ± 0.94
ICCV 3	0.05 ± 0.02	0.14 ± 0.06	0.18 ± 0.01	0.011 ± 0.005	2.15 ± 0.42	0.68 ± 0.13	1.26 ± 0.3	0.07 ± 0.02	3.24 ± 0.73
ICCV 89509	0.05 ± 0.01	0.17 ± 0.05	0.21 ± 0.05	0.01 ± 0.006	2.12 ± 0.24	0.77 ± 0.06	1.53 ± 0.28	0.07 ± 0.01	4.01 ± 0.55
ICCV 91302	0.05 ± 0.01	0.13 ± 0.05	0.18 ± 0.01	0.007 ± 0.001	2.11 ± 0.3	0.65 ± 0.13	1.24 ± 0.22	0.06 ± 0.02	3.31 ± 0.67
ICCV 93512	0.06 ± 0.01	0.16 ± 0.05	0.2 ± 0.02	0.007 ± 0.001	2.17 ± 0.28	0.81 ± 0.09	1.49 ± 0.25	0.08 ± 0.02	3.93 ± 0.59
ICCV 95311	0.04 ± 0.02	0.14 ± 0.08	0.2 ± 0.03	0.007 ± 0.001	2.24 ± 0.5	0.73 ± 0.22	1.36 ± 0.35	0.07 ± 0.02	3.7 ± 0.88
ICCV 95332	0.05 ± 0.02	0.14 ± 0.05	0.2 ± 0.04	0.008 ± 0.001	2.18 ± 0.26	0.72 ± 0.09	1.25 ± 0.3	0.07 ± 0.02	3.57 ± 0.53
ICC 17109	0.05 ± 0.01	0.13 ± 0.06	0.18 ± 0.03	0.008 ± 0.001	2.63 ± 0.24	0.78 ± 0.14	1.66 ± 0.4	0.09 ± 0.01	4.2 ± 0.9
ICCV 07118	0.04 ± 0.03	0.1 ± 0.09	0.2 ± 0.01	0.005 ± 0.0004	1.98 ± 0.21	0.77 ± 0.17	1.36 ± 0.45	0.07 ± 0.02	3.99 ± 0.43
ICCV 06301	0.04 ± 0.02	0.13 ± 0.05	0.22 ± 0.01	0.006 ± 0.001	2.4 ± 0.33	0.86 ± 0.08	1.47 ± 0.29	0.09 ± 0.02	4.05 ± 0.41
ICCV 06302	0.06 ± 0.01	0.15 ± 0.05	0.22 ± 0.02	0.013 ± 0.011	2.18 ± 0.16	0.84 ± 0.11	1.5 ± 0.31	0.07 ± 0.01	4.06 ± 0.49
ICCV 06306	0.05 ± 0.01	0.13 ± 0.06	0.21 ± 0.04	0.007 ± 0.001	2.49 ± 0.33	0.68 ± 0.04	1.3 ± 0.27	0.07 ± 0.02	3.35 ± 0.44
ICCV 07304	0.05 ± 0.02	0.16 ± 0.05	0.2 ± 0.02	0.007 ± 0.001	2.39 ± 0.34	0.78 ± 0.13	1.5 ± 0.32	0.09 ± 0.02	4.14 ± 0.7
ICCV 07311	0.05 ± 0.01	0.14 ± 0.06	0.22 ± 0.03	0.007 ± 0.001	2.42 ± 0.31	0.86 ± 0.17	1.59 ± 0.46	0.09 ± 0.03	4.23 ± 0.9
ICCV 07312	0.05 ± 0.02	0.14 ± 0.05	0.23 ± 0.05	0.007 ± 0.001	2.43 ± 0.22	0.8 ± 0.11	1.44 ± 0.24	0.09 ± 0.02	4.16 ± 0.55
ICCV 07313	0.04 ± 0.01	0.13 ± 0.06	0.21 ± 0.01	0.006 ± 0.001	2.31 ± 0.35	0.9 ± 0.18	1.72 ± 0.45	0.09 ± 0.02	4.6 ± 0.86
ICC 8261	0.04 ± 0.02	0.18 ± 0.05	0.21 ± 0.02	0.006 ± 0.001	2.53 ± 0.35	0.88 ± 0.12	1.71 ± 0.4	0.08 ± 0.02	4.43 ± 0.75