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# Changes in cell wall components during seed development in soybean (*Glycine max*) varieties

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**Abstract**
In the present study two soybean (*Glycine max*) varieties are selected; Large seeded variety (L seed) and Small seeded variety (S seed) for the growth and wall component analysis to understand the mechanism of sink size development in the developing seed. Changes in low and high molecular weight xyloglucan and pectic polysaccharide content were estimated during seed development in soybean. The seeds of both the varieties showed distinct difference in their growth phases, a significant difference was also observed in cell wall components during their developmental period. Pectic (Esterified and non-esterified) and xyloglucan (high and low molecular weight) remained higher in S seed variety compared to L seed during sink development phase. Overall, a maximum pectic substance was observed at the sink expansion and DMA phases in both seed varieties. While xyloglucan was observed higher in sink expansion phase in small and large seeds. The probable role of wall components in sink size development is discussed.

**Keywords:** Cell wall component, Growth, Pectins, Soybean, Xyloglucan

## 1. Introduction
Soybean is one of the most important crops in the world today and it contained 40 % protein and oil (~20%). Soybean is the 5th largest pulse crop of India, accounting for 4% of total pulse production (Gandhi, 2009) [11]. Desirable improvements of seed quality and yield may be achieved with understanding of the seed composition. Seed size is essential for evolutionary fitness in plants, and is also vital agronomic trait in crop domestication. Large seeds content adequate amount of nutritious substances for germination and have better tolerance to abiotic stresses, whereas small seeds are efficient at dispersing and colonizing (Westoby *et al.,* 2002; Moles *et al.,* 2005) [40, 24]. In crops, seeds are the major products for consumption, and seed size is one of the essentially important traits of seed yield. During the earlier part of seed development increase in weight may occur because of increase in size. Later, when the seed has attained its full size, the increase in dry weight is due to accumulation of storage material. This accumulation can be measured by changes in dry weight of seed (Lucas *et al.,* 2004) [22].
For high yield it is important to maintain a steady sink activity throughout the seed filling period (Liu *et al.,* 2006) [21]. In legume seeds the cell wall polysaccharides represent the significant portion of dry matter (Stombaugh *et al.,* 2000) [35]. In soybean cotyledons cell wall polysaccharides accounted 12% of dry matter and represent a substantial amount of dry matter deposited during seed development (Daveby and Aman, 1993) [7]. Plant cell walls play an important role in regulating physiological events in sink development (Thaker, 1998, Chudasama and Thaker, 2010) [37, 4].
The plant cell wall provides mechanical support and serves as a point of communication and defence to plant cells, and, as such, it is essential in many aspects of growth and development. Cell wall structure is continually modified to accommodate the development stage and the environment condition. The plant cell lays down the middle lamella and the primary wall during initial growth and expansion of the cell. In many cells the wall is thickened and further strengthened by the addition of a secondary wall (Caffall and Mohnen, 2009) [2].
Cell wall of the plant is composed of many complex carbohydrates and proteins, which undergoes rapid turnover during the process of cell elongation. Pectic compounds (pectic polysaccharides) form a group of complex and variable natural compounds. Pectins are an important component of primary plant cell wall and intercellular spaces, play important role in biological functions (Caffall and Mohnen, 2009) [2]. The hemicellulosic polysaccharide xyloglucan (XyG), found in the primary cell walls of most plant tissues, is important for structural organization of the cell wall and regulation of growth development (Somerville *et al.,* 2004) [34]. Xyloglucans were identified as seed storage polysaccharides from nasturtium, tamarind and other seeds (Kooiman, 1957) [20]. XyG is a major hemicellulosic component of the primary cell wall of flowering plants (up to 25%), but a minor concentration detectable,

constituent of grasses is less than 2% (Hsieh and Harris, 2009; Schultink *et al.,* 2014) [16, 30].

The growth of a plant cell is significantly influenced by the properties of the cell wall. Cell division and cell expansion are the two fundamental processes governing plant size and shape. Structural modification of the cell wall is important considering regulation of cell growth (Jan *et al.,* 2004) [17]. Considering to this, in this study, changes in wall components, i.e. pectic polysaccharides and xyloglucans were studied from two different varieties of *Glycine max* varying in seed size during the entire period of seed development.

## Materials and Methods

Certified seeds of *Glycine max* (Small (S seed) and large seed (L seed) size) varieties were selected for the study. The growth experiment of *Glycine max* was studied during the month of July to October 2016. Seeds were grown at Ansh Farm Jamvadi village, Gujarat, India. Standard agricultural practices including irrigation, application of fertilizers and insecticides etc., were maintained throughout the crop growth to maximize the yield. NPK fertilizer was applied to the soil before planting while pesticide was given twice during the flowering period. Irrigation applications were done at alternate day throughout the growth period. Flowers were tagged on the day of anthesis and numbers of the flower were recorded every day. Developing pods of equal size were harvested at the interval of seven days for growth analysis and estimation of cell wall components.

### Growth analysis

### Fresh and dry weight measurements

For the measurement of fresh and dry weights, pods were harvested at every seven days intervals (From the day of anthesis- 0 d to maturation - 63 d). Seeds were separated from the pods of different ages. A number of seeds per pod were calculated.

For the measurement of fresh and dry weights, freshly harvested pod were taken. Freshly separated seeds were weighed before and after oven drying to a constant weight at 65ºC for 72 hours. The water content of each stage was determined by a difference in fresh and dry weights. Data were taken in replicate and the calculated with $\pm$ standard deviations.

### Extraction and estimation of wall components

The method described by Selvendran *et al.,* (1985) [31], was followed for preparation of cell wall material as well as further extraction of the native wall components which can be summarized as follows:

### Preparation and purification of cell wall material

Freshly harvested seeds were powdered with liquid nitrogen in pre-chilled mortar-pestle and stored at -20ºC prior to use. From each developmental stage 500 mg of crushed seeds were suspended in 1.5% aqueous SLS (Sodium Lauryl Sulfate) containing 5mM $Na_2S_2O_5$, mixed thoroughly and centrifuged at 10,000 g for 10 min followed by washing with 0.5% SLS containing 5mM $Na_2S_2O_5$. The supernatant was discarded and residue was washed thrice with D/W and then suspended in 0.5% SLS containing 3mM $Na_2S_2O_5$ and incubated for 16 h at 4ºC. This treatment allowed optimum cell disruption and therefore homogenous product which can be readily separated by centrifugation, was obtained. The supernatant after centrifugation contained cold, water soluble pectic substances. The cold residue was sequentially extracted with two times with distilled water and re-suspended in (Phenol, Acetic acid, Water, 1:1:1 w/v/v) such extraction helped to remove intracellular compounds. This was followed by two washes of distilled water and two washes with 90% aqueous DMSO (Dimethyl Sulphoxide) to remove starch from residues. For removal of adsorbed DMSO the residue was then washed six times with distilled water. Washing was continued till the supernatant gave negative reaction with $I_2$/KI solution to ensure absence in the residue. The starch free residue was finally taken as purified cell wall material (CWM). Extraction and separation at each step in this method and other following methods were done by centrifugation at 10,000 g for 10 min.

### Extraction and estimation of pectic polysaccharides

Different fractions of pectic polysaccharides were extracted by using EDTA (Ethylene Diamine Tetra Acetic acid) as a chelating agent and $Na_2CO_3$. The CWM was stirred with 50mM EDTA (pH 6.5) for 6 h at 20-22ºC, centrifuged and washed with the distilled water. The pooled supernatants served as source of non-esterified pectic polysaccharides the residue was then suspended with 50 mM $Na_2CO_3$ (Sodium carbonate) containing 20mM $NaBH_4$ (Sodium borohydride,) incubated for 16 h at 1ºC. The supernatant was collected and the residue was washed once with distilled water. The pooled supernatant was used for estimation of soluble esterified pectic substances.

### Extraction of Hemicelluloses

The depectinated residue was then used for extraction of low and high molecular weight xyloglucan fractions. The residue was treated with 1 M KOH (Potassium hydroxide), containing 10mM $NaBH_4$; incubate for 2 h at 1ºC. The supernatant was collected that mainly contained cold 1 M KOH soluble low molecular weight xyloglucans. The pooled supernatant served as a source of KOH soluble low molecular weight xyloglucans. Thereafter, the residue was treated with 4M KOH containing 10mM $NaBH_4$ for 2 h at 20-22ºC. The supernatant served as a source of bulk of 'free' cold alkali/KOH soluble high molecular weight xyloglucans. For solubilisation and thereby the extraction of additional glucomannan rich xyloglucan fractions. The residue was finally treated with 4M KOH containing 3-4 % $H_3BO_3$ Boric acid for 2 h at 20-22ºC. The supernatant was collected and estimated for 4M KOH + Borate soluble high molecular weight xyloglucans. The pooled supernatant was used as a source of high molecular weight xyloglucans.

### Preparation of the extracts for estimation

The presence of alkali used for extraction of hemicelluloses and carbonate solution used for extraction of esterified pectic polysaccharides, could interfere with the estimation of the extracted xyloglucan and pectic polysaccharide contents of different fractions. Therefore, the acidification of such fractions was done prior to estimation and the pH was adjusted to 5.0 using acetic acid. After pH adjustment the final volume was made using the respective extracting solution for the specific fraction. Thereafter the fractions were used for estimation as follows.

### Estimation of pectic polysaccharides

The estimation of pectic substances in the entire four fractions was done by phenol-sulfuric acid method (Dubois *et al.,* 1956) [8]. Two ml of the extract was mixed with 2 mL of 5% phenol and 10 ml sulfuric acid. The mixture was incubated for 10 min at room temperature with stirring and 20 min at 30ºC

in water bath. Absorbance values were measured at 490 nm. The controls were prepared by addition of respective extractant and remaining additions were kept similar. The pectic polysaccharides were expressed as $\Delta A_{490}$ seed$^{-1}$.

**Estimation of xyloglucans**

Xyloglucan content of all different fraction estimation was done by Iodine staining method originally given and then slightly modified by Kooiman (1960) [19]. Two ml of the extract was mixed with 500 µl of $I_2KI$ and 4 ml 15% $Na_2SO_4$, incubated in dark for 1 h at 4°C and optical density of the resultant colour solution was measured at 640 nm. The controls were prepared by addition of the extractant instead of the xyloglucan fractions. The non-specific absorption measured from these controls was used for correction of the reaction. The low and high molecular weight xyloglucan contents were expressed as $\Delta A_{640}$ seed$^{-1}$.

**Statistical analysis**

Correlation coefficient between wall components and dry weight seed$^{-1}$ and water content seed$^{-1}$ were worked out during the entire period of seed development. P values significant at 0.05 or less were considered for data interpretation.

**Results and Discussion**

Changes in growth pattern i.e dry weight and water content mg seed$^{-1}$ and rate of dry matter accumulation and water content are presented in Fig. 1, 2. In small variety dry weight (DWt) per seed increased up to 9$^{th}$ week. Maximum DWt was 102.6 mg seed$^{-1}$ at 9$^{th}$ week. In large variety, DWt of seed increased up to 8$^{th}$ week and stabilized in later stages. Maximum DWt was 126.2 mg seed$^{-1}$ on 8$^{th}$ (Fig. 1A). In Small seed water content (WC) increased up to 8$^{th}$ week, and later it declined. The maximum WC per seed was 139.18 mg at 8$^{th}$ week. In large seed, WC per seed increased gradually up to 6$^{th}$ week, declined in later ages. Maximum value of water content was 157.25 mg seed$^{-1}$ at 6$^{th}$ week (Fig. 1B).

The rate of DMA and water content are presented in Fig. 2. The same pattern of DMA in small and large seed variety was observed. In small seed, rate of DMA increased up to 5$^{th}$ week (30.15), declined later on and in large seed, the rate of DMA increased up to 5$^{th}$ week (48.17), (Fig. 2A). The rate of water accumulation in small and large seed were increased up to 5$^{th}$ week and then after gradually decline. The maximum water accumulation was observed at 5$^{th}$ week in small seed 35.58 mg/seed and large seed 51.91 mg/seed (Fig. 2B).

Based on growth pattern soybean seed development is divided into four phase i.e. (i) Cell division (0-3 weeks), (ii) Cell elongation and sink expansion phase (3-5 weeks), (iii) DMA phase (3-8 weeks) and (iv) Maturation phases. Similar growth pattern of seed development was reported earlier in cotton plant (Thaker, 1999; Rabadia *et al.,* 1999) [38, 26] and *Cajanus cajan* (Chudasama and Thaker, 2007) [3] and in rice (Jhala and Thaker, 2015) [18].

Dry weight and water content between these two varieties showed a significant difference (P<0.001). Similarly rate of DMA and rate of water accumulation also showed a significant difference (P<0.001) Table 1. It was possible that high rate of water uptake is required for elongation growth and higher level of water content supports elevated rate of dry matter accumulation (Rabadia *et al*., 1999) [26]. In this study, rate of DMA and rate of water accumulation was higher in large seed compared to small seed (Fig. 2). Higher uptake of water content might be one of the factors for big size of seed. It has been suggested that seed water status play an important role in regulating its development (Egli, 1990) [10]. Earlier Xie *et al*., (2015) [41] found that larger carpels of wheat increased maximum grain water content. Water stress during seed filling in soybean reduced seed size and yield without directly affecting seed growth (Egli and Bruening, 2004) [9]. A strong and positive relationship between maximum grain water content and final grain weight was observed in wheat (Gonzalez *et al.,* 2014) [12], maize (Borra's *et al.,* 2003; Sala *et al.,* 2007) [1, 28], and sunflower (Rondanini *et al.,* 2009) [27].

In developing seed cell expansion determines the sink capacity of a seed. Many studies have demonstrated that an extensive turnover of cell wall polysaccharides occur during cell elongation in higher plants (Sharma and Thaker, 2006; Chudasama and Thaker, 2010) [32, 4]. The complex process of cell elongation is mediated by a series of metabolic events coordinated with wall polymer synthesis and secretion. Pectins and xyloglucans are the two major polysaccharides that change during seed development. In this work the content of pectins and xyloglucans were estimated during the entire growth period of soybean seed development. The changes in non-esterified and esterified pectic is presented in Fig. 3.

In S seed non esterified pectic polysaccharides remained higher throughout seed development. During cell-division phase amount of non-esterified pectics were low in S and L seeds (Fig.3A). Both seed showed same pattern of change during cell elongation and dry matter accumulation phase, where gradually increase was observed then decline at maturation phase. In S seed non esterified pectics substance were higher in elongation, dry matter accumulation and maturation phase compared to L seed (Fig. 3). Esterified pectic substance remained almost same during cell-division phase in both the seeds. In S seed variety pectic substance was increase during cell elongation, dry matter accumulation and decline at maturation stage (Fig. 4). While in L seed esterified pectic substance was low at cell division phase then gradually increased in elongation and dry matter accumulation phase than decreased at maturation phase (Fig. 4).

The DWt and WC data showed a significant correlation with esterified and non esterified pectins in S and L seed (Table 1). Seed cell wall component represent a significant portion of seed dry matter in soybean (Daveby and Aman, 1993; Stombaugh *et al.,* 2000) [7, 35]. Changes in cell wall components play a determinative role in establishing the size of the cell. Previously Crombie *et al.,* (2003) [6] reported that the pectic substances are main component of plant cell wall where they contribute to complex physiological process like cell growth and cell differentiation and so determine the integrity and rigidity of plant tissue. The epidermal cell layer of Arabidopsis seed coat undergoes complex cell differentiation, during which large quantities of pectinaceous mucilage are secreted between the plasma membrane and the outer tangential primary cell wall (Haughn and Western, 2012; North *et al.,* 2014) [14, 25]. Pectins have previously been reported in rice endosperm cell walls and in *B. distachyon* (Shibuya and Nakane, 1984; Guillon *et al.,* 2011) [33, 13].

In this work, the low molecular weight xyloglucans remained very low during cell-division phase in both varieties. The low molecular weight xyloglucans in S seed was gradually increased in cell elongation and dry matter accumulation phase and then declined (Fig. 4A). While in L seed low molecular weight xyloglucans was slowly increased in cell-elongation and dry matter accumulation phase and then became stable (Fig. 4A). Stabilization of low molecular weight xyloglucans substances at later stages of seed growth

suggests the deposition of xyloglucans as storage polysaccharides. In this work, low molecular weight xyloglucans was higher in S seed compared to L seed variety. The both variety of DWt and WC data showed a significant correlation with low and high molecular weight xyloglucans (Table-1). The high molecular weight xyloglucans showed low during initial cell-division phase in both seed variety (Fig. 4B). In both S and L seed high molecular weight xyloglucan was increased in elongation and dry matter accumulation phase. Deposition of xyloglucans in the later stages of seed development may function as storage polysaccharides, which was observed in cotyledonous cells of *Hymenaea courbari* that accumulate large amount of storage xyloglucans in the wall (Tine *et al.,* 2000; Santos *et al.,* 2004) [39, 29]. Most xyloglucans are subjected to turn over during growth (Terry *et al.,* 1981) [36]. The partial breakdown of hemicellulose may be required in addition to the degradation of pectic materials to bring about the extensive expansion (Maclachan and Brady, 1994) [23]. Xyloglucan is the major hemicellulose polymer in the primary cell walls of plant, and its binding to cellulose microfibrils by hydrogen bonding contributes to loosening or stiffening of the wall during cell elongation (Cosgrove, 2005; Hayashi and Kaida, 2011) [5,15].

The result of the present study reveals that the cell wall metabolism changes from pectin synthesis to the synthesis of hemicellulose and cellulose when young tissue becomes mature. In this study pectic substances (esterified and non esterified pectins) were higher during cell division phase as compared to xyloglucans which suggest that pectic polysaccharides are being synthesized during the early cell growth and transferred to new growing cells. Xyloglucans were higher during cell elongation and dry matter accumulation stages. From these results it is suggested that pectic polysaccharides are necessary for early seed growth and xyloglucans are essential in cell wall loosening in developing *Glycine max* seeds. However, in this study, during the entire growth development pectic amount still remained higher up to seed maturation, which suggests that it may be responsible for mechanical strength of cell wall.

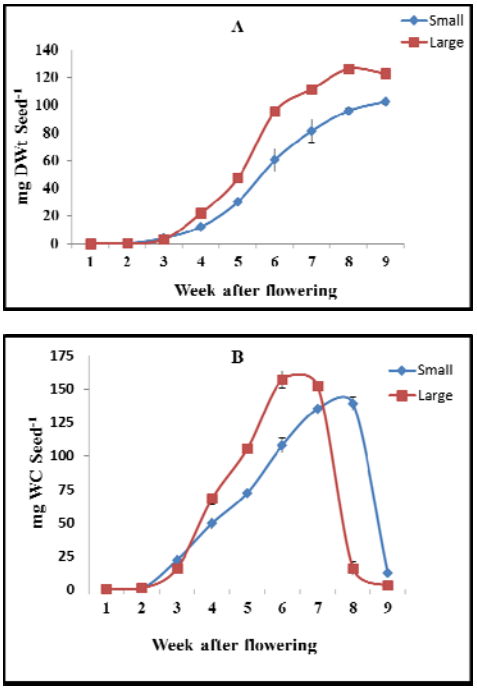


**Fig. 1:** Changes in Dry weight (A) and Water content (B) in developing seeds of *Glycine max*

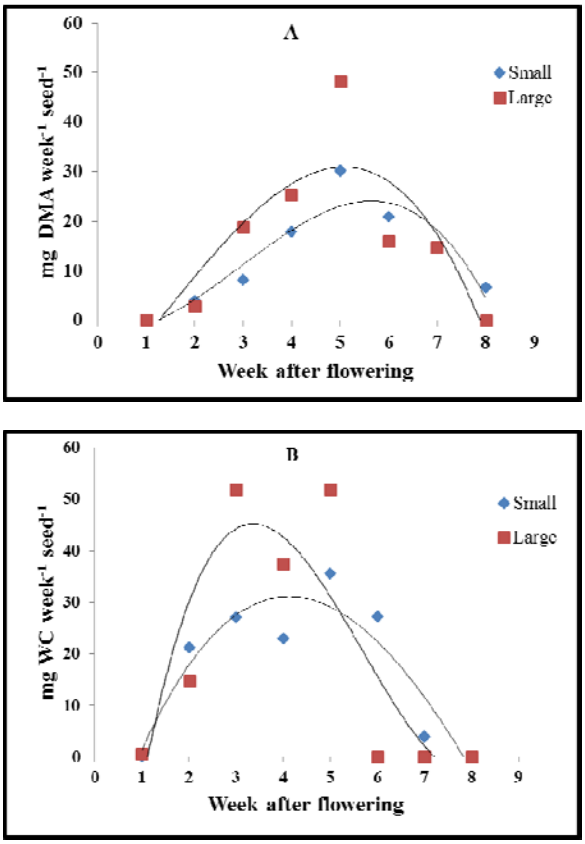


**Fig. 2:** Changes in rate of dry matter accumulation (DMA) (A) and rate of water accumulation (B) in developing seeds *Glycine max*

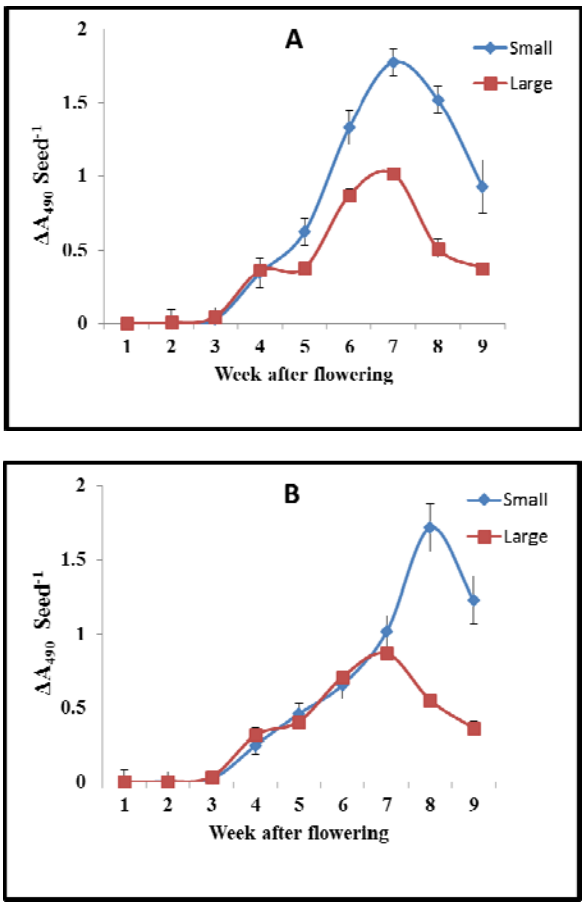


**Fig 3:** Changes in non-esterified pectins (A) and esterified pectins (B) in developing S and L seeds of *Glycine max*

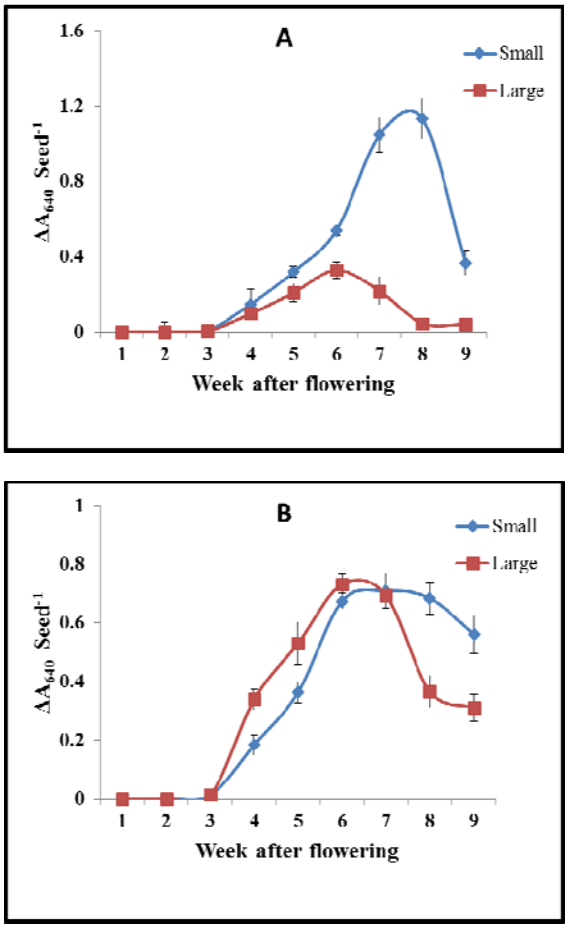


**Fig. 4:** Changes in low molecular weight xyloglucans (A) and high molecular weight xyloglucans (B) in developing S and L seeds of *Glycine max*

**Table 1:** Correlation coefficient between wall components and dry weight seed$^{-1}$ and water content seed$^{-1}$

| | Dry weight | | Water content | |
|---|---|---|---|---|
| | **Small Seed** | **Large Seed** | **Small Seed** | **Large Seed** |
| Non esterified pectins | 0.88*** | 0.82 *** | 0.88*** | 0.80*** |
| Esterified pectins | 0.95*** | 0.77*** | 0.67*** | 0.84*** |
| Low molecular weight xyloglucans | 0.82*** | 0.43** | 0.91 *** | 0.96*** |
| High molecular weight xyloglucans | 0.92** | 0.70*** | 0.82*** | 0.89*** |

**Note**: * Significant at $P<0.05$, ** Significant at $P<0.01$, *** Significant at $P<0.001$

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