International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(6): 625-631

Received: 16-09-2017
Accepted: 22-10-2017

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Changes in the activity of sucrose synthases and invertase in reproductive stage of lentil cultivars differing in seed size

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Abstract
Seed size is the most important component of seed yield and an important trait for consumer preference. To understand mechanisms governing seed size in lentil, the present investigation was carried out on sucrolytic enzymes in podwall and seeds of two small (PL 4 and LL 699) and two bold (DPL 62 and IPL 406) seeded lentil cultivars during crop development. The activities of sucrolytic enzymes *viz*. sucrose synthase and invertases were compared in developing pods and seeds of two genotypes of lentil differing in seed size. The lower activities of acid and alkaline invertases in pod wall of bold seeded as compared to small seeded might be helping to channel more sucrose towards the seeds. The rapid decrease in invertase activities in seeds of bold seeded at 21 DAF and higher and sustained activity of sucrose synthase in their seeds upto 35 DAF as compared to small seeded indicates longer seed filling duration and higher sink strength in them that might be responsible for their increased seed size at maturity.

Keywords: lentil, podwall, seed, seed size, invertases

1. Introduction
Lentil is the most ancient cultivated crops among the legumes (Rehman and Altaf 1994) [26]. Harlan (1992) [13] reported that lentil is one of the early domesticated plant species as old as barley and pea. It is indigenous to South Western Asia and the Mediterranean region. Lentil have thought to be originated in Mediterranean basin, Ethiopia, Afghanistan, India and Pakistan, China and later spread to Latin America (Duke 1981) [7]. Ahlawat (2012) [1] reported that India, Canada, Turkey, Bangladesh, Iran, China, Nepal and Syria are the important lentil growing countries of the world. According to report of IPGA (2015) [15] lentil production was 621.63MT and average yield was 471 kg/ha in India.Lentil can grow in a wide range of soil types. However, the heavy textured soils causes yield reduction whereas sandy-loam soils are the most suitable for lentil growth (Ozdemir 2002) [24]. Lentil (*Lens culinaris* L.) is one of the most nutritious winter season food legumes as from plant-based food; lentil has the third highest level of protein (26%). However, late sowing will decrease yield and increase protein content (Sehirali 1988) [31]. Lentils is a diploid species (2n = 14) (Muehlbauer 1991) [21]. It is self-pollinating annual species with a haploid genome size of an estimated 4063 Mbp (Arumuganathan and Earle 1991) [2]. Due to high protein content lentil is primarily used in foods. Bhatty (1988) [3] reported that lentil contains 28.6% protein, 3.1% ash, 4.9% crude fibre, 44.3% starch, 36.1%amylose and 63.1% total carbohydrates on dry basis. Swaminathan and Jain (1975) [35] reported a range of 20.4 to 30.5% in protein among lentil seed lots grown in India. Therefore, seed qualities are foremost criteria. In lentil, 93% of the phosphorus, 60% of the calcium and 79% of the iron are present in the cotyledon fraction (Singh *et al.* 1968) [33]. Therefore, it is preferred fodder for animals compared to wheat straw (Gupta *et al.* 2013) [12]. On cooking one half to two third amount of mineral nutrient were lost in the leach water. However, nutrient retention in cooked lentil may be governed by factors such as seed size and porosity of the seed coat (Meiners *et al.* 1976) [20]. Rathore (2002) [25] and Sinha *et al*. (2009) [34] reported two groups of lentil namely microsperma (2-6 mm diameter and < 2.5g/100seed) and macrosperma (6-9 mm diameter and > 2.5g/100 seed) on the basis of diameter and 100 seed weight respectively. Seed vigour is influenced by seed size hence large seeds have a better performance than small seeds under competitive conditions (Eriksson 1999) [8].

Ghassemi-Golezani *et al.* (2013) [10] observed that larger seed size reduces the deleterious effect of seed aging. Larger seed size is better over smaller ones in terms of seed quality, vigour and viability, better crop yield (Ghassemi-Golezani *et al*. 2012) [11]. Therefore it is essential to identify biochemical factor influencing seed size.

In grain legumes onset of pod formation profoundly changes source sink relationship (Salon *et al*. 2001) [29]. Growing pods not only attract a considerably higher amount of nitrogen than emerging leaves but significantly amount of carbon as well (Voisin *et al*. 2003a) [37]. Thus, during pod formation and pod filling nitrogen is in increasing demand from nodules. If nodules cannot meet the pods nitrogen requirements, additional nitrogen is attracted from older leaves, inducing progressive senescence (Schiltz *et al*. 2005) [30]. Fischinger and Schulze (2010) [9] reported that increasing nitrogen requirements of the growing pea pods were met by higher nitrogen fixation in pea plants due to more nodules. During seed filling the growth of both roots and nodules were limited by carbon supply because of large demand of assimilates for seed filling (Voisin *et al*. 2003b) [38]. Schiltz *et al*. (2005) [30] also reported that assimilates are preferentially furnished to the seeds during seed filling. Nitrogen remobilization is then exploited to satisfy the high nitrogen demand of filling seeds at the expense of vegetative organs and nodules. Weber *et al*. (2005) [39] reported that seed development is controlled by carbohydrate status with hexose signal inducing cell division in embryo and sucrose activates cell expansion and storage function.Sucrose is the primary organic carbon that is translocated through phloem from photosynthetic leaves (source) into non-photosynthetic tissues (sink) such as seed, fruit, and root. After phloem unloading in sinks, sucrose needs to be degraded into hexoses for diverse use by either invertase that hydrolyses sucrose into glucose and fructose or sucrose synthase that degrades sucrose into UDP-glucose and fructose (Ruan 2012) [27].

Keeping the factors in mind it is important to ascertain sucrose metabolizing enzymes in reproductive structures affecting the seed size in lentil. Hence, it is important to study the enzymes of sucrose metabolism at different reproductive stages in lentil cultivars differing in seed size.

2. Material and methods

Plants of lentil cv. PL 4 and LL 699 of small seeded cultivars and cv. DPL62 and IPL 406 of bold seeded cultivars were sown in the experimental area of Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, following recommended agronomic practices. The crop was sown in randomized block design with four replications of each cultivar. At flowering, uniformly growing plants were selected and their flowers were taggedand carbon metabolizing enzymes were estimated in podwall and seeds.Plants along with their pods were collected at 14, 21 and 28 days after flowering (DAF) and brought in the ice box to the laboratory. All the enzymes were extracted (triplicates) with relevant precooled extraction buffers at 4˚Cto minimize denaturation and assayed at 30°C.

2.1 Extraction of enzymes

Sucrose synthase (SuSy, EC 2.4.1.13) was extracted by crushing the required tissue (400-500 mg) in a chilled mortar with a pestle with 3-4 ml of 20 mM HEPES buffer (pH 8.2) containing 1 mM EDTA, 5mM $MgCl_2$ and 5mM β-mercaptoethanol. Insoluble polyvinyl polypyrrolidone (100 mg/ g tissue) was also added while extracting these enzymes. The extract was centrifuged at 10,000xg for 15 minutes at 4˚C. Acid invertase and alkaline invertases (AI, EC 3.2.1.27) were extracted in a similar manner as sucrose synthase except that 0.02M sodium phosphate buffer (pH 7.5) is used instead of HEPES buffer (pH 8.2).

2.2 Estimation of enzymes

Alkaline invertase was assayed by the method of Dey (1986) [6] with some modification. The assay reaction mixture contains 0.1 M sodium phosphate buffer (pH 8.0) and 0.5 M sucrose, 0.05 ml of enzyme extract was added. The contents were incubated at 37˚C for 2 h. The reaction was stopped by adding reagents like copper tartrate reagent and cooper sulphate reagent mixed in the ratio of 25:1. The tubes were kept in boiling water bath for 20 min. After cooling the tubes to room temperature, 1 ml of arsenomolybdate reagent was added. The intensity of blue colour developed was recorded at 510 nm against reagent blank. Sucrose synthase activity was determined by the assay mixture consisted of 150 µl of 1 M HEPES buffer (pH 6.5), 100 µl of UDP (4 mM) and 200µl of sucrose (100 mM). The reaction was initiated by adding 50µl of enzyme. In control assay system, UDP was not added. After incubation for 30 min at 37˚C and the reaction was stopped by adding 1ml of alkaline copper tartrate reagent and fructose released was estimated (Nelson 1944) [23]. Assay procedure for acid invertase was similar to that of alkaline invertase except 0.1 M sodium acetate buffer (pH 5.0) was used in place of 0.1 M sodium phosphate buffer (pH 8.0) in alkaline invertase and enzymes were estimated as described previously (Kaur *et al*. 2002) [17].

2.3 Extraction and estimation of Chlorophyll concentration

Chlorophyll concentration in fresh leaves was extracted with dimethyl sulphoxide and estimated by measuring absorbance at 645 nm and 665 nm (Hiscox and Israelstam 1979)[14]. The chlorophyll concentration was determined using formula:

Chl a $=12.19A_{665}- 3.45\ A_{645}$ (mg/ml)

Chl b $=21.99A_{645} – 5.32\ A_{665}$ (mg/ml)

Total Chl $= 20.2A_{645} + 8.02\ A_{665}$ (mg/ml)

2.4 Statistical analysis

The results were statistically analyzed by using factorial closed randomized design (CRD) to identify the significant relation between the cultivars and biochemical parameters at different days after flowering (DAF). In tables and figures, A represents relationship between cultivars, B represents relationship between days after flowering (DAF) and AB represents relationship between cultivars and DAF at CD (5%).

3. Results

Early flowering was observed in bold seeded cultivars at 75-80 DAS, while small seeded cultivars had initiation of flowering at 90 DAS.The changes in specific activities of sucrose metabolizing enzymes were estimated at weekly interval in the podwall and seeds of lentil cultivars during development starting from 14 days after flowering (DAF).

The acid invertase activity in pod wall increased up to 21 DAF and thereafter decreased in all the cultivars. At 21 DAF the activity was found to be maximum in IPL 406 (bold seeded cultivars) and the average activity during crop development was found to be minimum in DPL 62 (bold seeded cultivar) (Fig 1a). It was observed that alkaline invertase activity in pod wall was found to be maximum at 14

DAF in all cultivars and then decreased. At 21 DAF the activity was found to be more in small seeded cultivars as compared to bold seeded cultivars. At 28 DAF the activity of alkaline invertase decreased rapidly in all the cultivars. Alkaline invertase activity was found to be minimum in DPL 62 (bold seeded cultivar) (Fig 1b). It has been found that sucrose synthase activity was more at 14 DAF in pod wall of all cultivars after that it decreased till 28 DAF. Sucrose synthase activity was found to be minimum in DPL 62 (bold seeded cultivars) (Fig 1c). It was observed that chlorophyll content was more in pod wall of bold seeded cultivars compared to small seeded cultivars (Fig 1d).

The acid invertase activity in seeds increased upto 21 DAF and thereafter it decreased (Fig 2a). The specific activity of acid invertase was found to be significantly higher in PL 4 and LL 699 (small seeded cultivars) as compared to bold seeded cultivars. At later stage of seed development acid invertase decreased rapidly in bold seeded cultivars. Alkaline invertase activity increased upto 21 DAF in all cultivars and thereafter decreased (Fig 2b). It was observed that sucrose synthase activity in small seeded cultivars was maximum at 14 DAF and thereafter decreased whereas the activity of sucrose synthase in DPL 62 and IPL 406 (bold seeded cultivars) increased upto 21 DAF (Fig 2c). Even at 35 DAF the bold seeded cultivars still had higher activity.

It was observed that 100 seed weight was found maximum in IPL 406 followed by DPL 62 (bold seeded cultivars) and then LL 699 (small seeded cultivars). The minimum 100 seed weight was found in PL 4 (small seeded cultivars) Seed yield was found to be more in small seeded LL699 (3.5 kg) and large seeded IPL 406 (3.4kg) lentil cultivars (Table 1).

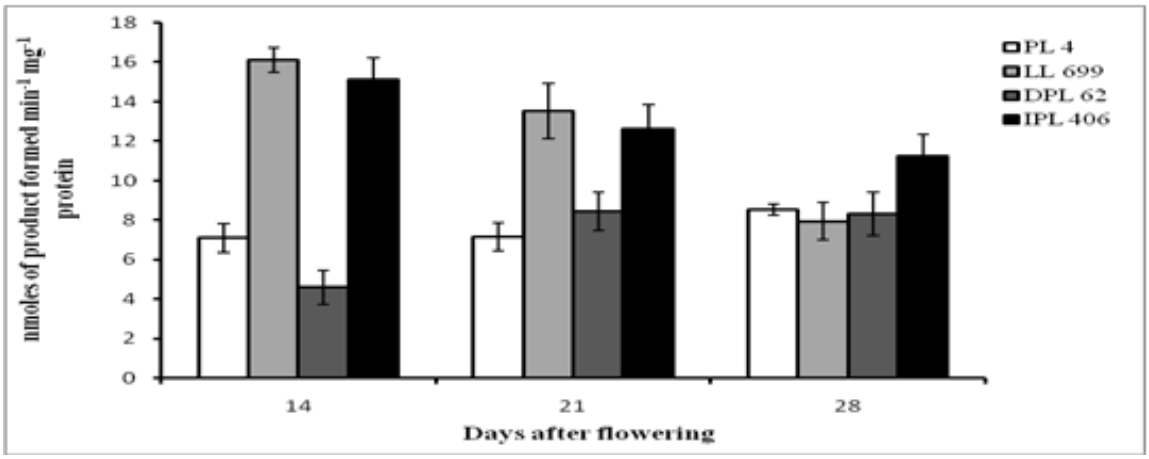
4. Discussion

Lentil is an important pulse crop containing high levels of protein (22-35%), including the essential amino acids such as isoleucine and lysine.Although low in methionine and cysteine, lentil is an excellent source of protein and amino acids to complement cereal protein. Seed size and shape are important factors influencing trade in pulse grains and determine market price as well as yield (Shahin *et al*. 2012) [32].

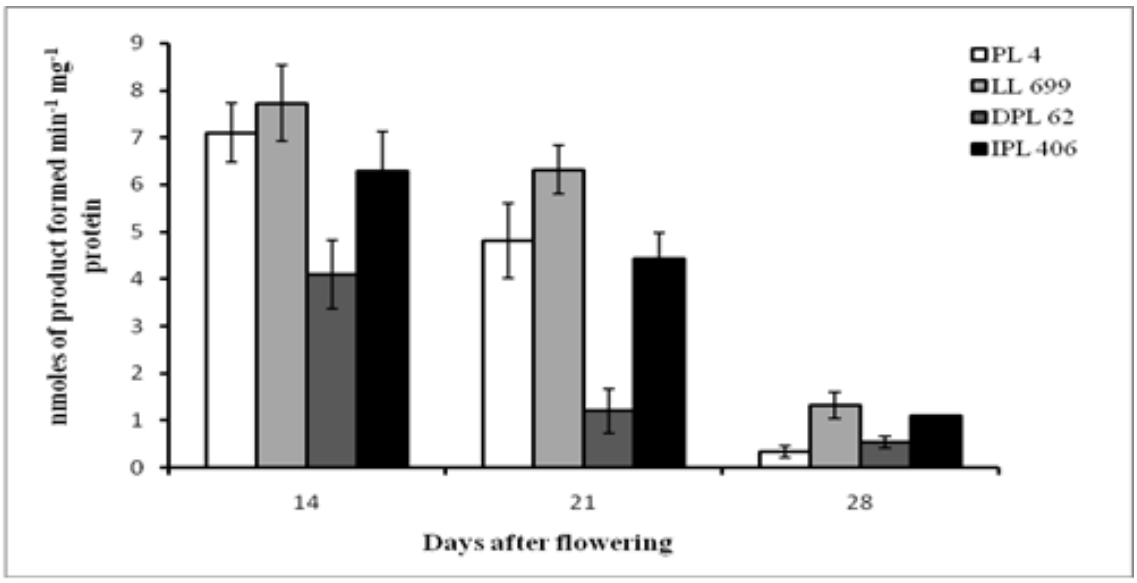
Sucrose is the predominant form in which photoassimilates are transported in the phloem from photosynthetic leaves (sources) to seeds (sinks) (Ruan *et al*. 2010) [28]. The higher activity of sucrose metabolizing enzymes in pod wall during initial stages indicates the utilization of photoassimilates for its growth and then decreased activity indicates its channeling into seeds. The reproductive establishments in legumes depend upon source sink relationships between pod wall and seeds during their development. Growth of legume pod requires translocation of photoassimilates from leaves to developing fruit. Lower activity of invertases and sucrose synthase in pod wall of bold seeded lentil cultivars as compared to small seeded cultivars might be helping them to transfer more sucrose towards seed that will be responsible for increasing seed size. Chopra *et al*. (2007) [5] observed that lower activity of sucrose synthase in pod wall of bold seeded cultivars indicated more assimilate channeling into seeds.

During filling, seeds are the main sinks to which assimilates are preferentially allocated at the expense of vegetative organs (Burstin *et al*. 2007) [4]. Seed weight in legumes depends upon two main factors. The first one corresponds to the number of cotyledon cells, which determines the potential seed weight as the cotyledon cell number is related to seed growth rate during seed filling and the second factor concerns carbon and nitrogen supply to the growing seed to support reserve accumulation (Munier-Jolain *et al*. 2008) [22]. Accumulation of more photosynthates during growth phases and their partitioning at later stages to storage organs had been reported to be responsible for increased seed weight and seed yield per plant in mungbean (Kumar *et al*. 2011) [18]. Higher chlorophyll concentration observed in podwall of bold seeded cultivars during initial stages of development results in synthesis of more photoassimilates by the podwall indicating its better source capacity. Therefore podwall in bold seeded cultivars acts as a better source as compared to small seeded cultivars. The higher activity of sucrose metabolizing enzymes in pod wall during initial stages indicates the utilization of photoassimilates for its growth and then decreased activity indicates its channeling into seeds. It was observed that specific activity of acid invertase was found to be significantly higher in PL 4 and LL 699 (small seeded cultivars) as compared to bold seeded cultivars. At later stage of seed development acid invertase decreased rapidly in bold seeded cultivars. Small seeded cultivars had comparatively higher activities as compared to bold seeded cultivars. It was observed that sucrose synthase activity in small seeded cultivars was maximum at 14 DAF and thereafter decreased. The activity of sucrose synthase in DPL 62 and IPL 406 (bold seeded cultivars) increased upto 21 DAF. Even the activity of sucrose synthase was found to be more in bold seeded cultivars (DPL 62 and IPL 406) at 35 DAF as compared to small seeded cultivars (LL 699 and PL 4). At 35 DAF the activity decreases more in small seeded cultivars whereas in bold seeded cultivars the activity was detected. It appears that in sink tissues the invertase pathway is directed towards growth and cell expansion whereas the sucrose synthase pathway is associates with storage product biosynthesis.

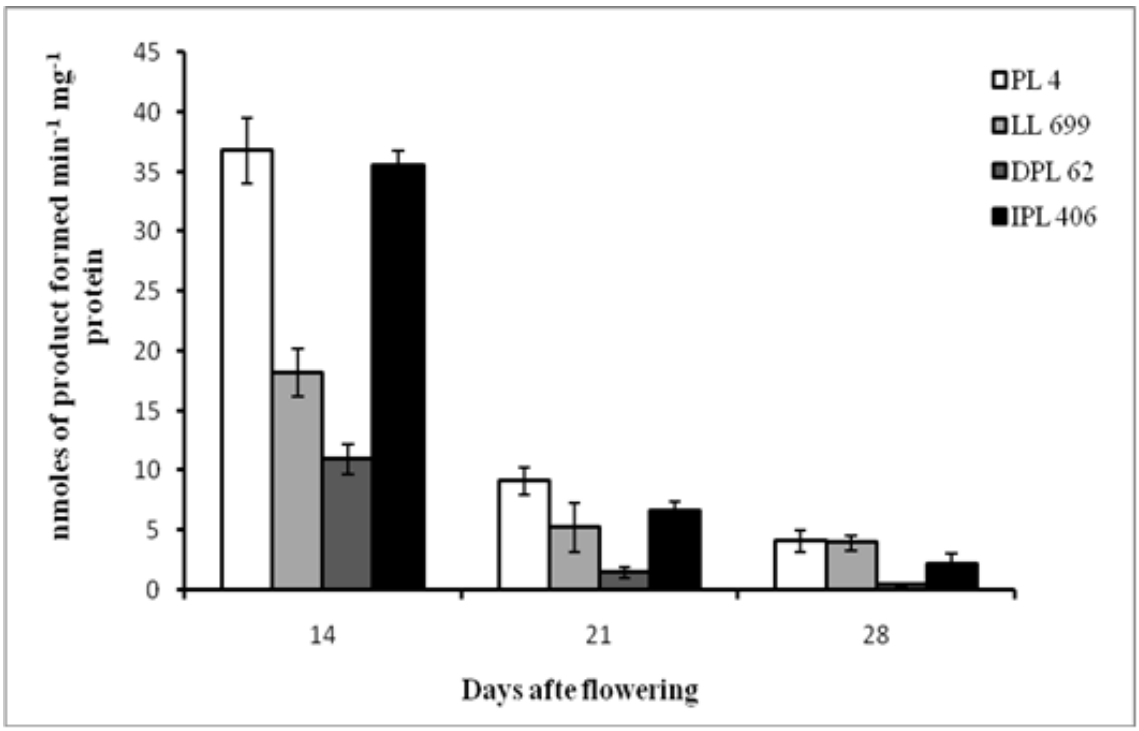
Turner *et al*. (2009) [36] observed that the maximum sucrose synthase activity is due to cessation of cell division and the rapid phase of dry weight accumulation by the cotyledon. Acid invertase activity is high during the early phase of seed development, as this enzyme is related with cell division. When significant cells are formed the activity of this enzyme decrease and sucrose synthase activity increases which is involved in formation of sugar nucleotides which can be taken up by amyloplast as such or after the conversion to glucose-1-phosphate, where these are utilized for starch synthesis. Chopra *et al*. (2007) [5] reported that sustained activity of sucrose synthase in large seeds in mungabean as compared to small seeds is responsible for increased seed size. Thus the sink activity of enzyme controlling carbon flux entering the seeds is required for the large seeds. Liu *et al*. (2010) [19] found that the large seeded cultivar had a strong and greater ability to accumulate photosynthate during seed filling. Kaur *et al*. (2012) [16] reported that high acid invertase activity for a prolonged period in the seeds is detrimental to seed filling and low sucrose synthase activity during seed storage phase cause detrimental effect on seed filling and resulting in highly reduced sink strength and productivity of wild species. Thus higher sink strength in bold seeded cultivars due to sustained activities of sucrose synthase for longer duration may be important factor responsible for their increased seed size. IPL 406 is the best lentil cultivar as it is bold seeded and is having higher yield.



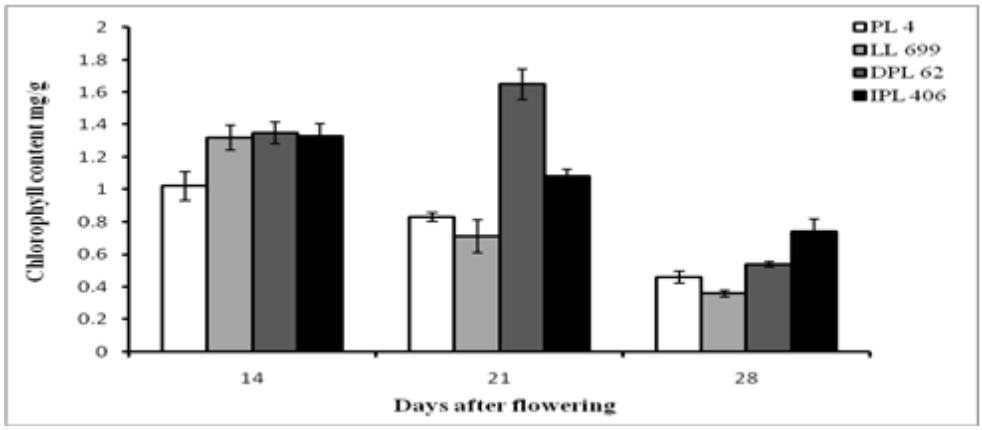
(a): CD (5%), A-3.5, B-NS, AB-NS



(b): CD (5%), A-2.0, B-1.7, AB-NS

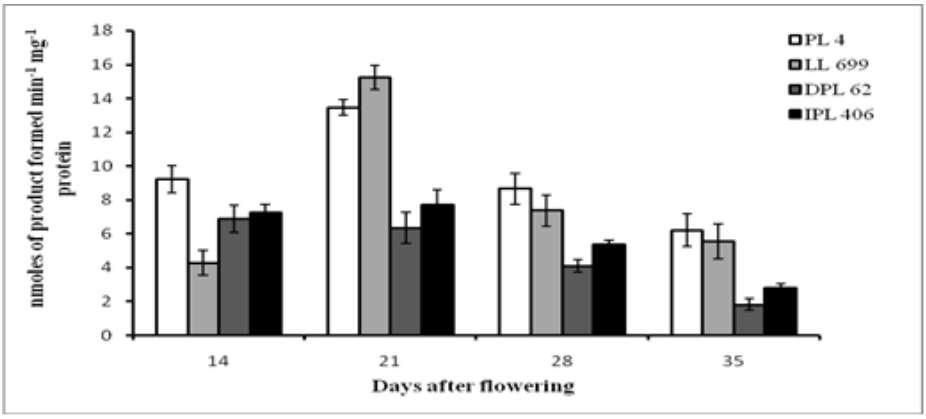


(c)

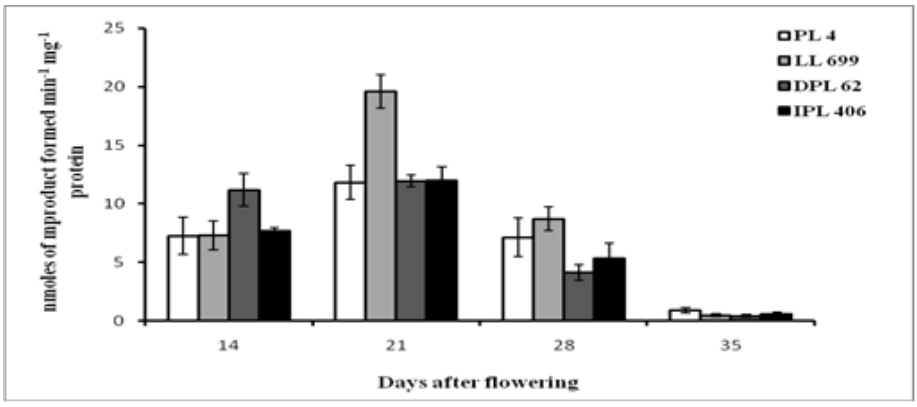


(d): CD (5%), A-NS, B-0.4, AB-NS

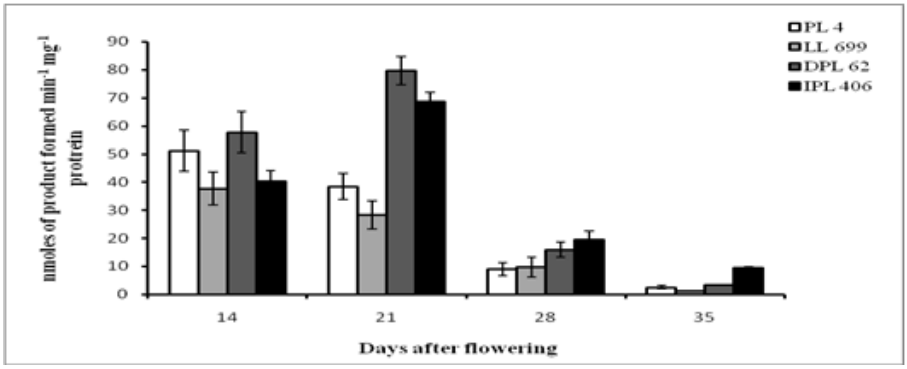
Fig 1: Specific activity of (a) acid invertase (b) alkaline invertase (c) sucrose synthase and (d) Chlorophyll content in pod wall of small (PL 4 and LL 699) and bold (DPL 62 and IPL 406) seeded lentil cultivars at different days after flowering (DAF).



(a): CD (5%), A-1.2, B-1.2, AB-2.4



(b): CD (5%), A-NS, B-1.5, AB-3.1



(c): CD (5%), A-3.8, B-3.8, AB-7.6

Fig 2: Specific activity of (a) acid invertase (b) alkaline invertase (c) sucrose synthase in seeds of small (PL 4 and LL 699) and bold (DPL 62 and IPL 406) seeded lentil cultivars at different days after flowering (DAF).

Table 1: Variation in weight of plant at maturity, 100 seed weight (g) and per seed weight (mg) of small and bold seeded lentil cultivars at maturity.

Cultivars	Weight of plants at maturity (g)	100 seed weight (g)	Per seed weight (mg)	Seed yield (kg)
PL 4	25	2.32	23.2	2.4
LL 699	35	2.44	24.4	3.5
DPL 62	37	3.40	34.0	2.5
IPL 406	45	4.26	42.6	3.4

Data represents mean of 10 plants taken randomly

5. Conclusion

It can be concluded that the reproductive establishments in legumes depend upon source sink relationships between pod wall and seeds during their development. The higher activity of sucrolytic enzymes in pod wall during initial stages indicates the utilization of photoassimilates for its growth and then decreased activity indicates its channeling into seeds. Accumulation of more photosynthates during growth phases and their partitioning at later stages to storage organs is responsible for increased seed size. Larger seed size is better over smaller ones in terms of seed quality, vigour and viability, better crop yield, market price and nutrient retention.

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