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Changes in the Activity of Carbon and Nitrogen Metabolising Enzymes in Nodules of Bold and Small Seeded Lentil Cultivars

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Seed size is the important component for seed yield and an important trait for consumer preference. The seed size is governed by the genetic as well as environmental factors; however the enzymes responsible for the C and N compounds constitute the major seed biomass. To understand mechanisms governing seed size in lentil, the present investigation was carried out on carbon and nitrogen metabolism in nodules of two small (PL 4 and LL 699) and two bold (DPL 62 and IPL 406) seeded lentil cultivars during crop development. In spite of early flowering in bold seeded, they have sustained activities of carbon and nitrogen metabolising enzymes in them as compared to small seeded cultivars at later stages of development indicates that nodules in them remain active for a longer time. Nodules are site for metabolism of sucrose to provide the energy and carbon skeletons for biological nitrogen fixation. The activity of carbon metabolizing enzymes in nodules of bold seeded cultivars was found to be high. The activity of nitrogen metabolizing enzyme in nodules was found to be higher in all the cultivars during development. The main route for the assimilation of fixed nitrogen is GS/GOGAT pathway while GDH function at higher NH4+ concentration in young and senescing lentil nodules.

Introduction

Lentil (*Lens culinaris* L.) is one of the earliest cultivated crops and most nutritious *rabi* season food legumes as from plant-based food; lentil has the third highest level of protein (26%) after soybeans and hemp and is an important constituent of the diet in many parts of the world, especially in Indian subcontinent which have large vegetarian populations (Tyagi and Khan, 2010). Lentil seeds contain 25% protein, 0.7% fat, 2.1% mineral, 0.7% fibre and 59% carbohydrate (Bhatty and Christison, 1984). It is rich in phosphorus, calcium, iron, zinc and carotene. Due to presence of more protein, calcium and phosphorus it is preferred fodder for animals also as compared to wheat straw (Gupta *et al.,* 2013). The seeds are consumed in North Africa, Middle East, West Asia and India as staple food as well as in vegetarian dishes worldwide providing essential nutrients such as proteins and micronutrients like iron and zinc. In developing countries, lentil straw is valued as animal feed (Erskine *et al.,* 1990). It plays an important role in human, animal and soil health improvement occupying a unique position in cereal based cropping system

(Hojjat 2011). Lentil ranks next only to chickpea in grain legume production (Dixit *et al.,* 2011). The important lentil-growing countries of the world are India, Canada, Turkey, Bangladesh, Iran, China, Nepal and Syria. In India, lentil production in 2014-15 was 621.63MT and average yield was 471 kg/ha representing total area of 1320 Ha (IPGA 2015). Lentil is divided into two sub-species including macrosperma (broad and large seeds with a diameter of 9-6 mm) and microsperma (concave shaped tiny seeds with a diameter of 6-2 mm) (Rathore, 2002). Singh *et al.,* (2009) reported two groups in lentil namely microsperma (< 2.5g/100 seed) and macrosperma (>2.5g /100 seed). Seed vigour is influenced by seed size since it is generally proportional to the amount of food reserves that will be destined to the embryo (Lloret *et al.,* 1999). Large seeds have an increased root/shoot ratio (Lloret *et al.,* 1999) and higher grain yields (Rao 1981). Large seeds have a better performance than small seeds, especially under competitive conditions (Eriksson, 1999; Moles and Westoby, 2004). Superiority of larger seed size over smaller ones in terms of yield, plant vigour and weight in lentil has been reported (Sinha *et al.,* 2009). Uniformity in seed size and constituents of seed lot has been emphasized for mechanical planting, better crop and determining the market price (Shahin *et al.,* 2012). So identification of biochemical factors affecting or governing the seed size is very important. Seed size is not controlled at one level as it is the coordination of a number of biochemical and physiological factors at all developmental stages of the plant including nodulation, vegetative growth and reproductive establishment. Photosynthesis activity supplied large amount of energy to allow symbiotic nitrogen fixation in legume root. Sucrose is the stable product of photosynthesis that is transported from the tissue via the phloem into all heterotrophic tissues and is a source of energy and carbon metabolites (Stitt *et al.,* 2010; Sun *et al.,* 2011). Sucrose is synthesized in the leaves and exported through the phloem to sinks such as the nodules. Legume nodules are primarily dependent on the import and metabolism of sucrose to provide the energy and carbon skeletons for biological nitrogen fixation (Gordon *et al.,* 1999). Photosynthates predominantly in the form of sucrose are translocated to nodules and are metabolized by cytosolic invertase and/or sucrose synthase (Aleman *et al.,* 2010). Chopra *et al.,* (1998) reported that alkaline invertase to be the major enzyme of sucrose metabolism. These reactions produce UDP-glucose and free hexoses, which, after phosphorylation by hexokinases enter the glycolytic or oxidative pentose phosphate pathways and are metabolized to Phosphoenol pyruvate (PEP). PEP is converted to oxaloacetic acid and then to L-malate by Phosphoenol pyruvate carboxylase (PEPC) (EC 4.1.1.31) and Malate dehydrogenase (MDH) (EC 1.1.1.37) respectively (Galvez *et al.,* 2005). Malate provides a significant portion of carbon skeleton for the assimilation of fixed nitrogen and is the preferred substrate for bacteroid respiration (Lodwig and Poole, 2003). Two enzyme systems are considered of primary importance in ammonium assimilation. Coupled glutamine synthetase (GS)/NADH-glutamate synthase (GOGAT) is the first pathway, where NH_4^+ is incorporated into glutamine by GS, which is then converted with 2-oxoglutarate to glutamate by GOGAT. The second and alternative pathway is glutamate dehydrogenase (GDH) pathway which mediates the reductive amination of a-ketoglutarate to yield glutamic acid (Masclaux-Daubresse *et al.,* 2006). Keeping these factors in mind it is important to ascertain the carbon and nitrogen metabolism in nodules affecting the seed size in lentil. Hence, it is important to study the enzymes of carbon and nitrogen metabolism in nodules of lentil cultivars differing in seed size.

Materials and Methods

Two small (PL 4 and LL 699) and two bold seeded (DPL 62 and IPL 406) lentil cultivars were sown in the experimental area of Department of Plant Breeding and Genetics, Punjab Agricultural University,(30˚54'N, 75˚48'E, elevation 247 m above sea level), Ludhiana, following recommended agronomic and crop management practices. The crop was sown in randomized block design with four replications of each cultivar.

Extraction of nitrogen and carbon metabolizing enzymes

The whole plants were uprooted in the morning from the wet field by random selection at 15 days interval between 50 and 110 days after sowing (DAS). The plants were brought to the laboratory buried in crushed ice, in an ice bucket. The roots bearing nodules were first thoroughly washed under tap water and then with distilled water so as to remove adhering soil particles on the nodules and then tapped dry in layers of filter paper. Fresh nodule samples were used for enzymatic analysis. All the enzymes were extracted (triplicates) with relevant precooled extraction buffers at 4˚C to minimize denaturation and assayed at 30°C. Sucrose synthase (SuSy, EC 2.4.1.13) was extracted by crushing the nodules (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. 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). Glutamine synthetase (GS, EC 6.3.1.2 L-glutamate:ammonia ligase), glutamate synthase or Glutamine 2-oxoglutarate aminotransferase (GOGAT, EC 1.4.1.14), glutamate dehydrogenase (GDH, EC 1.4.1.2), Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31), Malate dehydrogenase (MDH, EC 1.1.1.37) and Isocitrate dehydrogenase (IDH, EC 1.1.1.42) were extracted by homogenizing the nodules in 0.1 M Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 10,000xg for 20 minutes and the clear supernatant was used for the enzyme assay.

Estimation of nitrogen and carbon metabolizing enzymes

Glutamine synthetase was assayed by the method of Elliot (1953) with some modifications. The assay mixture contained 0.2 M Tris-HCl buffer (pH 7.5), 0.5 M $MgSO_4.7H_2O$, 0.5 M $NH_2OH.HCl$ and 0.5 M, 0.06 M ATP, enzyme extract and 0.6 M sodium glutamate. Incubate the reaction mixture at 30° C for 2 h and add $FeCl_3$ reagent. The clear supernatant obtained after centrifugation at 6,000×g for 10 min was measured at 540 nm. Glutamine 2-oxoglutarate aminotransferase was asssayed by the method of Misra and Oaks (1981) with some modification. GOGAT was assayed at 340 nm. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.6), 0.2 M L-glutamine, 0.2 M 2-oxoglutarate, 0.5 M KCl, 0.04 M EDTA, 2%, β-mercaptoethanol (v/v), 0.002 M NADH and 0.2 ml enzyme extract. Glutamate dehydrogenase was assayed by the method of Duke and Ham (1976). GDH was assayed at 340 nm. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.6), 3M NH_4Cl, 0.33 M 2-oxoglutarate, 1mM NADH and 0.2 ml enzyme extract. PEPC, MDH and IDH were from the rate of oxidation of NADH assayed spectrophotometrically at 340 nm by the methods of Christeller *et al.,* (1977), Vance and Stade (1984) and Kerr *et al.,* (1987)

respectively with some modification. Alkaline invertase and sucrose synthase were assayed by the method of Dey (1986) and Nelson (1944) respectively with some modification. The protein content of enzyme extract was measured by the method of Lowry *et al.,* (1951) using bovine serum albumin as a standard.

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 sowing (DAS).

Results and Discussion

The specific activity of sucrose synthase (SuSy) in PL 4 and LL 699 increases upto 80 DAS whereas in bold seeded cultivars it increased upto 95 DAS (Fig. 1a). It was observed that specific activity of alkaline invertase (AI) in nodules increased upto 80 DAS in all cultivars thereafter it decreased. At 80 DAS alkaline invertase activity was found to be maximum in small seeded cultivars (PL 4) and minimum in bold seeded cultivars (DPL 62). At 95 DAS the activity of AI was found to be more in nodules of LL699 (small seeded cultivars) and IPL 406 (bold seeded cultivars) as compared to other cultivars, but at 110 DAS the activity decreased in all cultivars (Fig. 1b). It was observed that PEPC in PL 4 and LL 699 increased upto 95 DAS and thereafter it decreased whereas in DPL 62 maximum activity was observed at 80 DAS and in IPL 406 it increased upto 110 DAS (Fig. 1c).

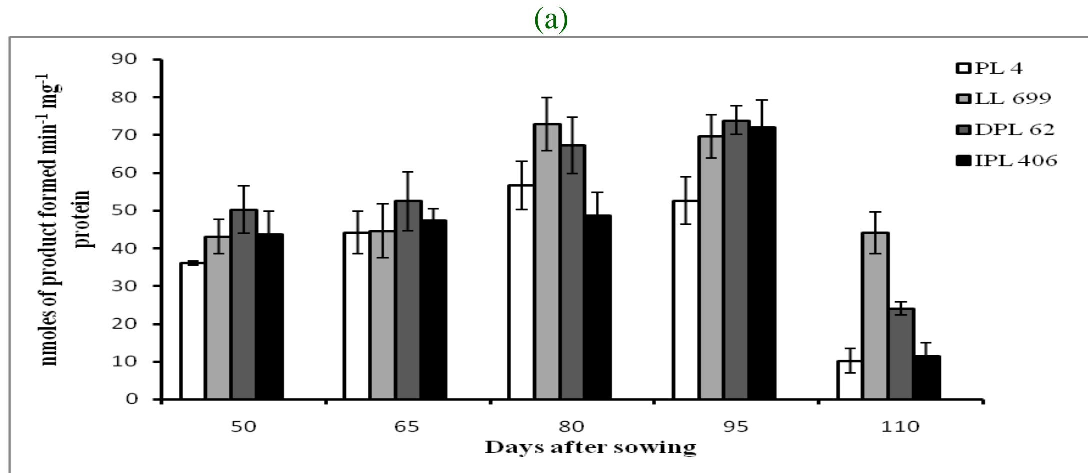
Malate dehydrogenase (MDH) was found to be more in IPL 406 at 65 DAS as compared to all cultivars thereafter it decreased (Fig. 2a). At 110 DAS the specific activity of MDH was found to be maximum in DPL 62 (bold seeded cultivars) followed by LL 699 (small seeded cultivars) and then PL4. The specific activity of isocitrate dehydrogenase (IDH) increases upto110 DAS and no significant differences were found in the cultivars (Fig. 2b).

The activity of glutamine synthetase (GS) and glutamate synthase (GOGAT) was found to be comparable in all the cultivars, however glutamate dehydrogenase activity was found to be maximum between 65-80 DAS in all the cultivars. GDH activity was found to be highest in bold seeded cultivars (DPL 62 and IPL 406) followed by small seeded cultivars (PL 4 and LL 699) at 80 DAS (Figs. 3a, b and c). Lentil is an important pulse crop containing high levels of protein (22-35%), including the essential amino acids such as isoleucine and lysine. Seed size and shape are important factors influencing trade in pulse grains and determine market price as well as yield (Shahin *et al.,* 2012). Seed size in lentil is the result of interlinked stages starting from nodule establishment; finally the source-sink relationship affecting seed size and the difference in these attributes will contribute towards difference in seed size in them.

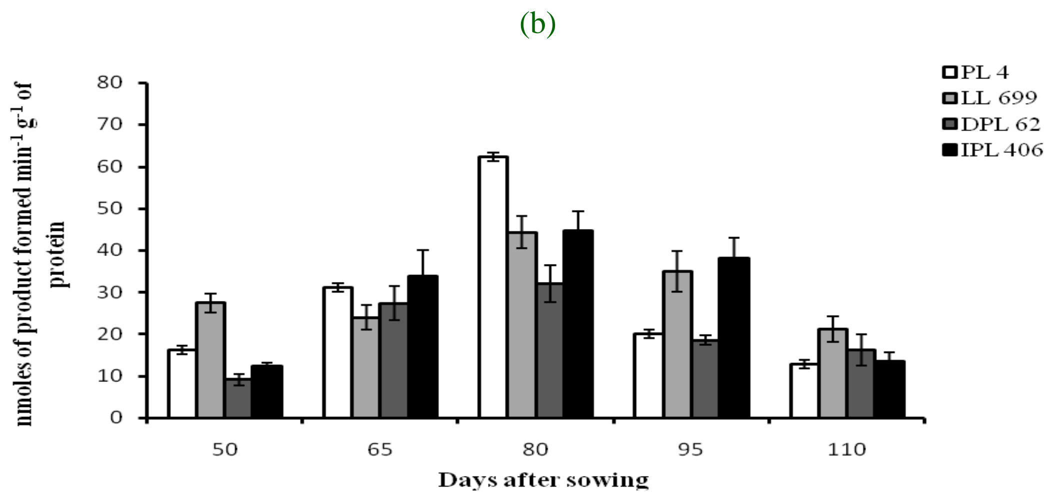
The higher activity of sucrose synthase in both the bold seeded cultivars; higher PEPC in DPL 62 at 80 DAS and in IPL406 at 110 DAS; higher MDH in IPL406 at 65 DAS and higher MDH and IDH in DPL 62 at 110 DAS; higher GDH in DPL 62 and IPL406 at 65 DAS and even comparable enzyme activities in bold seeded cultivars with small seeded cultivars were observed although the bold seeded cultivars flower 10-15 days earlier than small seeded cultivars, but the activity of carbon nitrogen assimilating enzymes in nodules of these cultivars even after flowering was comparable to small seeded cultivars. Thus the nodules in bold seeded cultivars remain active for longer time.

Fig.1 Specific activity of (a) sucrose synthase (b) alkaline invertase (c) phosphoenolpyruvate carboxylase in nodules of small (PL 4 and LL 699) and bold (DPL 62 and IPL 406) seeded lentil cultivars at different days after sowing (DAS)

(a)



(b)



(c)

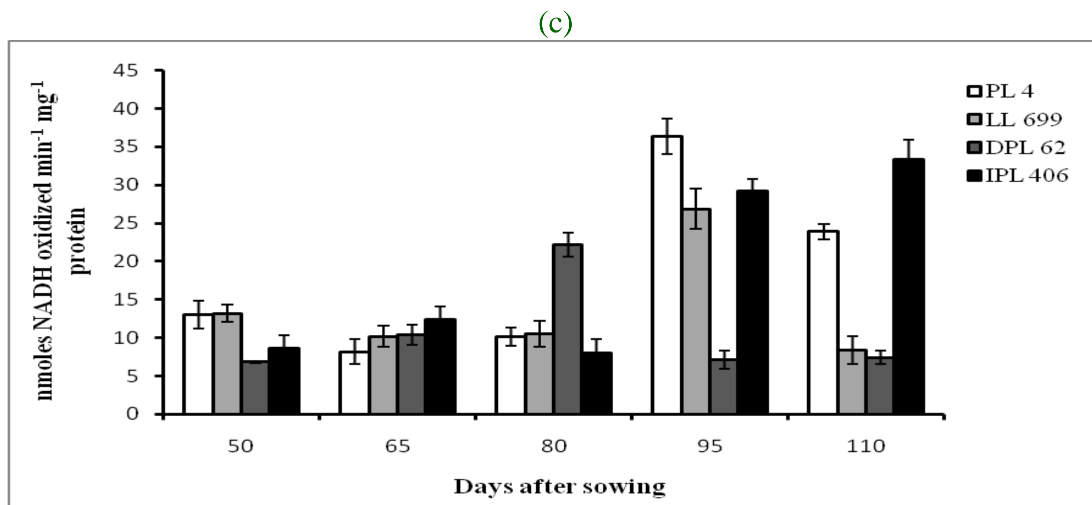
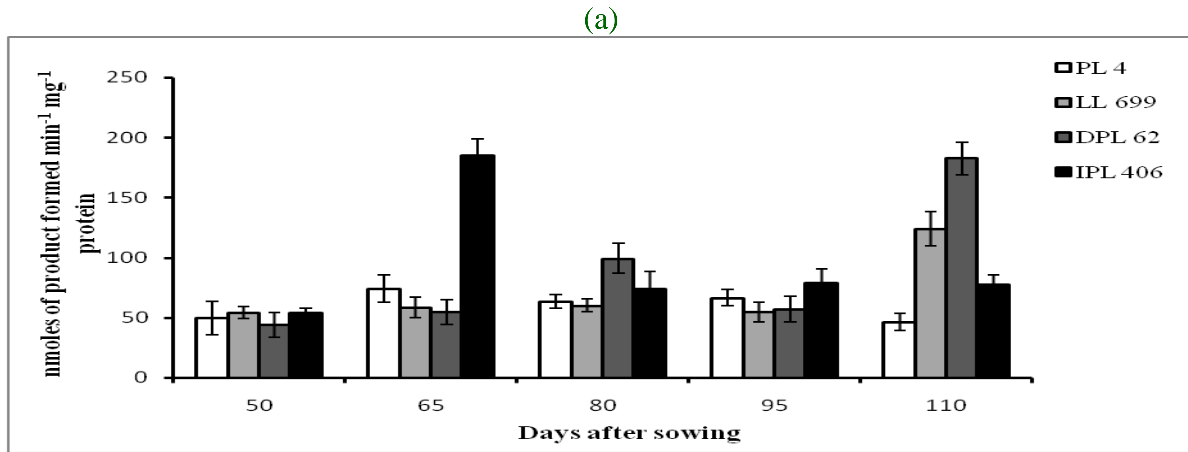


Fig.2 Specific activity of (a) malate dehydrogenase (b) isocitrate dehydrogenase in nodules of small (PL 4 and LL 699) and bold (DPL 62 and IPL 406) seeded lentil cultivars at different days after sowing (DAS)

(a)



(b)

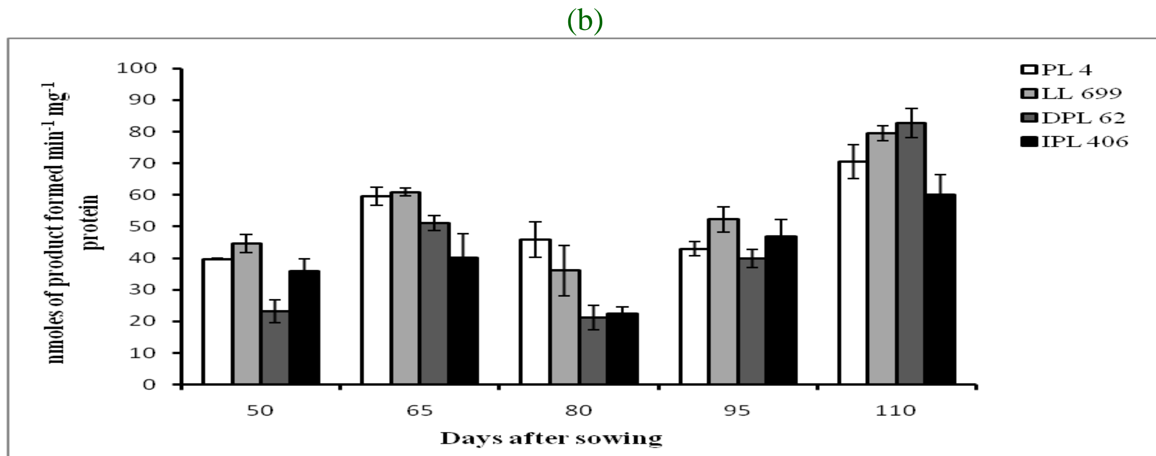
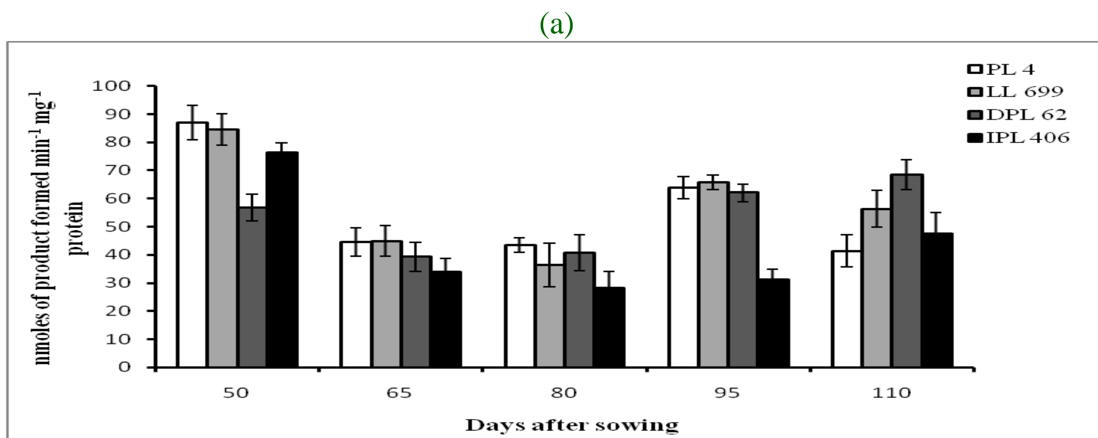
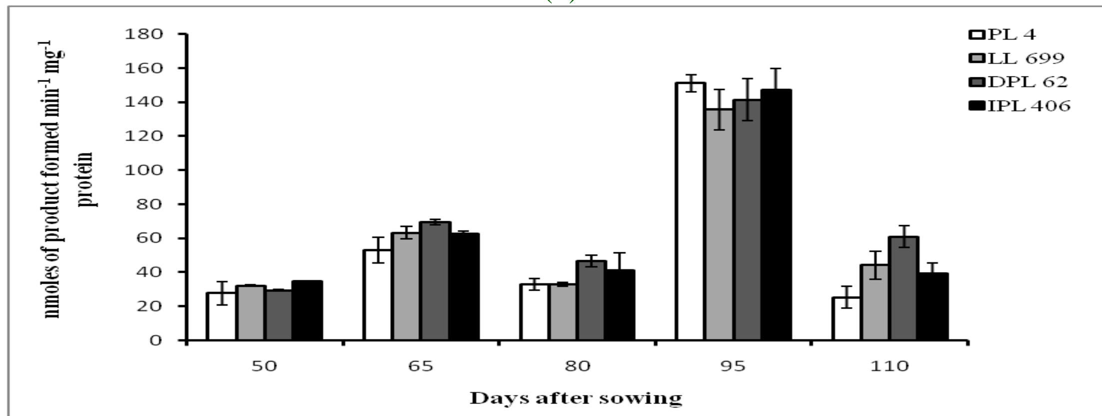


Fig.3 Specific activity of (a) glutamine synthatase (b) glutamate synthase (c) glutamate dehydrogenase in nodules of small (PL 4 and LL 699) and bold (DPL 62 and IPL 406) seeded lentil cultivars at different days after sowing (DAS)

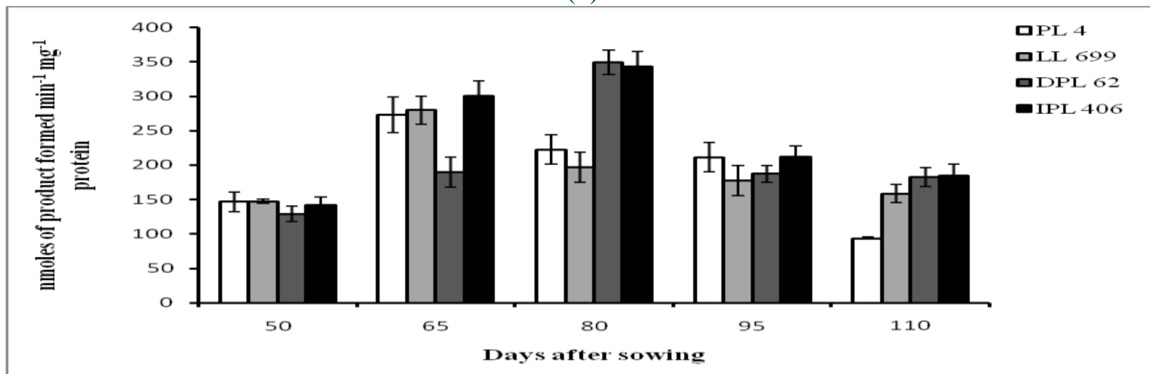
(a)



(b)



(c)



Sucrose is the main carbohydrate transported through phloem to sink tissues like nodules, where it is metabolized by either sucrose synthase (SuSy) or alkaline invertase (AI) and the breakdown of sucrose into glucose and fructose, are essential proteins in plant life (Aleman *et al.,* 2010; Mariana *et al.,* 2013).The higher sucrose synthase activity observed in the nodules of bold seeded cultivars ensures hexose supply for meeting the energy and reducing power demands of nodules during the stage of active nitrogen fixation and probably may regulate carbon metabolism and nitrogen fixation (Chopra *et al.,* 2003). In present investigation bold seeded cultivars showed higher status of PEP, MDH and IDH enzymes that indicate the utilization of glycolytic products for bacteroid respiration. The products of SuSy after phosphorylation by hexokinases enter the glycolytic pathway and are metabolized to phosphoenolpyruvate (Galvez *et al.,* 2005). PEP is converted to oxaloacetic acid and then to L-malate by phosphoenolpyruvate carboxylase and malate dehydrogenase, respectively (Gordon *et al.,* 1999). PEPC mediates the flow of carbon for the production of dicarboxylic acid which are the main products of sucrose degradation supplied to bacteroid to support nitrogen fixation in nodules (Lodwig and Poole, 2003). A down regulation of PEPC in nodules impairs the nitrogen fixation whereas up regulation of malate dehydrogenase has been reported to increase nitrogen fixation (Normura *et al.,* 2006; Schulze *et al.,* 1998). Isocitrate dehydrogenase (IDH) is NADPH-producing enzyme catalyzes the conversion of isocitrate to 2-oxoglutarate (2OG) and providing carbon skeleton for ammonium assimilation and therefore amino acid metabolism (Hodges, 2002). Two enzyme

systems are considered of primary importance in ammonium assimilation. First is glutamate dehydrogenase (GDH) and second is glutamine synthetase (GS)/glutamate synthase (GOGAT). Glutamine synthetase (GS) is the key enzyme in charge of glutamine biosynthesis in nature. The reaction catalyzed by glutamine synthetase involves the ATP dependent amination of glutamate to yield glutamine. Glutamate synthase (GOGAT) then catalyzes the transfer of the amide group from glutamine to α-ketoglutarate to yield two molecules of glutamate. This is referred as GS/GOGAT pathway (Miflin and Lea, 1980). The predominant GS/GOGAT enzymes are chloroplastic GS2 and Fd-GOGAT and cytosolic GS1 and NADH-GOGAT (Tobin and Yamaya, 2001). Chopra *et al.,* (2002) reported that GS and GOGAT function as the main route for the assimilation of fixed nitrogen while GDH may function at higher NH_4^+ concentration in young and senescing lentil nodules.

It was concluded that the activity of both C and N metabolizing enzyme were higher in bold seeded lentil cultivar. The higher sucrose synthase activity observed in the nodules of bold seeded cultivars ensures hexose supply for meeting the energy and reducing power demands of nodules during the stage of active nitrogen fixation and probably may regulate carbon metabolism and nitrogen fixation. The higher activity of PEPC, MDH and IDH ensure the utilization of glycolytic product for bacteroid respiration. GS and GOGAT function as the main route for the assimilation of fixed nitrogen while GDH may function at higher NH_4^+ concentration in young and senescing lentil nodules.

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