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Glutamine synthetase activity and root induction in callus and cell suspension cultures of *Vigna radiata* (L.) R. Wilczek grown under different nutritional conditions

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Abstract

The activity of enzyme Glutamine Synthetase (GS) was studied in the cell suspension cultures of *Vigna radiata* grown under normal MS (Murashige & Skoog) medium and different nutritional deficient media i.e., nitrate (NO_3) deficient, ammonia (NH_4) deficient and glycine deficient media to study the behaviour of two weeks and four weeks old cultures. The objective of this study was to assess the metabolism of nitrogen assimilation for the production of cell lines which are more productive and high yielding. In this study, maximum GS activity was observed in the cell suspension culture of normal MS medium (26.8±0.86) and minimum in that of ammonia deficient medium (17.9±0.38). The GS activity was higher in the culture of two weeks duration viz. 26.8±0.86, 24.0±0.42, 17.9±0.38 and 23.5±0.98 as compared to that of four weeks cultures as 24.7± 2.14, 21.8±1.54, 16.7±1.31, and 23.2±0.38, respectively for normal, NO_3, NH_4 and Glycine deficient media. Root induction was observed in both the callus and cell suspension cultures, which further highlights the potential for propagating more productive and high-yielding cell lines. The same hormonal concentrations used for callus initiation were employed for cell suspension cultures. These included 2,4-D (0.2 mg/l), IAA (0.25 mg/l), and Kn (0.25 mg/l), which also facilitated root induction in both culture types. These results provide valuable insights into optimizing culture conditions for the production of high-yielding and productive cell lines, which is crucial for future agricultural and biotechnological applications.

Keywords: Glutamine synthetase, Nutritional conditions, Root induction, Somaclonal variants, Suspension culture, *Vigna radiata*

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Introduction

Plant suspension cultures give the biochemists a relatively homogenous population of cells, readily accessible to applied chemicals and growing under defined aseptic conditions (Azam et al., 2019; Ali et al., 2021; Jabeen et al., 2021; Zafarullah et al., 2021; Jabeen et al., 2022). Cell suspension cultures are widely used as model systems for studying pathways of secondary metabolism, induction of enzyme and expression of gene, degradation of xenobiotics and as a basis of materials, and purification of enzyme. The most plant cell suspension cultures devoid of pigments like chlorophyll and carotenoid is of great benefit in isolation of enzymes and secondary products. For certain studies however, suspensions of isolated leaf mesophyll cells may be preferable as they are most representative of a differentiated tissue than cultured cells derived from callus (Callow & Dow, 1980). Hormonal regulation of root morphogenesis in callus culture of cowpea (*Vigna unguiculata* L. WALP) was studied for the establishment of routine system for callus induction and root regeneration from primary leaves. Embryonic axes were used for seedling initiation using different concentrations of 2, 4-D (Dichlorophenoxyacetic acid). Calli were further sub-cultured on hormone free media or media fortified with 2.22 µM concentrations of (BAP) N^6-Benzylamino purine (Sani, 2018).

The effects of nitrogen were examined for NADH-dependent glutamate synthase activity and for the examination of proteins in cell suspension cultures of rice by (Watanabe et al., 1996). Plant cell cultures have been regarded as an alternative supply of fine chemicals of those extracted from whole plants (Scragg et al., 1988). Isolated cells in cell suspension cultures are used for the isolation of protoplasts. The removal of cell walls from cells and production of Protoplasts established a profitable instrument to study the physiology and biochemistry of higher plants and the genetic advancement in crops (Burgess, 1978). Morphogenically competent cell suspension cultures may be used to generate somaclonal variations with desirable traits (Gamborg et al., 1983).

Studies on efficient callus induction and cell suspension culture proliferation from leaf explants of cowpea was conducted using different concentrations of 2,4-D (Anand et al., 2000; Ramakrishna et al., 2005). Physiological conditions

of donor plants not only determine the callogenic tendency of explant but also affect the morphogenic potential of callus formation in cereal crops (Souza Canada & Beck, 2013). Plants can be regenerated from long term suspension cultures being used as a convenient source in all genetic treatments and choosing the somaclonal variants. Grain legumes that can be regenerated in cultures usually lose their totipotency within 3-4 months (Kysely et al., 1987). Suspension cultures of grain legumes have been successfully employed and reported by many researchers (Hammatt et al., 1986; Kumar et al., 1988). Nitrogen is an essential part of important compounds such as proteins, nucleic acids, some of the plant growth regulators and most of the vitamins. It is involved in almost all the biochemical reactions of living organisms. All plants absorb Nitrogen in the form of nitrate, ammonia or some organic compounds. Normally nitrate is the pre-dominant cause of nitrogen for the leguminous plants (Crafts-Brander & Harper, 1982). The role of glutamine synthetase and glutamate dehydrogenase was studied in nitrogen assimilation and possibilities for the improvement in nitrogen utilization in plants. The studies suggested the considerable evidence for a glutamate dehydrogenase shunt to return the carbon in amino acids back into reactions of carbom metabolism and TCA cycle. In studies transgenic plants with glutamine synthetase (GS) genes evidenced the possible improvement in crops nourished with nitrogen.

All inorganic nitrogen has to be converted into ammonia before its entry into organic pool through an "ammonia bridge". Ammonia even in low concentration is toxic for plants because it uncouples electron transport system, promotes the oxidation of pyridine nucleotide and inhibits the electron transport system (impairs important processes like photosynthesis and respiration) (Beevers & Hageman, 1980). So, in plants there must be an effective and efficient system of ammonia assimilation to avoid its acute toxicity. Response of nitrogen assimilating enzymes during in vitro culture of *Argyrolobium roseus* was studied to investigate the role and pattern of glutamine synthetase, nitrate reductase and glutamate dehydrogenase. Glutamine synthetase and NR activities were noticed to promote the growth of root and shoot during *in vitro* culture while GDH showed adverse effect (Darima et al., 2015). Although a number of enzyme systems are known to be capable of ammonia assimilation, but Glutamate Dehydrogenase (GDH) and Glutamine Synthetase/ Glutamate Synthase (GS/GOGAT) systems are considered to be the most significant as revealed by the studies of mycorrhizal and non-mycorrhizal plants i.e., *Trifolium subterraneum* L. and *Allium cepa* L. roots and shoots extracts (Smith et al., 1985). The enzyme glutamine synthetase catalyses the conversion of ammonia into glutamine. Enzyme glutamine synthase (E.C 1.4.1.13) catalyses the conversion of glutamine to glutamate (Tempest et al., 1970). In *Klebsiella aerogens* the kinetic studies revealed that the level glutamine decreased as the glutamate concentration increased suggesting the following sequence.

Ammonia → Glutamine → Glutamate

Glutamine synthetase is the first enzyme of this pathway with universal occurrence and thus has a unique significance in plant nitrogen metabolism. A key characteristic of this route is its cyclic nature (Miflin & Lea, 1980). This enzyme appears to be present throughout the plants and animal kingdom as studied for their characteristics, properties and behavior when isolated from mitochondria of lettuce leaves, mungbean seedlings, fronds of *Lemna minor* etc (Stewart et al., 1980). The glutamine synthetase has been reported to be present both in chloroplast and cytosol fractions in higher plants (Wallsgrove et al., 1979) and in green algae Euglena (Chaudhary & Merrett, 1984; Chaudhary et al., 1985). Evidence in favour of glutamine synthetase/ glutamate synthase pathway of ammonia assimilation was further strengthened using L-Methionine-S-Sulphoximine (MSO) an irreversible and specific inhibitor of glutamine synthetase (Ronzio et al., 1969). Labeling studies with NH_4, use of metabolic inhibitors (MSO) and kinetic studies have clearly shown that GDH did not have any significant role in ammonia assimilation and glutamate synthase is the only major pathway of ammonia assimilation (Miflin & Lea, 1980). Present investigation was initiated to study the glutamine synthetase activity in the cell suspension cultures of *Vigna radiata* grown under various nutrient deficient conditions thus providing a base for future studies keeping in view the importance of mung bean in the agriculture of Pakistan.

Materials and Methods

Preparation of culture media

Murashige & Skoog (MS, 1962) was used as basal medium for the induction of callus and subsequent preparation of cell suspension culture (liquid medium) from callus and was prepared according to the protocol of (Gamborg's & Wetter, 1975) with different modifications to grow the cell suspension culture under different nutritional deficiencies. First of all, stock solutions were prepared and stored under appropriate conditions for the preparation of culture medium.

Preparation of explants and initiation of callus

For the preparation of explants seedlings were grown from healthy seeds soaked for three hours at 30 ˚C. They were surface sterilized by 0.1 % $HgCl_2$, followed by distilled water washings. They were inoculated into separate test tubes containing 0.8 % plain agar under aseptic conditions and kept the tubes at 28 ˚C for 24 hours in darkness. Germinated seeds were then transferred to 18/6 hours light/ dark cycle. 0.2-0.3 cm pieces of hypocotyls of these three days old seedlings, as explants, were transferred to 100 ml pyrex flasks containing 40 ml MS medium supplemented with growth hormones (Auxins 2,4-D; IAA) and Cytokinins (Kinetin and Zeatin). Flasks were placed in growth room at 25 ˚C (±2 ˚C) under fluorescent light of 2000 lux and 18/6 light dark regime.

Initiation of cell suspension cultures

Suspension cultures were initiated using 6 weeks old green hypocotyls callus cultures. Callus was taken in sterilized petri dish, slightly mashed and was transferred to 250 L Erlenmeyer flasks containing 70 ml MS medium (prepared under different nutritional treatments ie, normal MS, NO_3, NH_4 and glycine deficient media) so that each flask may receive 2-3 gm callus for 100 cm^2 (Helgeson, 1979). A large number of hormonal combinations and concentrations were tried but the best combinations and concentrations are given in Table 1. After inoculation the flasks were transferred in an incubator shaker (Model EFM-60 SEIWA SIKO CO Ltd) at 25 ˚C (±2 ˚C) and at 100 rpm in dark. After 2 and 4 weeks, suspension cultures were sub- cultured using 0.5 mm wide pore pipettes to remove the large clots of cells. At each sub-culture cells were observed under the microscope.

Determination of Glutamine synthetase (GS) activity

The enzyme activity was determined according to (Row et al., 1970). It consisted of two steps:

Preparation of crude extract

The crude extract was prepared by grinding 5 gm of cell suspension culture tissue for 5 minutes in pre-chilled pestle and mortar.10 ml of ice-cold imidazole buffer (100 mM, pH7) containing 2 µ moles Dithiothreitol (DTT) and 100 µ moles $MgCl_2$ was used as the extraction medium during grinding. Homogenate was centrifuged at 10000 rpm in a refrigerated centrifuge (Kokusan H-251 CS) by using angle head rotor for 15 minutes at 40 ˚C.

Preparation of reaction medium and reaction

The reaction medium was contained in a final volume of 1 ml containing Na glutamate (100 mM, pH 7) 0.1 ml, ATP (200 mM) 0.1 ml, Hydroxylamine freshly prepared (1M, pH 7) 0.1 ml, $MgCl_2$(100 m M)20 µl, distilled water 0.18 ml, crude enzyme extract 0.5 ml. The reaction was started by the addition of 0.5 ml of suspension culture extract to the pre-incubated reaction medium at 37 ^{0}C in a shaking water bath. After 15-20 minutes 1.5 ml of Ferric nitrate (Fe $(NO_3)_3$ solution (0.37 M (Fe $(NO_3)_3$, 0.67 N HNO3 and 0.2 N Trichloroacetic acid (TCA) was added, and the tubes were transferred to ice for for 15 minutes. Precipitated proteins were removed by centrifugation at 3000 rpm for 30 minutes in a refrigerated centrifuge at 4 ˚C. The absorbance of L-glutamic acid-Ɣ-monohydroxamate in the supernatant was measured on spectrophotometer (Shimadzu UV 120-01) at 535 nm against a control which contained all the reagents except crude enzyme extract of the suspension culture.

Preparation of standard curve

Standard curve was prepared (Fig. 1) by adding all the reagents of enzyme assay to the known quantities (0.0-2.0 µ moles) of L-glutamic acid-Ɣ-monohydroxamate against the control containing all the reagents except the L-glutamic acid-Ɣ-monohydroxamate.

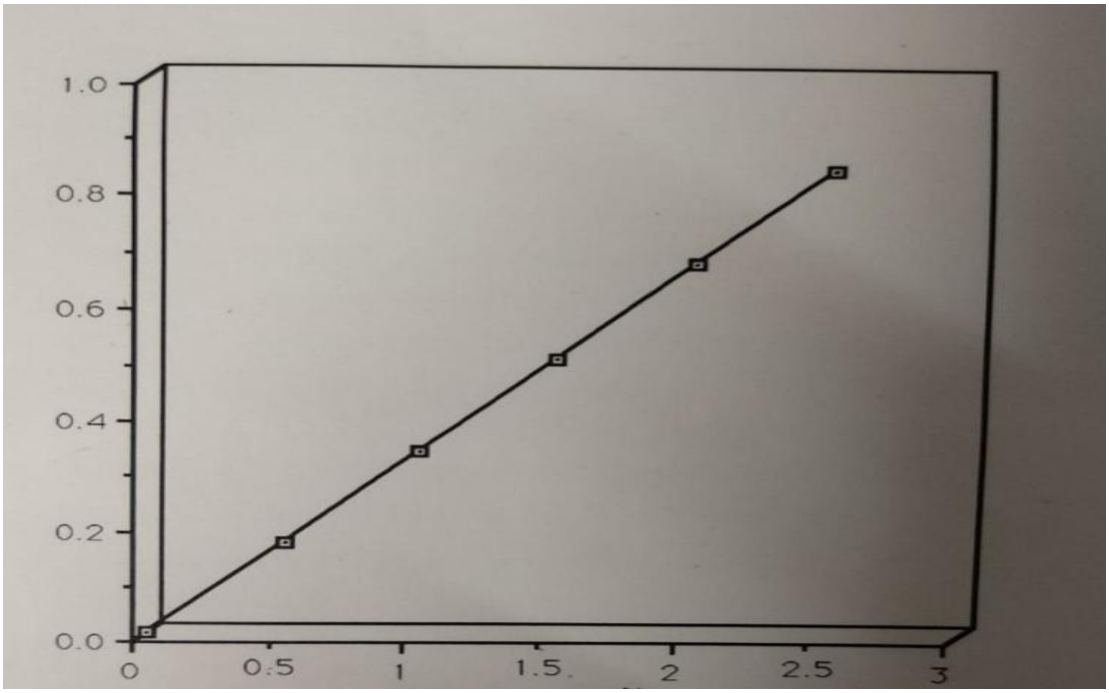

Fig. 1 Standard calibration Curve For the determination of L-glutamic acid-Ɣ- monohydrate Absorbance at 535 nm

Results

Various combinations and concentrations of plant hormones were tried to prepare the callus and cell suspension cultures, but the most useful combinations are given in Table 1. Same hormonal combination was used to initiate the cell suspension cultures as used in the callus. Cell suspension cultures were initiated under four different nutritional treatments i.e. Normal MS medium, NO_3 deficient, NH_4 deficient and glycine deficient MS media to study the glutamne synthetase activity in two- and four-weeks old cultures and the results were compared. Root initiation was also observed in the combination and concentration 2, 4-D (0.2 mg/l), IAA (0.25 mg/l), Kn (0.25 mg/l) (Fig. 2 and 3).

Table 1 Combinations and concentrations of hormones which proved useful in callus, suspension cultures initiation and root induction in callus and cell suspension cultures

Callus culture	Hormone	Concentration (mg/l)	Hormone	Concentration (mg/l)	Hormone	Concentration (mg/l)
	2,4-D	0.2	IAA	0.25	Kn	0.25
	2,4-D	0.2	-	-	Kn	0.25
	2,4-D	0.2	IAA	0.3	Kn	0.3
	2,4-D	2	IAA	3	Kn	0.3
Cell suspension culture	2,4-D	0.2	IAA	0.3	Kn	0.3
	2,4-D	0.2	IAA	0.25	Kn	0.25
	2,4-D	2	IAA	3	Kn	0.3
Root induction in callus and in cell suspension cultures	2,4-D	0.2	IAA	0.25	Kn	0.25

2, 4-D (2, 4-Dichlorophenoxy acetic acid); IAA (Indole acetic acid); (Kinetin) Kn (0.25)

Table 2 shows that in two weeks cell suspension culture of normal MS medium, the glutamine synthetase activity was 26.8 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour which was reduced to 24.7 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour for the next two weeks. In two weeks, cell suspension culture of NO_3 deficient MS medium, the GS activity was 24.0 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour which was reduced to 21.8 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour for the next two weeks. In two weeks, cell suspension culture of NH_4 deficient MS medium, the GS activity was 17.9 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour which was reduced to 16.7 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour for the next two weeks. Similarly in two weeks cell suspension culture of glycine deficient MS medium, the GS activity was 23.5 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour which was reduced to 23.2 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour for the next two weeks.

The result in comparison reveals that maximum glutamine synthetase activity (26.8 µ moles of L-glutamic acid-Ɣ-monohydroxamate was produced per gram of fresh weight per hour) was observed in cell suspension culture of normal MS medium and minimum (17.9 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour in that of NH_4 deficient medium for two weeks of culture because the most suitable inorganic source for growing plant cells is a mixture of ammonium and nitrate (Filner, 1966; Gamborg, 1968). The glutamine synthetase activity in glycine deficient medium was higher (23.5 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour than that of NH_4 deficient MS medium (17.9 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour but little bit lower than that of NO_3 deficient MS medium (24 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour). It indicates the inhibitory effect of glycine confirming the previous report (Stewart & Rhodes, 1978). It is also reported that amino acids like alanine, serine and aspartate inhibit the glutamine synthetase activity (Kimura et al., 1986). The glutamine synthetase activity in NO_3 deficient medium was higher (24 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour than that of NH_4 deficient medium (17.9 µ moles L-glutamic acid-Ɣ-monohydroxamate produced per gram of fresh weight per hour gm. It indicates that plant cells in cell suspension cultures can grow with nitrate as sole nitrogen source (Gamborg, 1970) and the high concentration of ammonium inhibited growth in *Sphagnum magellanicum* while studying the effects of ammonia nitrogen and nitrate nitrogen on its growth and metabolism (Rudolph et al., 1986). 2.5-3 times decrease in enzyme activity in roots of ammonium fortified medium grown seedlings as compared to nitrate fortified medium grown seedling (Shirshova et al., 1986). The activity of glutamine synthetase was also increased 2-3 folds when concentration of NH_4 decreased from 2-0.2 m M (Ahmad et al., 1986; Bhatnagar et al., 1986). It is also indicated that in Chlorella, the specific activity of glutamine synthetase was higher in nitrate compared with ammonium grown cells and nitrate starved cells always exhibited higher specific activity of

glutamine synthetase than did the nitrate medium grown cells (Sumar et al., 1984).

Table 2 Glutamine Synthetase activity in cell suspension cultures of *Vigna radiata* grown under different nutritional deficiencies for 2 and 4 weeks of sub-culturing

Nutritional status of MS medium	GS activity (µ moles L-glutamic acid-ɣ-monohydroxamate produced g fresh wt^{-1} h^{-1}) ± SD	
	2 Weeks	4 Weeks
Normal	26.8±0.86	24.7± 2.14
NO_3 deficient	24.0±0.42	21.8±1.54
NH_4 deficient	17.9±0.38	16.7±1.31
Glycine deficient	23.5±0.98	23.2±0.38

Hormonal Concentrations (mg/l): 2, 4-D (0.2), IAA (0.25),

The values of Glutamine synthetase activity in all the four treatments in two weeks culture was higher than that of four weeks culture. It means that enzyme activity is decreased during the most weeks of culture. It is comprehended that pulses lose their regeneration abilities within 3-4 months when regenerated in laboratory cultures (Kysely et al., 1987). For one-way (Analysis of Variance) ANOVA the F test accomplishment value is 156.24 for the glutamine synthetase activity of two weeks culture. To determine the statistically significant value we consult the F distribution table to compare this value to the F critical value found in the table where α (Significant level) is 0.001, DF1 (numerator degrees of freedom) is df treatment and is equal to 3 and DF2 (denominator degrees of freedom) is df error and is equal to 8. The F critical value found in the table was 130.62. Similarly, F test statistic value for this one-way ANOVA is 54.05 for the glutamine synthetase activity of four weeks culture. To determine the statistically significant value we consult the F distribution table to compare this value to the F critical value found in the table where α (Significant level) is 0.01, DF1(numerator degrees of freedom) is df treatment and is equal to 3 and DF2 (denominator degrees of freedom) is df error and is equal to 8. The F critical value found in the table was 27.489. It is observed that the F test accomplished values in the ANOVA are more than the F critical values in the F distribution table in both the cases, hence we decline to accept the null hypothesis. The results exhibit sufficient evidence to conclude the statistically significant differences and sufficient evidence for the observed values (Tables 3 a, 3b).

Table 3(a) Statistical analysis of two weeks data of glutamine synthetase activity using one way-ANOVA

Source	Sum of squares (SS)	Degree of freedom (df)	Mean squares (MS)	F
Treatment	154.70	3	51.56	156.24
Error	2.64	8	0.33	
Total	157.34	11		

Table 3(b) Statistical analysis of four weeks data of glutamine synthetase activity using one way-Anova

Source	Sum of squares (SS)	Degree of freedom (df)	Mean squares (MS)	F
Treatment	108.66	3	36.22	54.05
Error	5.375	8	0.67	
Total	114.03	11		

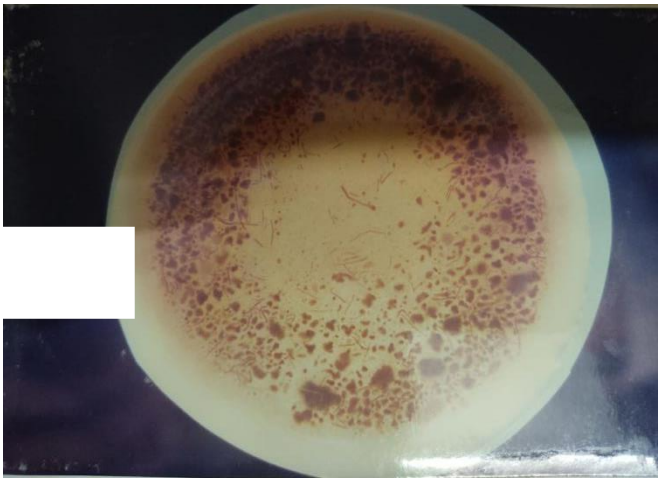

Fig. 1 Root induction in cell suspension culture

Fig. 2 Root induction in callus

Discussion

Many researchers worked on the propagation and initiation of callus and suspension cultures in different crops, medicinal and ornamental plants. Maximum production of flavonoids from Ginkgo leaves under different nutritional media with addition of adenine sulphate and pyruvic acid (each 0.1, 0.2, 0.3 mg/l), cadmium and lead acetate (each 2 and 4 mg/l) were studied (Sherif et al., 2023). Antibacterial, antioxidant and anti-inflammatory activities of leaf and cell cultures extracts of *Randia aculeata* L. and, its chemical components evaluation by GC-MS, was conducted by Martinez-Ceja et al. (2022). The studies of antioxidant activity and Lupeol acetate production in cell suspension culture established from the leaves of *Cnidoscolus chayamansa* was carried out by Perez-Gonzales et al. (2019). The cell suspension cultures of *Catharanthus roseus* grown under nitrogen- limiting conditions was studied for its growth and stoichiometry of biomass production (Rho & Andre, 1991).

Cell suspension culture of *Ageratina pichinchensis* (Kunth) for the improved production of anti-inflammatory compounds was established by Sanches-Ramos et al. (2020). The kinetics of the inhibition of Glutamine synthease activity was determined by the compounds DL-phosphinothricin and L-methionine sulphoxomine in the cultures prepared from the leaves of *Pisum sativum* as Ki values of 0.0073mM and 0.16 mM respectively, and both compounds showed mixed-competitive response apparently (Leason et al., 1982). The studies of enzymes glutmine, glutamate, and asparagines for their metabolism was worked out by, Lea et al., 1990. Nitrogen is the most essential nutrient from the quantitative point of view and a vital component of diverse cellular metabolites viz-viz amino acids, proteins, chlorophylls, and nucleic acids, in all organisms (Crawford & Forde, 2002). For the efficiency of nitrogen uptake and assimilation the intensity of the carbon cycle is a key determinant (Cross et al., 2006; Shofield et al., 2009). The assimilation of nitrogen is imperative parameter for the growth and development of plants and is chiefly important for the biomass production and yield of crops (Xu et al., 2012. Nitrogen assimilation is an intricate biochemical method catalyzed by a couple of reactions of glutamine synthetase (GS:EC6.3.1.2) and glutamate synthase (Masclaux-Daubresse et al., 2010; Xu et al., 2012). The studies in rice have suggested that Ferredoxin-dependent GS regulates N-C metabolomes and is the cause of genetic differentiation between *japonica* and *indica* subspecies. The analysis of metabolites revealed the accumulation of undue amount of high N/C ratio amino acids (Glutamine & Asparagine) and several intermediate compounds in the tricarboxylic acid cycle (TCA), thus suggesting that ABC1 plays a decisive part in nitrogen assimilation and C-N balance (Yang et al., 2016). The studies also reveal the appearance of genes during carbon metabolism and are considered very closely to the level of nitrogen (Krapp et al., 2011). The result of the present investigation is in conformity with the findings of various researchers who worked on nutritional medium preparation with different supplements, enzymes activities in callus and cell suspension cultures and their regeneration capabilities and organogenesis under different media protocols. During shoot regeneration from calli the glutamine dehydrogenase activity was reduced, and it showed the reduction even from the start of root regeneration to acclimatization stages. It is evident that during the developmental stages the plants need amino acids and proteins thus the activity of enzymes is reduced like glutamine dehydrogenase (Lea & Azevedo, 2007). In *Argyrolobium reseum* glutamine synthetase activity was found higher in comparison to that in stem and root during in vitro germination and the same is observed in other plants (Mack & Schjoerring, 2002). The studies revealed that plants assimilate most of the nitrogen in leaves during the growth period with the increase in enzyme activities (Kiomiya et al., 2001; Tabuchi et al., 2005). Our results also showed increased

activities in callus and in suspension cultures of two weeks as compared to that of four weeks for glutamine synthetase activities.

Conclusion

The present investigation achieved a standardized nutritional medium protocol for the establishment of callus culture, cell suspension culture and root induction in both the cultures and enzyme glutamine synthetase (GS) activity and this data will be useful for further research to enhance the growth, yields and productivity in *Vigna radiata* L. being an important protein rich pulse used globally as a staple food.

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Conflict of Interests: The authors declare no conflict of interest.

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