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Standardisation of Assay of L-glutaminase Activity in Arid and Semi Arid Tropical Soils of South India

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Abstract: L-glutaminase (L-glutamine amidohydrolase E.C. 3.5.1.2) is the soil enzyme that catalyzes the hydrolysis of L-glutamine and produces L-glutamic acid and ammonia. Thirty surface soil samples of varying physico-chemical properties representing, various cropping systems were collected from the Rajendranagar campus of Hyderabad. The procedure for assay of L-glutaminase activity in soils was standardized and quantification was compared between the modified indophenol blue method and the steam distillation method. A linear regression analysis (R^2 = 0.997) was carried out for the activity of enzymes obtained by the modified indophenol method against steam distillation. Assay its activity is described, which involves determination of the NH_4^+ released by L-glutaminase activity when 10 grams of soil is incubated with L-glutamine, THAM buffer (pH 8) and toluene at 37°C for 4 h. Incubation was terminated by the addition of 2.5 M KCl containing a L-glutaminase inhibitor (100 ppm Ag_2SO_4). The NH_4^+-N released soil suspension is determined modified indophenol method. Correlation analysis between the soil properties and L-glutaminase activity indicated that L-glutaminase was significantly and positively correlated with organic carbon, total nitrogen, clay content (and available nitrogen for red soils. Similarly, in black soils, these were positive and significant also, coefficient correlation with O.C, total N, clay content and available N. For all the soils (red and black soils together) highly significant correlation was with O.C, total nitrogen, clay content and available nitrogen. However, there was no significant correlation between L-glutaminase activity and pH, total soluble salt content.

Keywords: L-glutaminase, Modified indophenol blue method, O.C, Total N, Clay content

Soil is the medium for crop growth through which nitrogen is provided to the plants. The nitrogen in the organic forms is mineralized and made available to crop growth through the action of microorganisms and soil enzymes. The organic form of N is a major component of soil organic matter and may account for greater than 95% of the total N in most of the surface soils. About half of this organic N has not yet been identified. It has been estimated that about 20-40% of the total N in soils is present in the form of amino acids, but only a small portion of the amino acids are present in a "free" state and the major portion is bound to soil organic matter (Bremner 2006). The amino acids bound to the soil organic matter are most likely in the form of proteins or associated with the clay-organic matter complexes as peptides, amides or arylamides. These amino acids are released from the soil organic matter by the activities of enzymes such as arylamidase, and later the "free" amino acids are hydrolyzed by specific enzymes, producing NH_4^+, which in turn is nitrified for plant uptake. Among the aminohydrolases, L-glutaminase (L-glutamine amidohydrolase E.C. 3.5.1.2) is one that acts on free amino acids in soils. L-glutaminase is the enzyme that catalyzes hydrolysis of L-glutamine to glutamic acid and ammonium, thus it is important in making the amide form of nitrogen available to plants (Hojjati and Nourbakhsh 2007). For the assessment of amidase enzyme activity, the quantification of ammonical nitrogen is an essential requirement. Quantification of NH_4^+-N in KCl extracts of soils has been attempted by the steam distillation method (Frankenberger and Tabatabai 1991a) and the modified indophenol blue method (Dorich and Nelson 1983). A number of soil properties like organic carbon, pH, total and available N, CEC, clay content, and nutrient content have influenced L-glutaminase activity. Studies on the distribution of L-glutaminase in soil profile samples have shown that the activity decreased with soil depth, which was accompanied by decreasing O.C content. A significant relationship among soil L-glutaminase and L-asparginase activity and O.C has been observed by Frankenberger and Tabatabai (1991b) in diverse soils of Iowa State. Further, they observed a strong relationship between the soil nitrogen content and the activity of enzymes, mainly because of their association with O.C (Nahidan and Nourbakhsh 2018). Increasing soil enzyme activity is important to increasing plant and microbial access to nutrients, leading to more efficient cycling of nutrients. The

available literature on L-glutaminase activity is scanty and no systematic investigation seems to have been carried out on the L-glutaminase activity, its determination and methodology, especially under Indian conditions (low organic matter content soils), and hence the present research work was carried out.

MATERIAL AND METHODS

Thirty soil samples (10 red soils and 20 black soils) of varying physico-chemical properties representing, various cropping systems were collected from the Rajendranagar campus of Hyderabad by the quartering method. These soil samples were air dried and passed through a 2 mm sieve before use. The samples were analyzed for soil properties, viz., physical, physico-chemical and chemical properties, by using standard procedures (Jackson, 1973). Soil pH, electrical conductivity (dSm^{-1}), available potassium (kg ha^{-1}) and total nitrogen (%) were determined as described by Jackson (1973). The mechanical composition (particle size analysis) of soils was determined by the Bouyoucos hydrometer method. The relative proportions of sand, silt and clay soils were determined to describe their textural classes (Singh, 1980). Organic carbon (%) in soil was determined by the chromic acid wet digestion method given by Walkley and Black (1934). The available nitrogen was determined by a Macro Kjeldahl distillation method using alkaline potassium permanganate as described by Subbiah and Asija (1956) and expressed in kg ha^{-1}. The available phosphorus was determined by Olsen's method (Olsens et al 1954). The intensity of the blue colour was developed by L-ascorbic acid and was measured using a UV-1800 spectrophotometer at 660 nm and expressed in kg P_2O_5 ha^{-1}.

Assay of soil L-Glutaminase (L-glutamine amidohydrolase E.C. 3.5.1.2): L-glutaminase activity was assayed by incubating the soil samples with L-glutamine as described by Frankenberger and Tabatabai (1991a), with some modifications. For the quantification rate of release of NH_4^+-N estimated by the ammonical distillation method and the modified indophenol method. Soil sample (10 g) was taken in a 150 ml conical flask and adds 0.4 ml of Toluene, to which 12 ml of 0.1 M THAM buffer of pH 8 was added. The flasks were gently swirled to mix the contents, followed by the addition of 8 ml of 0.125 M L-glutamine (pH 8) was added, so that the concentration substrate was 50 mM. The flasks were gently shaken for a few seconds and covered with polythene paper. Then the contents were incubated at 37±0.5°C for 4 hours in the BOD incubator. After incubation, the reaction was terminated by the addition of 30 ml of KCl-Ag_2SO_4 solution. The contents were agitated on a mechanical shaker for 30 min to release all NH_4^+ formed and the suspension was allowed to settle and filter. In the controls, the same procedure described above was followed, but the L-glutamine solution was added after deactivating with the KCl-Ag_2SO_4 reagent.

Steam distillation of KCl extract: The activity of L-glutaminase was assayed by the steam distillation method, which was followed as given by Frankenberger and Tabatabai (1991a).

Modified Indophenol method: This method was followed as given by Dorich and Nelson (1983). One ml of supernatant from the soil suspension after incubation with L-glutamine and deactivation with KCl-Ag_2SO_4 was transferred to a 25 ml volumetric flask. To this, 1 ml of 6% EDTA (EDTA complexes di and trivalent cations present in the extract and prevents precipitate formation) was added, followed by the addition of 2 ml of phenol-nitroprusside and 8 ml of buffered hypochlorite reagent (this was prepared by dissolving 14.8 g of NaOH and 49.8 g of Na_2HPO_4 in 400 ml of distilled water, adding 400 ml of NaOCl (4-5%), adjusting the pH to 11.8 and making up to 1 liter). The volume was then made up to the mark with double distilled water, mixed thoroughly by inverting several times and placed in a water bath for 30 min at 40 °C for color development. The flasks were removed and brought to room temperature and the absorbance of the blue color was measured at 636 nm using UV-1800 spectrophotometer. The L-glutaminase activity was measured with respect to the amount of NH_4^+ liberated and expressed as µg of NH_4^+ released g^{-1} soil $4h^{-1}$.

RESULTS AND DISCUSSION

Thirty soil samples (10 red soils and 20 black soils) of varying Physico chemical properties representing various cropping systems were collected from the Rajendranagar campus of Hyderabad. The details of soil samples collected are presented in Table 1. In Table 2, the physical, physico-chemical, and chemical properties of soil samples were presented.

Standardization of assay of L-Glutaminase: In the present investigation, an attempt was made to standardize the assay of L-glutaminase activity and compare the amount of NH_4^+ liberated by the steam distillation method (Frankenberger and Tabatabai, 1991a) and the modified Indophenol method as given by Dorich and Nelson (1983). The preliminary studies carried out in the laboratory indicated that 10 grams of soil, 4 hours of incubation and incubation of soil at pH 8 were optimum for the assay of this enzyme. Another problem associated with the assay of L-glutaminase was the elimination of enzymes produced by the growing population of soil microorganisms and the assimilation of reaction products during the assay of abiontic enzymes, as they don't

include the enzymes accumulated by growing microorganisms. To overcome this problem, the use of toluene was recommended (Frankenberger and Tabatabai 1991b). Hence, in the present study, toluene was used as a microbial biostatic agent. However, the results reported on the use of toluene were contradictory, with several authors showing increased activity of enzymes by the addition of toluene and a decrease in activity, especially for soil urease and phosphatase (Tabatabai and Bremner 1972, Skujins 1978). However, in the present study, toluene was used as a microbial biostatic agent because of its incubation period of 4 hours, which might induce proliferation of microbial cells during assaying.

The L-glutaminase activity determined by the above two methods is presented in Table 3. The activity of L-glutaminase by steam distillation varied from 3.13 to 23.41 µg of NH_4^+ released g^{-1} soil $4h^{-1}$ and for the modified Indophenol method, the values varied from 3.25 to 23.50. A linear regression analysis (R^2 = 0.997) was carried out for the activity of enzymes obtained by the modified indophenol method against steam distillation. The paired t-test was carried out between these two methods and results indicated no significant difference between the values obtained by these two methods, suggesting that both these methods can be used for the assay of L-glutaminase activity. However, considering the sensitivity of the modified indophenol blue

Table 1. Details of soil samples collected

Sample number	Crop	Soil type	Location of sample collected
V1	Forest	Black	Agricultural Research Institute
V2	Mango	Black	Agricultural Research Institute
V3	Paddy	Black	Rice Section (ARI)
V4	Sapota	Black	Agricultural Research Institute
V5	Maize	Black	All India Coordinated Research Project Maize (ARI)
V6	Maize	Black	Soil Test Crop Response field (ARI)
V7	Paddy	Black	Agricultural Research Institute
V8	Maize	Black	Integrated Farming Systems, ARI
V9	Aerobic paddy	Black	College Farm
V10	Paddy	Black	College farm
V11	Cotton	Black	College farm
V12	Agro forestry	Black	AICRP on Agro Forestry
V13	Paddy	Black	Student farm
V14	Sesame	Black	Indian Institute of Oil Seeds Research, Rajendranagar
V15	Safflower	Black	Indian Institute of Oil Seeds Research, Rajendranagar
V16	Sunflower	Black	Indian Institute of Oil Seeds Research, Rajendranagar
V17	Castor	Black	Indian Institute of Oil Seeds Research, Rajendranagar
V18	Korra	Black	College Farm
V19	Cauliflower	Black	College Farm
V20	Paddy	Black	Indian Institute of Rice Research, Rajendranagar
A1	Teak block	Red	Bio diversity park, Rajendranagar
A2	Pomogranate	Red	Horticultural university, Rajendranagar
A3	Guava	Red	Horticultural university, Rajendranagar
A4	Capsicum	Red	Shade net (ARI)
A5	Mahu block	Red	Bio diversity park, Rajendranagar
A6	Ground nut	Red	Seed Research and Technology Center, Rajendranagar
A7	Brinjal	Red	Horticulture Garden
A8	Red gram	Red	Student farm
A9	Custard apple	Red	Horticultural university, Rajendranagar
A10	Mango	Red	NAARM

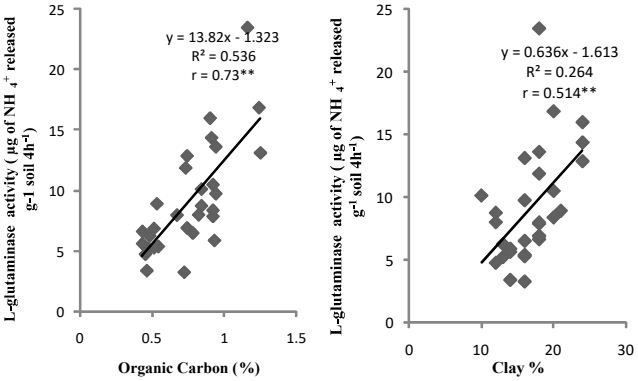

Fig. 1. Relationship between L-glutaminasc activity and % organic C in soils

Fig. 2. Relationship between L-glutaminasc activity and clay

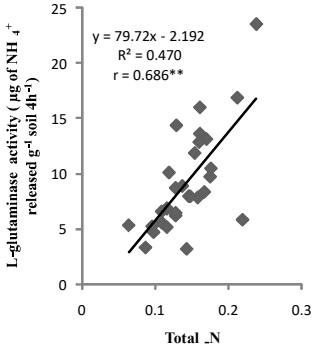

Fig. 3. Relationship between L-glutaminasc activity and Total-N

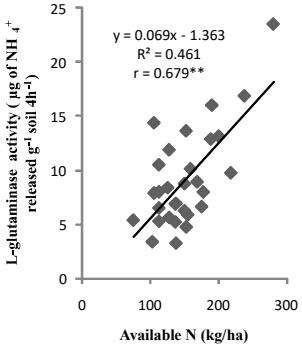

Fig. 4. Relationship between L-glutaminasc activity and Available N

Table 2. Physical, physico-chemical and chemical properties of surface soil samples

S.No.	pH	EC (dsm^{-1})	OC%	Available N kg ha^{-1}	Available P_2O_5 kg ha^{-1}	Available K_2O kg ha^{-1}	Total N (%)	Clay (%)	Silt (%)	Sand (%)	Texture
V1	7.98	0.152	1.25	200.70	81.24	734.72	0.170	16	24	60	Sandy loam
V2	7.81	0.154	1.16	280.70	68.23	675.36	0.238	18	28	54	Sandy loam
V3	8.23	0.479	0.84	150.50	92.30	659.68	0.128	12	16	72	Loamy sand
V4	7.86	0.136	1.24	238.30	53.76	704.48	0.212	20	44	36	Loam
V5	7.93	0.490	0.94	218.50	72.36	607.04	0.175	16	22	62	Sandy loam
V6	7.64	0.149	0.74	137.98	61.50	323.68	0.116	18	18	64	Sandy clay loam
V7	7.67	0.343	0.43	175.61	35.84	211.68	0.109	18	12	70	Sandy loam
V8	8.32	0.127	0.92	112.90	71.80	331.52	0.176	20	26	54	Sandy clay loam
V9	7.86	0.116	0.93	155.30	71.80	272.16	0.219	14	21	65	Sandy loam
V10	7.96	0.302	0.48	150.50	87.20	189.28	0.128	13	25	62	Sandy loam
V11	7.80	0.174	0.51	137.00	51.29	374.08	0.116	18	16	66	Sandy clay loam
V12	7.58	0.066	0.72	138.00	51.29	812.00	0.143	16	8	76	Sandy loam
V13	7.74	0.664	0.46	103.20	56.50	610.40	0.087	14	16	70	Loamy sand
V14	7.92	0.272	0.91	105.60	46.17	775.04	0.129	24	22	54	Sandy clay loam
V15	7.52	0.294	0.94	152.80	51.30	680.96	0.161	18	36	56	Sandy loam
V16	8.06	0.312	0.92	105.80	66.70	482.72	0.158	18	12	70	Sandy loam
V17	7.69	0.291	0.92	125.80	61.50	465.92	0.167	20	14	66	Sandy loam
V18	6.80	0.179	0.43	128.00	44.57	312.00	0.109	14	16	70	Sandy loam
V19	7.20	0.182	0.47	137.00	45.14	298.00	0.116	13	12	75	Sandy loam
V20	7.90	0.240	0.53	169.00	44.80	346.00	0.137	21	24	55	Sandy clay loam
A1	6.98	0.080	0.90	190.53	56.40	514.08	0.161	24	10	66	Sandy clay loam
A2	7.56	0.153	0.51	113.00	41.03	285.60	0.096	16	4	80	Sandy loam
A3	7.58	0.163	0.82	113.00	41.03	294.50	0.146	12	8	80	Loamy sand
A4	7.58	0.722	0.45	153.07	85.30	1043.84	0.098	12	24	64	Sandy loam
A5	7.18	0.064	0.74	189.00	66.68	445.76	0.160	24	6	70	Sandy clay loam
A6	6.98	0.375	0.78	113.00	61.50	618.24	0.128	16	18	66	Sandy loam
A7	7.18	0.325	0.84	159.30	66.68	450.24	0.119	10	14	76	Loamy sand
A8	7.55	0.174	0.73	87.80	56.50	564.48	0.154	18	22	60	Sandy loam
A9	7.62	0.196	0.54	75.26	46.16	303.52	0.064	16	4	80	Sandy loam
A10	6.92	0.167	0.67	178.00	46.20	321.00	0.148	18	21	61	Sandy loam

method, its accuracy and its capability to carry out the assay of a large number of samples at a given time, was higher.

Distribution of L-glutaminase and its correlation with physico-chemical properties of soils: The L-glutaminase activity of surface soils (NH_4^+ released g^{-1} soil $4h^{-1}$) varied from 3.25 to 23.50 with an average value of 9.15. Simple correlation analysis (Table 4) indicated that L-glutaminase was significantly and positively correlated with organic carbon, total nitrogen and clay content and available nitrogen for red soils. Similarly, in black soils, there was also a positive and significant coefficient correlation with O.C, total N, clay content and available N. For all the soils (red soils and black soils together) highly significant correlation was noticed with O.C, total nitrogen, clay content and available nitrogen. However, there was no significant correlation among L-glutaminase activity, pH and total soluble salt content. Soil enzyme activities are, in general, significantly correlated with soil organic carbon because of possible immobilization on soil organic matter (Boerner et al 2005). The enzyme L-glutaminase might be immobilized on humus and organic polymers or entrapped in their polymeric networks. In the present study, though the organic matter content was low to medium, it played a predominant role in enhancing the activity of L-glutaminase (Frankenberger and Tabatabai 1991c, Hu and Cao 2007, Zimmermanet and Ahn 2011). The significant and positive correlation with clay content indicates that the tropical soil, which contains large quantities of clay, could play an important role in immobilizing and entrapping the enzymes either on the edges or clay surfaces, and sometimes getting entrapped in between layers of the crystal lattice. Further, there could be a possibility of the formation of stable clay and humus complexes on which the immobilization of enzymes occurs and expresses their activity. The significant and positive correlation of L-glutaminase with total nitrogen and available nitrogen indicates that the considerable portions of nitrogen present in the amide or amine form are easily subjected to hydrolysis. Similar results were reported for other amidases (Frankenberger and Tabatabai 1991b, Frankenberger and Tabatabai 1991c, Vandana et al 2012).

Table 3. L–glutaminase activity (µg of NH_4^+ released g^{-1} soil $4h^{-1}$) in soil samples determined by Modified indophenol Blue method and Steam distillation method

Soil samples	Modified indophenol blue method	Steam distillation method
V1	13.13	13.41
V2	23.5	23.41
V3	8.75	8.92
V4	16.87	16.53
V5	9.75	10.20
V6	6.92	6.78
V7	6.63	6.76
V8	10.50	10.81
V9	5.88	6.13
V10	6.25	6.41
V11	6.87	6.73
V12	3.25	3.13
V13	3.38	3.91
V14	14.38	14.50
V15	13.63	13.72
V16	7.88	8.00
V17	8.38	8.13
V18	5.62	5.50
V19	5.21	5.41
V20	8.92	9.14
A1	16.00	16.00
A2	5.28	5.41
A3	8.00	7.80
A4	4.75	4.72
A5	12.87	13.12
A6	6.50	6.88
A7	10.13	10.50
A8	11.88	11.88
A9	5.38	5.50
A10	7.98	8.43
Mean	9.15	9.26

Table 4. Coefficient of correlation between different soil properties and soil L-glutaminase enzyme activity in different soils

Soil properties	Coefficient of correlation for L-glutaminase		
	Red soils	Black soils	Red + Black soils
OC %	0.75*	0.74**	0.73**
Total N	0.78**	0.688**	0.686**
Available N	0.664*	0.69**	0.679**
Clay %	0.664*	0.475*	0.514**
pH	0.11	0.1	0.1
E.C	-0.55	-0.27	-0.3

CONCLUSION

For the assay of L-glutaminase 10 grams of soil, 4 hours of incubation and incubation of soil at pH 8 were optimum. Linear regression analysis indicated no significant difference between values obtained by these two methods, suggesting that the modified indophenol blue method was simple, accurate and the number of samples analysed can be

increased at any given time. Correlations between the soil properties and L-glutaminase activity indicated that L-glutaminase activity was significantly and positively correlated with OC, clay content, total nitrogen and available nitrogen. The high correlation between enzyme activity and organic carbon content is because the increase in organic carbon and total nitrogen content could serve as a basis for an increase in soil enzyme activity. Therefore, it can be concluded that the organic matter content of the soil is the main factor controlling variations in enzyme activity. So, this study helps to find some cause-effect relationship between soil properties and enzyme activities. However, there was no significant correlation with pH and EC.

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