J. Phytol. Res. 17(2) : 141-146, 2004

IDENTIFICATION AND CHARACTERIZATION OF L-*MYO*-INOSITOL-1-PHOSPHATE SYNTHASE FROM *TAXUS BACCATA* L.

D. R. CHHETRI* and PO FONG CHIU
Plant Physiology and Biochemistry Laboratory, Post Graduate Department of Botany, Darjeeling Government College, Darjeeling-734 101, India.
*P. O. Box No. 79, Darjeeling-HPO, Darjeeling-734 101, WB, India.
E-mail: munal@sancharnet.in

Myo-Inositol is an important metabolite for normal growth and development of all living organisms. The cellular level of *myo*-inositol is controlled by the enzyme L-*myo*-inositol-1-phosphate synthase (MIPS) [EC 5.5.1.4]. Appreciable level of MIPS activity has been detected from *Taxus baccata* L. during the present studies. The enzyme has been partially purified from the young needles of *Taxus baccata* L. and about 85 fold purification was obtained. The final enzyme preparation shows pH optima between 7.0 and 7.5 while the temperature maximum was at 30 °C. The enzyme activity was remarkably stimulated by NH_4^+; slightly inhibited by Na^+ and Mn^{2+}; highly inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+}. The K_m values for D-glucose-6-phosphate was found to be as 1.05 mM and while the V_{max} value was 2.95 mM. The present study indicates the universal occurrence of this enzyme in all plant groups.

Keywords: Inositol synthase; L-*myo*-inositol-1-phosphate synthase; *myo*-inositol; *Taxus baccata*.

Introduction

Myo-Inositol serve as an essential nutrient for growth and development of all living organism, for the formation of sex units and as integral part of storage phosphates in seeds[1-3]. It also forms cell wall polysaccharides and essential component of myelin in the central nervous system[4]. The biosynthesis of *myo*-Inositol from Glucose-6-phosphate has been documented from a number of biological systems. D-glucose-6-phosphate (G-6-P) irreversibly isomerizes to L-*myo*-inositol-1-phosphate by L-*myo*-inositol-1-phosphate synthase (MIPS). The product of this enzyme generates free *myo*-inositol on dephosphorylation. The enzyme, MIPS have been isolated and characterized from a number of systems covering bacteria[5], protozoa[6], lower plants[7-12], higher plants and animals [13,14]. Despite this, there is only one report on *myo*-inositol from gymnosprerms[15]. The present study is concerned with the study on the occurrences of free *myo*-inositol and MIPS (EC: 5.5.1.4) from gymnosperms. Purification of MIPS and characterization of its properties have been reported here from a suitable gymnosperm, *Taxus baccata* studied (Family-Taxaceae) for the first time.

Materials and Methods

Plant material : Fresh specimens of gymnosperms viz., *Taxus baccata* L., *Abeis webbiana* Lindl, *Cedrus deodara* (Roxb. ex D. Don.) G. Don., *Pinus longifolia* Roxb. ex Lamb., *Cryptomeria japonica* (L. f.) D. Don., *Cupressus torulosa* D. Don.,*Juniperus recurva* Ham., *Podocarpus neriifolius* D. Don., *Ginkgo biloba*, *Thuja plicata* D. Don., *Araucaria sp.*, *Cephalotaxus sp.* were collected from their natural habitats in and around Darjeeling hills (2134 m.) in the Eastern Himalayas. All collections were done in the morning before 9.00 A.M.

Enzyme preparation : Different samples (100 g each) were collected fresh in the morning, washed twice with cold, sterile distilled water and homogenized in a chilled mortar and pestle in double volume of 50 mM tris-acetate buffer (pH 7.5) containing 0.2 mM of ME. The crude homogenate was passed through four layers of muslin and the liquid was centrifuged at 1,000 x *g* for 5 minutes in a Plasto Crafts Superspin-R centrifuge. The supernatant was again centrifuged at 11,400 x *g* for 20 minutes and the supernatant collected. The supernatant was dialyzed overnight against 50 mM tris-acetate (pH 7.5) buffer containing 0.2 mM ME and the clear supernatant was collected from the dialysis bag (10k Sup).

Enzyme activity assay : The MIPS activity was assayed by the procedure of Barnett *et al.*,[16] with slight modifications[17]. The assay mixture contained 500 mM tris-acetate (pH 7.5), 140 mM NH_4Cl, 8 mM NAD, 50 mM ME, 50 mM G-6-P and an appropriate aliquot (100-200 μg) of enzyme protein in a total volume of 500 μl. Suitable blanks (without the enzyme) and zero minute controls were also run. After incubation at 37 °C for 1h, the reaction was terminated by 200 μl of 20 % chilled TCA. An equal volume of 0.2 M $NaIO_4$ was added to the deproteinised supernatant followed by a second incubation at 37 °C for 1h for the oxidation of MIPS reaction product *myo*-

insositol-1-phosphate, with concomitant release of inorganic phosphate. The excess periodate was destroyed by 1M Na_2SO_3. Simultaneously, with the periodate set, appropriate non-periodate controls, in which $NaIO_4$ and Na_2SO_3 treatments were omitted were also run. The activity of the enzyme was determined by estimating the product-specific release of inorganic phosphate from *myo*-inositol-1-phosphate by MIPS reaction.

Inorganic phosphate determination : Inorganic phosphate was determined by the method of Chen *et al.*[18] with slight modifications. A freshly prepared Pi-reagent (2.8 ml.) containing H_2SO_4 (6N), ascorbic acid (10%, w/v), chilled ammonium molybdate (2. 5%, w/v) and H_2O mixed in 1:1: 1: 2 ratio was added to the reaction mixture and incubated at 37 °C for 1h. The blue colour developed was measured at 820 nm in a Beckman DU-64 Spectrophotometer. The inorganic phosphate released was quantified with a standard curve prepared using K_2HPO_4.

Protein determination : Protein was determined according to the method of Lowry *et al.*,[19] with BSA as a standard. A reagent blank was also prepared with 100 μl of distilled water. The protein content in fractions obtained from column chromatography was also determined by the same method.

Purification of MIPS : The enzyme was purified from the needles of *Taxus baccata* following the method outlined below:

The clear supernatant (LSS) was subjected to Streptomycin sulphate treatment to a final concentration of 2% (w/v) with constant stirring (using Remi cyclomixer). The mixture was kept in ice-bucket at 0 °C for 15 minutes and then centrifuged at 11,400 x *g* for 15 minutes. The supernatant (SSP) was collected which was fractionated with 0-70% $(NH_4)_2SO_4$. The precipitated protein fraction was dialysed against 500 volumes of extraction buffer with 2 changes. The dialysed fraction (A_2S fraction) was adsorbed in DEAE-cellulose and the preparation was loaded on a glass column (1.2 x 8.0 cm). The effluent was collected. After this, the column was washed with one bed volume of the same buffer and the adsorbed proteins were eluted from the column with a linear gradient of 0-5.0 M KCl. The active DEAE-cellulose fractions (DE fraction) were pooled and loaded on top of a column (0.8 x 7.5 cm) of Sephadex-G-200. The column was eluted with the equilibration buffer. The active Sephadex-G-200 were pooled together and the pooled active fractions (Spx fraction) was concentrated and used as the ultimate preparation in this experiment.

Free myo-inositol determination : Free *myo*-inositol was isolated by the method of Charalampous and Chen[20]. The extracted sample was passed through a mixed bed column of Dowex-1-Cl (100-200 mesh) and Amberlite IR-120 (Na-form) and the free *myo*-inositol was ultimately isolated by one dimensional descending chromatography through Whatman no.1 paper. Free *myo*-inositol was estimated spectrophotometrically according to the method of Gaitonde and Griffiths[21] using a standard curve prepared using known concentrations of pure *myo*-inositol.

Results and Discussion

Determination of free myo-inositol from gymnosperms : Appreciable quantity of free *myo*-inositol (the final product of *myo*-inositol biosynthesis) was detected from juvenile needles of different gymnosperm species (Table 1) employing the techniques of its isolation using filtration through a mixed bed column consisting of Dowex-1-Cl and Amberlite-IR-120 followed by paper chromatography and spectrophotometric assay. It has been revealed that the quantities of free *myo*-inositol in almost all plant parts are moderately high. Maximum free *myo*-inositol content was detected in the needles of *Juniperus recurva* (9.76 mg g^{-1} FW) and *Podocarpus neriifolius* (7.6 mg g^{-1} FW). However, the content of the same in the needles of *Cedrus*

Table 1. Free *myo*-Inositol content in different gymnosperm species.

Botanical name	Family	Order	Free *myo*-inositol mg g^{-1} FW
Taxus baccata	Taxaceae	Coniferales	3.48
Abeis webbiana	Pinaceae	Coniferales	2.08
Cedrus deodara	Pinaceae	Coniferales	7.20
Pinus longifolia	Pinaceae	Coniferales	2.20
Cryptomeria japonica	Taxodiaceae	Coniferales	3.92
Araucaria sp.	Araucariaceae	Coniferales	6.88
Cupressus torulosa	Cupressaceae	Coniferales	4.56
Juniperus recurva	Cupressaceae	Coniferales	9.76
Thuja plicata	Cupressaceae	Coniferales	2.32
Podocarpus neriifolius	Podocarpaceae	Coniferales	7.60
Cephalotaxus sp.	Cephalotaxaceae	Coniferales	5.04
Ginkgo biloba	Ginkgoaceae	Ginkgoales	2.48

Table 2. Partial purification of L-*myo*-inositol-1-phosphate synthase from *T. baccata.*

Fraction	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Sp. activity [n mol I-1P-produced (mg protein)$^{-1}$ h^{-1}]	Total activity [n mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]	Recovery (%)	Fold purification
Low speed supernatant fraction(LSS)	200	0.615	123.000	16.77	2062.71	100.00	1.00
Streptomycin sulphate Fraction (SSP)	182	0.681	123.942	15.57	1929.77	93.55	1.07
0-70% $(NH_4)_2SO_4$ fraction (A_2S)	16	0.761	12.176	32.2	392.06	19.00	5.26
DEAE-cellulose fraction (DE)	5.2	0.096	0.499	59.13	29.50	1.43	69.92
Sephadex-G-200 fraction (Spx)	4.5	0.079	0.355	68.48	24.31	1.17	84.85

Table 3. Requirements for L-*myo*-inositol-1-phosphate synthase activity from *T. baccata.*

Condition	Sp. activity [n mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]	Percent activity
Complete set	33.2	100.00
Minus substrate (G-6P)	0.0	0.0
Minus tris buffer	27.9	84.03
Minus NAD	13.8	41.56
Minus NH_4Cl	16.2	48.79
Minus ME	20.6	62.04
Heat-killed enzyme	0.0	0.0

Table 4. Effect of incubation with different ions (5 mM) on MIPS activity in *T. baccata.*

Sl. No.	Type of ions	Sp. activity [μ mol I-1-P produced (mg protein)$^{-1}$ h^{-1}]	Percent activity
1.	Control	6.65	100.00
2.	K^+	7.03	105.71
3.	Na^+	5.50	82.70
4.	NH_4^+	10.98	165.11
5.	Mg^{2+}	7.15	107.51
6.	Mn^{2+}	5.32	80.00
7.	Ca^{2+}	7.93	119.24
8.	Zn^{2+}	3.15	47.36
9.	Cu^{2+}	3.60	54.13
10.	Hg^{2+}	0.91	13.68

deodara and *Araucaria sp.* is also worthy of mention.

Purification of enzyme : The cytosolic enzyme, L-*myo*-inositol-1-phosphate synthase was isolated and purified from the young needles of freshly collected *T. baccata* employing the techniques of low speed centrifugation, streptomycin sulphate precipitation, ammonium sulphate fractionation, and chromatography on DEAE-cellulose, Sephadex-G-200. The summary on the purification of MIPS is given in Table 2. The ultimate chromatography on Sephadex-G-200 column resulted in about 1.2 fold purification over the penultimate purification step employing DEAE cellulose column. Chromatography on DEAE column in turn resulted in 13.3 fold purification over the $(NH_4)_2SO_4$ fraction. An overall purification of the enzyme to about 85 fold with about 1.17% recovery based on total activity could be achieved in the present study.

Characterization of the purified enzyme

Requirements for T. baccata MIPS activity : The *T. baccata* MIPS, when assayed in presence of 50 mM tris-acetate buffer (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD, 5 mM 2-mercaptoethanol (ME) and 5 mM glucose-6-phosphate

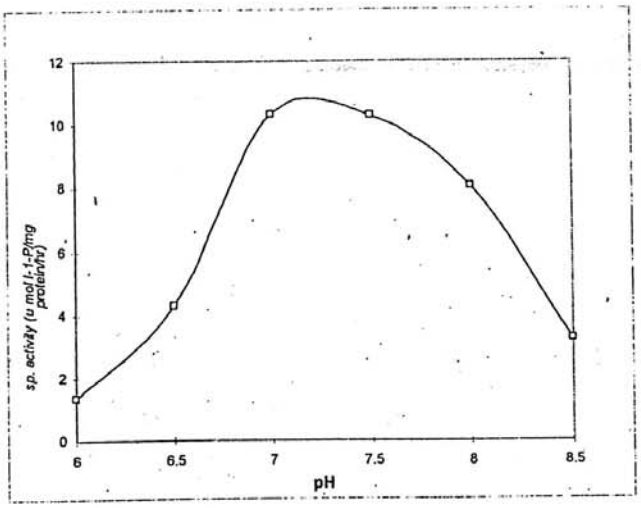

Fig. 1. Effect of different pH on MIPS activity in *T. baccata.*

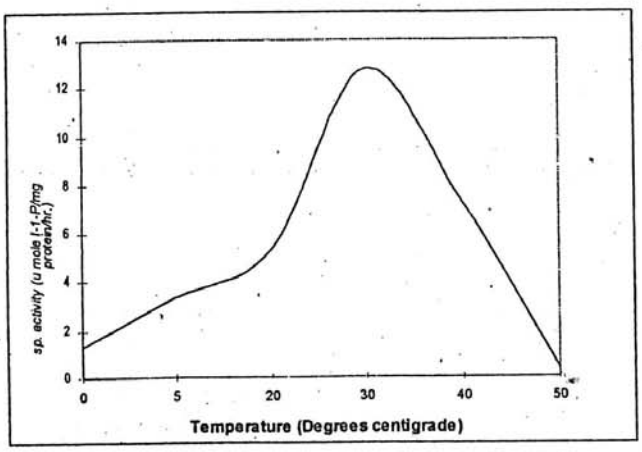

Fig. 2. Effect of various incubation temperatures on *T. baccata* MIPS activity.

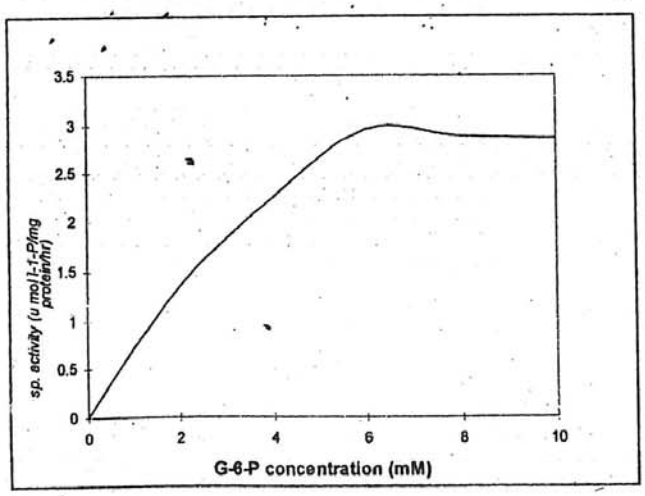

Fig. 3. Effect of varied substrate concentration on MIPS activity in *T. baccata.*

(G-6-P) recorded maximal activity (Table 3). When the specific substrate (G-6-P) was not added in the incubation mixture, the enzymatic synthesis of L-*myo*-inositol-1-phosphate could not be detected. About 16% activity was lost when tris buffer was omitted from the reaction mixture. Deduction of NAD, NH_4Cl or ME resulted in the loss of enzyme activity by about 59%, 51% and 38% respectively.

Stability of enzyme : An important feature of *T. baccata* MIPS is the moderate stability of its catalytic activity. Stability varies with the enzyme at different stages of purification. While the low speed supernatant remained active for 20-22 days when stored at -20°C, the Sephadex-G-200 purified fractions maintained its activity only up to 5-7 days when stored at identical temperature. However, repeated freezing and thawing resulted in remarkable loss of activity. Addition of enzyme stabilizer, 2-mercaptoethanol (ME) or dithiothritol (DTT) considerably increased the activity of the enzyme.

pH – activity relationship : The *T. baccata* MIPS exhibited optimum activity at a pH range of 7.0 – 7.5 when 50 mM tris-acetate buffer at a pH range of 6.0 – 8.5 were employed (Fig. 1).

Effect of temperature and metal ions : The effect of temperature was studied in the temperature range of 0 - 50°C. The activity of the enzyme was least both at 10°C and 50°C. However, it was remarkably active between the temperature ranges of 20-40°C with maxima at 30°C. (Fig. 2). Effect of different metal ions was tested in 1-10 mM concentrations using chloride salts of metals. Of the monovalent cations tested K^+ had a little effect, Na^+ played a minor inhibitory role while NH_4^+ was an appreciable stimulator (165.11% activity) of the enzyme. Using the similar concentrations of divalent cations it was revealed that Ca^{2+} exhibited mild (119.24% activity) stimulatory effect, Mg^{2+} slightly inhibited (20%) and Zn^{2+}, Cu^{2+} and Hg^{2+} strongly inhibited (53%, 46% and 86% respectively) the enzyme activity (Table 4).

Reaction rate-substrate concentration relationship : Kinetic studies were carried out using G-6-P (substrate) in the range of 0-10 mM. The reaction rate was found to increase with respect to G-6-P upto a concentration of 6 mM (Fig. 3). The K_m value for G-6-P calculated from Michaelis-Menten equation was 1.05 mM and the V_{max} value was calculated as 2.95 mM.

In the present communication the occurrence and partial purification of MIPS has been reported for the first time from *T. baccata*. Table-2 depicts the presence of this enzyme from *T. baccata* . Though the enzyme exhibits its optimal activity in presence of co-enzyme NAD, still it could maintain about 42% of the total activity when NAD was not added externally. This emboldens us to conclude the presence of bound NAD in the molecular architecture

is enzyme which has also been reported earlier[22]. S is involved in the metabolic utilization of G-6-P it also generates Ribulose-5-phosphate as a product. activity of this enzyme also seems to be related to of Fructose-1,6-bisphosphatase and coupled with this myriad of biochemical activities of the phosphate esters *yo*-inositol[23-29]. All these points towards a central role ed by this enzyme in metabolism and make this study thwhile in understanding the basic metabolism in its[30-35].

erences

Murashige T and Skoog F 1962, A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* **15** 473-497.

Maiti I B and Loewus F A 1978, *Myo*-inositol metabolism in germinating wheat. *Planta* **142** 53-60.

Chiu T T, Rogers M S, Briton-Jones C and Haines C 2003, Effects of *myo*-inositol on the *in-vitro* maturation and subsequent development of mouse oocytes. *Hum. Reprod.* **18** 408-416.

Adhikari J and Majumdar A L 1988, L–*Myo*–Inositol–1-phosphate synthase from mammalian brain: partial purification and characterization of the fetal and adult enzyme. *Ind. J. Biochem. Biophys.* **25** 408-412.

Pittner F and Hoffmann-Ostenhof O 1979, Preparation of homogeneous crystals of myo-inositol 1-phosphate synthase from rat testicles—further data on the chemical and catalytic properties of the enzyme (studies on the biosynthesis cyclitols, XXXIX). *Mol. Cell. Biochem.* **28** 23-26.

Lohia A, Hait N C and Majumdar A L 1999, L-*Myo* Inositol 1-phosphate synthase from *Entamoeba histolytica*. *Mol. Biochem. Parasitol.* **98** 67-79.

Loewus M W and Loewus F 1971, The isolation and characaterisation of D-Glucose 6-phosphate cycloaldolase(NAD dependent) from *Acer pseudoplatanus* L. cell cultures. *Plant Physiol.* **48** 255-260.

Donahue T F and Henry S A 1981a, Inositol mutants of *Saccharomyces cerevisiae*: Mapping the INO1 locus and characterizing alleles of INO1, INO2 and INO4 loci. *Genetics.* **98** 491-503.

Donahue T F and Henry S A 1981b, Myo inositol 1-phosphate synthase: Characteristics of the enzyme and identification of its structural gene in Yeast. *J. Biol. Chem.* **256** 7077-7085.

Dasgupta S, Adhikari J and Majumdar A L 1984, *Myo*-inositol–1–phosphate synthase from lower plant groups: purification and properties of the enzyme from *Euglena gracilis*. *Physiol. Plant.* **61** 412-416.

RayChoudhury A, Hait N C, Dasgupta S, Bhaduri T J, Deb R and Majumdar A L 1997, L-myo-Inositol 1-phosphate synthase of plant sources. *Plant Physiol.* **115** 727-736.

12. Chhetri D R, Mukherjee A K and Adhikari J 2004, Studies on myo-Inositol and the principal enzyme for its biosynthesis, L-myo-inositol-1-phosphate synthase of pteridophytic origin. *Ind. J. Appl. &Pure Biol.* **19** 67-73
13. Loewus F A and Loewus M W 1983, Myo–Inositol: its biosynthesis and metabolism. *Ann. Rev. Plant Physiol.* **34** 137-161.
14. Majumdar A L, Johnson M D and Henry S A 1997, 1L–myo-Inositol–1-phosphate synthase. *Biochim. Biophys. Acta.* **1348** 245–256.
15. Gumber S C, Loewus M W and Loewus F A 1984, Myo-inositol-1-phosphate synthase from pine pollen: Sulphahydryl involvement at the active site. *Arch. Biochem. Biosphys.* **231** 372–377.
16. Barnett J E G, Brice R E and Corina D L 1970, A colorimetric determination of inositol monophosphates as an assay for D-Glucose–6-phosphate–1L-myo-Inositol-1- phosphate cyclase. *Biochem. J.* **119** 183-186.
17. Adhikari J, Majumdar A L, Bhaduri T J, Dasgupta S and Majumdar A L 1987, Chloroplast as a locale of L–myo-Inositol–1-phosphate synthase. *Plant Physiol.* **85** 611-614.
18. Chen R S, Toribara T Y and Warner H 1956, Microdetermination of phosphorous. *Anal. Biochem.* **28** 1756-1758.
19. Lowry O H, Rosebrough N J, Farr A L and Randall R J 1951, Protein measuremeant with Folin-phenol reagent. *J Biol. Chem.* **193** 265-275
20. Charalampous F and Chen I W 1966, Inositol-1-phosphate synthase and Inositol 1-phosphatase from Yeast. *Methods Enzymol.* **9** 698-704.
21. Gaitonde M K and Griffiths M 1966, A spectrophotometric method for the determination of microquantities of free inositol in biological material. *Anal. Biochem.* **15** 532.
22. Pittner F and Hoffmann-Ostenhof O 1976, Studies on the biosynthesis of Cyclitols. XXXV. On the mechanism of action of myo-Inositol 1-phosphate synthase from rat testicles. *Hoppe-Scylers Z. Physiol. Chem.* **357** 1667-1671.
23. Joseph S K, Esch T and Bonner W D Jr. 1989, Hydrolysis of inositol phosphates by plant cell extracts. *Biochem. J.* **264** 851-856.
24. Drobak B K 1992, The plant phosphoinositide system. *Biochem. J.* **288** 697-712.
25. Martinoia E, Locher R and Vogt E 1993, Inositol triphosphate metabolism in sub-cellular fractions of

barley (*Hordeum vulgare* L.) mesophyll cells. *Plant Physiol.* **102** 101-105.

26. Huang C H, Yan A K F, Crain R C and Cote' G C 1994. Phosphoinositide specific Phospholipase C in oat roots. *Plant Physiol.* **105** 105.
27. Murthy P P N 1996, Inositol phosphates and their metabolism in plants. In: *Sub-cellular Biochemistry*, Vol 26 (Eds.) Biswas B B, Biswas S., Plenum Press, New York pp 227–255.
28. Voglmaier S M, Bembenek M E, Kaplin AI, Dorman G, Olszewski J D, Prestwich G D and Snyder S H 1997, Purification and assay of inositol hexakisphosphate kinase and diphosphoinositol pentakisphosphate kinase. In: *Signalling by Inositides* (Ed.) Shears S., Oxford University Press, New York, pp. 195-201.
29. Ogawa S 1999, Chemical components of Rice bran: *Myo* Inositol and related compounds, a review. *Anticancer Res.* **19** 3635-3644.
30. Loewus F A, Loewus M W, Maity I B and Rosenfield C L 1978, Aspects of myo-Inositol metabolism and biosynthesis in higher plants. In: *Cyclitols and Phosphoinositides.* (Ed.) Wells WW, Eisenberg F Jr. Academic Press, New York. pp. 249-267.
31. Verma D C and Dougall D K 1979, Biosynthesis of myo-inositol: its role as a precursor of cell wall polysaccharides in suspension cultured wild carrot cell. *Planta.* **146** 55-62..
32. De B P and Biswas B B 1979, Evidence for the existence of novel enzyme system; myo-Inositol 1-phosphate dehydrogenase in *Phaseolus aureus. J. Biol. Chem.* **254** 7817-8719.
33. Culberston M R and Henry S A 1975, Inositol requiring mutants of *Saccharomyces cerevisiae. Genetics* **80** 23-40.
34. Raychoudhury A and Majumdar A L 1996, Salinity induced L-myo-Inositol–1-phosphate synthase in rice (*Oryza sativa*). *Plant Cell Environ.* **19** 1437–1442.
35. Iqbal J, Afzal J, Yaegashi S, Ruben E, Triwitayakorn K, Njiti N, Ahsan R, Wood J and Lightfoot A 2002, A pyramid of loci for partial resistance to *Fusarium solani* f. sp. *glycines* maintains *Myo*-inositol-1-phosphate synthase expression in soybean roots. *Theor. Appl. Genet.* **105** 1115-1123.