TRANSCRIPTOME CHARACTERIZATION TO IDENTIFY PUTATIVE GENES RELATED TO AMINO ACID BIOSYNTHESIS PATHWAY AND D-AMINO ACID METABOLISM IN *LATHYRUS SATIVUS*

M.Sc.(Ag.) Thesis

By

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INDIRA GANDHI KRISHI VISHWAVIDYALAYA,
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Thesis

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By

Supriya Nayak

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CERTIFICATE

This is to certify that the thesis Viva-voce in respect of **Supriya Nayak** student of M.Sc. (Ag.) **Department of Plant Molecular Biology and Biotechnology**, has been conducted under the chairmanship of Head of the Department/Dean (in case of out campii) along with Advisory committee on 12/09/2020The necessary corrections have also been made as per comments/suggestions made by the Advisory Committee and Head of the Department/Dean.

Two copies of his/her thesis (in final bound form) are being forwarded separately to the Dean through Head of Department for its onward transmission to the Vishwavidyalaya.

Date: 18/09/2020

Signature of Major Advisor
Dr. Shubha Banerjee

CERTIFICATE – II

This is to certify that the thesis entitled **"Transcriptome characterization to identify putative genes related to amino acid biosynthesis pathway and d-amino acid metabolism in *Lathyrus sativus*"** submitted by **Supriya Nayak** to the Indira Gandhi KrishiVishwavidyalaya, Raipur, in partial fulfilment of the requirements for the degree of **Master of Science in Agriculture** in the Department of **Plant Molecular Biology and Biotechnology** has been approved by the external evaluator and Student's Advisory Committee after oral examination under the chairmanship of head of the Department/Dean (in case of outcampii).

Signature of Head of theDepartment/Dean

(Name: **Dr. S. B.Verulkar**)

Date: 12 | 10 | 2020

Major Advisor _____

Faculty Dean _____

Approved/Not approved

Director of Instructions _____

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%	Per cent
ºC	Degree Celsius
β-ODAP	β-N-oxalyl-L-α,β-diaminopropionic acid
DAPA	β-2-3, diaminopropionic acid
SAT	serine acetyltransferase
RT-PCR	Real Time PCR
RNA	Ribonucleic acid
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
PLPD	Pyridoxal 5-Phoaphate depenedent
OPT	o-phthalaldehyde
NGS	next generation sequencing
KEGG	Kyoto Enclyclopedia of Gene and Genomes
IDT	Integrated DNA technology
CS	Cysteine synthase
CAS	Cyanoalanine synthase
BLAST	Basic Local Alignment Search Tool
et al	And other/co-workers
PCR	Polymerase Chain Reaction
Fig.	Figure
P5CDH	Delta-pyrroline-5- carboxylate dehydrogenase

ROS	Reactive oxygen species
ALDH	aldehyde dehydrogenase
SRO5	overlapping gene in the sense orientation
OAS	o-acetyl serine
H_2O_2	hydrogen peroxide
D-Tyr-tRNA(Tyr)	D-aminoacyl-tRNAdeacylase or D-Tyr-tRNA (Tyr) deacylase.

THESIS ABSTRACT

a) **Title of thesis:** "Transcriptome characterization to identify putative genes related to amino acid biosynthesis pathway and d-amino acid metabolism in *Lathyrus sativus*."

b) Full name of student : Supriya Nayak

c) Major Subject : Plant Molecular Biology and Biotechnology

d) Name and address of Major Advisor : Dr. Shubha Banerjee (Assistant Professor), Department of Plant Molecular Biology and Biotechnology, College of Agriculture, IGKV, Raipur

e) Degree to be Awarded: M.Sc. (Ag.) Plant Molecular Biology and Biotechnology

Signature of Major Advisor

Signature of Student

Date: 19/03/2020

Signature of Head of the Department

ABSTRACT

Grasspea (*Lathyrus sativus L.*) is considered as a hardy and wonderful crop owing to its ability to withstand abiotic stress like drought, waterlogging, salinity and cold conditions and some insect herbivory. It can be grown as mixed crop or as "*utera"* crop with minimal agronomical inputs. Although it is highly nutritious but its cultivation is highly restricted due to its association with a neurotoxic disorder – Neurolathyrism which causes paralysis of lower limbs. The neurolathyrism is caused by a non-protein amino acid β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP). β-ODAP is considered to form from the substrate o-acetyl serine (OAS) (Malathi *et al*.,1970). The immediate precursor of β-ODAP is the BIA which is formed from a ringed structure isoxazolin-5-one. β-ODAP biosynthesis also follows a cyanide detoxification pathway which is supposed to be interlinked with nitrogen and sulphur metabolism pathway. Although the β-ODAP biosynthesis pathway was investigated long back but the genes associated with it are still not known. So in a view to identify the putative genes related to amino acid biosynthesis pathway in *Lathyrus sativus*, functional annotation of transcripts of Mahateora and RLK-1950 was performed.

To functionally annotate the transcriptome data of Mahateora and RLK-1950, a new pipeline MapMan Mercator was approached. This pipeline was specially designed to functionally categorize the orphan plant *omics* data. The Mercator output (excel sheet) functionally described total 94,793 transcripts and assigned a specific functional category to the 20,562 transcripts in to 34 functional category. This pipeline provided us with more functionally described orphan *Lathyrus sativus* transcripts as compared to previously approached CLC Genomics Workbench which described functions to only 28,132 transcripts.

The Mercator output (excel sheet) was screened was enzymes related to the amino acid biosynthesis, cyanide metabolism, ROS metabolism and d-amino acid metabolism pathways. Among amino acid biosynthesis, alanine aminotransferase, Cysteine synthase, asparagines metabolism and ornithine decycloaminase. Alanine aminotransferase was selected as earlier study suggested a differential correlation between L-alanine synthase and β-ODAP. Cysteine synthase was selected due to its involvement in the cyanide metabolism pathway which leads to formation of Cyanoalanine from OAS which subsequently forms isoxazolin-5-one (precursor for

BIA) via asparagine. Consequently, Cyanoalanine synthase, asparaginase and asparagine synthase is also selected. The selection of ROS is dependent due to the fact that whenever the content of ROS increases in the leaves, β-ODAP content reduces significantly and vice-vesa (Jiao *et al*., 2011). One enzyme associated with d-amino acid namely D-aminoacyl-tRNAdeacylase or D-Tyr-tRNA (Tyr) deacylase was selected due to the fact that β-ODAP is a d-amino acid. Also the cyanoamino pathway from KEGG showed interrelation between Cyanoalanine synthase, D-aminoacyl-tRNAdeacylase and Cysteine synthase.

Consequently, 17 transcripts were selected to assess the expression of primers designed from corresponding transcripts in different genotypes of *Lathyrus.*

सारांश

a) थीसिसकाशीर्षक: "तापिरासीटिसमेएमि-बीएसिडबायोसोर्बेंसिसमार्गओरडी अमीनोएसिडचयापनयसोरांबंधितपाचनसंबंधीजी-नएपहचानकरनेकेलिएट्रांसक्रिप्टोमलक्षणवर्णन।"

b) छात्र कापूरा नाम: सुप्रिया नायक

c) प्रमुख विषय: प्लांट आणविक जीव विज्ञान और जैव प्रौद्योगिकी

d) प्रमुख सलाहकार का नाम और पता: डॉ।पुष्पा बलगी (सहायक प्रोफेसर), पाठ्य आणविक जीव विज्ञान और जैव प्रौद्योगिकी विभाग, कृषि महाविद्यालय, IGKV, रायपुर

ई) उपाधि से सम्मानित किया जाएगा: M Sc (Agri) प्लांट आणविक जीव विज्ञान और जैव प्रौद्योगिकी

प्रमुख सलाहकार का हस्ताक्षर

छात्र का हस्ताक्षर

तारीख: 14/09/2020

विभाग के प्रमुख का हस्ताक्षर

सारांश

ग्रासपीया (लैथिरस सैटिवस एल) को सूखा और जलभराव, खारेपन और ठंड की स्थिति और कुछ कीटशाकभक्षी जैसे अजैविक तनाव को झेलने की क्षमता के कारण एक हार्डी और अद्भुत फसल माना जाता है। इसे मिश्रित फसल के रूप में या न्यूनतम कृषि आदानों के साथ

"गर्भाशय" फसल के रूप में उगाया जा सकता है। हालांकि यह अत्यधिक पौष्टिक है लेकिन न्यूरोटॉक्सिक डिसऑर्डर – न्यूरोलैथिरिज़्म के साथ इस की खेती के कारण इस की खेती अत्यधिक प्रतिबंधित है, जो निचले अंगों के पक्षा घत का कारण बनता है। न्यूरोलैथिरिज़्म एक गैर-प्रोटीन अमीनो एसिड N-N-oxalyl-L-α,-diaminopropionic एसिड (β-ODAP) के कारण होता है। considered-ODAP को सब्सट्रेट ओ-एसिटाइल सेरीन (OAS) (मलाथी एट अल., 1970) से माना जाता है। B-ODAP का तत्कालअग्रदूत BIA है जो एक रिंगेड संरचना isoxazolin-5-one से बनता है। β-ODAP जैवसंश्लेषण भी साइनाइड डिटॉक्सिफिकेशन पाथवे का अनुसरण करता है जिसे नाइट्रोजन और सल्फर मेटाबॉलिज्म मार्ग से इंटरलिंक किया जाता है। हालांकि ODAP जैव संश्लेषण मार्ग की लंबी जांच की गई थी, लेकिन इस से जुड़े जीन अभी भी ज्ञात नहीं हैं।अतः लैथिरस सैटिवस में अमीनो एसिड बायोसिंथेसिस पाथवे से संबंधित पुटीय जीन की पहचान करने के लिए, महातेरा और आर.एल.के -1950 के लिपियों के कार्यात्मक एनोटेशन का प्रदर्शन किया गया था।

महातेरा और आर.एल.के -1950 के ट्रांसक्रिप्शनल डेटा को कार्यात्मक रूप से एनोटेट करने के लिए, एक नई पाइपलाइन मैपमैन मर्केटर से संपर्क किया गया था। इस पाइपलाइन को विशेष रूप से अनाथ संयंत्र ओमिक्स डेटा को कार्यात्मक रूप से वर्गीकृत करने के लिए डिज़ाइन किया गया था। मर्केटर आउटपुट (एक्सेलशीट) को कार्यात्मक रूप से कुल 94,793 लिपियों के रूप में वर्णित किया गया और 34 कार्यात्मक श्रेणी में 20,562 लिपियों को एक विशिष्ट कार्यात्मक श्रेणी सौंपी गई। इस पाइपलाइन ने हमें अधिक कार्यात्मक रूप से वर्णित अनाथ लाथिरस sativus टेपों के साथ प्रदान किया जो पहले सी.एल.सी. जीनोमिक्स कार्यक्षेत्र की तुलना में था जो केवल 28,132 लिपियों के लिए कार्यों का वर्णन करताथा।

मर्केटर आउटपुट (एक्सेलशीट) जांच की गई थी जो एमिनो एसिड बायोसिंथेसिस, साइनाइड चयापचय, आर.ओ.एस. चयापचय और डी-एमिनो एसिड चयापचय पथ से संबंधित एंजाइम थे। अमीनो एसिड बायोसिंथेसिस के बीच, एलेनिन एमिनोट्रांस्फरेज़, सिस्टीन सिंटेज़, एस्पेरेगिन्स मेटाबॉलिज्म और ऑर्निथिन

डिकाइक्लामिनाज़। पहले अध्ययन के रूप में एल-अमाइन सिंथेज़ और ODAP के बीच एक अंतरसहसंबंध का सुझाव दिया गया था। सिस्टीन सिंथेस को साइनाइड मेटाबॉलिज्म मार्ग में शामिल होने के कारण चुना गया था जो OAS से Cyanoalanine केगठनकीओरजाताहैजोबादमें Iparazolin-5-one (BIA केलिएअग्रदूत) केमाध्यमसे Asparagine बनाताहै।नतीजतन, साइनाओलानेनसिंथेज़, शतावरीऔरशतावरीसिंथेज़काचयनभीकियाजाताहै। ROS का चयन इस तथ्य केकारण निर्भरकरता है कि जब भी पत्तियों में ROS की सामग्री बढ़ती है, तो ROS-ODAP सामग्री काफी कम हो जाती हैऔरउप-वेसा (Jiao et al।, 2011)।डी-अमीनो एसिड से संबंधित एक एंजाइम अर्थात्डी-अमीनोसिल-टी आर एएन एडीसीएलेज़ या डी-टायर-टी आर एन ए (टीआईआर) डी इसी लैस को इस तथ्य केकारण चुना गयाथा कि β-ODAP एक डी-एमिनो एसिड है। इसके अलावा के.ई.जी.जी. से सायनामैनिनो मार्ग ने साइनाओलेनिन सिन्थेज़, डी-अमीनोइल-टी.आर.ए.एन.डी.सी.एलेस और सिस्टीन सिंटेज़ के बीच अंतर्संबंध दिखाया।

नतीजतन, लाथियस के विभिन्न जीनोटाइप में संबंधित टेप से डिज़ाइन किए गए प्राइमरों की अभिव्यक्ति का आकलन करने के लिए 17 टेप का चयन किया गया था।

CHAPTER-I

INTRODUCTION

Grass pea (Lathyrus sativus L.) is an annual, diploid, winter season pulse crop with chromosome number 2n = 14. It is the third most important cool season pulse crop of India, grown mainly in the states of Chhattisgarh, Madhya Pradesh, Orissa, Uttar and Maharashtra. It's a self-pollinated crop having hypogeal germination with attractive blue, pink, red and white coloured flowers. The pods of lathyrus usually have 3-5 seeds having white, grayish-brown or yellowish and usually spotted. It is commonly called khesari, lakhadi, torea or teora, lakhori, Lakhodi. Lathyrus sativus is usually grown as "utera" i.e fellow crop after rice, with remnant water and nutrients. In India its seeds or split seeds are used to prepare dhal, a type of soup, or the flour of seeds basan are used to prepare bada or pakodi (Yadov, *et a*l., 1992). Lathyrus is a hardy crop as it can withstand inordinate dry conditions, poor soils, cold waterlogging, salinity, heat stress and insect herbivory makes it choice pulse crop for cultivation in challenged soils. It can likewise be cultivated in different altitude regions up to 1300 m altitude in India; in some parts of Ethopia it can also grow upto 2500-3000 m height. Being leguminous Lathyrus productively fixes atmospheric nitrogen (Jiao *et al*., 2011a; Drouin *et al*., 2000) thus its cultivation adds about nitrogen to the field at the rate of 124Kg/ha/year, consequently making it a more efficient crop.

Besides, it is also nutritious too providing 362.3 calories energy, protein 31.6 % (raging from 18.2-34.6% in seeds); fat 2.7%; nitrogen-free extract 51.8%; crude fibre 1.1% and ash 2.2-4.3% crude protein averaged 32.5% (Rahman *et al*., 1974). Like other cool season crops it is deficient in methionine, cysteine and tryptophan but rich in lysine (18.4-20.4 mg/kg) which is quite higher than other pulses (Ravindran & Blair, 1992), essential PUFA αlinolenic, linoleic and γ-linolenic acids are the most abundant. Therefore it is considered as an 'insurance crop' to subsistence farmers.

Even though Lathyrus is a hardy pulse crop with good protein content and taste but the production of Lathyrus is restricted due to the presence of an antinutritional factor. The presence

of β-N-oxalyl-L-α, β-diaminopropionic acid (β-ODAP), also known as β-N-oxalyl-amino-L-alanine (BOAA) in Lathyrus seeds (16.2±0.5 g/Kg seeds) makes it unsuitable for human consumption for longer periods (Rao et al, 1964; Ross et al, 1985; Nunn et al, 1987; Kuo et al, 1998). Prolonged consumption of Lathyrus for 3-4 months triggers characteristic motor neuron system diseases which is a form of spastic paraperesis known as "Neurolathyrism". It causes paralysis of lower limbs. β-ODAP is found in 21 species of Lathyrus, 17 species of Acacia & 13 species of Crotolaria which are leguminous (Quereshi *et al.*, 1977). Only one non-legume species is found in which ODAP is biosynthesized (*Panax ginseng*, *P. notoginseng* and *P. quinquefolius* (Long et al., 2000).Past studies have demonstrated that there are 2 different types of lathyrism caused by different Lathyrus species as Neurolathyrism (paralysis of leg muscles and seizures) and Osteolathyrism (bone structures particularly of legs goes through obsessive changes and miss happening). *L. sativus* is mainly cultivated and is used as food which causes neurolathyrism. Naturally, 2 isomers of ODAP are found in plants: α-ODAP and β-ODAP, which are at equillibrium when heated. α -isomers are present @ of 5% of the total ODAP concentration in the seeds (Roy and Rao 1968). The α-isomers are less toxic then the β-isomers (Wu *et al*, 1976). The amount of β-ODAP increases on exposure to stress such as heat or arid conditions, deficiency of zinc, Iron toxicity etc. (Lambein *et al*., 1996). Increasing levels of Reactive Oxygen Species (ROS) especially, (H2O2) and O-2 leads to diminishing the level of β-ODAP (Jiao *et al.*, 2011). Although all parts of L.sativus contains β-ODAP yet maximum content is reported in leaves at vegetative stage and ripening seeds in pods. Treatments such as washing, boiling the seeds of L. sativus and discarding water have shown to eliminate up to 90% of β-ODAP content (Padmajaprasad *et al*., 1997). Similarly, dry roasting of seeds also lowers the content of seeds upto 50-60% but still the occurrence of lathyrism is recurrently reported in the population consuming Lathyrus in routine.

For decreasing the content of β-ODAP several mutational and conventional breeding strategies were devised and some varieties with low β-ODAP content are also released (Addis & Narayana, 1994; Santha & Mehta, 2001). Some somaclones of L. sativus having low β-ODAP concentration up to 0.03% than their parents were also obtained but they were stable only for 3 generations (Mehta *et al*., 1994). Some somaclones were P-24 and Bio I22, the P-24 parents have 0.3% ODAP content while the somaclone of P-24 have ODAP content of 0.03%. With the

advent in genomics and gene editing tools new strategies aiming at genetic manipulation of the β-ODAP synthesis pathway have the potential to develop low or zero ODPA containing genotypes.

Researchers in past few decades on elucidation of β-ODAP synthesis pathway suggests that the immediate precursor of β-ODAP is β-isoxazoline-5-one-2yl-l-alanine (BIA), a ringed structure which is formed from O-acetyl serine (Ikegami *et al*., 1995). The BIA opens to give a brief intermediate: diamino-propionic acid (DAPA) which is further oxalylated by oxalyl CoA to form β-ODAP. Recent studies (Xao *et al*., 2012, Liu *et al*., 2017) have shown interaction of nitrogen and sulfur metabolism with β-ODAP bio synthesis pathway and its association with the metabolism alanine, cysteine, ornithine, purine and pyrimidine. The enzyme β cyanolalanine synthase (β-CAS) is considered to be a major regulator of β-ODAP synthesis because it is involved in retaining cysteine molecules within the β-ODAP pathway. Still much has not been deciphered due to lack of Lathyrus sativus genome sequence information and very less information on sequence of the genes involved in β-ODAP. The novel gene Cysteine Synthase (LsCSase) is also recognized recently that is helps in the formation of BIA in the pathway pathway with maximum similarity to O-acetyl serine (thiol) lyase of *Medicago truncatula*., with a potential connection between LsCSase gene activation and β-ODAP biosynthesis to manage external stresses in grass pea under some stress condition (Chakarborty *et al*.,2018).

The analysis of leaf tissue transcriptome sequence of two Lathyrus sativus genotypes with high and low ODAP content (Bhariya *et al*.,2018) and analysis have also led to identification of the putative transcripts associated with β-ODAP content in the *L.sativus* seeds. Nayak *et al*., 2019 have also reported association of 2 enzymes encoding genes with ODAP biosynthesis pathway. The enzymes Serine acetyl transferase (SAT) and L-alanine synthase encoded by Paay and PLPD. Pyridoxal phosphate dependent enzyme (PLPD) were assumed to be involved in the pathway using the O-acetyl-L-serine as the common precursor, converting O-acetyl-L-serine to β-isoxazoline-5-one-2yl-l-alanine (BIA) and its isomer β-isoxazoline-5-one-4-yl-L-alanine which does not forms ODAP. The expression analysis of the transcripts corresponding to bacterial homologue of Serine acetyl transferase (SAT) did not show correlation while the level of expression of transcript corresponding to enzyme L-alanine Synthase was reported to be corelated to the seed β-ODAP content. Their study indicated that

two transcripts corresponded to the enzyme L-alanine synthase, and the expression of 2 transcripts was mutually exclusive in low and high ODAP containing genotypes. So, there can exist in 2 isoforms, out of which one proceeds for formation of β-ODAP while other plays role in alanine synthesis.

Based on the previous studies suggesting interaction with nitrogen, alanine, cystine and sulphur metabolism (Jiao *et al*, 2011). with β-ODAP pathway and the leads obtained in the previous studies (Bhariya *et al*.,2018 and Jajati *et al*.,2019) to annotate transcriptome sequence Mahateora (low ODAP) and RLK-1950 (high ODAP) genotypes, analyse the expression of putative candidate transcripts and validate their role in β-ODAP biosynthesis pathway the present study was undertaken to identify the transcripts corresponding to the enzymes involved in cyanide detoxification pathway in relation to ROS metabolism, metabolism of O-acetyl serine and cysteine amino acid pathways. The analysis would be useful to identify and validate the role of enzymes and their corresponding genes that have significant role in the ODAP biosynthesis pathway in Lathyrus sativus. The major objectives of the study were:

1. Characterization and annotation of transcriptome sequences of Lathyrus sativus using Mercator pipeline.

2. Sequence Homology based prediction of function of transcripts to select transcripts related to amino acid biosynthesis pathway and D-amino acid metabolism.

3. Validation of expression of selected genes related to amino acid biosynthesis pathway in high and low ODAP containing genotypes.

CHAPTER-II

REVIEW OF LITERATURE

Grass pea (Lathyrus *sativus L.*) is an annual, herbaceous and cool season crop belonging tofamily Leguminoceae (=Fabaceae). It is a food, feed and forage crop. It's low agricultural input requirement with good yield and high nutritional contents under adverse climatic conditions too make this crop an economically important crop. But due to its antinutritional factor- β-ODAP which cause neurolathyrism.

So the review of literatures found in the head of **"Transcriptome characterization to identify putative genes related to amino acid biosynthesis pathway and d-amino acid metabolism in *Lathyrus sativus*"** is divided under following heads:-

2.6. Area, consumption and breeding of Grasspea

2.7. Nutritional contents

2.8. Antinutritional factor - β-ODAP and factors affecting it

2.9. Biosynthesis of β-ODAP

2.10. Relation of β-ODAP with sulphur metabolism, radical scavenging and different amino acids.

2.11. Functional annotation with the help of different software

2.1 Area, consumption and breeding of Grasspea

Grasspea has large morphological variation mainly in the vegetative especially in the vegetative characters. It has slender trailing or climbing stem having a height of 60cm with multibranches. The leaves are opposite, pinnately compound and have 2 pairs of leaflets which are linear-lanceolate, sessile and are 5-7.5cm long×1cm broad. The flowers are bright-blue, reddish-purple, red, pink or white in colour which are solitary and are on the axillary bud. The pods of Grasspea are flat, oblong, slightly curved, 2.5-4.5 cm long and 0.6-1.0cm wide. Pods have 3-5 seeds in number which are white, brownish-gray or yellow in color with mottled forms too. Seeds are angled or wedge-shaped having 4-7cm in diameter. Grasspea seeds are staple food in many Asian and African countries. In India, spilt pulse is prepared as *dhal* or vada.In Ethiopia, ground seeds are used to prepare a sauce with are eaten with the flat bread or injera. The flour of seeds are also used to prepare *pakoda* in Nepal and flatbread or *roti* in Bangladesh (Campbell, 1994).

The tribe vicieae or fabeae (which contains *L.sativus*) includes many major grain legumes containing genus *Lens* (lentils- 4 sp.), *Vicia* (the vetches &faba beans~140 sp.), *Pisum* (pea – 2/3 sp.), monotypic genus- *Vavilovia* and genus *Lathyrus* (most diverse group- 160 sp.). Genus *Lathyrus* contains species which are used as fodder (eg. *L. ochrus, L. clymenum*), used as ornamentals (*L. odoratus, L. latifolius*, etc.) and also for human consumption *L.sativus* and *L.cicera*). Genus *Lathyrus* has following characteristics:-

- It has adaxially pubescent styles.
- Most of the species has truncate staminal tube tip.
- Supervolute leaflet vernation.
- Some species has winged stems.
- Leaflet veins in some species are parallel, and in reticulate-veined species, they do not loop back from the margins of the leaflet.

According to DNA based molecular phylogenetic evidence, the vicieae or fabeae tribe is paraphyletic. (Kenicer *et al*., 1996).

Graasspea is grown in Rabi season which acqiures the third most important pulse crop grown in India. It is grown in an area of 0.58 million hectare with a production of 0.43 million tonnes (Kaul *et al*., 1986). It is mainly grown in the regions of Chhattisgarh, Madhya Pradesh, Maharashtra, Bihar and West Bengal. Majorly it is grown in Chhattisgarh and Vidharbha region of Maharashtra, where it is grown as relay crop or *utera* crop alongwith paddy.

Grasspea is a very hardy and sustainable crop. It can withstand extreme conditions, it thrives well in the drought conditions, mainly because of its deep penetrating roots. It also grows in floody areas too. The crop is tolerant to extremely dry conditions in drought prone areas as in Ethiopia and also tolerant to excessive floods as in Bangladesh (Smartt *et al*., 1994). It can be grown in areas with rainfall from 300-1200mm. It can be grown in different altitudes upto 1300mts. In Ethiopia, it can be grown up to 2500-3000mts of height. Lathyrus is suited well to different types of soil ranging from very poor to heavy clay soil. Besides all this, Grasspea can also withstand salinity, heat stress and cold stress too. It is also tolerant to some insect and fungal diseases.

Yadav (2006) investigated that Grasspea is grown in spring in temperate regions and in winter season in the sub tropical regions. It can be mainly grown between temperature range of 10-25℃. It can be cultivated in areas recieving 400-650mm of rainfall and an altitude of 1200m in India. This crop grows well in heavy rainfall during early growth stage and can withstand prolonged drought during grain filling stage. Also tolerant to water-logging, salinity and alkalinity conditions.The immature seeds and leaves of young plants are eaten as vegetable curry in India.

In *Lathyrus* sp., natural outcrossing also occurs. Rate of outcrossing is important for deciding the breeding method for improvement of the crop. Although Grasspea is a self-pollinating crop, some percentage of cross-pollination also occurs. The percentage of outcrossing varies from 1.65-2.7% with an average of 2.17%. Therefore, this suggests that some heterozygosity is present in the grasspea (Chowdhury et al,).

Rahman *et al*, found that there is a relation between outcrossing and the flower color. The red colour genotypes has the highest outcrossing rate with 27.8% and the genotypes with white clour flower has the lowest outcrossing rate with 9.8%. The fact that red colour flowers has highest outcrossing rate might be due to that red colour attracts more insects than white colour.

An experiment was conducted in the 3 districts of Chhattisgarh: - Raipur, Durg and Bilaspur to know the present scenario of Grasspea consumption, incidences of lathyrism, nutritional and antinutritonal factors of Grasspea in both rural and urban areas. The results show that urban areas has more β-ODAP content than in rural areas of these 3 districts. The β-ODAP content of seeds collected from Bilaspur, Durg and Raipur respectively were 0.66 ± 0.16 g%, 0.67 ± 0.13 g% and 0.67 ± 0.13 g% from urban areas and 0.62 ± 0.14 g%, 0.64 ± 0.14 g% and 0.59 ± 0.10 g% from rural areas. Protein content were 27 ± 2.39 g% in Bilaspur, 27.0 ± 1.99 g% in Durg and 26.7 ± 1.90 g% in Raipur districts. The mean consumption was higher in Bilaspur (20.9 g/CU/day) then in Raipur districts. The mean consumption was nil in the urban Raipur areas. This study suggests that the β-ODAP content was less, so that the low-toxin varieties can be introduced in the different states of India (Khandere *et al*., 2014).

Khandere *et al*., (2015) also tried to find out the Grasspea consumption and its effect on the people of Maharashtra. They grow it in the Rabi season. It was conducted in the Gondia

district. Here litreracy level is high (81%) but are mainly depended on agriculture. Maximum households (HH) (61%) consume graspea in different forms (gravy or vada or dal). OBCs majorily consume Grasspea by cultivating it in their respective fields only. Some socio-economically backward classes (6HH) consume it in high quantity (25g/day) and others consume it less (12-25g/day). Out of 32 samples collected from different villages maximum concentration of β-ODAP was found from Bora village with amount 1254.5+528.21 mg % and minimum was found from Menda village with concenteration of 102.6±248.38 mg %. This study reveals that consumption of low amount of Grasspea does not leads to neurolathyrism.

G.B.Polignano *et al*, in their experiment showed that the most variable agronomic traits were Seed yield, biomass, leaf width and no.of seed and pod among the 16 agronomic quantitative traits (1. Time to emergence 2.Time to flowering 3.Length of longest stem 4. Height of 1st podded node 5. Length of internode 6.Leaf length 7.Leaf width 8.Pod length 9.Pod width 10.Pedicel length 11.No.of seeds and pods 12.Seed length 13.Seed width 14.Seed thickness 15.Seed yield 16.Biomass. The lowest variability was seen for Time to emergence & time to flowering and rest traits were of intermediate variability.

Earlier *L.sativus* is cultivated vividly from the Mediterranean, near Temparate as well as from Tropical countries too. In contrast, *L.cicera* is cultivated only in the Southwestern Europe. Also from the combined studies of Archaeobotanical and Phylogeographical evidences, it is concluded that the origin of *L.sativus* is at Balkan Peninsula in the early Neolithic period at the 6000BC. It is also suggested that *L.sativus* may be the first crop to be domesticated in the Europe as a consequence of expansion of agriculture from near east. (Kislev, 1988)

The genetically nearest wild species to *L.sativus* is *L.cicera*. So it is hypothesized that *L.sativus* is the derived from*L.cicera* .Also during expansion of domestication of *L.sativus,* evidences of cultivation of *L.cicera* were found (Hopf, 1986).

From the studies of electron micrograph, the difference between *L.sativus* and *L.cicera* is shown. While *L.cicera* have dense papillae projected from the epidermis; *L.sativus* has less dense papillae protruding from a common base structure. These characters are specific for both of them. It is also suggested that small seeds have more dense papillae than larger ones. As *L.sativus* has wide and more rounded papillae with a blunt summit having long, radial and

prominent ridges which interconnects neighbouring papillae but seed coat of *L.cicera* has conical papillae with pointed summit having short and temporary ridges which are not connected to neighbouring papillae. So there is some relationship size of seed and the density of papillae. (Kislev and Hopf, 1985).

Grasspea has a good tolerance capacity in Lead (Pb) accumulated soils. Eleven-days old Grasspea plants were grown in the hydroponic solution containing 0.5 mM lead nitrate (Pb(NO3)2). The survival rate is 100% for this hydroponic plants contaminated with Pb. However, plant growth is affected. The root length was more affected then the shoot length by lead accumulation. Also it leads to change of colour in roots from whitish to light brown and root dry biomass is reduced too. Pb pollutant does not affects leaves per plant and water content (Relative Water Content) in the leaf tissues. Compared with control plants, lead-exposed plants showed a six-fold, two-fold and three and a half-fold reduction in their root calcium, zinc and copper contents, respectively but amount of sodium increases. On the basis of ICP-OES measurement, the mean lead content was 153 mg Pb g^{-1} in the root tissues. This shows that lead is tightly retained by the roots of *L.sativus*. So it can be used as potential phytoextracting species in rhizofiltration setups.

Genetic Resources & Breeding of *Lathyrus*:-

- ➔ The ICARDA of Syria with its main research centre at Morocco has the highest germplasm collection of *Lathyrus* with around 4200 accessions.
- ➔ In the Royal Botanical Garden, Kew of UK also has a large germplasm collection with 1115 accessions.
- ➔ The National Bureau of Plant Genetic Resources (NBPGR) of India has 2600 accessions which are being maintained at Indira Gandhi KrishiVishwavidyalay (IGKV).
- ➔ ICARDA has the largest *L.sativus* collection with 2175 accessions; also has maximum around 1900 accessions of species collection.
- ➔ The Genesysdatabase supported by the Crop Diversity Endowment Fund contains 6580 accessions of grass pea.

53 accessions of *L.sativus* from India were analysed for morphology and variability of seeds. As a result on the basis of coefficient of variation (CV) 58% seeds are of rhomboid shape

(15.38 ±1.23), 94%CV seeds are oblate shape (11.25±1.46). Also square and oblong triangular seeds were found. Mostly seeds are of Black, gray, gray mottled, grayed white and yellow white coloured. Frequently the seeds are of yellowish brown and brown coloured and rarely are of red purple and pink coloured seeds. Smooth seed coat accessions were minimum. Small sized seeds were frequent in this accessions followed by medium sized seeds which is followed by large sized seeds. The 100 seed weight of these accessions showed the mean value of 5.95g ± 0.13 with range of 4.03g ± 0.07 to 8.82g± 0.09. The protein content has a mean value of 25.6% with range of 21.9 to 33.7%. There is a positive significant correlation between 100 seed weight and total seed protein content on the basis of multivariate linear regression analysis. (Surendra Barpete, 2015).

Tiwari and Campbell (1995) studied the inheritance of ODAP concentration with flower colour and seed coat colour. 5 Grasspea with low to high ODAP concentration were taken. They are then intercrossed with each other. Results showed that most of the progenies of Low ×Low ODAP concentration lines have low ODAP concentrations. Similarly the progenies of cross Low× High ODAP concentration has intermediate ODAP concentration suggesting that it is being quantitatively inherited. Also reciprocal crosses resulted heterosis that is reciprocal crosses produces progenies with higher ODAP concentration which is more than high ODAP concentration lines. This indicates that there is a maternal effect on ODAP concentration. Blue coloured flowers has coloured or speckled seed coat white colur flower has white seed coat. A cross between white and blue coloured flowers produces blue colour F1 progeny and in F2 generation it segregates in a ratio of 13:3. So blue flower colour are dominant over white flower colour suggesting that there are interaction 2 genes responsible for flower colour. Similar pattern was observed in seed coat colour with 13:3 segregation ration in F2 progenies.

A set of 50 genotypes of *L.sativus* available at IGKV Raipur which includes germplasm lines and vareities was analysed for morphological characters, protein content and β-ODAP contents. Results revealed a high variation among all the studied genotypes for all traits. The β-ODAP ranged from 0.07-0.57 mg/g with an average of 0.29mg/g and protein content of seed was recorded to be an average of 24.29mg/g. observations showed a strong positive correlation between pod yield and seed yield/plant and between seed yield/plant and biological yield. There

is a negative or non-significant correlation between β-ODAP and most of the traits (Minakshi*et al.,* 2015).

13 lines from F2 population and 14 varieties as parents of *L.sativus* was observed for different morphological characters like days to flower, plant height, branches per plant, pods per plant, 100 seed weight and seed yield per plant. Pods per plant and seed yield per plant showed highest coefficient of variation among all morphological characters. Broad sense heritability was high for pods per plant (0.70-0.90), seed yield per plant (0.74-0.95) and low to high heritability for plant height (0.03-0.78). There was significant progress under selection for the characters – plant height (0.82-32.66cm) and pods per plant (30.56-56.51) for the expected genetic advances (Chakrapani *et al.,* 2008).

Ambade *et al*., (2014) used 24 ISSR markers to find the genetic diversity of *L.sativus* at molecular level for 48 genotypes. 12 ISSR markers out of 48 expressed polymorphism. So the selected genotypes were grouped into 2 clusters with a similarity coefficient of 0.61 which ranges from 0.61-0.92. The maxium similarity coefficient was observed between RLK-637 and RLK-466 with 0.92 similarity coefficient while the minimun was observed between BIOL-203 and RLK-120 with 0.63 similarity coefficient.

2.2Nutritional contents

- Seeds of *L.sativus*contain protein -18.2-34.6%, carbohydrate -58.2% (about 35% starch), fat-0.6% (Duke, 1981; Williams et al., 1994).
- The seeds of *Lathyrus* are deficient in lysine, methionine, isoleucine, iodinevitamins C, D, E and B1, fluorine, lipids, sodium chloride, cobalt, phosphorus and sulphur.
- Another study reveals that 100g edible portion of seeds contain : moisture- 10g, protein- 25.0g, fat- 1.0g, total carbohydrate- 61.0g, fibre- 15.0g, ash- 3.0g, calcium- 110mg, Fe- 5.6mg, Vitamin A- 70 IU, thiamine- 0.1g, riboflavin- 0.4 mg & starch- 34.8% (of which 30.3% is amylose and 69.7% is amylopectin).
- Also the seeds contain: sucrose- 1.5%, pentosans- 6.8%, phytin- 3.6%, lignin-1.5%, albumin- 6.6%, prolamine- 1.5%, globulin- 13.3% and glutelin- 3.8%.
- Lysine and phytate are in high amount in the seeds of *L.sativus* and *L.cicera* (Enneking).

- Some condensed polyphenols are also present which has some correlation with the seed coat colour. (Deshpande and Campbell, 1992).
- In *Lathyrus* the content of saturated fatty acids were higher (369.32 g/100 g; 53.69%) then the amount of unsaturatedacids (322.44 g/100 g; 46.61%).
- There is a low lipid content (<2%) in the seed of *L.sativus* (Buchanan, 1904).
- It has 40 IU kg^1 of Vitamin-E in the seeds (Grela and Günter, 1995).
- Amounts of some essential amino acids are (in grams per 16 grams of nitrogen): arginine-7.85, leucine-6.57, histidine-2.51, lysine-6.94, isoleucine-6.59, methionine-0.38, threonine-2.34, phenylalanine-4.14, tryptophane-0.40, and valine-4.68.
- The leaf of *L.sativus*contains: moisture-84.2%; fat (ether extraction)-1.0%; crude protein-6.1%; carbohydrates-7.6%; ash-1.1%; Ca-0.16%; Fe-7.3 mg and P-0.1%; and carotene (as vitamin A),-6,000 IU/100 g.
- Also when Grasspea seeds are germinated some of its vitamin content increases especially folic acid, pyridoxine and (Dukeriboflavin, 1981; Williams et al., 1994).
- A neurotoxin β-ODAP is also present in the Grasspea which is about 16.2±0.5 g/Kg in quantity.
- The seeds of *L.sativus*are used in homeopathic medicines in local areas.

2.3Antinutritional factor - β-ODAP and factors affecting it

Although being a hardy, nutritious, sustainable and an advantageous crop, L.sativus also contain an antinutritional factor-β-N-oxalyl-L-α,β-diaminopropionic acid (β-L-ODAP); also known as β-N-oxalyl-amino-alanine (BOAA)(Aletor *et al*., 1994; Gannon and Terrian, 1989; Ormandy and Jope, 1990; Pai and Ravindranath, 1993; Ross *et al*., 1989; Weiss *et al*., 1989) in many literature which is a non-standard, non-proteinaceous amino acid. It is found in all part of the plant which are its seeds, vegetative and reproductive parts (Murti *et al*., 1964; Jiao *et al*., 2006.). It is a neurodegenerative disease which causes paralysis of lower limbs (a form of spastic paraperesis) known as neurolathyrism not only in humans but animals too. Over consumption or consuming Grasspea as a staple food for 3-4 months causes this neurodegenerative disease (Spencer *et a*l., 1986; Yan *et al*., 2006).

ODAP occurs in 2 isomeric forms – α -ODAP and β –ODAP. When heat is provided, the 2 forms of ODAP eqillibrates i.e., interconversion of β and α forms. This interconversion is achieved via an intermediate till an equilibrium ration of 3:2 is attained ((Bell and O Donovan, 1966) Naturally, in the seeds of *L.sativus*α-form occurs only 5% 0f total ODAP content and rest is the β- form (Roy & Rao,1968). The α-form is less toxic then β- form or non-toxic form of ODAP (Wu et al., 1976). When α-form is injested in the cerebrospinal fluid, it is non-neurotoxic. Also when this form is analysed in tissue culture, it lacks neuro toxic property (Chase *et al*., 1985; Nunn *et al*., 1987).

β-ODAP act as the structural analogue of a class of glutamate receptor of neuron - the AMPA-receptors (alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propanoic acid). Thereby inhibiting the uptake of glutamate again which enhances the duration of excitotoxicity (Kusama *et al*., 2000).

Besides β-ODAP which is found in *L.sativus,* there are other neurotoxins too which leads to neurolathyrism in the laboratory animals. The other neurotoxin found in n *L.odoratus* (sweet pea), *L. hirsutus, L.pusillus and L. roseus* is thethe c-glutamyl derivative of b-aminopropionitrile (BAPN) -d b-(N-c-glutamyl)-aminopropionitrile. And another neurotoxin found in *L.sylvestris* and *L. latifolius* is L-2, 4-diaminobutyric acid (DABA) (Murti and Seshadri, 1964; Barrow et al., 1974; Spencer, 1989&Foster, 1990.).

β-ODAP is not only found in the *L.sativus* but also in the 21 species of the *Lathyrus*. This is found mainly in the *L.sativus, L. cicera and L. clymenum.*β-ODAP is also found in other genera of legumes which includes 17 species of Acacia and 13 species of Crotalaria. There is only one non-legume in which β-ODAP is found- the older ginseng roots containing P. ginseng, P. notoginseng and P. quinquefolius (Quereshi et al., 1977; Long et al., 1996).

ODAP is considered as the excitotoxicity substance. It is one of the most anionic amino acid and a good metal chelator.in some studies L-ODAP is considered to act as agonist for α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor or glutamate receptor agonist (Lambein et al,. 1994; Watkins et al,.).

Some studies also reveal that L-ODAP inhibits the action of enzyme Tyrosine Aminotransferase (TAT) both in vitro and in vivo. After ODAP treatment in the black mice, the DOPA content and catecholamine of brain significantly increases. Also the mice which normally does not respond to L-ODAP, when treated with tyrosine becomes susceptible to it. So it is suggested that the cause of neurodegeneration might be the excessive production of catecholamine (6-OH DOPA) which are the good neurotoxins. This explains the difference in susceptibility for L-ODAP between different species (Shashivardhan et al, 1994; Cha et al,.).

The variation in theamount of ODAP content in *Lathyrus* after milling, roasting and boiling is studied. To study its effect 12 L.sativus genotypes were taken of which 6 are having low ODAP concentration and 6 have quite high ODAP concentration. In this genotypes, the ODAP concentration ranges from 0.42 to 1.18 mg g^{-1}. Dehusking of grains leaves *dahl* considered as the most digestable form. After dehusking 17.3% of total β-ODAP increases which is in the range of 0.53 to 1.29 mg g^{-1}. This is due to that most of β-ODAP lies in the cotyledons and dehusking is simply removal of seed coats which results in the increase of this compound. The dehusked grains of L.sativus when subjected to roasting (dry roasting) at 120°C and 150°C resulted in a significant decrease by 43.18% and 44.32% respectively. Cooking of unprocessed dehuskedgrain also marginally reduces the ranging from 0.81 mg g^{-1} to 0.77 mg g^{-1}.

Shrivastava and Khokhar (1996) investigated that when pre-soaked *Lathyrus* seeds were cooked and pressure cooked, levels of natural toxic compounds in this plant is reduced. While the germination and fermentation of its seeds helps in destroying of the enzyme inhibitors (amylase inhibitors-69-71%; trypsin inhibitors- 65-66%). Lectins are only marginally effected by cooking and fermentation. When pre-soaked seeds were dehusked, the β-ODAP content was reduced significantly but other anti-nutrients were not much effected. So they suggested on the basis of both their findings and high-water solubility that this simple and effective means can be practically used for reducing the β-ODAP content in *L.sativus*.

Selye (1958) investigated that consumptiom of *Lathyrus* causes 3 different types of symptoms in laboratory animals. 2 symptoms are caused due to the consumption of *L.sativus*: 1st in small laboratory animals like chicks and rodents, a neurotoxic disorder with convulsions appears. Second, in primates also neurolathyrism occurs due to the consumption of β-ODAP develops a central motor pathway and hindlimb deficits (similar to that occurs in humans). Third

syndrome occurs as osteolathyrism and angiolathyrism due to the consumption *L. odoratus* (sweet pea) which has b-aminopropionitrile (BAPN) as the neurotoxin which inhibits the activity of lysyl oxidase, (enzyme helping in collagen formation). Its effect results in the collapse of vertebral column due to abnormal connective tissue which leads to secondary paralysis and abnormal blood vessel walls (Barrow et al., 1974; Roy, 1988; Spencer et al., 1986).

29 varieties of *L.sativus* from different places of India were analysed for some association between biochemical content (BOAA content, and trypsin inhibitory activity (TIA), moisture and extractable ether fraction, etc.) and color and size of the seeds. There are 5 white, 8 light grey or light brown, 14 were grey or brown, and 2 black coloured seeds. The white coloured seeds had 0.41% β-ODAP content, 0.49% in light grey or light brown coloured seeds, 0.58% in grey or brown and 0.74% in black coloured seeds. Statistically, no correlation is found between TIA content and size and colour of seeds; between extractable ether fraction and seed colour and size. So, no stastical correlation was found between them. (Roy. 1978).

The leaf explant of *Lathyrus sativus L*. were cultured in the synthetic media under different environmental and stress conditions with different concentrations of micronutrients to find the effect on the biosynthesis of the neuro-excitatory b-ODAP (b-N-oxalyl-L-a,b-diaminopropionic acid) was induced by feeding the precursor BIA, (b-isoxazolin-5-on-2-yl)-alanine. When the amount of copper was increased, the biosynthesis of β-ODAP decreases in the nutrient medium. Increasing the concentration of Aluminium also enhaces the conversion of BIA into β-ODAP. Iron and zinc were supplemented in combination, increasing amount of zinc decreases the formation of β-ODAP. Increasing amount of iron also increases the production of β-ODAP suggesting that it might have a role in the β-ODAP biosynthesis pathway. But increasing amount of zinc leads to lower the formation of β-ODAP. When salinity increases by increasing amount of NaCl, the formation of β-ODAP from BIA decreses. (Haque et al.,)

β-ODAP content in the plant of *Lathyrus sativus L*. vary with the stages of its life. Although it is present on all parts of plant but with age of plant its formation alters. The nitrogen rich fields have less β-ODAP accumulation than the nitrogen poor fields. The younger seedlings (6-days old) were to have 3 times more β-ODAP content then the original seeds. The amount of β-ODAP gradually decreases from all parts (leaves, stem and roots) of Lathyrus plant. Its content in the yellowing leaves, stems and roots were virtually devoid at the seed maturation execpt stage

except the maturing seeds (125days). But β-ODAP content slightly increases during flowering and pod formation stage. (Jiao et al.,).

Jiao et al., suggests that Nitrogen and Phosphate are the crucial macronutrients not only for growth of plant but also for the β-ODAP biosynthesis. Their study reveals that the β-ODAP formation is much less in the N_2- rich fields than in the N_2-poor fields. Due to the high-levels of free- N_2 compounds during germination and young seedling stage, the levels of β-ODAP increases. So they suggested on the basis of their hydroponics experiment that increasing β-ODAP content in N_2 were related to N-metabolism. Also both in phosphorus-rich and phosphorus-poor fields there is a sharp increase in the β-ODAP content.

Berger et al. reported that increasing salinity level also decreases the β-ODAP content up to a certain level in both low and high varieties. Bur further increasing the salinity level now help in increasing the formation of β-ODAP content.

The lathyrism epidemic or problem generally occurs during the drought condition. Although *L.sativus* is known as drought-tolerant crop but it effects the physiological processes. So in experiments, researchers found that increasing drought conditions also increase the β-ODAP content in plants.

Grasspea also fixes atmospheric N_2 with the help of Rhizobia. These rhizobia forms nodules in the roots in the early seedling stage then gradually forms symbiotic association with the roots. Alongwith higher β-ODAP accumulation in the early seedling stage nodule formation starts. Furthermore it is also seen that rhizobium growth is more when there is less synthesis of β-ODAP. Also, no traces of β-ODAP were found in the nodules of *L.sativus.* So there exists a relation between the growth of rhizobia and β-ODAP. Also this can explain why the roots have lower levels of β-ODAP then shoots. (Jiao et al.,)

2.4 β-ODAP biosynthesis

Zhang et al., (2003) proposed that there is relationship between the activity of glycolate oxidase (GO) and the content of β-N-oxalyl-L-α, β-diaminopropionic acid (ODAP) under high light. In the experiment, they sprayed 2-hydroxy-3-butanoate (BHB, inhibitor of GO) on the leaves of a 15- days old seedlings. They analysed that 32% of GO activity decreases during BHB

treatment and 55% ODAP concentration also decreases when kept in darkness. So they are positively correlated by value r = 0.97. Also it was proposed that oxalate was a precursor in the ODAP formation. So they treated the seedlings with oxalate and found that as the concentration of oxalate increases, the ODAP content and the activity of GO also increases at steady level under high light. So they suggested that *L.sativus* by scavenging the hydroxyl ions protects the activity of GO using oxalate as precursor to form ODAP.

Malathi et al., (1969) showed the biosynthesis of neurotoxin - β-N-oxalyl-L-α, β-diarninopropionic acid (ODAP) in *L.saivus*. They showed that in the first reaction oxalyl-CoA is formed from oxalate, coenzyme A and ATP synthesized by oxalyl-CoA Synthase. This enzyme has similar properties as that of enzyme in Peas. Second reaction is catalysed by an enzyme which is specific to *L.sativus*-oxalyl-CoA-~, fl-diaminopropionic acid oxalyltransferase. The reaction is as follows:

$$\text{Oxalate} + \text{ATP} + \text{Coenzyme A} \leftrightarrow \text{Oxalyl-CoA} + \text{AMP} + PP_t$$

Oxalyl-CoA + L-, β-diaminopropionic acid ODAP + Coenzyme A. ODAP synthase

B-ODAP is formed from the oxyalylation ofoxalyl coenzyme A and β (isoxazolin-5-on-2-yl)-L-alanine (BIA) which converts into a short-lived intermediate 2, 3-diaminopropionic acid (DAPA). (Malathi et al., 1970; Kuo et al,. 1998).

As earlier suggested that BIA is the precursor for ODAP in its biosynthesis pathway and DAPRO was just a short lived intermediate. To investigate this tissue culture plantelets were taken and incubated with radioactive [^{14}C] BIA alone and also with DAPRO. So the rate of formation of ODAP in alone [^{14}C] BIA was more as compared to with DAPRO. Also as the concenteration of DAPRO increases the incorporation [^{14}C] BIA decreases. But its incorporation was stimulate by the oxalates which was analysed in paper chromatography. So, this suggests that the inhibition by DAPRO was concentration dependent and was only a short-lived intermediate in the β-ODAP biosynthesis pathway (Kuo& Lambein., 1991).

Ikegami et al., (1999) confirmed *in vivo* that the direct precursor of β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) is β(isoxazolin-5-on-2-yl)-L-alanine (BIA). BIA enzymatically

breaks down into 2, 3-diaminopropionic acid (DAPA, which is a short-lived intermediate) and oxalyl CoA by the process of oxylation as proposed by Lambein.

Ikegami et al., (1994) suggested that there are two isoforms of enzyme Cysteine synthase (CSase, also known as *O*-acetylserine (thiol) lyase [EC 4.2.99.8]. Naturally, they catalyses many reactions, it catalyzes the reaction in which neurotoxic amino acid *gb*-cyano-l-alanine is formed from*O*-acetyl-l-serine OAS and cyanide. Also CSase mainly catalyse the formation of l-quisqualic acid and *β*-(pyrazol-1-yl)-l-alanine (heterocyclic *β*-substituted alanines) from OAS. This activity in higher plants are different in which 3-cyanoalanine synthase (BCASase) catalyses the reaction of 3-cyano-L-alanine with L-cysteine and cyanide as a substrate.

The Cysteine formed in the ODAP biosynthesis pathway catalysed by Cysteine Synthase (CS). This Cystein when catalysed by Cyanoalanine synthase (CAS) gives β-Cyanoalanine which is then converted to the isoxazolin-5-one having asparagine as an intermediate. This pathway also helps in the cyanide detoxification as HCN is utilized here to form β-Cyanoalanine (Machingura, M., 2006).

Chakarborty et al., (2018) proposed that the down regulation or deactivation of Cystein synthase (LsCSase) gene can help in development of toxin-free genotypes. They identified a novel cysteine synthase gene (LsCSase) and characterized with the help of bioinformatics approaches from *Lathyrus sativus* L. The bioinformatics analysis showed that LsCSase has maximum similarity with the O-acetyl serine (thiol) lyase of *Medicago truncatula* which also has many sequence-specific motifs like cysK, CBS like, ADH_zinc_N, PALP, etc which are localized subcellularly in the cytoplasm or mitochondria. They also suggested that there is a relation between activation of LsCSase gene and the synthesis of β-ODAP due to external stress management on the basis of studies of the tissue-specific regulation of the LsCSase and its transcriptional activation under certain stressed conditions like low Zn^{+2}-high Fe^{+2}, osmotic stress.

Rao*et al*., (1978) illustrated a spectrophotometric procedure for determining the neurotoxic non-protein amino acid 3-Noxalyl-2,3-diaminopropionic acid (β-ODAP) which involves the alkaline hydrolysis of β-ODAP to produce α,β-diaminopropionic acid. This product is then

reacted with o-phathaldehyde (OPT) in presence of etahnethiol to produce a coloured compound which quantifies at 420 nm. This procedure is applicable to calculate the content of β-ODAP in *L.sativus*seeds and in the tissues of rat which is infused with the β-ODAP. But this procedure doesn't differentiate between the 2 isoforms of ODAP- β-ODAP& α-ODAP as α-ODAP is less neurotoxic or non-neurotoxic then the β-ODAPand can be easily converted to α-form by heating.

A modification in the spectrophotometric method of Rao et al., was detailed by Briggs *et al.*, for small samples. They analysed only a small amount (0.5grams) for determining the β-ODAP content in the Grasspea seeds by utilizing o-phathaldehyde (OPT) as a reagent followed by the alkane hydrolysis of β-ODAP. This method is as sensitive and specific as the Rao*et a*l's calorimetric method for 3-4 seeds only. This method isstill utilized in the production of low-toxic breeding programmes.

Cristina Megías*et al*,.(2006) analysed the content ofneurotoxic non-protein amino acid 3-Noxalyl-2,3-diaminopropionic acid (β-ODAP) and other free amino acids in *the Lathyrus cicera*and *Lathyrus sativus* with the help of reverse-phase High Performamnce Liquid Chromatography (RP-HPLC). Before analysing in the HPLC, seed extricates were derivitizedby reacting it with diethyl ethoxymethylenemalonate (DEEMM). This procedure showed a good linearity of response in the calibration curve and an exceptional accuracy of 99% in spiked samples with a high intra- (RSD<0.42%) and inter-repeatability (RSD= 2.01-2.33%). The limits of detection (LOD) and quantification (LOQ) were 0.15 and 0.50 μM, respectively. This procedure yields a similar result when compared to the calorimetric method with the simple HPLC euipments. It can perform a large number of samples simultaneously and is easy to handle.

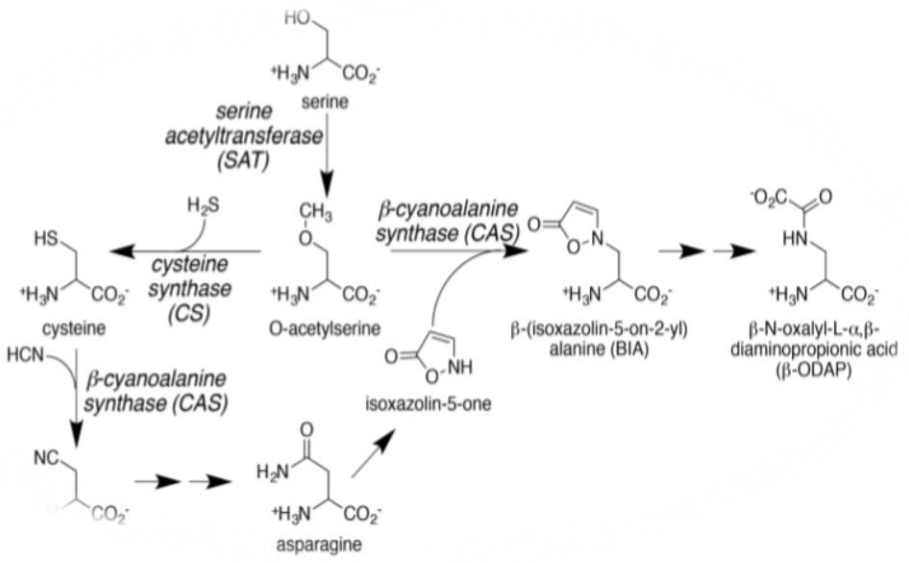
2.5 Relation of β-ODAP with sulphur metabolism, radical scavenging and different amino acids

Kusama-Eguchi et al., (2011) suggested that the lack of sulphur-containing amino acids like cysteine and methionine in diet effects the toxicity of β-ODAP. They investigated this experiment by preparing a media in which these amino acids are either present or absent and a primary motor neuron culture and a motor neuron cell line NSC-34 (these cells are immunostaining using a motor neuron-specific antibody SMI32). When these cultures were

deprived of amino acids the toxicity of β-ODAP is alleviated by 66%. In the amino acid deficient medium, glutathione content greatly declines. By adding reduced glutathione ethyl ester or N-acetylcysteine to the media, the toxicity is neutralized. This suggests that under sulfur-deficient amino acid condition the importance of oxidative stress of mitochondria which is induced by β-ODAP.

Xing et al., (2001) investigated that the association between scavengers of oxidases like superoxide dismutase (SOD), catalase, peroxidase, and glutathione reductase and Hydrogen peroxide and β-ODAP. The activities of these enzymes were measured on the basis of changes in lipid peroxidation during different duration of PEG treatment (oxidative stress). As the duration of PEG treatment increases, the activity of superoxide dismutase, catalase and peroxidase decreases significantly while the activity of glutathione reductaseincreases. Also the concentration of β-ODAP, hydrogen peroxide and extent of lipid peroxidation is increased. ThediethyldithiocarbamateDDC (inhibitor of SOD) and aminotriazole AT (inhibitor of catalase) strongly inhibits the activity of SOD and catalase but extent of lipid peroxidation increases significantly. Mannitol decreases the extent of lipid peroxidation. But the accumulation of β-ODAP is independent of activity of mannitol, DDC and AT. This results suggests that there is no direct relation between the accumulation of ODAP and the metabolism of free radicals.

When the formation of hydroxyl radical (·OH) increases the content of β-ODAP in Grasspea seedlings, when ·OH is provided exogenously. ·OH initiates the lipid peroxidation. Lipid peroxidation activity also increases when ·OH is supplemented exogenously in the Grasspea seedlings. ODAP also competes with salicylate for hydroxyl radicals in the hydroxyl radical generating/detecting system in vitro. This suggests that β-ODAP may act as hydroxyl radical (·OH) scavenger (Gongke et al., 2001).



Hatzfeld*et al.,* (2000) hypothesized that the isoform of Cysteine Synthase (CS), Bsas 3 (β-substituted Ala synthase) is similar toβ-Cyanoalanine synthase (CAS) in the spinach and Arabidopsis. It is hypothesized that CAS (Bsas 3) is a mitochondrial enzyme. B-cyanoalanine is formed from Cysteine and HCN (hydrogen cyanide) which is being catalysed by mitochondrial localized β-Cyanoalanine synthase (CAS).

HS-CH2CH (NH2) COOH+ HCN 3 → NC-CH2CH (NH2) COOH +H2S
(L-cysteine) (Cyanide) (3-cyano-l-Ala)

A novel Cysteine synthase (LsCSase) gene was identified from the *L.sativus* which is localized within the mitochondria or plastid. This enzyme belongs to the superfamily Tryptophan synthase beta (Trp-Synth-beta_II superfamily) which has significant conserved domains like Zinc-binding dehydrogenase (ADH_zinc_N) with other essential motifs like PALP_1 (Pyridoxalphosphatedependent enzymes), PKC/CK2_PHOSPHO_SITE (Protein kinase C phosphorylation site/ Casein kinase II phosphorylation site), CYS_SYNTHASE (Cysteine synthase/cystathionine beta synthase P-phosphate attachment site). According to bioinformatics approaches this enzyme LsCSase has found to be similar with the O- acetyl serine (thiol) lyase of *Medicago truncatula*. Also this study uncovers that there is some connection of the activation of LsCSase enzyme and β-ODAP biosynthesis to deal with the abiotic stress conditions (Chakarborty*et al.,*).

According to literature of Yi *et al.,* (2012) it is suggested that there is a link between S-associated Cysteine and ODAP biosynthesis pathway. The heterocyclic BIA (β-isoxazolin-5-on-2-yl) is the precursor of ODAP which is formed from the β-cyanoalanine catalysed by the enzyme β-cyanoalanine synthase. The 2 enzymes CS and CAS both follows up on the similar substrate O-acetyl serine. Additionally both have the pyridoxal dependent activity too which comes under superfamily β-subsituted alanine synthase (BSAS).

The consumption of sulphur-containigamino acids like methionine and cysteine reduces the effect of β-ODAP and neurodegenerative diseases. Depletion of these amino acids in the cultured cells increased the intensity of neurolathyrism. This study demonstrates that methionine and cysteine act as antioxidants in the oxidative stress and assist in the neurolathyrism (Kusama-Eguchi*et al.,* 2011 Getahun*et al.,* 2005, 2003).

Jiao *et al.,* (2011) hypothesized that inhibition of synthesis β-ODAP or degradation of β-ODAP might be due to the increased levels of ROS. It is also suggested that the primary cause of neurolathyrism is the thiol oxidation which is due to ROS (mainly superoxide ions O_2^- and hydrogen peroxide H_2O_2) which leads to mitochondrial dysfunction. Leaves having low levels of O_2^- and H_2O_2 have the hogher concentration β-ODAP and vice-versa.

López-Martín*et al,*.hypothesized that during the low ROS content in leaves in the young leaves, the O-acetyl serine(OAS) which is the presursor of β-ODAP biosynthesis pathway proceeds towards the formation of BIA. While OAS is being used to form Cysteine and then glutathione in the mature leaves. So in the mature leaves Cysteine and glutathione are in abundance which are recognized as the key metabolites during high ROS contents.

The metabolic regulation of sulphur metabolism is very important for the synthesis of many metabolites including. So, Cysteine is the first molecule which contains both Nitrogen and Sulphur in the sulphur metabolism. Cysteine is catalysed by CAS to form cyanoalanine and hydrogen sulphide. So there might be some possible effect in maintaining sulphate status in plants during stress conditions. Also hydrogen sulphide is responsible for regulating many physiological processes like germination, abiotic stress tolerance, and senescence (Kopirav et al., 2012,2014;Alvarez et al,.2010).

Nitrogen nutrition has a significant effect on the content of β-ODAP in the *L.sativus*. So CAS might be the enzyme that links cyanide and cyanogenic compounds to the Nitrogen metabolism. So the cyanide detoxification pathway involves formation of Cyanoalanine from cysteine and CN^- which is being catalysed by mitochondrial localized CAS (Goudey, J.S.*et al*,. 1989; Gleadow, R.M. *et al.*, 2014).

2.12. Functional annotation with the help of different software

The enormous amount of gene sequence data generated by Next-generation technologies were needed to be functionally annotated for the further experiments to be carried on. For this an efficient bioinformatics software is developed namely – MapMan 'BIN' Mercator (http://mapman.gabipd.org/web/guest/app/Mercator) which is based on the MapMan BIN ontology that customizes the functional annotation to plant 'Omics'data. This software designates functional categories to the orphan protein or amino acid or nucleotide sequences on the basis of homology sequences searches against reference 3 organism's gene sequence data mainly Arabidopsis, Chlamydomonas, rice, various other plant species that have areviewed SwissProt annotation, and more than 2000 protein domain and family profiles at InterPro, CDD and KOG. The functional categories envisaged to the orphan sequences by Mercator attains an accuracy of above 90%. The data sequences should be uploaded in the FASTA format. The Mercator software provides result in a user-friendly interface with graphical overview charts, detailed annotation information in a MapMan-to-GO translation table to export results as GO terms (Lohse*et al*., 2014).

Schwacke*et al*,.(2019) revised or redesigned the Mercator software and named as Mercator4 for functionally annotating the rapidly increasing orphan protein and nucleotide sequences due to advances in gene sequencing technologies. This version of Mercator has incorporated the recent knowledge of various land plants of high quality. Recently, this version consists of 27 top level Bins which are of strong biological context.

A bioinformatics approach for the prediction of functional names to orphan nucleotide sequences of plant resources is developed namely: GoMapMan (http://www.gomapman.org).

This tool provides genes functional annotation in the plant sciences and is an open web-accessible resource. It encourages improvement, consolidation and visualization of gene annotations over several plant species. GoMapMandescribes gene functions which depends on the MapManontology, organized in the form of a hierarchical tree of biological concepts. In this tool reference genes were included of *Arabidopsis* and three crop species (potato, tomato and rice). The principle highlights of GoMapMan are

(I) Dynamic and intuitive gene product annotation through different curation choices

(II) Combination of gene annotation for diverse plant species through the combination of orthologue bunch data.

(III) Discernibility of gene ontology changes and annotations.

(IV) Combination of outer information about genes from diverse open resources.

(V) Giving assembled data to high-throughput investigation instruments by means of dynaically produced export records.

All of the GoMapMan functionalities are straightforwardly accessible, with the limitation on the curationwhich require prior registration to ensure traceability of the implemented changes (Z ivaRamsak*et al*., 2013).

Thimm*et al*., (2003) illustrated a bioinformatics approach – MAPMAN; it is a user-friendly tool that exhibits immense data sequences on to illustrations of metabolic pathways or other processes. MAPMAN comprises of 2 modules: 1.) SCAVANGER and 2.) IMAGE ANNOTATOR. SCAVANGER module allocates the measured parameters in to heirarchial tree (i.e. categorizes parameters in to BINs, subBINs…, particular enzymes). The SCAVANGER module has further sub modules; TRANSCIPT SCAVANGER categorizes the genes on *Arabidopsis*Affeymatrix 22K array into groups of heirarchial BINs and subBINs which provides an overview of central metabolism, secondary metabolism and other processes. METABOLITE SCAVANGER classifies several metabolites into specific pathways or sets of structurally related metabolites. The IMAGE ANNOTATOR module utilizes this classification to assemble and exhibits diagrams according to the user's choice. Its modular structure helps to modify the present categories and incorporation of new categories and develop SCAVANGER modules into other sort of data.

In the review literature of FatihOzsolak and Patrice M. Milos (2010) the advances of massive RNA sequences and parallel their cDNA were developed. This huge amount of RNA sequences or cDNA sequences permitted characterization and quantification of the trenscriptomes. Recent improvements in the RNA sequencing strategies has provided a much more complete characterization of transcriptomes. These advancements incorporate upgrades insmall RNA characterization and detection of alternative splicing events, transcription start site mapping, gene fusion detection, strand-specific measurements.

BJÖRN USADEL (2009) reviewed about the MAPMAN tool which is utilized to envisagition of profiling data. Its SCAVANGER module creates hierarchial and non-redunctant‘mapping files’ (Gene Ontology). Then the IMAGE ANNOTATOR module picturizes these mapping data into schematic ‘maps’ or diagrams of biological processes. Then there is a PAGE MAN module which statistically evaluates the processed mapping files to know the responses at pathway or processing levels. In this literature they compared the Arabidopsis transcriptome with the sequences of Maize to explore the transcriptome responses during diurnal changes. They showed that this tool can be utilized to examine and distinguish the global transcriptonal responses between distantly related species.

CLC Genomics workbench is tool of CLC Bio’s which lessened the barriers to functionally annotate the *de novo* assembled orphan sequences. It is considered as the first comprehensive analysis package which analyzses and visualizes data from all major NGS platforms, like SOLiD, 454, Sanger, Illumina and Ion Torrent. It is a multi- technology multi-platform which can provide us with results: • Read mapping of Sanger, 454, Illumina Genome Ana- lyzer and SOLiD sequencing data • De novo assembly of genomes of any size (only limited by RAM available) • Color space mapping • Advanced visualization, scrolling, and zooming tools • SNP detection using advanced quality filtering • Support for multiplexing with DNA barcoding Genomics • RNA-seq incl. support for paired data and transcript- level expression • Small RNA analysis • Expression profiling by tags • EST library construction • Advanced visualization, scrolling, and zooming tools • Gene expression analysis Transcriptomics • ChIP-seq analysis • Peak finding and peak refinement • Case/control analysis Epigenomics • Primer design • Molecular cloning • BLAST • Alignments • Phylogenetic trees • Advanced RNA structure

prediction and editing • Integrated 3D molecule analysis • Secondary protein structure predictions.

Gusberti, M.et *al.*, (2013) found a horizontal resistance in Apple against apple scab (*Venturiainaequalis*). In his studies, they identified 5 putative candidate gene which are involved in the ontogenic disease resistance. In their experiment they extracted RNA then sequenced it with the help of IlluminaHiSeq 2000. Then for functional annotation they utilized both CLC Genomics Workbench (CLC GWB) and MapMan Mercator. The sequence files from IlluminaHiSeq 2000 were then analysed in the CLC GWB which compared its sequences with unannotated *M. x domestica* gene files which then normalized its expression value using RPKM value. Also principal component analysis was done using this tool with the diferentially expressed genes (DEG's) which was selected on the basis of RPKM values. They then also uploaded the sequences of these DEG's in FASTA format which provided them the functional category for pathway analysis.

CHAPTER-III

MATERIALS AND METHODS

The current theory entitled **"Transcriptome characterization to identify putative genes related to amino acid biosynthesis pathway and d-amino acid metabolism in *Lathyrus sativus*"** was directed at the department of Plant Molecular Biology and Biotechnology, Indira Gandhi KrishiVishwavidyalaya, Raipur, India during the session of 2019-2020. This part of thesis contains the experimental materials utilized over the span of experiment and the methodology for the equivalent. The particulars of the materials utilized and methods embraced were followed under the heading and subheadings:-

3.1. Experimental site

The analysis was directed in the field and at the different laboratories of Department of Plnat Molecular Biology and Biotechnology, College of Agriculture, Indira Gandhi KrishiVishwavidyalaya, Raipur.

3.2. Materials Used

Nine diverse genotypes of *L.sativus* were selected on the basis of their ODAP concentration in the seeds. Out of which five were sorted out for the identification of gene related to amino acid biosynthesis pathway and d-amino acid metabolism. 277 lines of cross Mahateora×RLK-1950 were analysed for their ODAP content in the seeds.

Table 3.1 List of 9 *Lathyrus sativus*diverse genotype

SL.No.	Variety
1	Mahateora
2	Prateek
3	Pusa-24
4	BioR-231
5	BioL-203
6	RLS-2
7	SEL-527
8	SEL-516
9	SEL-504

3.3 PHENOTYPIC characterization of 277 line of cross Mahateora×RLK1950

The F2 population of cross Mahateora×RLK1950 having 277 line were sown on 12 December 2020 in the Rabi season under rainfed condition. 4 observation viz., leaf pigmentation, flower colour, seed pigmentation and ODAP content of seed were recorded. 90% of the germination was observed on 17 December 2020.

3.3.1 Leaf pigmentation

Leaf pigmentation was recorded 25 days after sowing asnon-pigmented(NP), slightly pigmented(SP) , moderately pigmented (MP) and highly pigmented (HP) on the basis of purple colouration of leaves.

3.3.2 Flower colour

Flower colour was recorded 55-60 days after sowing as purple coloured or pink coloured flowers.

3.3.3 Seed pigmentation

This was recorded after harvesting and drying of pods on the basis of spots on the seeds as higly pigmented (HP), moderately pigmented (MP), pigmented (P) and slightly pigmented (SP) and non-pigmented (NP)

(Fig.3.1. 25 days old plants of 277 lines.)

3.4 METHODOLOGY

3.4.1 Biochemical characterization of *Lathyrus sativus* diverse genotypes

ODAP ESTIMATION

The biochemical analysis for the ODAP estimation was conducted according to the procedure of Rao*et al.*, (1964) in the dry Lathyrus seeds in which o-phathaldehyde (OPT) is utilized as a reagent in the alkaline hydrolyzing medium.

3.4.2. Chemicals and materials used:

i. Potassium hydroxide (KOH) -3N
ii. Potassium tetraborate ($K_2B_4O_7$)- 0.5M
iii. O-phathaldehyde (OPT)
iv. β-mercaptoethanol
v. 60% ethanol
vi. Absolute ethanol
vii. Grounded Lathyrus seeds
viii. Eppedroff tubes-2ml
ix. Falcon tubes-15ml
x. Borosilicate bottels

3.4.3 Preparation of BLANK solutions

As per the protocol of Rao*et al.*, (1964) 3 blank solutions were prepared which were as follows**:-**

i. Sample blank = 2ml $K_2B_4O_7$ + 750µL double distilled water + 250µL non-hydrolysing solution (60% ethanol)
ii. Buffer Blank = 2ml $K_2B_4O_7$ + 750µL double distilled water + 250µL non-hydrolysing solution (2:1 of KOH and 60% ethanol solution).
iii. OPT Blank = 2ml OPT+ 750µL double distilled water + 250µL non-hydrolysing solution (60% ethanol).

3.4.4 Preparation of OPT reagent

The OPT reagent for the ODAP estimation should always be freshly prepared. For it preparartion Potassium tetraborate ($K_2B_4O_7$) is taken as the solvent. So for preparing 100mL of OPT solution, dissolve properly 100mg of OPT reagent in 90mL of $K_2B_4O_7$ along with 1mL 99% ethanol and 0.2 mL (200 µL) of β-mercaptoethanol, then make up its volume to 100mL. The buffer solvent $K_2B_4O_7$ should have a pH of 9.9.

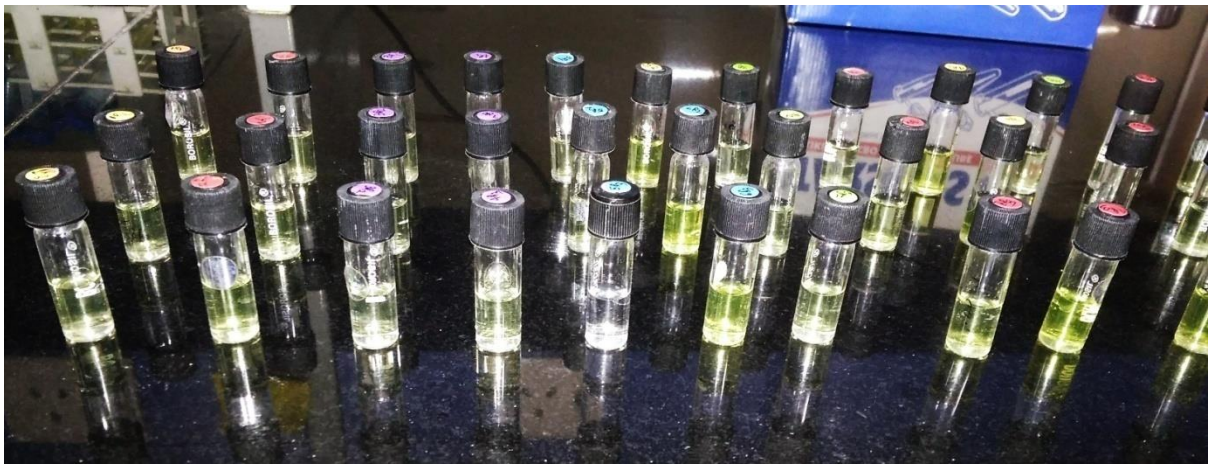
3.4.5. Procedure

- 0.5gm or 500mg of firmly grounded seed samples were allowed to rest in the 10mL 60% ethanol overnight.
- After adding grounded seeds to the 10mL 60% ethanol vortex it well and leave it for overnight.
- 2ml of supernatant or aliquot was pippeted in the 2ml eppenderoff tubes.
- Centrifuge this aliquot at 4500rpm for 15 minutes at 25°C.
- Then this supernatant was hydrolyzed with 4ml of 3N KOH, sustain this hydrolyzed samples in the waterbath at 95°C for 30 minutes.
- After cooling, 250µL of hydrolyzed sample solutions taken in the borosilicate bottles along with 2mL OPT solution and 750µL double distilled water.
- Prepare the blank solution as directed above then allow all the sample solutions along with blank to incubate for 2 hours at 37°C-38°C.
- A yellow colour develops in the sample solution which was absorbed 425nm in the Systolic spectrophotometer.

The absorbance of blank solution was also taken by substracting sample blank and buffer blank and substractingOPT blank and sample solutions. The final absorbance was obtain by the formula:-

Final absorbance = (Sample Blank-Buffer blank) – 1/3(OPT Blank – Sample solution)

This final absorbance is then multiplied by a correction factor of 1.67 to obtain the ODAP content of seeds.



(Fig.3.2. ODAP estimation; yellow colour develops corresponding to the ODAP concentration of seeds.)

3.4.6. Standard curve

- To calculate the standard curve, LDAP was used which is analogues to ODAP content in the seeds
- 1% stock slolution of LDAP was prepared by dissolving 1gm LDAP in 100 mL of water.
- This stock solution was again dissolved in 4ml of 60% ethanol and 2ml of KOH to make a total volume of 6mL.
- In this 6ml solution tubes add 6µL LDAP stock solutions and make up the volume to 6ml which makes a concentration of 0.001 (6mg/6ml). Similarly prepare solutions of 0.002,0.003,0.004 and 0.005 concentration with a blank.
- Then incubate this solutions in waterbath at 95℃ for 30 miutes, now observe the absorbance in spectrophotometer.

Table 3.2 – concentration and absorbance of LDAP for standard curve

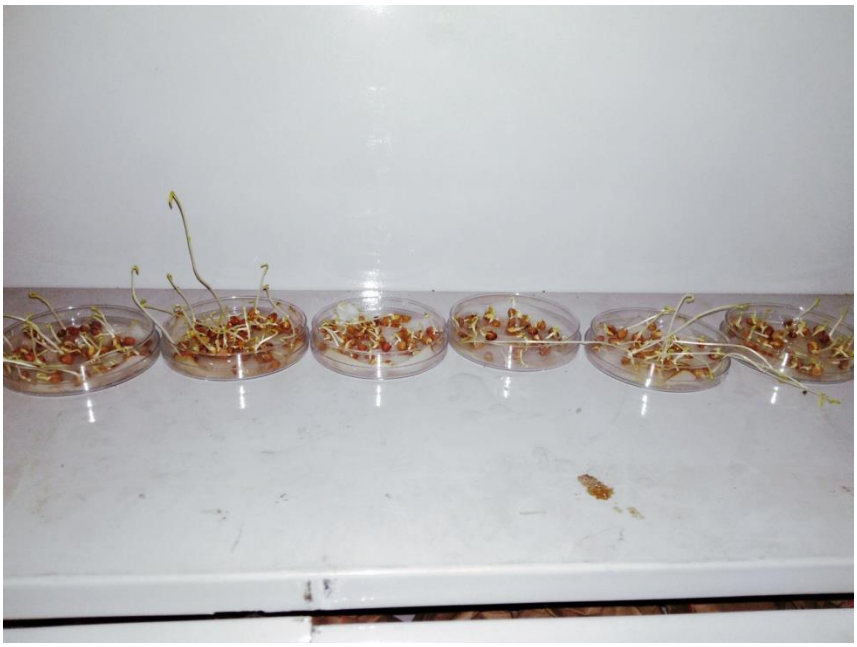
SL.No.	Conc.(6mg/ml) LDAP	Absorbance (425nm)
1	0.001	0.228
2	0.002	0.423
3	0.003	0.592
4	0.004	0.772
5	0.005	0.902
6	0.006	1.112

On the basis of ODAP estimation 5 genotypes were selected:-

SL.No.	Variety
1	Mahateora
2	Prateek
3	PUSA-24
4	BIOR-231
5	RLS-2

(Table 3.3 – genoypes selected)

Seed of these genotypes were firstly germinate in the petriplate then grown in the field condition for 10 days. Then RNA was extracted from 8^{th} and 10^{th} day plants.



(Fig.3.3. Germination of 9 genotypes of L.sativus)

(Fig.3.4. Growing of germinated seeds in the field condition.)

3.5. Semi-quantitative expression analysis of candidate genes responsible for amino acid biosynthesis and D-amino acid biosynthesis pathway in diverse *Lathyrus* genotypes.

3.5.1. RNA EXTRACTION

Leaf tissue collection and isolation of RNA:

Fresh leaf samples of 5 diverse *Lathyrus* genotypes were collected from 8 and 10 days old plants as the accumulation of ODAP content from 6-10 days were highest. Healthy and tender

leaves were choosen and diseased or damaged leaves were diascraded. These leaves were crushes in the liquid nitrogen for RNA extraction.

RNA extraction was performed using TRIZOL reagent.

Materials Required:-

- Trizol
- Chloroform:Isoamyl Alcohol mixture (24:1,v:v)
- Isopropanol
- DEPC water
- 75%ethanol

Methods:

Preparation of DEPC Water

- 0.1gram of DEPC (diethyl pyrocarbonate) is dissolved in the 1Lit. of double distilled water to make a 0.1% DEPC water. Mix it well by stirring it for 2 hours. This DEPC should be handled very carefully and with proper protection as DEPC IS HIGHLY TOXIC COMPOUND.
- DEPC gets hydrolyzed while autoclaving process, so it should be double autoclaved. Then it can be handled easily and can be stored at room temperarture for several days.

Preparation of utensils for RNA extraction

- The mortar pestels, spatula, eppendroff tubes and other glasswares were soaked in 30% hydrogen peroxide for 15 minutes for RNA extraction procedure. Then rinse it with DEPC treated water and keep it overnight and dry it in the hot air oven before use.
- Always use RNAase free microcentrifuge tubes of 1.5ml or RNAase free filter tips.

Procedure:-

- 8 and 10 day old leaves were collected and crushed in the liquid nitrogen to form a fine powder with the help of mortar pestle which is then transferred to a 2ml eppendroff tube.
- To this crushed leaf smaples, add 500µl of TRIZOL reagent, vortex it well. Then incubate it for 10 minutes.
- Now add 200µl of Chloroform: Isoamyl Alcohol (24:1) then vortex it again. These tubes are agai incubated for 10 minutes at room temperature.

- Centrifuge it at 4°C at maximum speed for 15 minutes in a bench top centrifuge.
- Pipette out the aqueous phase of samples in a clean eppendroff tubes after centrifugation.
- Add 500µl of isopropanol and mix it gently. Now incubate it for 10 minutes.
- Again centrifuge it for 15 minutes at maxium speed at 4°C. Now the pellets appear at bottom of tubes.
- Dispose the isopropanaol.
- Now add 70% DEPC treated ethanol and resuspend the pellets by gently tapping the tubes. Centrifuge for 5 minutes then decant the ethanol.
- Now air dry the pellets by inverting the tubes on a paper towel or blotting paper untill every single droplet is dried.
- Resuspend the pellets in 20µl of RNAase free DEPC treated water for dilution.

After dilution the RNA samples can be quantified and qualified in the Nanodrop Spectrophotometer and gel electrophoresis respectively.

3.5.2 cDNA synthesis

The Bio-RADiScriptTMcDNA synthesis kit was utilized for synthesis of cDNA from the quantified RNA which is isolated as per manufacturer's instruction. The reaction mix and temperature profile are presented in table for cDNA synthesis. This kit is applicable for 1pg to 1µg of RNA and consists of following components:-

1. 5X iScript Reaction Mix
2. iScript Reverse Transcriptase
3. Nuclease free water

Table 3.4: cDNA synthesis (reverse-transcription) reaction components.

Components	Volume reaction	Final concentration
5X iScript reaction mix	4 µl	1x
iScript Reverse Transcriptase	1 µl	-
Nuclease free water	Variable	-
RNA template	1-15 µl	1 µg
Total volume	20 µl	

Table 3.5: Reverse Transcriptase cycling program

Step	Temperature	Time	No.of cycles
Priming	25°C	5min	1
Reverse Transcription	46°C	20min	1
RT inactivation	95°C	1min	1
Optional step	4°C	hold	

cDNA synthesis procedure:

- The template RNA samples should be thawed on the ice. The amount/volume of RNA to make it 1µg of concentration should calculated by adjusting the volume of water. Dispense the required quantities of water and RNA accordingly in the tubes of 0.2ml capacity.
- Mix all the given amounts of 5X iScript Reaction Mix, iScript Reverse Transcriptase and Nuclease free water for preparing the master mix.

- Gently mix by pippeting.
- 10µl of master mix along with RNA samples and water should be dispensed in the corresponding tubes then gently tap the tubes for mixing of all contents and spin it down.
- Then incubate the tubes in PCR machine on reverse transcription cycle as depicted in the table 3.5.

Quantification and dilution of cDNA

cDNA is qualified and quantified by the measuring the absorbance with the help of Nanodrop spectrophotometer (ND 100). At the tip of Nanodrop1µlof cDNA is pipetted and absorbance was recorded at 260nm. For each sample absorption ratio (A260/280) and (A260/280) was recorded for evaluating the quantity and quality of cDNA.

3.6. Functioal annotation of *L.sativus* transcriptomes using Mercator

The MapMan Mercator (http://mapman.gabipd.org/web/guest/app/ Mercator) is an open, user-friendly resources for the functional annotation of orphan genes. It is specially designed for the plant researchers to functionally categorize the unknown gene sequences. The MapMan Mercator provides functional categories on the basis of MapMan Ontology which is termed as "BIN" (Thimm *et al*., 2004). BINs are structured to form a hierarchical tree describing about the various biological terms of plant metabolic pathways and process including secondary metabolism and metabolism in stress (Rotter, A.et *al*., 2007; Rotter, A A.*et al*., 2009). The BIN ontology is very flexible as it can also accomodate a more detailed specification which is further classified as subBINs.

The Mercator predicts the fuctional categories of orphan gene sequences on the basis of following reference data (*Lohse* et al., 2014):

1. ~33000 Arabidopsis proteins (TAIR10 protein).
2. ~6000 plant protein from the Swiss Prot database
3. ~57000 rice proteins (TIGR5)
4. ~17000 *Chlamydomonas reinhardtii* protein models
5. 2169 domains and profiles from InterPro database, Conserved Domain Database (CDD) and KOG database.

The functional categories were assigned on the basis of a combination of reviews, literatures, database researches, sequence analysis and personal expertise. It requires to upload the sequences in the FASTA format. Mercator can analyse upto 150million nucleotides or amino acid sequences in ne go which corresponds to 1,00,000 sequences of 1.5Kb each. The sequences should be uploaded as assembled transcripts or derived protein sequences and not in a raw next-generation sequencing (NGS) data. It provides us with an accuracy of above 90%.

3.6.1. **Proccesing of Mercator** (Lohse et al., 2014)

- The query or orphan sequences are clustered or screened on the basis various reference databases.
- All the results of searches are compiled and filtered on the basis of cut-off score provided by various databases which were then passed on to the Reasoner module.
- The Reasoner module assigns the reference BIN categories to each query sequences based on the above-threshold hits.
- Each reference hits were supported by the experimental evidence documented by publications receive extra weight.
- Consequently, on the basis of all above processes, the results were provided in the form of excel sheet and chart.

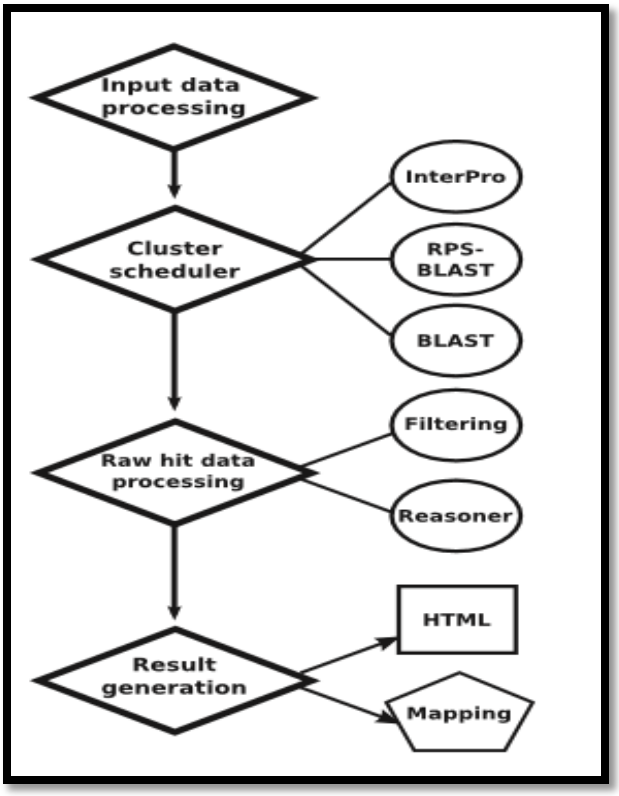
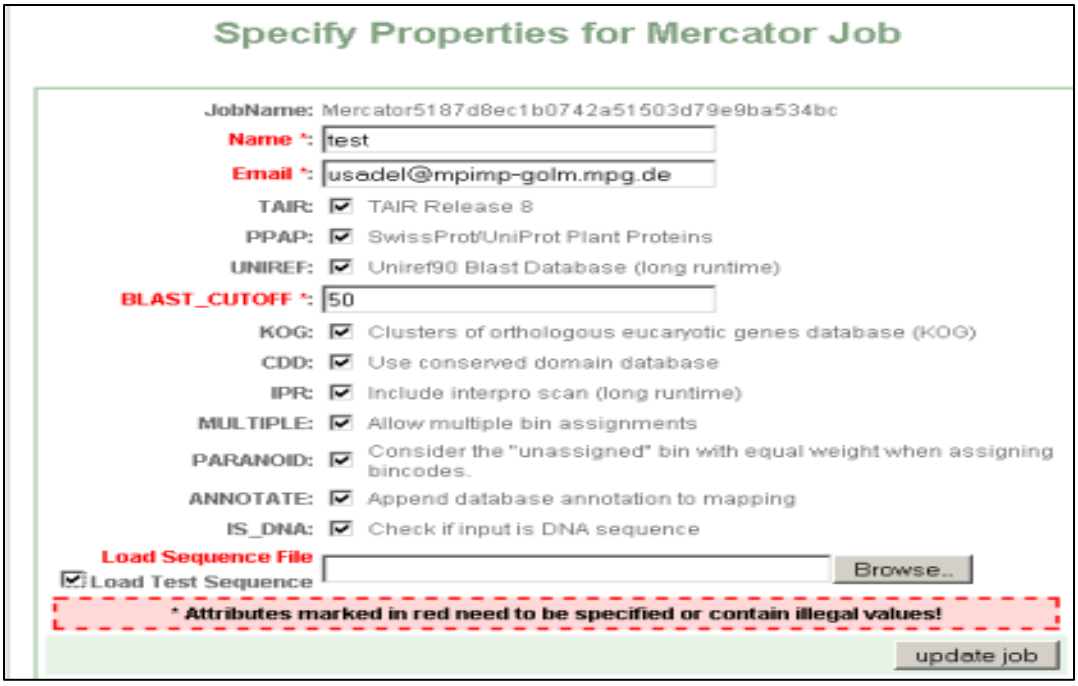


Fig.3.5. Processing of Mercator
Source: Lohse *et al*.,; Plant, Cell and Environment, 37, 1250–1258

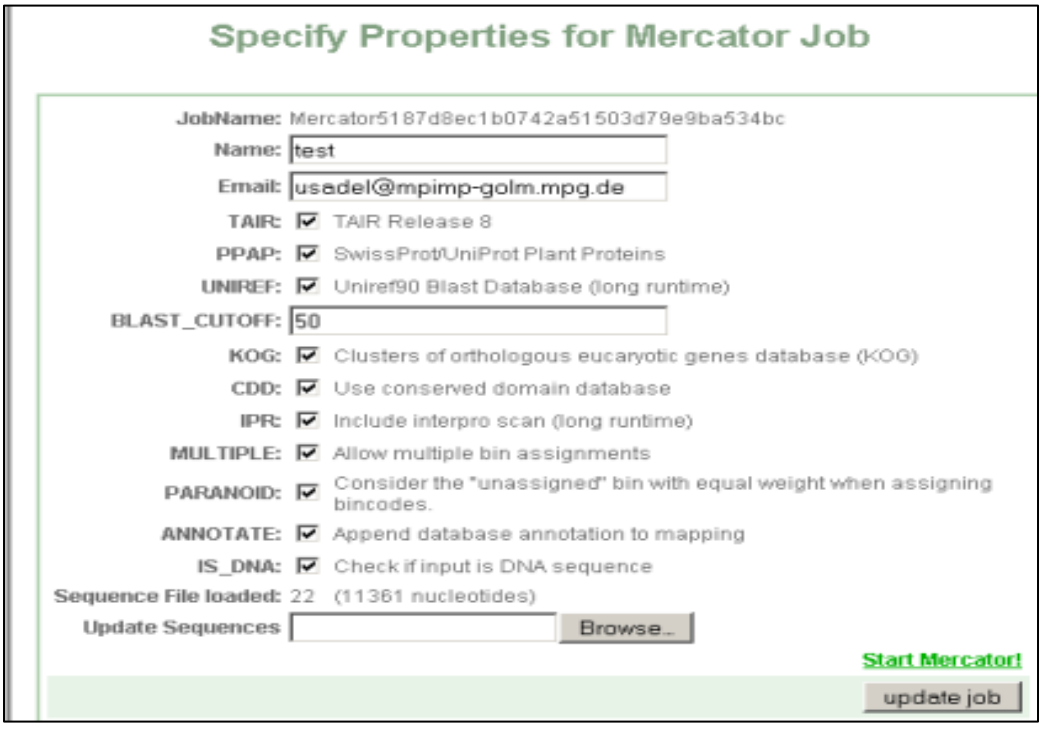
3.6.2 Uploading of orphan sequences in the Mercator

- Upload the sequence file and specify the properties to be given.



(Fig. 3.6. filling of all necessary requirements to run the Mercator)

- After filling the necessary options, check further if everthing is correct then click the update button to start the processing.



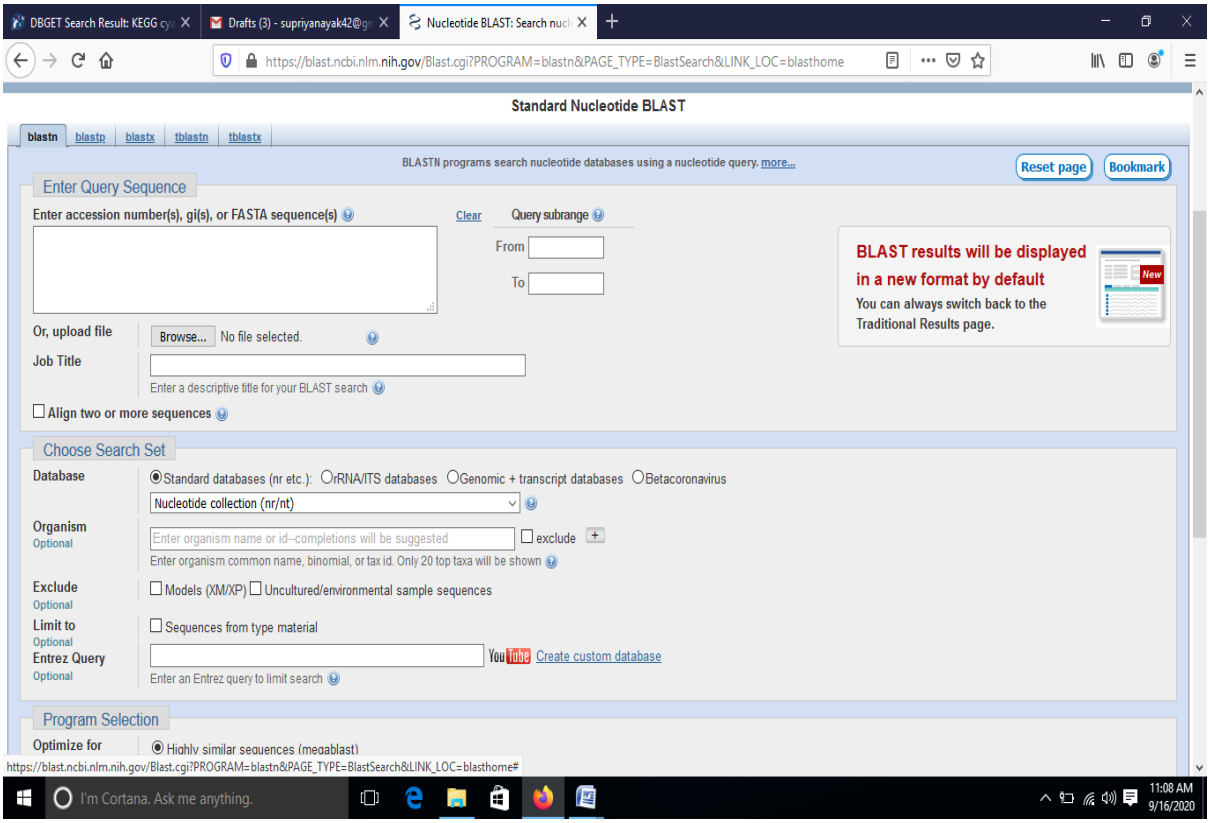
(Fig.3.7. checking of the specifications)

- On the completion of job, a download link will appear for viewing of the results (excel sheet and pie chart).

(Fig.3.8. After completion of the process, this page appears to download result)

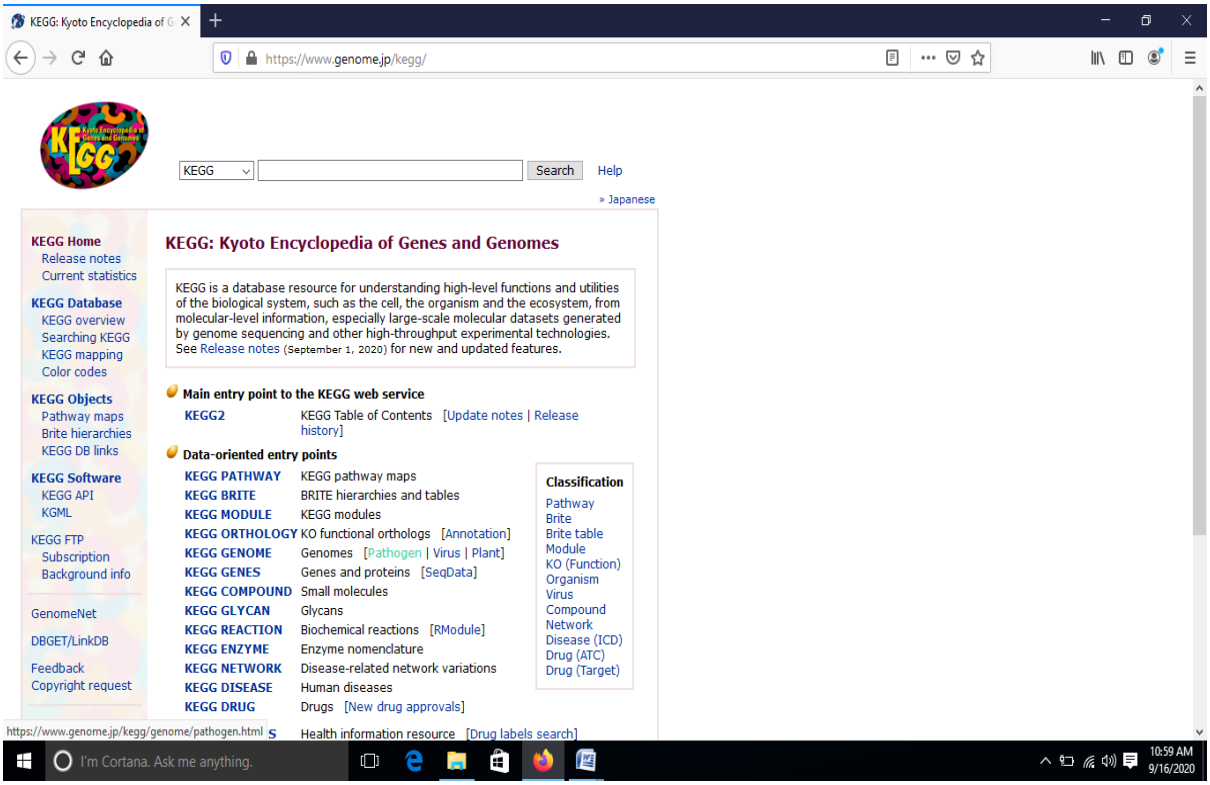
3.7. In silico designing Primers:

The concerned genes related to amino acid and d-amino acid biosynthesis pathway were selected on the basis of Mercator output (excel sheet) for Cyanoalanine synthase, alanine aminotransferase, Asparagine synthase, asparaginase, and. These functional categories from Mercator output (excel sheet) were then screened for its corresponding sequences in the Mahateora and RLK-1950 transcriptome sequence data. The corresponding sequences is also screened for homology sequences searches in the NCBI BLAST (https://blast.ncbi.nlm.nih.gov).

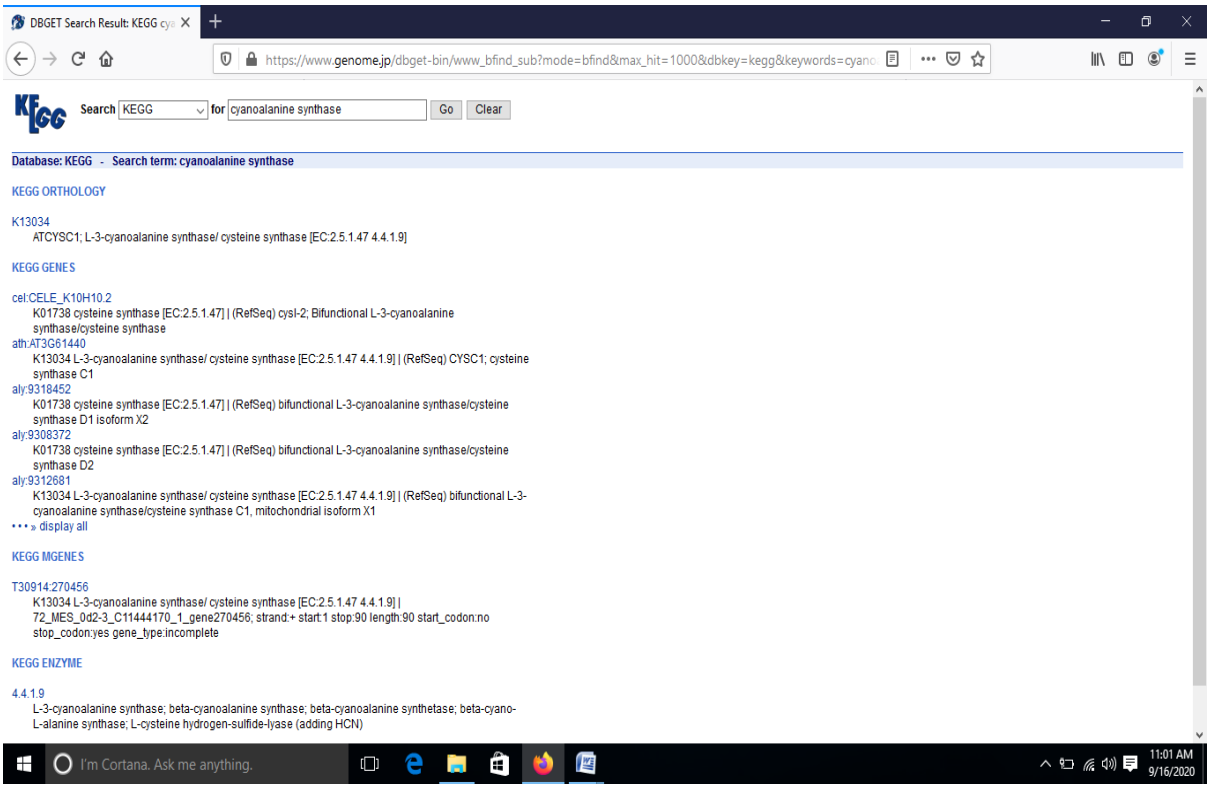


(Fig.3.9. - Homepage for NCBI BLAST)

The candidate genes were also observed for there corresponding pathways, contig ID's and functions in the Kyoto Encyclopedia of Genes and Genomes (KEGG). (https://www.genome.jp/kegg/.).

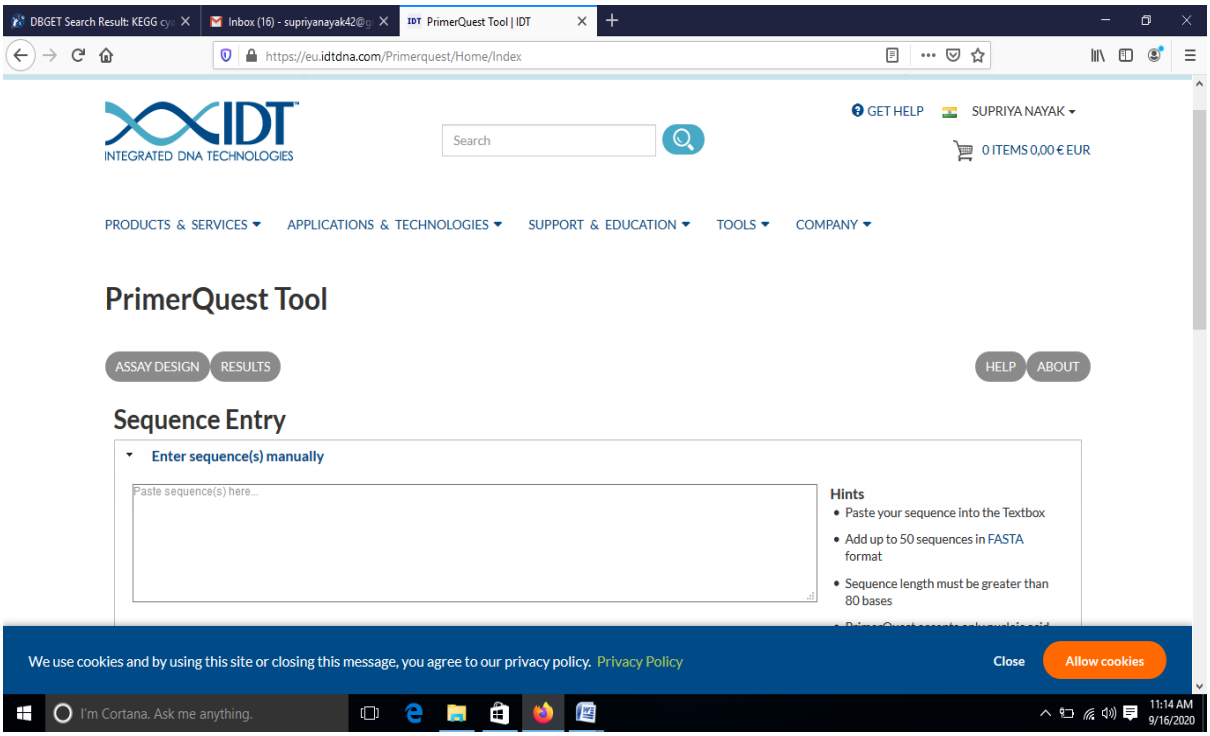


(Fig.3.10- Homepage of KEGG)



(Fig.3.11. – Results of KEGG showing results for an enzyme.)

Consequently, along with the homology sequences of *Medicago truncatula*, the *L.sativus* sequences were subjected for Primer designing utilizing the Integrated DNA Tecechnology's (IDT) PrimerQuest tool (https://www.idtdna.com).



(Fig.3.12. Home page of Primer Quest tool)

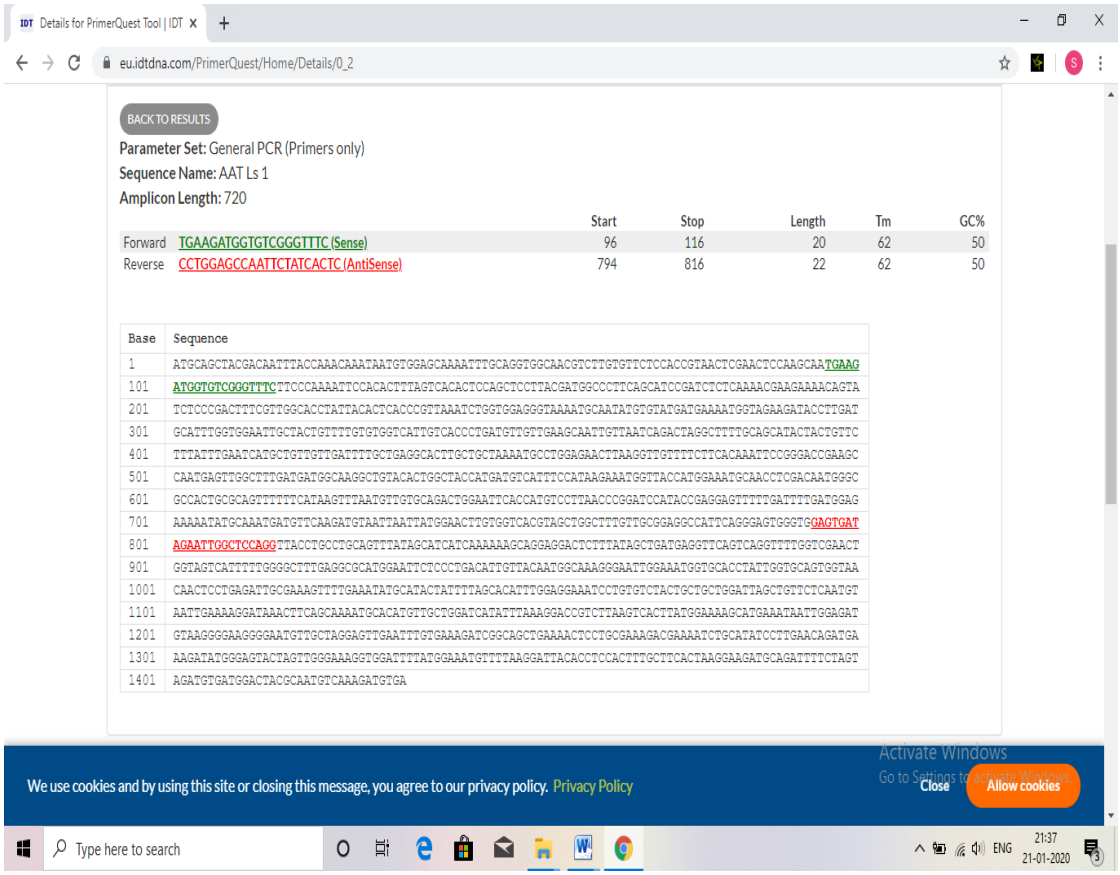
Precautions for Primer Designing

The important factors for prier designing includes Melting temperature, Annealing temperature (T_a) and GC content.

- The length of primers (both forward and reverse) for PCR and sequencing should be between 18-25 nucleotides. It should amplify atleast an 80 kb sequence.
- The GC content of designed primers should be between 35% to 65 % with an average of 50% to promote complexity during maintaining a unique sequence. Primers 3′ should have G or C to enhance binding but should not accommodate 4 or more consecutive G residues to avoid mispriming.
- Primer Melting Temperature (T_m) by definition is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability.

Ideally, the Melting Temperature T_m value is between 60ºC-62ºC. The Melting Temperature T_m of 2 primers (forward and reverse) should not vary by more than 2 ºC, so that both primers can bind simultaneously and efficiently to the amplicon.

- The Annealing temperature (T_a) of primer depends on the length, composition and primer Melting Temperature (T_m). The Annealing temperature (T_a) is 5ºC less than the T_m ovalue of both the primers. If T_a value id too low then one or both primers can anneal to sequences other than the targeted ones because of internal single-base mismatches or of partial annealing which can lead to non-specific PCR amplification and reduces the yield of desired products. And if T_a value is too high, the reaction efficiency may be reduced due to the probable primer annealing is reduce significantly.



(Fig.3.13. Designing of primers)

Table 3.6. List of Primers

Sl.no.	Name	Forward primer	Reverse primer	Tm	Amplicon length
1	ODA Ls 1a	TGAGAAAGGGAAAG GGTGTAAG	CTCCAGGCATAAC AGGTTCAA	62	382
2	ODA Ls 1b	CGAAAGAGGTGATT GAGGATGA	TGCAAGTTCTACCT GAGGAATAG	62	254
3	AAT Ls 1a	CGACTTTCGTTGGC ACCTATTA	GCACCATTTCCAAT TCCCTTTG	63	775
4	AAT Ls 1b	TGAAGATGGTGTCG GGTTTC	CCTGGAGCCAATT CTATCACTC	62	720
5	AAT Ls 2a	GTTGAACTCGTACC TGGGTATT	TTCCATGCAGTCCT CCTTTC	62	544
6	AAT Ls 2b	CCAATACCGGAGGG TGAAAT	GGCAAACCGTTGC CAATAC	62	356
7	AAT Ls 3a	GCCGAAACAACGAG GACTAT	AGCTGAATGGAAG GAGTGTATG	62	679
8	AAT Ls 3b	CGCCTGGAGATCGT ATTGTATC	GAGACCGTAGAGA AGCTGAATG	62	533
9	AAT Ls 4a	TCTTCCCAGCATAA CCGATTC	TGGACATCTCTTGC ATACTTCTC	62	692
10	AAT Ls 4b	GGGAGTATCTTAGC CCTTCAATC	ATAGGCAGCAGAC AAGTAACC	62	641
11	CYAS Ls 1a	GGCTGCCATGAAGG GATATAA	GTGTTAGCTCCGG ACGATATG	62	567
12	CYAS Ls 1b	CTCAAGCGAACCTC TTCTTCA	CTCAGCTCCAAAT GCTCTCATA	62	492
13	CS Ls 1a	TGCAGTGTCAAAGA CAGGATAG	CACCAAAGCTCGG GAATACA	62	772
14	CS Ls 1b	CCGAGAAGAAAGGA GCCATAAC	CTCGGGAATACAA CCGCAATAA	63	721

15	CS Ls 2a	CAATGCCTGTTTCG ATGAGTTTAG	GTTGTCCACCGCTA AGTATGT	62	366
16	CS Ls 2b	GAGCATTTGGAGCT GAACTTG	CACCGCTAAGTAT GTTGCTTTC	62	318
17	CS Ls 3a	TGGCGCTATCATCTT CTTCATC	CCCATATCTCTGGT CCTGTAGT	62	699
18	CS Ls 3b	CCGTAACCTCAAAC CCTACTTC	GTTCCAATAGCAG CAACCAATAC	62	652
19	Asparaginase Ls 1a	TGGTGCTGTTTCTGG TCTTAC	CTACAGTCGCGCG GATTATT	62	479
20	Asparaginase Ls 1b	TCGATTGGTTATGG ATCGTACTC	GCGGATTATTGCTT CACCTATTC	62	421
21	Asparaginase Ls 2a	TCTCTGCTCTTCGTT CCAATC	CGCAATCAGTCCA GCAAATC	62	767
22	Asparaginase Ls 2b	TCTCTGCTCTTCGTT CCAATC	GCAAATCCTTCATC CAACCTATTC	62	763
23	Asparaginase Ls 3a	ATCTTGGCATCTCTG CTCTTC	TCTACAGCCCTTAA CCTTGATTT	61	408
24	Asparaginase Ls 3b	GAGCCTCTAGTGGT GGTATTG	TCGGCAACAGAGC GTAAA	62	247
25	ASN Ls 1a	GTTGATGACTGATG TGCCTTTC	CTGCTCTACCAGA AGGATCAAG	62	974
26	ASN Ls 1b	TAAGGGTGCTCCTG ACCTAA	ATCCCATCAATCC AGCCATATC	62	588
27	ASN Ls 2a	GTACTTTCACAAGG CACCAAAC	CCGAGTTCTGAGG GAAGAAAC	62	473
28	ASN Ls 2b	GCTATTCCATCGGCT CCTTATG	CCTTCACCAGAGA TCACCATTT	62	437
29	ASN Ls 3a	TGCCATTGTTGACCC TACTT	GACTTCCTCGAGT GCATCTATAC	62	766

30	ASN Ls 3b	CATCGAACTCTCTC GCAGATT	GTCGGATTGTACC ACCTTCTT	62	519
31	DTD Ls a	GGCATCAGCAACAA CAGAATC	GCATTCCTAAACCT GTCCACTA	62	428
32	DTD Ls b	TGTAGACTTGCGTG CGTATG	TCCCATGCTTTGCC AGTATC	62	296
33	P5CD Ls 1a	GCTGTCGCTGCTTTC AATTC	CAGTCAAACCCAG CATCTTCTA	62	860
34	P5CD Ls 1b	CCGCTAGGGTTTCTT CTCTTAC	CCAACTTCTCTGCC ACTCTT	62	843

3.8. Semi quantitative RT-PCR based validation

The semi-quantitative reverse transcriptase RT-PCR was accomplished to evaluate the expression of genes related to amino acid and d-amino acid biosynthetic pathway in *L.sativus*. To observe the expression of concerned genes, the cDNA obtained from the total RNA isolated from the 5 diverse *Lathyrus* genotypes having differential ODAP content were subject to semi-quantitative RT-PCR.

The expression profiling was prepared for a 10 µl of reaction mixture by utilizing the above designed primers from the transcriptomes of RLK-1950. The PCR product were then resolved on 1.5% agarose gel utilizing 1X TAE buffer along with 100bp ladder at 70Volts for 30 minutes. The expression of candidate genes with the designed primers and their corresponding intensities were observed under gel documentation system. Actin (*LsActin*, designed from L.sativus transcriptomes) was used as an internal control for normalization of the cDNA. The details of PCR components and temperature profile are depicted below in the table no. 3. And 3.

Table 3.7. Quantity of PCR components used for semi-quantitave PCR

Components	Concentration	Quantity
cDNA	1000 ng/µl	1 µl
PCR buffer	10X	1 µl
dNTP Mix	10 mM	1 µl
Primer (forward)	10 mM	0.5 µl
Primer (reverse)	10 mM	0.5 µl
Taq polymerase	5U/ µl	0.1 µl
$MgCl_2$	50 mM	0.25 µl
Nanopure water	-	5.65 µl
Total		10 µl

Table 3.8. Temperature profile used for semi-quantitave PCR

Activity	Temperature	Duration	Cycles
Initial Denaturation	94ºC	5 min	1
Denaturation	94ºC	45 sec	
Annealing	55 ºC	30 sec	35
Extension	72 ºC	1 min	
Final extension	72 ºC	7 min	1
Store	4 ºC	infinity	1

3.9. Agarose Gel Electrophoresis

1.5% Agarose gels (horizontal) were utilized for visualizing the expression of the resultant PCR amplicons. Gels were casted in the Boras gel electrophoresis unit. The casting trays and combs were thoroughly cleaned before using.

3.9.1. Pouring of Agarose Gel

- To prepare 1.5% of agarose gel, 1.5gram of agarose was dispensed in the 100ml of 1X TAE buffer in a conical flask. Then agarose was melted in the oven by heating for 2 minutes.
- After slightly cooling of the gel, 2.5-3µl of EtBr was added to the heated gel.
- Fix the comb in the casting tray, then pour the agarose gel in the casting tray and leave it for about 30 minutes for solidification.
- After solidification of gel, the casting tray with solidified gel was places properly in the casting tank filled with 1X TAE buffer then remove the comb.
- 3 µl of loading dye was added to the amplified PCR product which was then loaded in the wells formed by the comb along with 100bp ladder.
- Electrophoresis was run at 70-80 volts for about 30-45 minutes.

CHAPTER-IV

RESULTS AND DISCUSSION

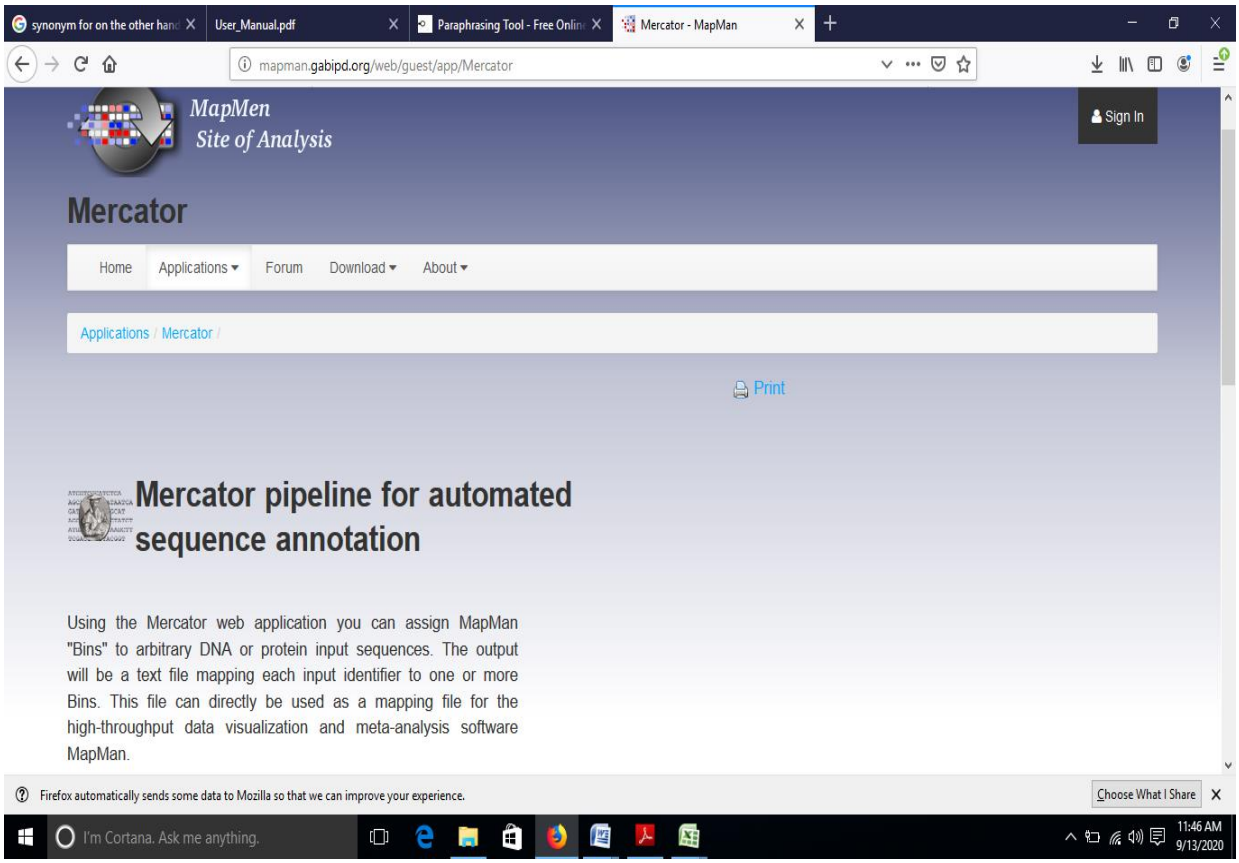
The presence of β-N-oxalyl-L-α, β-diaminopropionic acid (β-ODAP), also known as β-N-oxalyl-amino-L-alanine (BOAA) in *Lathyrus* seeds makes it unsuitable for human consumption due to its association with neurolathyrism. Studies conducted till now on elucidation of β-ODAP synthesis pathway have suggested that the immediate precursor of β-ODAP is synthesized as an ancillary byproduct of O-acetyl serine metabolism in interaction with reactive oxygen species (ROS) and cross talk with nitrogen and sulfur metabolism (Ikegami *et al*., 1995; Xao *et al*., 2012, Liu *et al*., 2017). Enzymes such as β-cyanolalanine synthase (β-CAS), cysteine synthase have also been reported to participate in the pathway in *Glycine max, Phaseolus vulgaris and Medicago truncutala*. However, the genes encoding these enzymes or enzymes involved in key steps in β-ODAP synthesis reaction are not identified till date. Identification of the key genes in the pathway will be significant in genetic improvement of the crop. The present study entitled **"Transcriptome characterization to identify putative genes related to amino acid biosynthesis pathway and d-amino acid metabolism in *Lathyrus sativus*"** was conducted to analyze and characterized the putative genes involved in the amino acid biosynthesis pathway. The leaf tissue transcriptome sequence of two *Lathryus* genotypes Mahateora and RLK1950 was used for the analysis (Banerjee, 2018). This transcriptome sequence data used in the present study where the transcriptome sequence was analyzed using an MapMan Mercator tool to annotate and functionally characterize all the transcripts obtained from transcriptome sequence and compared with the annotation obtained based on comparison with *Phaseolus vulgaris*.

4.1. CHARACTERIZATION OF TRANSCRIPTOME SEQUENCES USING MERCATOR PIPELINE

Transcriptome is the total RNA (mRNA) content which is present in the particular cell or tissue of an organism. The transcriptome data of RLK-1950 and Mahateora was obtained from previous experiment conducted where transcriptome sequence of 10 days old leaf tissue was done by Illumina sequencing, with 150bp read length with de-novo assembly and annotation done by using CLC Genomics Workbench (CLC GWB) and trinity (Banerjee, 2018). Total number of nucleotides obtained from both the transcriptome were 11,964,309,538. Out of which genotype Mahateora transcriptome (201M) comprised of 40,068,526 sequence pairs and RLK-1950 transcriptome (202R) comprised of 39,165,312 sequence pairs. The earlier studies on this transcriptome data analysis resulted in the functional annotation of only 30,502 sequences which are common in both Mahateora and RLK-1950 sequences in which only 28132 sequences were functionally categorized. The CLC GWB de-novo assembled the *Lathyrus* transcriptome while annotation was done by comparison with *Phaseolus vulgaris*, being closely related to *L. sativus*. Homology based annotation has the limitation of loss of the sequences that are less represented in the reference genome or are completely different. *L. sativus* is genetically much diverse. Moreover, the B-ODAP biosynthesis occurs only in two (*L. sativus* and *L. odoratus*) of the species among 187 species falling in the genus Lathyrus, none of which are completely sequenced till today (Campbell,C.G., 1997), making it almost impossible to find a homologous sequence for the genes under question. Therefore, a large fraction of the transcripts remained un-annotated and hence could not be processed in the previous study based on homology search for functional characterization (Bhariya *et al*., 2018).

To further dig out the informative transcripts the original assembly of transcriptome was again analyzed for functionally annotation using bioinformatics pipeline – MapMan Mercator (http://mapman.gabipd.org/web/guest/app/Mercator) so that probably some important transcriptome sequences must have outreached (Nayak *et al*., 2019 personal communication). Mercator provides researchers with convenient applications for high-throughput plant *omics* data by utilizing the MapMan 'BIN' Ontology (May *et al*,. 2014). It is specially designed for the online functional annotation of plant orphan sequences. This pipeline allocates functional terms to orphan protein or nucleotide sequences automatically. The sequences need to be uploaded in FASTA format and can accommodate upto 150 million residues (nucleotides or amino acids). It look through obscure sequences against a few reference data base utilizing a BLAST searches

accompanied by protein domain searches and eventually evaluates BIN (ontology concepts) assignments dependent upon significant similarity to reference proteins or domain hits (Ramšak,Z *et al*., 2013). BIN code is a name provided by the MapMan4 ontology indicating functional classes of gene of a strong biological context. Totally there 50 BINs in which 27 comprises of top-level functional categories (that is they have a renowned protein function in the pathway) (Schwacke, R.*et al*., 2019). It provides us an accuracy of above 90% (92.3%) and can process a whole proteome in few hours (May *et al*., 2014).

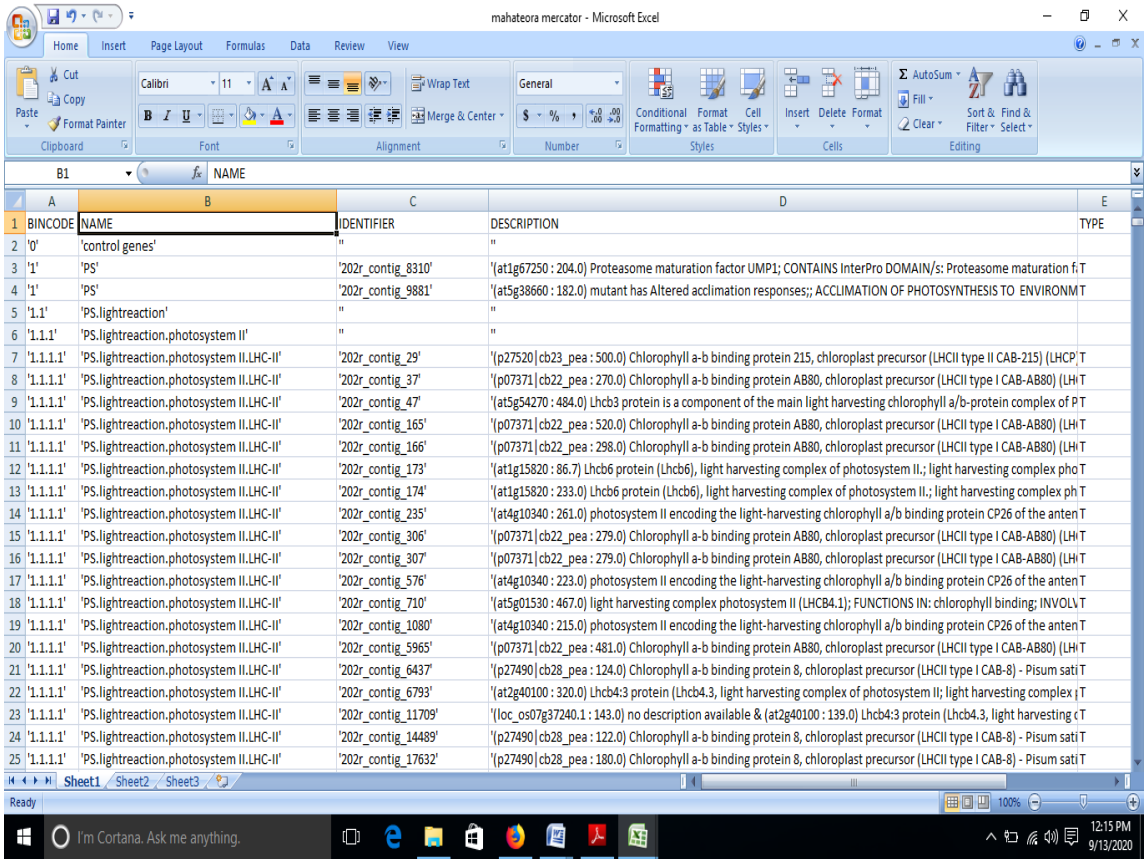


(Fig.4.1. MapMan Mercator home webpage)

On Contrary to Mercator, CLC genomics workbench needs to be installed on the personal workspace and acquires a lot of space for its installation including memory for read maps (>20GB). Additionally, it requires a license from license manager for installing it. Basically, CLC GWB is biomedical tool which also compares orphan sequences to human and mouse genomics. Although CLC GWB can hold as many sequences as Mercator can but it

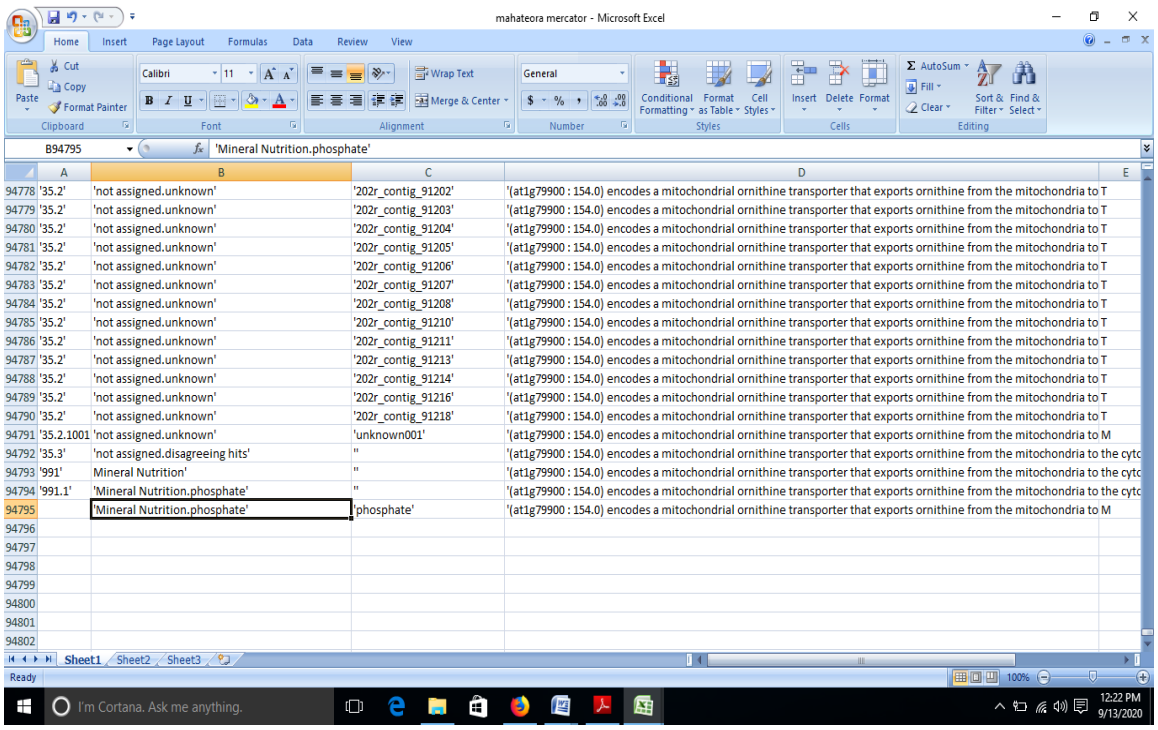
requires memory corresponding to the sequence data file (User manual CLC Genomics Workbench 20.0.4, 2012; Stark *et al*., 2010).

The Mahateora and RLK-1950 transcriptomes when processed in the CLC GWB, it annotated only 30,502 transcriptome sequence while comparing it with the sequences of *Phaseolus vulgaris* in which only 20,592 were functionally categorized in both the genotypes. While the MapMan Mercator predicted the functions of 94,793 transcriptome sequences in both the genotypes (Mahateora and RLK-1950) along with BIN code on the basis of MapMan BIN ontology and description of corresponding enzyme. The results were obtained in a excel format after processing as shown in Fig.4.3. The functional categories of transcriptomes sequences along with the number of sequences in the particular functional category were depicted in the Table no.4.1 seen in the figure below:



	A	B	C	D	E
1	BINCODE	NAME	IDENTIFIER	DESCRIPTION	TYPE
2	'0'	'control genes'	''	''	
3	'1'	'PS'	'202r_contig_8310'	'(at1g67250 : 204.0) Proteasome maturation factor UMP1; CONTAINS InterPro DOMAIN/s: Proteasome maturation fi	T
4	'1'	'PS'	'202r_contig_9881'	'(at5g38660 : 182.0) mutant has Altered acclimation responses;; ACCLIMATION OF PHOTOSYNTHESIS TO ENVIRONM	T
5	'1.1'	'PS.lightreaction'	''	''	
6	'1.1.1'	'PS.lightreaction.photosystem II'	''	''	
7	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_29'	'(p27520|cb23_pea : 500.0) Chlorophyll a-b binding protein 215, chloroplast precursor (LHCII type II CAB-215) (LHCP	T
8	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_37'	'(p07371|cb22_pea : 270.0) Chlorophyll a-b binding protein AB80, chloroplast precursor (LHCII type I CAB-AB80) (LH	T
9	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_47'	'(at5g54270 : 484.0) Lhcb3 protein is a component of the main light harvesting chlorophyll a/b-protein complex of P	T
10	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_165'	'(p07371|cb22_pea : 520.0) Chlorophyll a-b binding protein AB80, chloroplast precursor (LHCII type I CAB-AB80) (LH	T
11	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_166'	'(p07371|cb22_pea : 298.0) Chlorophyll a-b binding protein AB80, chloroplast precursor (LHCII type I CAB-AB80) (LH	T
12	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_173'	'(at1g15820 : 86.7) Lhcb6 protein (Lhcb6), light harvesting complex of photosystem II.; light harvesting complex pho	T
13	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_174'	'(at1g15820 : 233.0) Lhcb6 protein (Lhcb6), light harvesting complex of photosystem II.; light harvesting complex ph	T
14	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_235'	'(at4g10340 : 261.0) photosystem II encoding the light-harvesting chlorophyll a/b binding protein CP26 of the anten	T
15	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_306'	'(p07371|cb22_pea : 279.0) Chlorophyll a-b binding protein AB80, chloroplast precursor (LHCII type I CAB-AB80) (LH	T
16	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_307'	'(p07371|cb22_pea : 279.0) Chlorophyll a-b binding protein AB80, chloroplast precursor (LHCII type I CAB-AB80) (LH	T
17	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_576'	'(at4g10340 : 223.0) photosystem II encoding the light-harvesting chlorophyll a/b binding protein CP26 of the anten	T
18	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_710'	'(at5g01530 : 467.0) light harvesting complex photosystem II (LHCB4.1); FUNCTIONS IN: chlorophyll binding; INVOLV	T
19	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_1080'	'(at4g10340 : 215.0) photosystem II encoding the light-harvesting chlorophyll a/b binding protein CP26 of the anten	T
20	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_5965'	'(p07371|cb22_pea : 481.0) Chlorophyll a-b binding protein AB80, chloroplast precursor (LHCII type I CAB-AB80) (LH	T
21	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_6437'	'(p27490|cb28_pea : 124.0) Chlorophyll a-b binding protein 8, chloroplast precursor (LHCII type I CAB-8) - Pisum sati	T
22	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_6793'	'(at2g40100 : 320.0) Lhcb4:3 protein (Lhcb4.3, light harvesting complex of photosystem II; light harvesting complex	T
23	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_11709'	'(loc_os07g37240.1 : 143.0) no description available & (at2g40100 : 139.0) Lhcb4:3 protein (Lhcb4.3, light harvesting c	T
24	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_14489'	'(p27490|cb28_pea : 122.0) Chlorophyll a-b binding protein 8, chloroplast precursor (LHCII type I CAB-8) - Pisum sati	T
25	'1.1.1.1'	'PS.lightreaction.photosystem II.LHC-II'	'202r_contig_17632'	'(p27490|cb28_pea : 180.0) Chlorophyll a-b binding protein 8, chloroplast precursor (LHCII type I CAB-8) - Pisum sati	T

(Fig.4.2. Results of MapMan Merctor in a excel sheet)



	A	B	C	D	E
94778	'35.2'	'not assigned.unknown'	'202r_contig_91202'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94779	'35.2'	'not assigned.unknown'	'202r_contig_91203'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94780	'35.2'	'not assigned.unknown'	'202r_contig_91204'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94781	'35.2'	'not assigned.unknown'	'202r_contig_91205'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94782	'35.2'	'not assigned.unknown'	'202r_contig_91206'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
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94785	'35.2'	'not assigned.unknown'	'202r_contig_91210'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94786	'35.2'	'not assigned.unknown'	'202r_contig_91211'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94787	'35.2'	'not assigned.unknown'	'202r_contig_91213'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94788	'35.2'	'not assigned.unknown'	'202r_contig_91214'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94789	'35.2'	'not assigned.unknown'	'202r_contig_91216'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94790	'35.2'	'not assigned.unknown'	'202r_contig_91218'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to T	
94791	'35.2.1001'	'not assigned.unknown'	'unknown001'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to M	
94792	'35.3'	'not assigned.disagreeing hits'	''	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to the cyto	
94793	'991'	Mineral Nutrition'	''	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to the cyto	
94794	'991.1'	'Mineral Nutrition.phosphate'	''	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to the cyto	
94795		'Mineral Nutrition.phosphate'	'phosphate'	'(at1g79900 : 154.0) encodes a mitochondrial ornithine transporter that exports ornithine from the mitochondria to M	
94796					
94797					
94798					
94799					
94800					
94801					
94802					

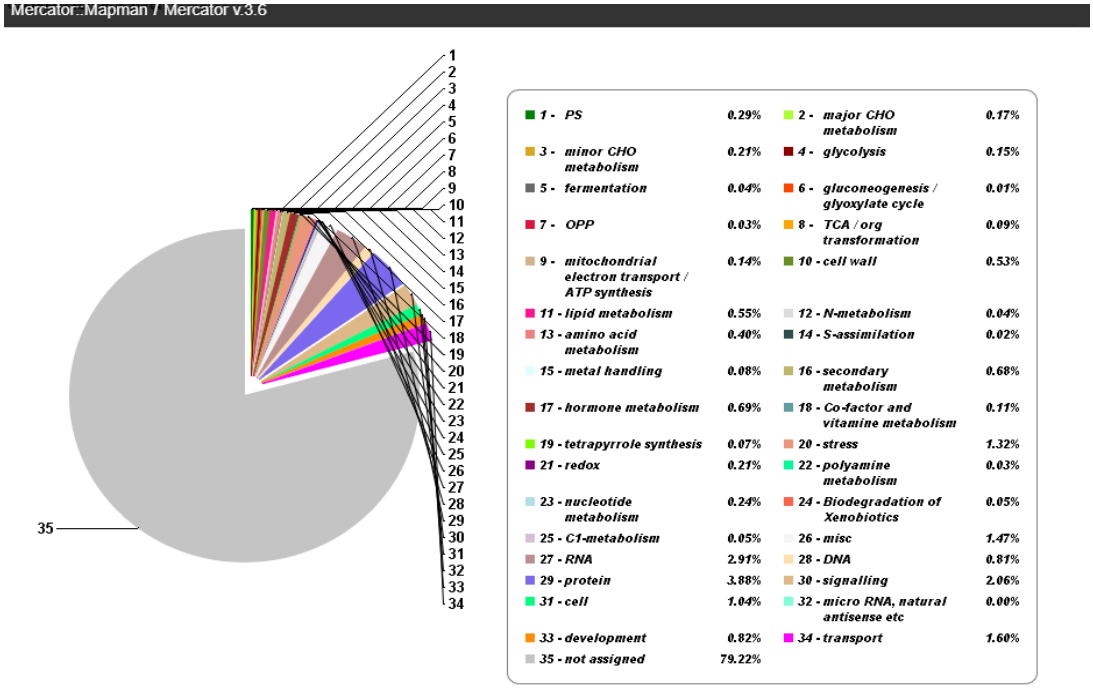
(Fig.4.3. 94,794 sequences were annotated)

Table: 4.1. Functional category of Lathyrus transcriptomes with the number of sequences under each category

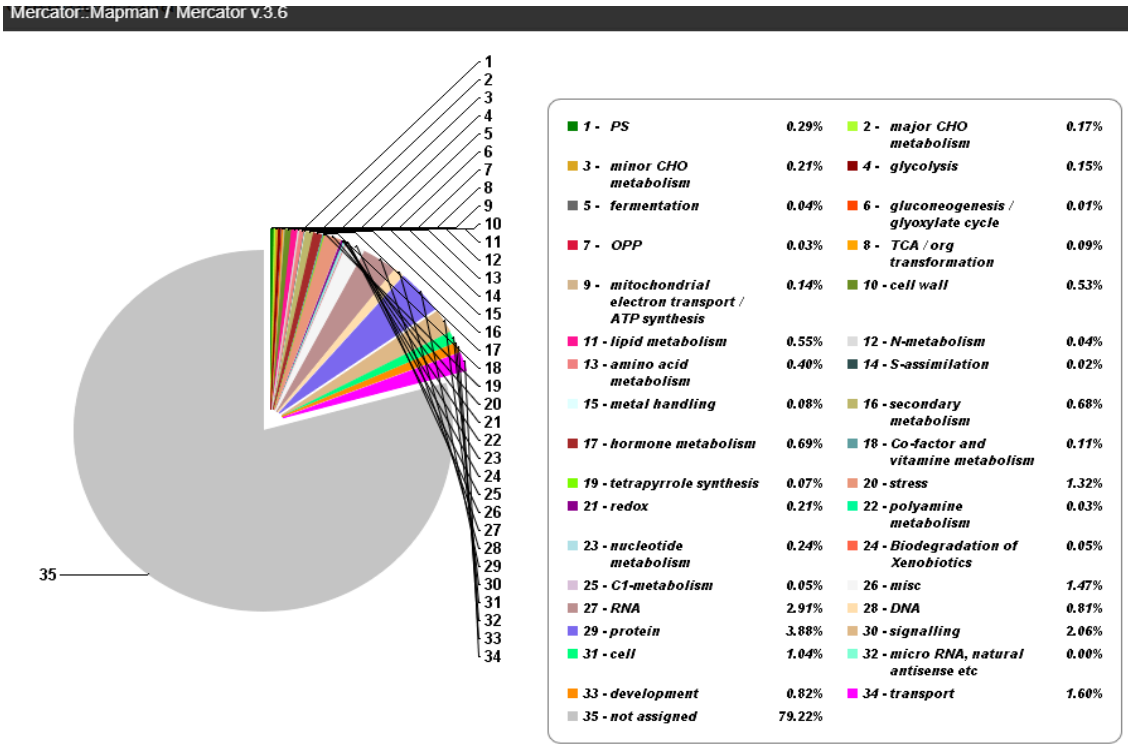
Sl.No	Enzymes identified in the functional categories	Total no. of sequences corresponding to the category
1.	Photosynthesis Light Reaction	307
2.	Photosynthetic Reductive pathway	194
3.	Minor Carbohydrate metabolism	220
4.	Glycolysis	162
5.	Fermentation	42
6.	Glyconeogenesis/Glyoxalate cycle	14
7.	OPP	34
8.	Tricarboxylate (TCA) cycle	101
9.	Mitochondrial electron transport chain(ETC)	136
10.	Cell wall synthesis	524
11.	Lipid metabolism	588
12.	Nitrogen metabolism	45
13.	Amino acid metabolism	496
14.	Sulphur-assimilation	25
15.	Metal handling	72
16.	Secondary metabolism	812
17.	Hormone metabolism	696
18.	Co-factor and vitamin metabolism	127
19.	Tetrapyrrole synthesis	64
20.	Stress	1248
21.	Redox reactions	207
22.	Polyamine metabolism	37
23.	Nucleotide metabolism	270
24.	Biodegradation of xenobiotics	44
25.	Carbon 1 metabolism	78
26.	Miscellaneous	1352
27.	RNA processing	2750

28.	DNA synthesis	769
29.	Protein metabolism	3862
30.	Signaling	1949
31.	Cell organization	1017
32.	Micro RNA synthesis	1
33.	Development	769
34.	Transport	1506

Although the remaining transcriptomes were not functionally categorized but there functional descriptions were provided.



(Fig.4.4. Pie chart showing the functional categories of genotype Mahateora.)



(Fig.4.5. Pie chart showing the functional categories of genotype RLK-1950.)

4.2. Selection of transcripts based on the functional annotation of Mercator.

The β-ODAP biosynthesis pathway, although is not fully understood (Ikegami, F. *et al*., 1996; Malathi, K.*et al*., 1967) but the present knowledge on the pathways indicated that the synthesis start with formation of β - (isoxazolin-5-on-2-yl) alanine from the precursor, O-acetylserine and isoxazolin-5-one. Here β -cyanoalanine synthase (CAS) uses acts on isoxaolin-5-one and use it as an alternative nucleophile in the reaction (Ikegami, F.*et al*., 1996; Ikegami, F.*et al*., 1997). In the next step β-(isoxazolin-5-on-2-yl)alanine (BIA) is believed to be converted to the short-lived intermediate 2,3,-L-diaminopropanoic acid (DAPA), which is further oxalylized by oxalyl-coenzyme A to form β -ODAP (Malathi, K.*et al*., 1967; Malathi, K.*et al*., 1970; Kuo, Y.H.*et al*.,1994 ;Kuo, Y.H.*et al*.,1991). Thus the formation of isoxazolin-5-one is essential for β -ODAP synthesis which is perceived to be linked to general activities of enzyme cysteine synthase (CS; also known as either O-acetylserine sulfhydrylase or O-acetylserine (thiolyase) and CAS (Ikegami, F.*et al*., 1992; Ikegami, F.*et al*., 1993). The CS enzyme is important in cysteine biosynthesis, where it catalyzes formation of cysteine from O-acetylserine and hydrogen sulfide. The cysteine so formed is then used by enzyme CAS as a substrate for cyanide detoxification in plants (Machingura, M. *et al*., 2016). In the same series of reaction the

conversion of β-cyanoalanine to asparagine occurs that leads to the formation of isoxazolin-5-one. It indicates that the β-ODAP synthesis and its content in L. sativus is regulated through integration of nitrogen and sulfur metabolism where the enzyme CAS plays a major role.

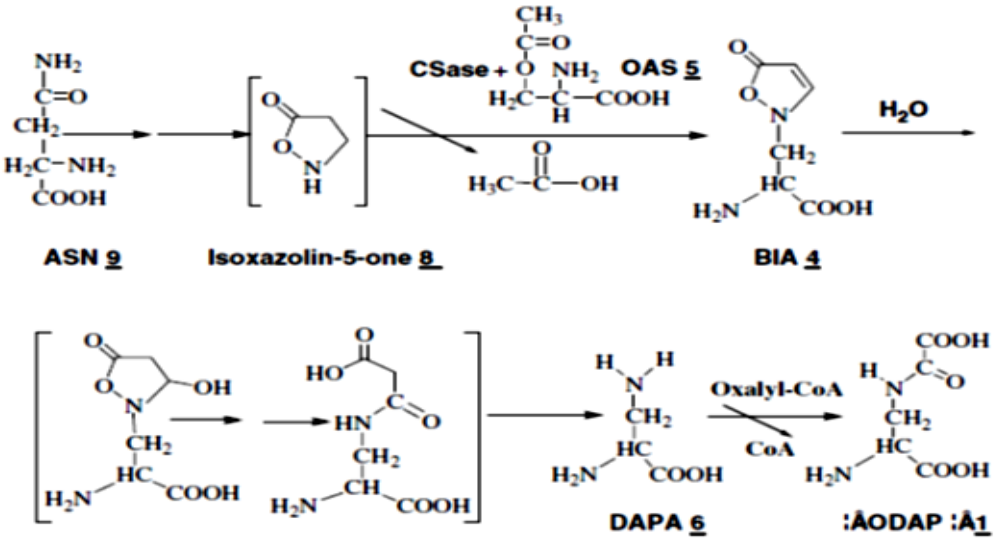


Fig.4.6. Proposed biosynthetic pathway of β –ODAP in *Lathyrus sativus*
Source: Z.-Y. Yan et al / Phytochemistry 67 (2006) 107-121.

The neurotoxicity of ODAP increases in the isolated neurons when the growth medium is depleted with methionine and Cysteine, Sulphur-containig amino acids (Kusama-Eguchi, K. *et al*., 2011). Additionally, consuming grasspea with Sulphur-containig vegetables reduces the neurotoxicity of β –ODAP (Getahun, H. *et al*., 2003; Getahun, H. *et al*., 2005). The oxidative stress induced by the lack of Sulphur-containig amino acids might be associated with the human neurolathyrism (Hanbury, C.D. *et al*., 2000). Consequently, it is suggested that increase in the Sulphur-containig amino acids in Grasspea might help in lowering the human neurotoxicity even without considerable decrease in the levels of β –ODAP (Hanbury, C.D. *et al*., 2000; Lambein, F. *et al*., 2009).

The relation between Nitrogen availability, accumulation of β –ODAP and increasing levels of Reactive Oxygen Species (ROS) is suggested as the leaves of *L.sativus* containing low levels of β-ODAP has high levels of O^{2-} and H_2O_2 and vice versa. Consequently, the inhibition

or degradation of β –ODAP might be caused by the presence of increased levels of ROS, especially O_2^- (Jiao, C.J. *et al.*, 2011).

On the basis of above cited literature and proposed schemes and reaction of biosynthesis pathways the sequence search in the present study was focused on 4 main metabolic pathways as followed:

4.2.1. Cyanide metabolism

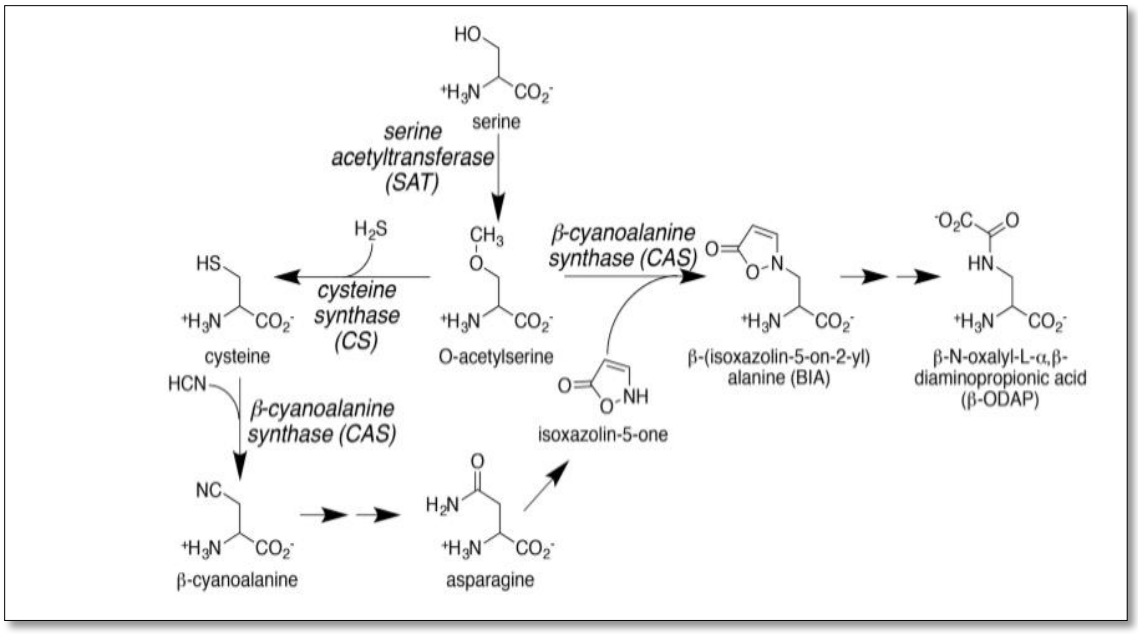
4.2.2. Amino acid metabolism

4.2.3. d-amino acid metabolism

4.2.4. Reactive Oxygen Species (ROS) metabolism

4.2.1 Selection on the basis of Cyanide metabolism:-

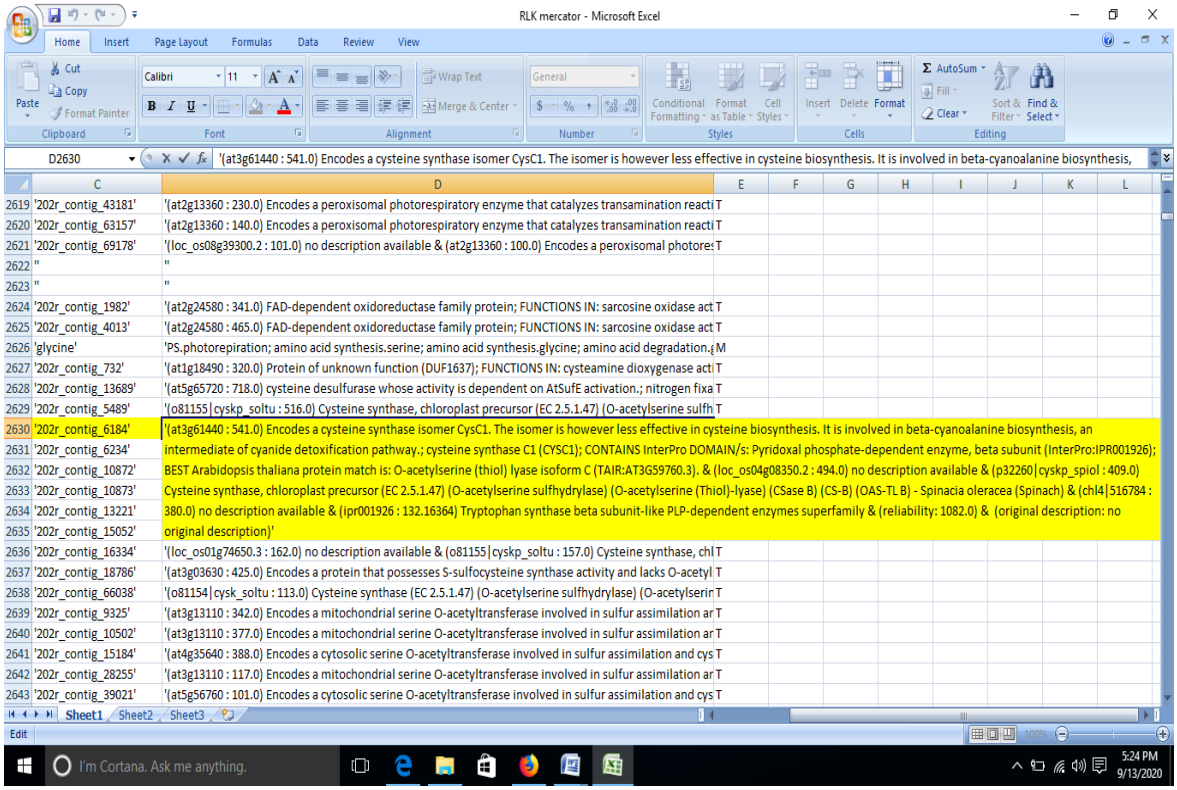
Ther enzyme cyanoalanine synthase (CAS) which catalyzes the formation of cyanoalanine from the precursor Cysteine and hydrogen cyanide (HCN) which forms a ringed structure isoxazolin-5-one, precursor of β-(isoxazolin-5-on-2-yl)alanine (BIA) which further forms β-ODAP (Machingura *et al*,. 2016). This pathway is suggested as the cyanide detoxification pathway localized in the cytoplasm or mitochondria. The enzyme CAS also directly catalyzes the formation of β-(isoxazolin-5-on-2-yl) alanine (BIA) from the substrate O-acetylserine. CAS also plays an important role in the integration of nitrogen and sulphur metabolism and β-ODAP biosynthesis.



(Fig.4.7. β-ODAP biosynthetic pathway in grass pea.
Source: Xu, *et al.*, Int. J. Mol. Sci. 2017, 18, 526)

Only one enzyme predicted as Cyanoalanine synthase has been observed in the Mercator output (excel sheet) which is also an isomer of Cysteine synthase as suggested by Wirtz, M.et *al*., (2004). The functional description also predicts that this enzyme is localized in the chloroplast or mitochondria.

The Mercator output predicts this enzyme as 'Encoding a cysteine synthase isomer CysC1. The isomer is however less effective in cysteine biosynthesis. It is involved in beta-cyanoalanine biosynthesis, an intermediate of cyanide detoxification pathway. Cysteine synthase C1 (CYSC1); CONTAINS InterPro DOMAIN/s: Pyridoxal phosphate-dependent enzyme, beta subunit (InterPro:IPR001926); BEST Arabidopsis thaliana protein match is: O-acetylserine (thiol) lyase isoform C (TAIR:AT3G59760.3). & (loc_os04g08350.2: 494.0) (p32260|cyskp_spiol: 409.0) Cysteine synthase, chloroplast precursor (EC 2.5.1.47) (O-acetylserine sulfhydrylase) (O-acetylserine (Thiol)-lyase) (CSase B) (CS-B) (OAS-TL B) - Spinacia oleracea (Spinach) & (chl4|516784: 380.0) no description available & (ipr001926: 132.16364) Tryptophan synthase beta subunit-like PLP-dependent enzymes superfamily & (reliability: 1082.0).



(Fig.4.8. Mercator output (excel sheet) showing 1 gene for Cyanoalanine synthase localized in chloroplast)

4.2.2. Amino acid metabolism

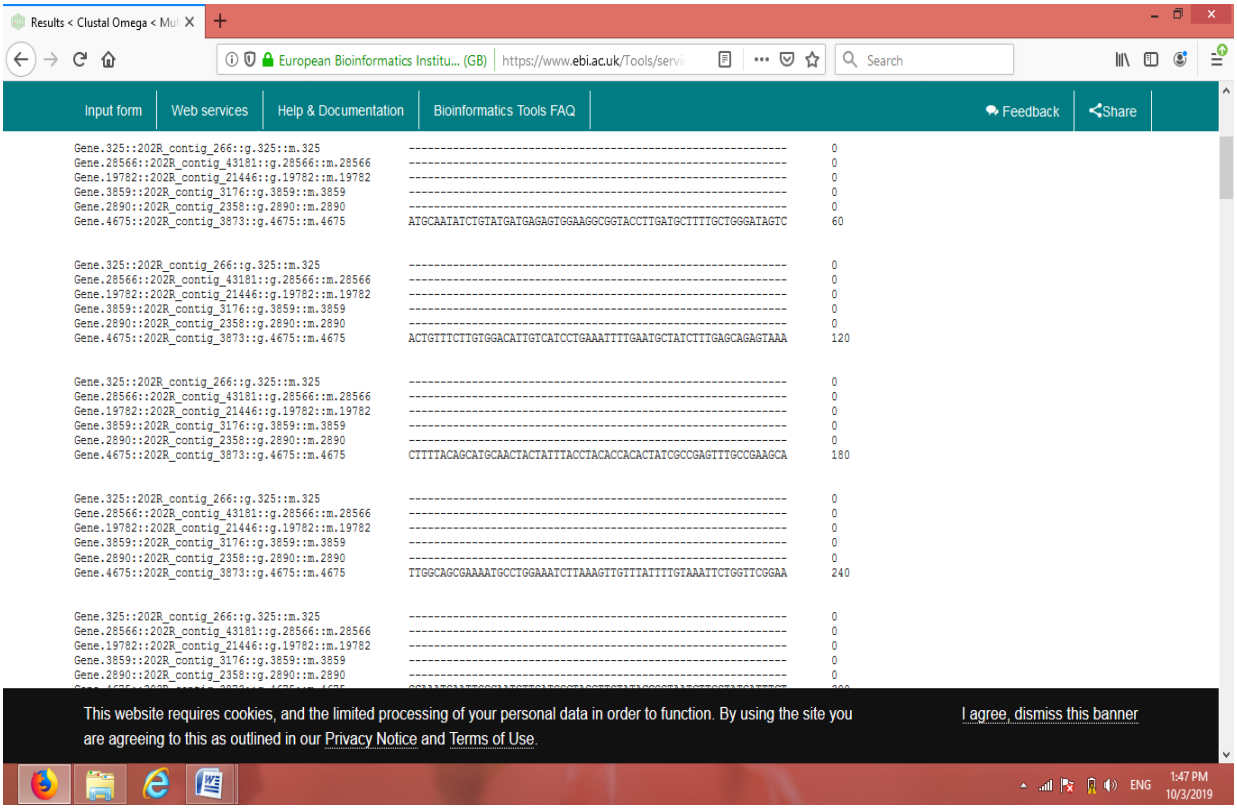
As it is known that β-ODAP is a non-proteinaceous amino acid (Hussain,M.*et al*., 1994; Jiao *et al*., 2011; Yan *et al*., 2005; Yadav *et al*., 1995), henceforth all functional annotations predicted in Mercator that were corresponding to amino acid metabolism (496) were short listed. Further this were classified in the following subcategories to identify their function in relation to ODAP biosynthesis reactions:-

4.2.2.1. Alanine Aminotransferase:-

Malathi *et al*., (1970) predicted that the main precursor β-ODAP biosynthesis pathway is O-acetyl serine formed from the L-serine by the catalytic action of O-acetyltransferase whch further converts to 3-(5-oxoisoxazolin-2-yl)-L-alanine. The final steps of biosynthesis of β-ODAP from OAS (O-Acetyl serine) involves breakdown of 3-(5-oxoisoxazolin-2-yl)-L-alanine to form a short lived intermediated, presumably β-2,3 diaminopropionic acid (DAPA). This short lived intermediate is then perceived to be converted into N3 L-oxalyl β-2-3, diaminopropionic

acid, by activity of an enzyme 2,3-diaminopropionate N- oxalyltransferase. Also, the study carried out by Nayak *et al*., (2019) on Alanine Synthase enzyme identified from bacterial genome origin showed differential expression of the corresponding gene in high and low ODAP containing Lathyrus genotypes. They hypothesized that the gene which is homologous to L-alanine synthase (Pyridoxal phosphate dependent enzyme (PLPD) can catalyze the pathway using the O-acetyl-L-serine as the common precursor, converting O-acetyl-L-serine to either β-isoxazoline-5-one-2yl-l-alanine (BIA) or its isomer β-isoxazoline-5-one-4-yl-L-alanine synthase. The isomer β-isoxazoline-5-one-4-yl-L-alanine does not forms ODAP but proceeds to form another compound L-Willardine. Additionally, they hypothesized that the enzyme PLPD when proceeds to form willardine, its β-isoxazoline-5-one-2yl-l-alanine synthase activity decreases.

In this study, the Mercator functional annotation did not predicted transcripts encoding for L-alanine synthase, yet it accommodated 7 sub-categories for the Alanine aminotransferase. The transcript sequence were retrieved from the transcriptome data and were compared for sequence similarity amongst them. The Multiple sequence alignment of these sequence performed using Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) showed no similarity. On the basis of gene functions, length and localization in chloroplast and mitochondria 4 sequences were selected with contig Id's 202R_contig_17606, 202R_contig_3873, 202R_contig_3873 and 202R_contig_2358.

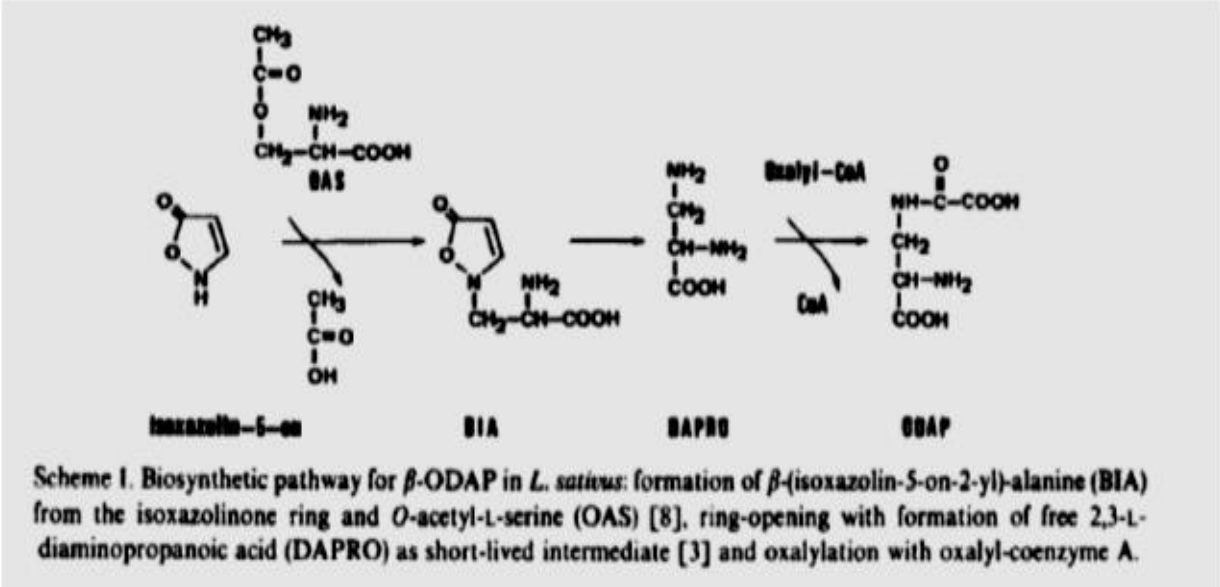


(Fig.4.9. Clustal omega results showing no similarity between 7 genes sequences of alanine aminotransferase)

4.2.2.2. Cysteine Synthase (CS)

Cysteine synthase (CS) is considered as principal regulatory enzyme that catalyze the reaction to form Cysteine from O-acetyl serine in the β-ODAP biosynthesis pathway. Also studies from native-polyacrylamide gel electrophoresis reported that L.sativus has five Bsas (I-V) isoenzymes in which I is classified as Cyanoalanine synthase (CAS) and II-V were classified as Cysteine synthase (CS) (Jiao *et al*,. 2014). Additionally, 2 isoenzymes of cysteine synthase purified from the young seedlings of L. sativus that catalyse the formation of BIA, one of them localized is the mitochondria and the other in the chloroplast. Further both the isoenzymes showed similar catalytic activity to form β-(isoxazolin-5-on-2-yl) alanine from O-acetyl-serine (OAS) (Ikegami *et al*., 1993; Wirtz et al,. 2004). Cysteine synthase has also known to the sulphur and nitrogen assimilatory pathway and in the regulation of these 2 pathways (Leustek, T.et *al*., 1999; Takahashi *et al*., 1996).

As a consequence of above cited information Mercator output (excel sheet) was screened and 4 genes associated with Cysteine synthase (CS) was observed. Among these 4 genes one predicts the Cyanoalanine synthase (CAS). Consequently the contig Id's of selected Cysteine synthase enzymes were 202R_contig_5489, 202R_contig_13221 and 202R_contig_18786.



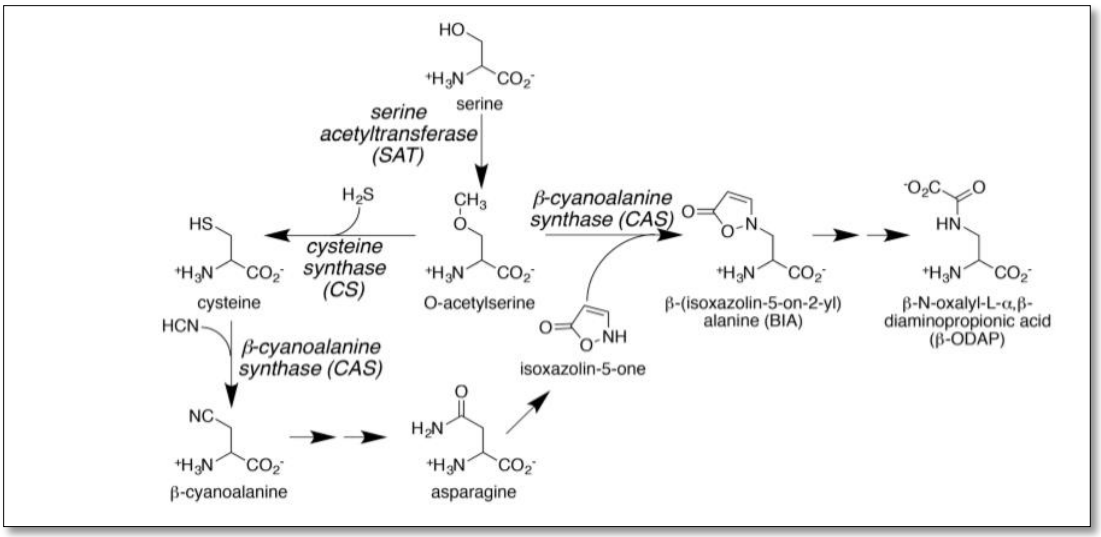
Scheme 1. Biosynthetic pathway for β-ODAP in *L. sativus*: formation of β-(isoxazolin-5-on-2-yl)-alanine (BIA) from the isoxazolinone ring and *O*-acetyl-L-serine (OAS) [8], ring-opening with formation of free 2,3-L-diaminopropanoic acid (DAPRO) as short-lived intermediate [3] and oxalylation with oxalyl-coenzyme A.

(Fig.4.10. Biosynthetic pathway for ODAP in *L.sativus.*
Source: Ikegami et al., (1993) Phytochemistry 33, 93.)

4.2.2.3 Aaparagine Metabolism

The cyanide metabolism pathway in higher plant involves the formation of Asparagine from β–cyanoalanine catalyzed by β–cyanoalanine hydrolases (Castric.P.A.*et al.*, 1974). Similarly, the biosynthetic pathway of β-ODAP also manifests that asparagine is formed from β-Cyanoalanine which subsequently converted to a ringed structure isoxazolin-5-one. Henceforth the enzymes related to Asparagine metabolism might also be involved in the β-ODAP biosynthetic pathway.

The Mercator output (excel sheet) was screened for genes related to asparagines metabolism. As a result 3 genes related to asparagine synthase and 3 genes related to L-asparagine. A total of 6 enzymes were found out which might help in the β-ODAP biosynthesis pathway. The contigs of Asparaginase were 202R_contig_15653, 202R_contig_17961and 202R_contig_31383 and that of Asparagine synthase were 202R_contig_427, 202R_contig_8260 and 202R_contig_18458.



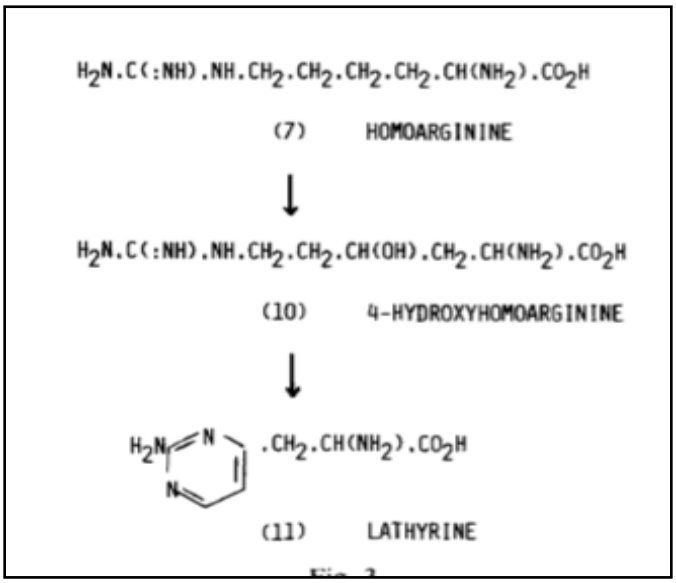
(Fig.4.11. β-ODAP biosynthetic pathway in grass pea.
Source: Xu, *et al*., Int. J. Mol. Sci. 2017, 18, 526)

4.2.2.4. Ornithine Cyclodeaminase

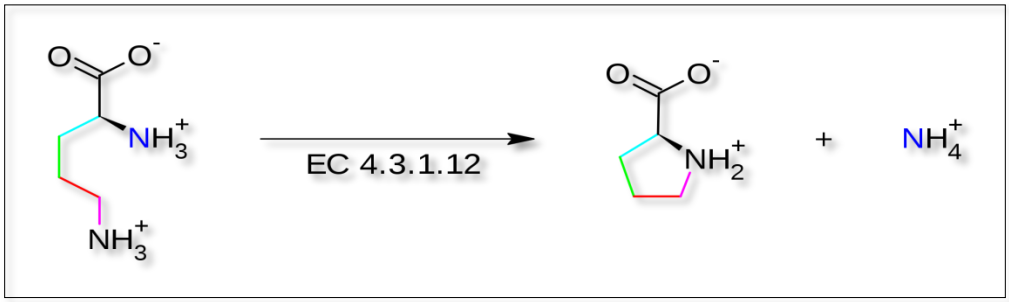
Lathyrus, in addition to β-ODAP, also synthesizes other lathyrogens like oxalyldiaminopropionic acid, L-homoarginine, 4-hydroxyhomoarginine, lathyrine, tetrahydrolathyrine, L-2, 4-diaminobutyric acid and 3-aminopropionitrile. Every *Lathyrus* sp. Ehich accumulates β-ODAP also synthesizes L-homoarginine (neurotoxic). This L-homoarginine is synthesized from the ornithine which converts to arginine then to L-homoarginine(Bell, 1984). Further this L-homoarginine acts as a precursor for the synthesis of 4-hydroxyhomoarginine and lathyrine. Additionally this helps in the formation of by L-2, 4-diaminobutyric acid (Ressler *et al*., 1961; Bell, 1962) which is a lower homologue of ornithine and is highly toxic to the experimental animals and resulted in death due to inhibition of ornithine transcarbamylase (O'Neal *et al*., 1968). The homologues of diamino acids endures a transamination reaction at a decreasing rate as: ornithine > α, β-diaminopropionate > α, y-diaminobutyrate > lysine. The α, β-diaminopropionate is suggested to form from orinithine which is very much similar to β-ODAP. Consequently, ornithine might have involved in the β-ODAP biosynthesis pathway.

The biosynthetic pathway of β-ODAP has still many uncatalyzed reactions whose enzymes are unknown. The reaction in which asparagine is converted to isoxazolin-5-one also converts a straight chain to cyclic compound and has unknown enzymes involved. The enzyme ornithine cyclodeaminase acts on the Carbon –Nitrogen bond other then the peptide bonds and

also cyclizes the staright chain (Fig.13). Hence hypothesizing that this enzyme might be involved in the β-ODAP biosynthesis pathway to form a cyclic product isoxazolin-5-one.



(Fig.4.12. Structures of homoarginine, 4-hydroxyhomoarginine and lathyrine)

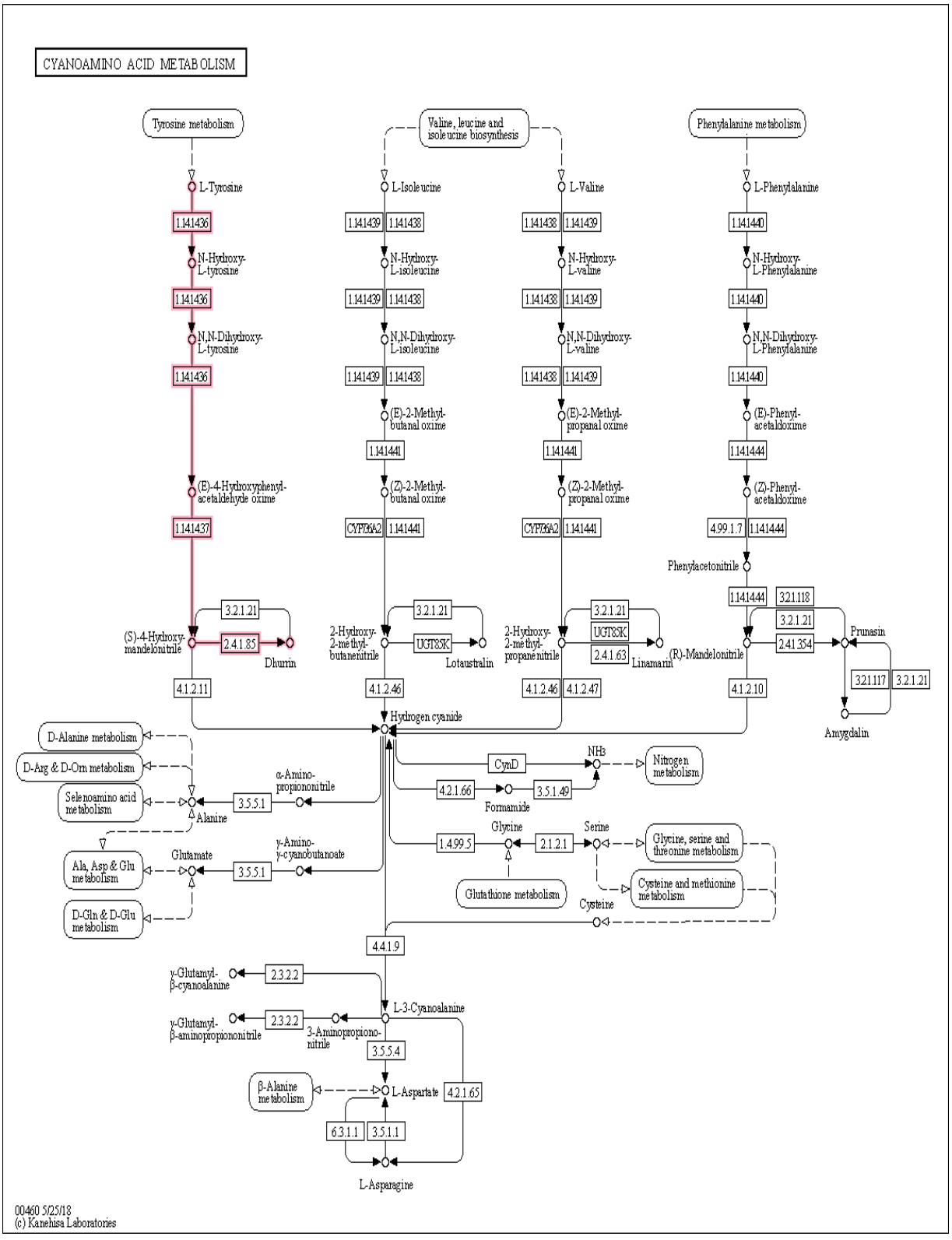


(Fig .4.13. Reaction performed by ornithine cyclodeaminase)

4.2.3. D-amino acid metabolism

The alanine synthase investigated by Nayak *et al*., (2019) when screened for homology sequences showed a result for D-alanaine synthase. Moreover the KEGG (https://www.genome.jp/kegg/) results also shows the metabolism of D-alanine, D-glutamate D-arginine and D-ornithine in the Cyanoalanine pathway depicted in the fig. Consequently, it is hypothesizes that β-ODAP can be formed from D-form of amino acid (alanine). This D-form alanine might be involved in the β-ODAP biosynthesis pathway.

As a result Mercator output (excel sheet) is searched for enzymes involved in the d-amino acid metabolism. Consequently, only 2 sequences were identified corresponding to the search. Among the 2, one sequence is associated with the RNA regulation specifically in D-aminoacyl-tRNAdeacylase or D-Tyr-tRNA (Tyr) deacylase. This enzyme belongs to acylase family acting on the ester bonds. And from tyrosine, Cyanoalanine is also synthesized (from KEGG) depicted in fig.4.14.



(Fig.4.14. Pathway of Cyanoalanine metabolism which also forms D-alanine.

Source: KEGG map00460)

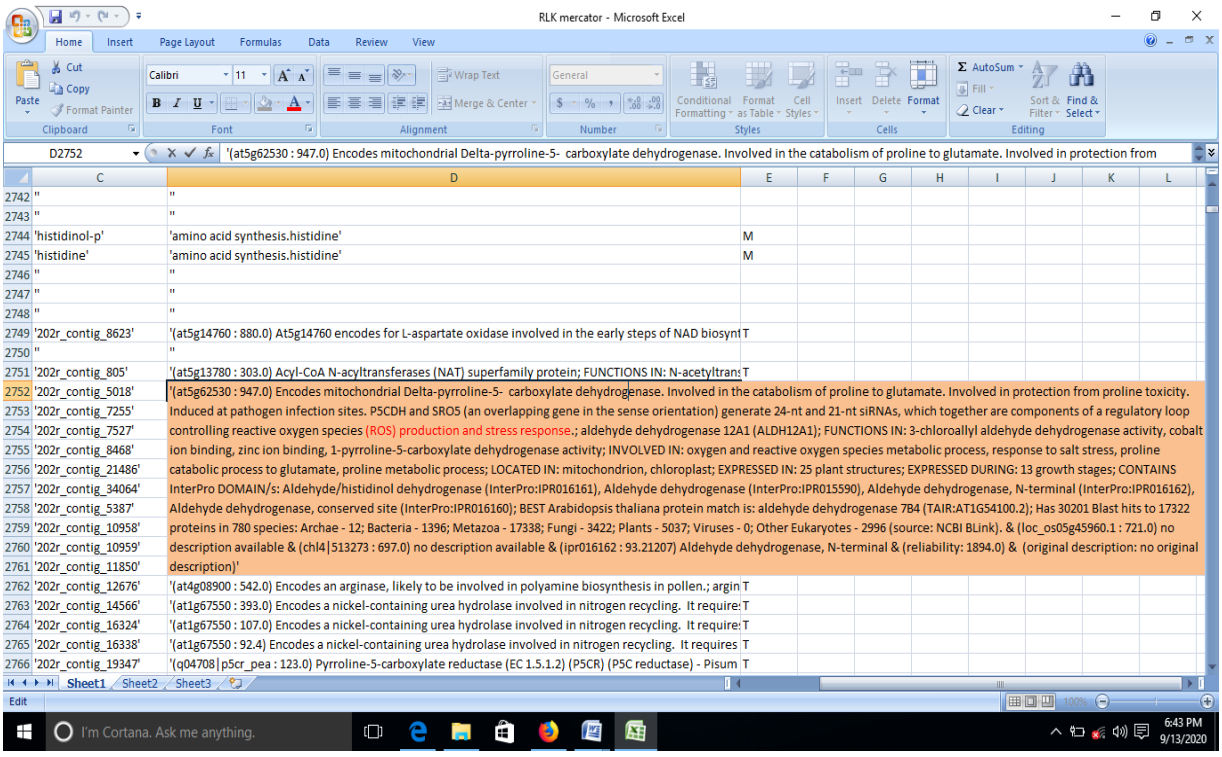
4.2.4. ROS Metabolism

Ravindranathan *et al*., (2002) suggested that mitochondrial dysfunction resulted due to the thiol oxidation caused by reactive oxygen species ROS (mainly superoxides O^{2-} and hydrogen peroxide H_2O_2) leads to neurolathyrism. Moreover, Jiao *et al*., (2011) suggested that ROS might be involved in the inhibition or degradation of β-ODAP due to the fact that leaves containing high levels of β-ODAP had low levels of O^{2-} and H_2O_2 and vice- versa. Further it is suggested when the leaves are young, β-ODAP concentration is high due to the O-acetylserine to BIA which further converts to β-ODAP while in the older leaves, the antioxidant activity increases as a consequence of increasing Reactive oxygen species content in the older leaf tissue. The antioxidant activity involves the formation of Cysteine from OAS which subsequently forms glutathione, principal metabolite formed during ROS antioxidant activity (López-Martín *et al*., 2008).

When Mercator output (excel sheet) was screened for enzymes involved in ROS metabolism 43 BIN codes were identified, yet only 1 named delta-1-pyrroline-5-carboxylate dehydrogenase (P5CD)enzyme was involved in both ROS metabolism and amino acid metabolism too. This enzyme helps in the stress condition and has localization in the mitochondria and chloroplast. Hence, it might be involved in the β-ODAP biosynthesis pathway.

The Mercator output describes this transcriptomes as 'Encoding a mitochondrial Delta-pyrroline-5- carboxylate dehydrogenase which is involved in the catabolism of proline to glutamate and in protection from proline toxicity (produced during stress) which is induced at pathogen infection sites. P5CDH and SRO5 (an overlapping gene in the sense orientation) generate 24-nt and 21-nt siRNAs, which together are components of a regulatory loop controlling reactive oxygen species (ROS) production and stress response.; aldehyde dehydrogenase 12A1 (ALDH12A1); FUNCTIONS IN: 3-chloroallyl aldehyde dehydrogenase activity, cobalt ion binding, zinc ion binding, 1-pyrroline-5-carboxylate dehydrogenase activity; INVOLVED IN: oxygen and reactive oxygen species metabolic process, response to salt stress, proline catabolic process to glutamate, proline metabolic process; LOCATED IN: mitochondrion, chloroplast; EXPRESSED IN: 25 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: Aldehyde/histidinol dehydrogenase (InterPro:IPR016161), Aldehyde dehydrogenase (InterPro:IPR015590), Aldehyde

dehydrogenase, N-terminal (InterPro:IPR016162), Aldehyde dehydrogenase, conserved site (InterPro:IPR016160); BEST Arabidopsis thaliana protein match is: aldehyde dehydrogenase 7B4 (TAIR:AT1G54100.2); Has 30201 Blast hits to 17322 proteins in 780 species: Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants - 5037; Viruses - 0; Other Eukaryotes - 2996 (source: NCBI BLink). & (loc_os05g45960.1: 721.0) no description available & (chl4|513273: 697.0) no description available & (ipr016162: 93.21207) Aldehyde dehydrogenase, N-terminal & (reliability: 1894.0).'



(Fig.4.15. Mercator excel sheet showing ROS metabolism under glutamate metabolism.)

4.3. Homology sequences searches using NCBI BLAST

NCBI (National Centre for Biotechnology Information; http://www.ncbi.nih.gov/) is an online, database, user-friendly bioinformatic tool containing all the nucleotide and protein sequence which has been sequenced till date. It gives the result by comparing the query sequences with the sequences present already in the database.

Hence, comprehensive database screening of above selected sequences was done with the default settings in the BLASTn. Results predicted highest identity with the *Medicago truncatula* followed by *Cicer arienatum* with the similar functions as predicted by Mercator functional annotation tool.

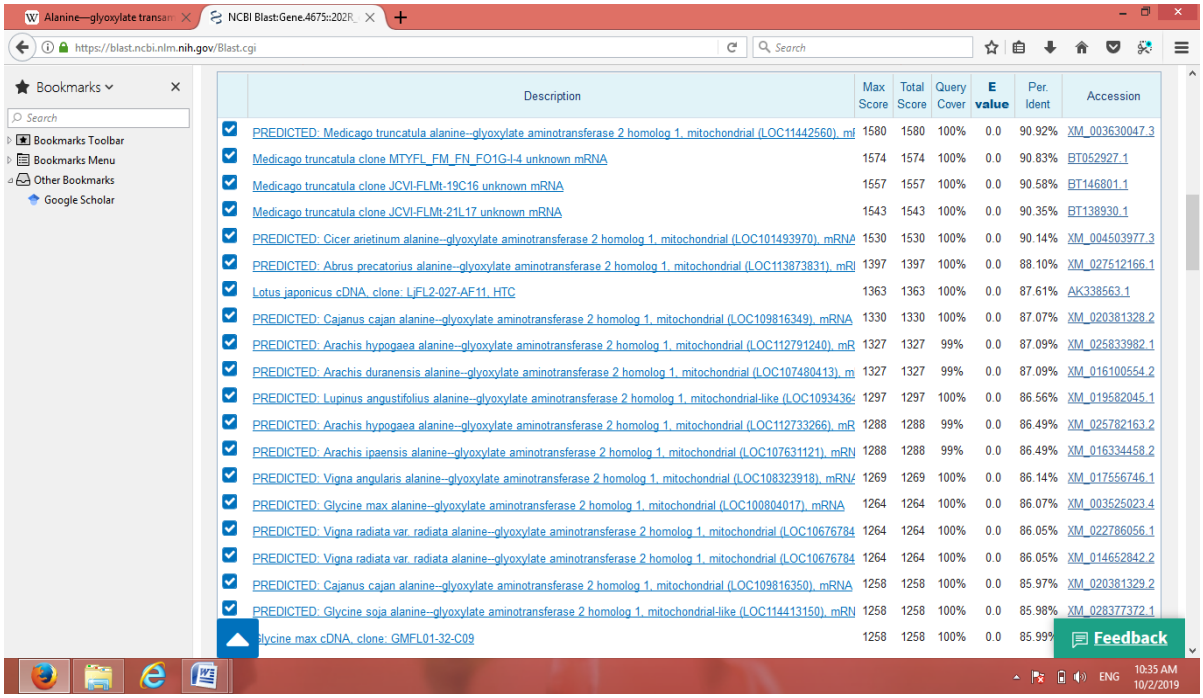
Table: 4.2.Functions predicted by Mercator to selected transcripts and fuctions predicted by NCBI BLAST to corresponding transcript

Sl. No.	Function assigned by Mercator	Similarity with (in NCBI BLAST)	Query cover	EC value
1.	**Cysteine synthase isomer (CAS)**	a. Lathyrus sativus beta-cyanoalanine synthase (Bsas3;1) b. Medicago truncatula Cysteine Synthase	100% 100%	0 0
2.	**Cysteine synthase**			
	• 202R_contig_5489	Medicago truncatula cysteine synthase, chloroplastic	99%	0
	• 202R_contig_6234	Medicago truncatula cysteine synthase, chloroplastic	98%	0
	• 202R_contig_18786	Medicago truncatula probable S-sulfocysteine synthase, chloroplastic	98%	0

	3.**Alanine:glyoxylate aminotransferase** • 202R_contig_17606	Medicago truncatula alanine--glyoxylate aminotransferase	96%	0
	• 202R_contig_3873	Medicago truncatula alanine--glyoxylate aminotransferase	100%	0
	• 202R_contig_266	Medicago truncatula serine--glyoxylate aminotransferase	98%	0
	• 202R_contig_2358	Medicago truncatula alanine--glyoxylate aminotransferase	100%	0
	4. **L-Asparaginase** • 202R_contig_15653	Medicago truncatula isoaspartyl peptidase/L-asparaginase	100%	0
	• 202R_contig_17961	Medicago truncatula probable isoaspartyl peptidase/L-asparaginase 2	99%	0
	• 202R_contig_31383	Medicago truncatula putative threonine aspartase	96%	0

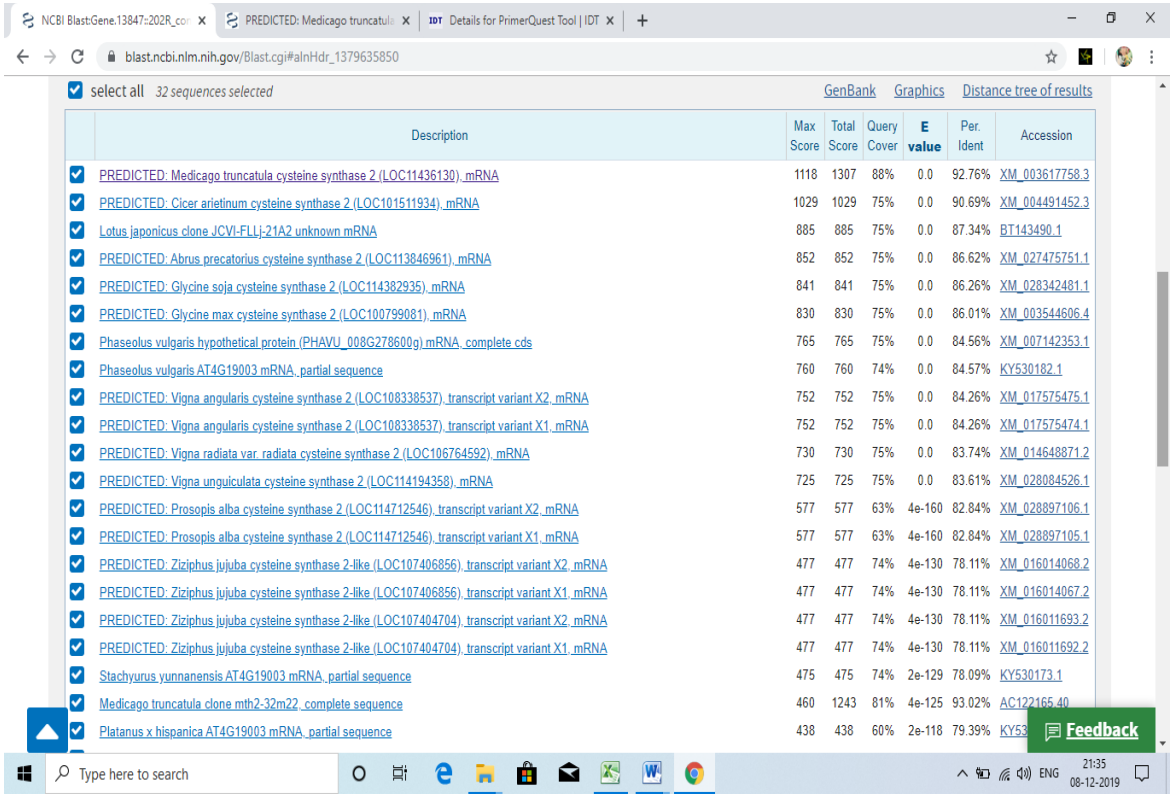
	5. **Asparagine Synthase** • 202R_contig427	Medicago truncatula asparagine synthetase	99%	0
	• 202R_contig_8260	Medicago truncatula asparagine synthetase, root [glutamine-hydrolyzing]	100%	0
	• 202R_contig_18458	Medicago truncatula asparagine synthetase [glutamine-hydrolyzing] 2	99%	0
	6. **Ornithine Cyclodeaminase** • 202R_contig_31662		98%	0
	7.**D-Tyr-tRNA(Tyr) deacylase(D-amino acid metabolism)** • 202R_contig_20127	Medicago truncatula D-aminoacyl-tRNAdeacylase	85%	-
	8.**Delta-pyrroline-5-carboxylate dehydrogenase (ROS Metabolism)** • '202r_contig_5018'	Medicago truncatula delta-1-pyrroline-5-carboxylate dehydrogenase 12A1, mitochondrial	100%	0

Figures showing the results of BLAST:



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
PREDICTED: Medicago truncatula alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC11442560), mI	1580	1580	100%	0.0	90.92%	XM_003630047.3
Medicago truncatula clone MTYFL_FM_FN_FO1G-I-4 unknown mRNA	1574	1574	100%	0.0	90.83%	BT052927.1
Medicago truncatula clone JCVI-FLMt-19C16 unknown mRNA	1557	1557	100%	0.0	90.58%	BT146801.1
Medicago truncatula clone JCVI-FLMt-21L17 unknown mRNA	1543	1543	100%	0.0	90.35%	BT138930.1
PREDICTED: Cicer arietinum alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC101493970), mRNA	1530	1530	100%	0.0	90.14%	XM_004503977.3
PREDICTED: Abrus precatorius alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC113873831), mRI	1397	1397	100%	0.0	88.10%	XM_027512166.1
Lotus japonicus cDNA, clone: LjFL2-027-AF11, HTC	1363	1363	100%	0.0	87.61%	AK338563.1
PREDICTED: Cajanus cajan alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC109816349), mRNA	1330	1330	100%	0.0	87.07%	XM_020381328.2
PREDICTED: Arachis hypogaea alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC112791240), mR	1327	1327	99%	0.0	87.09%	XM_025833982.1
PREDICTED: Arachis duranensis alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC107480413), m	1327	1327	99%	0.0	87.09%	XM_016100554.2
PREDICTED: Lupinus angustifolius alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial-like (LOC1093436	1297	1297	100%	0.0	86.56%	XM_019582045.1
PREDICTED: Arachis hypogaea alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC112733266), mR	1288	1288	99%	0.0	86.49%	XM_025782163.2
PREDICTED: Arachis ipaensis alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC107631121), mRN	1288	1288	99%	0.0	86.49%	XM_016334458.2
PREDICTED: Vigna angularis alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC108323918), mRNA	1269	1269	100%	0.0	86.14%	XM_017556746.1
PREDICTED: Glycine max alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC100804017), mRNA	1264	1264	100%	0.0	86.07%	XM_003525023.4
PREDICTED: Vigna radiata var. radiata alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC10676784	1264	1264	100%	0.0	86.05%	XM_022786056.1
PREDICTED: Vigna radiata var. radiata alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC10676784	1264	1264	100%	0.0	86.05%	XM_014652842.2
PREDICTED: Cajanus cajan alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial (LOC109816350), mRNA	1258	1258	100%	0.0	85.97%	XM_020381329.2
PREDICTED: Glycine soja alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial-like (LOC114413150), mRN	1258	1258	100%	0.0	85.98%	XM_028377372.1
Glycine max cDNA, clone: GMFL01-32-C09	1258	1258	100%	0.0	85.99%	

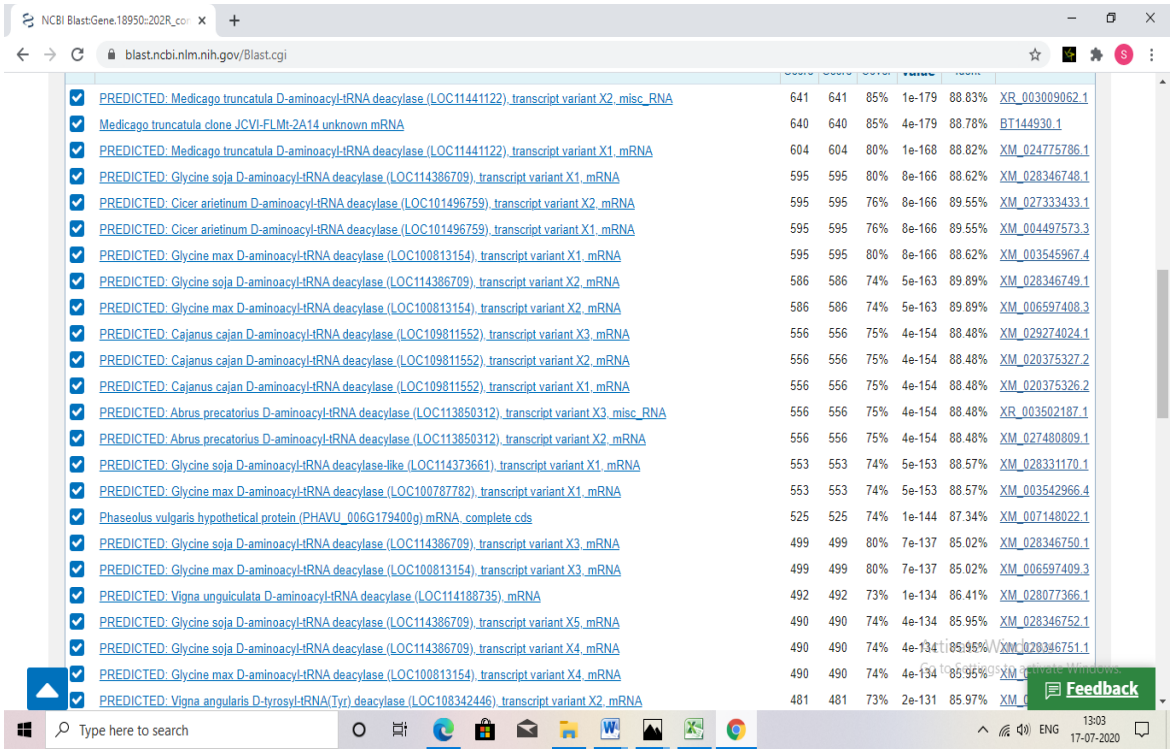
(Fig.4.16. BLAST results of Alanine amino transferase)



select all 32 sequences selected — GenBank | Graphics | Distance tree of results

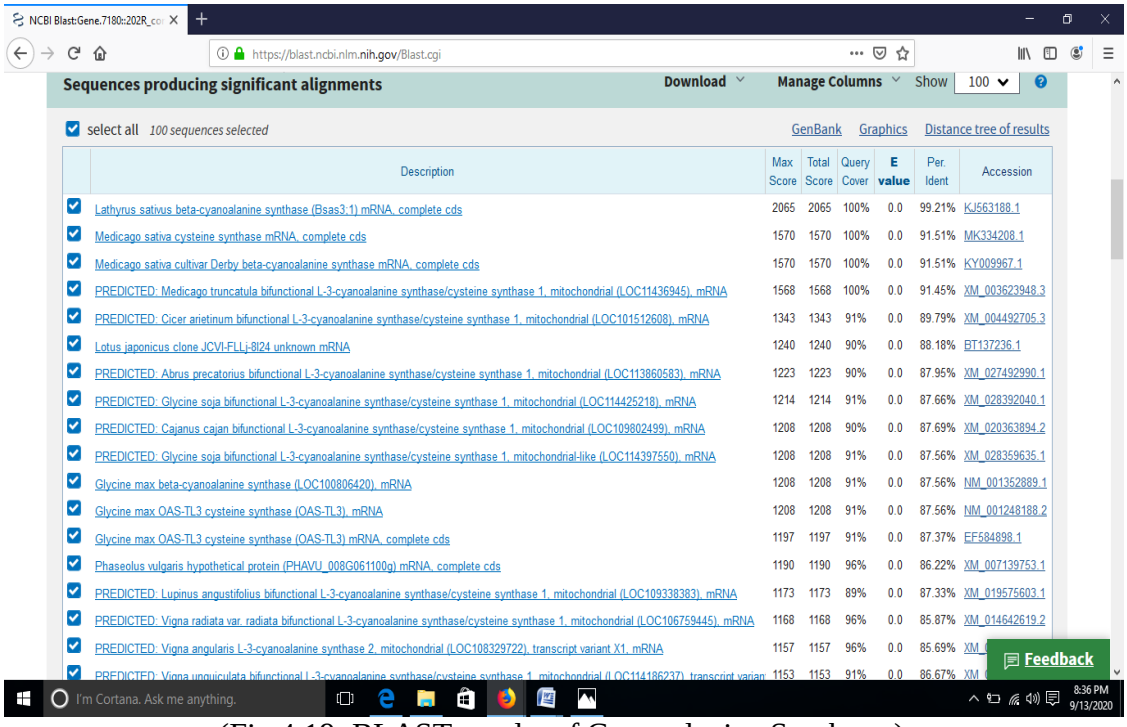
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
PREDICTED: Medicago truncatula cysteine synthase 2 (LOC11436130), mRNA	1118	1307	88%	0.0	92.76%	XM_003617758.3
PREDICTED: Cicer arietinum cysteine synthase 2 (LOC101511934), mRNA	1029	1029	75%	0.0	90.69%	XM_004491452.3
Lotus japonicus clone JCVI-FLLj-21A2 unknown mRNA	885	885	75%	0.0	87.34%	BT143490.1
PREDICTED: Abrus precatorius cysteine synthase 2 (LOC113846961), mRNA	852	852	75%	0.0	86.62%	XM_027475751.1
PREDICTED: Glycine soja cysteine synthase 2 (LOC114382935), mRNA	841	841	75%	0.0	86.26%	XM_028342481.1
PREDICTED: Glycine max cysteine synthase 2 (LOC100799081), mRNA	830	830	75%	0.0	86.01%	XM_003544606.4
Phaseolus vulgaris hypothetical protein (PHAVU_008G278600g) mRNA, complete cds	765	765	75%	0.0	84.56%	XM_007142353.1
Phaseolus vulgaris AT4G19003 mRNA, partial sequence	760	760	74%	0.0	84.57%	KY530182.1
PREDICTED: Vigna angularis cysteine synthase 2 (LOC108338537), transcript variant X2, mRNA	752	752	75%	0.0	84.26%	XM_017575475.1
PREDICTED: Vigna angularis cysteine synthase 2 (LOC108338537), transcript variant X1, mRNA	752	752	75%	0.0	84.26%	XM_017575474.1
PREDICTED: Vigna radiata var. radiata cysteine synthase 2 (LOC106764592), mRNA	730	730	75%	0.0	83.74%	XM_014648871.2
PREDICTED: Vigna unguiculata cysteine synthase 2 (LOC114194358), mRNA	725	725	75%	0.0	83.61%	XM_028084526.1
PREDICTED: Prosopis alba cysteine synthase 2 (LOC114712546), transcript variant X2, mRNA	577	577	63%	4e-160	82.84%	XM_028897106.1
PREDICTED: Prosopis alba cysteine synthase 2 (LOC114712546), transcript variant X1, mRNA	577	577	63%	4e-160	82.84%	XM_028897105.1
PREDICTED: Ziziphus jujuba cysteine synthase 2-like (LOC107406856), transcript variant X2, mRNA	477	477	74%	4e-130	78.11%	XM_016014068.2
PREDICTED: Ziziphus jujuba cysteine synthase 2-like (LOC107406856), transcript variant X1, mRNA	477	477	74%	4e-130	78.11%	XM_016014067.2
PREDICTED: Ziziphus jujuba cysteine synthase 2-like (LOC107404704), transcript variant X2, mRNA	477	477	74%	4e-130	78.11%	XM_016011693.2
PREDICTED: Ziziphus jujuba cysteine synthase 2-like (LOC107404704), transcript variant X1, mRNA	477	477	74%	4e-130	78.11%	XM_016011692.2
Stachyurus yunnanensis AT4G19003 mRNA, partial sequence	475	475	74%	2e-129	78.09%	KY530173.1
Medicago truncatula clone mth2-32m22, complete sequence	460	1243	81%	4e-125	93.02%	AC122165.40
Platanus x hispanica AT4G19003 mRNA, partial sequence	438	438	60%	2e-118	79.39%	KY53

(Fig.4.17. BLAST results of Cysteine Synthase)



PREDICTED: Medicago truncatula D-aminoacyl-tRNA deacylase (LOC11441122), transcript variant X2, misc_RNA	641	641	85%	1e-179	88.83%	XR_003009062.1
Medicago truncatula clone JCVI-FLMt-2A14 unknown mRNA	640	640	85%	4e-179	88.78%	BT144930.1
PREDICTED: Medicago truncatula D-aminoacyl-tRNA deacylase (LOC11441122), transcript variant X1, mRNA	604	604	80%	1e-168	88.82%	XM_024775786.1
PREDICTED: Glycine soja D-aminoacyl-tRNA deacylase (LOC114386709), transcript variant X1, mRNA	595	595	80%	8e-166	88.62%	XM_028346748.1
PREDICTED: Cicer arietinum D-aminoacyl-tRNA deacylase (LOC101496759), transcript variant X2, mRNA	595	595	76%	8e-166	89.55%	XM_027333433.1
PREDICTED: Cicer arietinum D-aminoacyl-tRNA deacylase (LOC101496759), transcript variant X1, mRNA	595	595	76%	8e-166	89.55%	XM_004497573.3
PREDICTED: Glycine max D-aminoacyl-tRNA deacylase (LOC100813154), transcript variant X1, mRNA	595	595	80%	8e-166	88.62%	XM_003545967.4
PREDICTED: Glycine soja D-aminoacyl-tRNA deacylase (LOC114386709), transcript variant X2, mRNA	586	586	74%	5e-163	89.89%	XM_028346749.1
PREDICTED: Glycine max D-aminoacyl-tRNA deacylase (LOC100813154), transcript variant X2, mRNA	586	586	74%	5e-163	89.89%	XM_006597408.3
PREDICTED: Cajanus cajan D-aminoacyl-tRNA deacylase (LOC109811552), transcript variant X3, mRNA	556	556	75%	4e-154	88.48%	XM_029274024.1
PREDICTED: Cajanus cajan D-aminoacyl-tRNA deacylase (LOC109811552), transcript variant X2, mRNA	556	556	75%	4e-154	88.48%	XM_020375327.2
PREDICTED: Cajanus cajan D-aminoacyl-tRNA deacylase (LOC109811552), transcript variant X1, mRNA	556	556	75%	4e-154	88.48%	XM_020375326.2
PREDICTED: Abrus precatorius D-aminoacyl-tRNA deacylase (LOC113850312), transcript variant X3, misc_RNA	556	556	75%	4e-154	88.48%	XR_003502187.1
PREDICTED: Abrus precatorius D-aminoacyl-tRNA deacylase (LOC113850312), transcript variant X2, mRNA	556	556	75%	4e-154	88.48%	XM_027480809.1
PREDICTED: Glycine soja D-aminoacyl-tRNA deacylase-like (LOC114373661), transcript variant X1, mRNA	553	553	74%	5e-153	88.57%	XM_028331170.1
PREDICTED: Glycine max D-aminoacyl-tRNA deacylase (LOC100787782), transcript variant X1, mRNA	553	553	74%	5e-153	88.57%	XM_003542966.4
Phaseolus vulgaris hypothetical protein (PHAVU_006G179400g) mRNA, complete cds	525	525	74%	1e-144	87.34%	XM_007148022.1
PREDICTED: Glycine soja D-aminoacyl-tRNA deacylase (LOC114386709), transcript variant X3, mRNA	499	499	80%	7e-137	85.02%	XM_028346750.1
PREDICTED: Glycine max D-aminoacyl-tRNA deacylase (LOC100813154), transcript variant X3, mRNA	499	499	80%	7e-137	85.02%	XM_006597409.3
PREDICTED: Vigna unguiculata D-aminoacyl-tRNA deacylase (LOC114188735), mRNA	492	492	73%	1e-134	86.41%	XM_028077366.1
PREDICTED: Glycine soja D-aminoacyl-tRNA deacylase (LOC114386709), transcript variant X5, mRNA	490	490	74%	4e-134	85.95%	XM_028346752.1
PREDICTED: Glycine soja D-aminoacyl-tRNA deacylase (LOC114386709), transcript variant X4, mRNA	490	490	74%	4e-134	85.95%	XM_028346751.1
PREDICTED: Glycine max D-aminoacyl-tRNA deacylase (LOC100813154), transcript variant X4, mRNA	490	490	74%	4e-134	85.95%	XM_
PREDICTED: Vigna angularis D-tyrosyl-tRNA(Tyr) deacylase (LOC108342446), transcript variant X2, mRNA	481	481	73%	2e-131	85.97%	XM_

(Fig.4.18. BLAST result of D-aminoacyl-tRNAdeacylase)

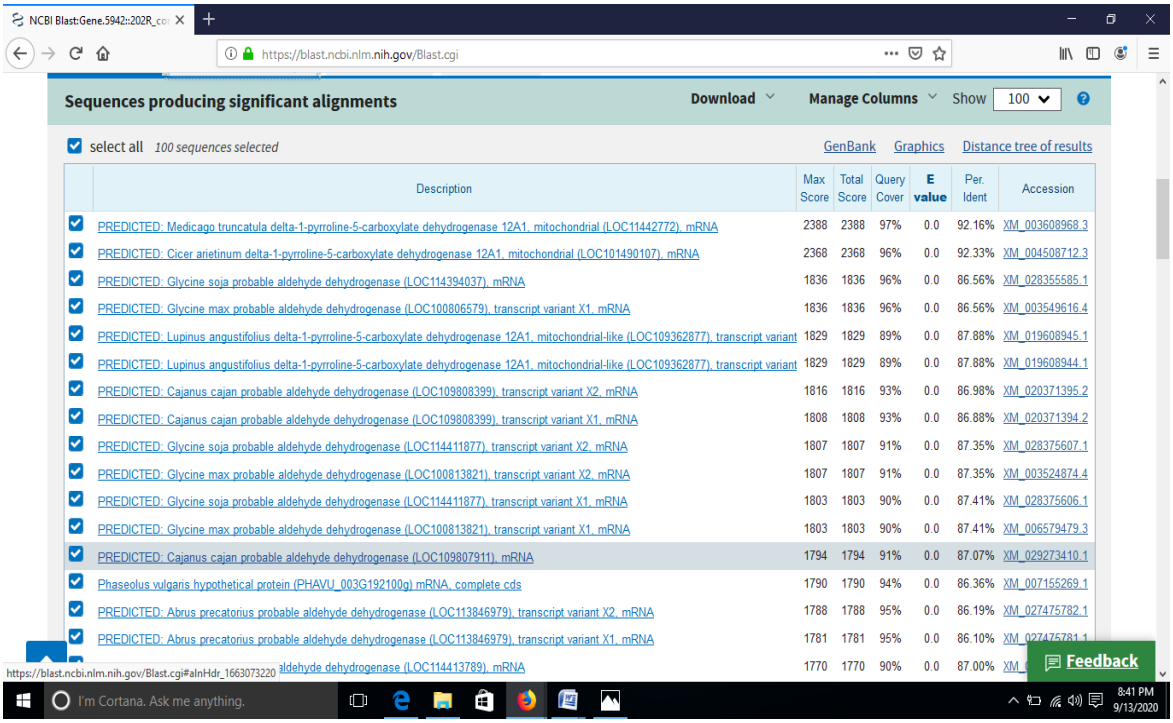


Sequences producing significant alignments Download Manage Columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Lathyrus sativus beta-cyanoalanine synthase (Bsas3;1) mRNA, complete cds	2065	2065	100%	0.0	99.21%	KJ563188.1
Medicago sativa cysteine synthase mRNA, complete cds	1570	1570	100%	0.0	91.51%	MK334208.1
Medicago sativa cultivar Derby beta-cyanoalanine synthase mRNA, complete cds	1570	1570	100%	0.0	91.51%	KY009967.1
PREDICTED: Medicago truncatula bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial (LOC11436945), mRNA	1568	1568	100%	0.0	91.45%	XM_003623948.3
PREDICTED: Cicer arietinum bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial (LOC101512608), mRNA	1343	1343	91%	0.0	89.79%	XM_004492705.3
Lotus japonicus clone JCVI-FLLj-8I24 unknown mRNA	1240	1240	90%	0.0	88.18%	BT137236.1
PREDICTED: Abrus precatorius bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial (LOC113860583), mRNA	1223	1223	90%	0.0	87.95%	XM_027492990.1
PREDICTED: Glycine soja bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial (LOC114425218), mRNA	1214	1214	91%	0.0	87.66%	XM_028392040.1
PREDICTED: Cajanus cajan bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial (LOC109802499), mRNA	1208	1208	90%	0.0	87.69%	XM_020363894.2
PREDICTED: Glycine soja bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial-like (LOC114397550), mRNA	1208	1208	91%	0.0	87.56%	XM_028359635.1
Glycine max beta-cyanoalanine synthase (LOC100806420), mRNA	1208	1208	91%	0.0	87.56%	NM_001352889.1
Glycine max OAS-TL3 cysteine synthase (OAS-TL3), mRNA	1208	1208	91%	0.0	87.56%	NM_001248188.2
Glycine max OAS-TL3 cysteine synthase (OAS-TL3) mRNA, complete cds	1197	1197	91%	0.0	87.37%	EF584898.1
Phaseolus vulgaris hypothetical protein (PHAVU_008G061100g) mRNA, complete cds	1190	1190	96%	0.0	86.22%	XM_007139753.1
PREDICTED: Lupinus angustifolius bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial (LOC109338383), mRNA	1173	1173	89%	0.0	87.33%	XM_019575603.1
PREDICTED: Vigna radiata var. radiata bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial (LOC106759445), mRNA	1168	1168	96%	0.0	85.87%	XM_014642619.2
PREDICTED: Vigna angularis L-3-cyanoalanine synthase 2, mitochondrial (LOC108329722), transcript variant X1, mRNA	1157	1157	96%	0.0	85.69%	XM_
PREDICTED: Vigna unguiculata bifunctional L-3-cyanoalanine synthase/cysteine synthase 1, mitochondrial (LOC114186237), transcript varian	1153	1153	91%	0.0	86.67%	XM_

(Fig.4.19. BLAST results of Cyanoalanine Synthase)

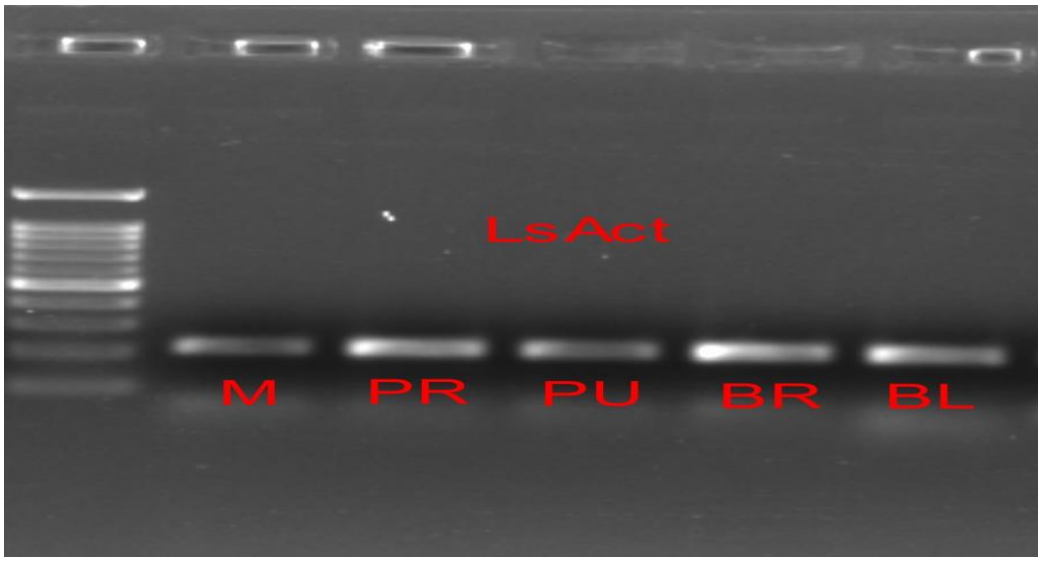


(Fig. 4.20.BLAST results delta-1-pyrroline-5-carboxylate dehydrogenase (P5CD)

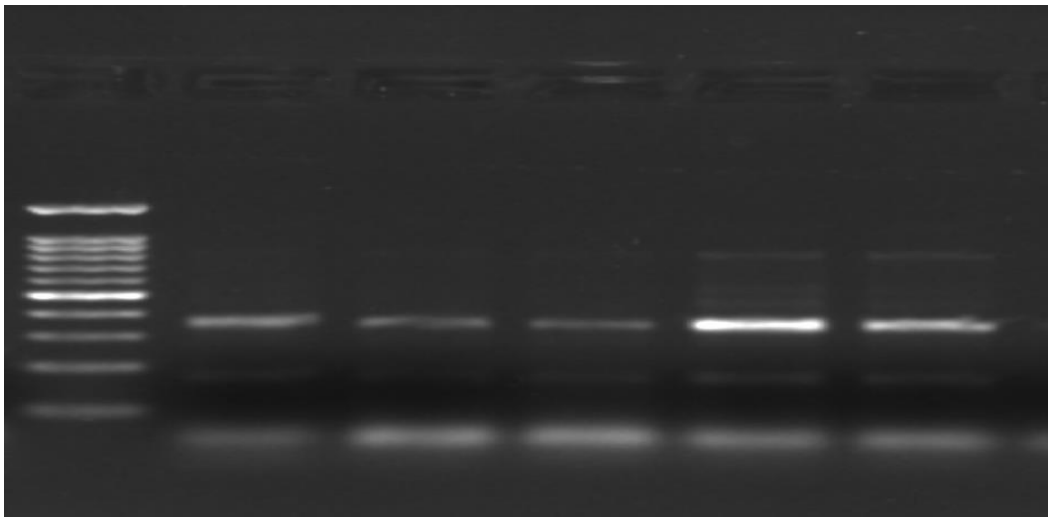
4.5 Amplification of cDNA with the primer PLPD

The RNA was extracted from 8 days and 10 days old seedlings from the five selected genotypes after germination for the evaluation of expression of primer PLPD due to the fact that ODAP biosynthesis pathway occurs in the early seedling stage particularly in the leaves (Jiao *et al*., 2012; Xu *et al*., 2017) which was designed from the IDT's primer quest tool (https://www.idtdna.com). While the mature plants leaves, stem and roots shows a reduced level of accumulation of ODAP content which suggests that the age of plant and ODAP content are inversely propotional.

The cDNA formed from RNA were first normalized using the primer house-keeping gene *Ls*Actin which was designed from the *Lathyrus* transcriptomes in the selected 5 genotypes. The amplification of *Ls*Actin resulted in similar expression in all the genotypes as expected.



(Fig. 4.21. Gel image of LsActin on the selected genotypes.)


(Fig. 4.22. gel image of amplification of PLPD in selected genotypes)

The 10th day seedlings cDNA was more correlated than the 8th day cDNA in house-keeping gene and candidate gene prime-PLPD too.

The results showed 2 bands which was differentially correlated with the seed ODAP content in the 5 genotypes. This result also correlates with the pathway proposed by Malathi *et al*., (1970) showing that the L-alanine synthase occurs in 2 isoforms in the *Lathyrus sativus* viz. β-isoxazoline-5-one-2yl-l-alanine synthase and isomer β-isoxazoline-5-one-4-yl-L-alanine synthase.

The results also depicted that the 2 isoforms of L-alanine synthase were inversely proportional to each other. When the expression of isoxazoline-5-one-4-yl-L-alanine is then the expression of β-isoxazoline-5-one-2yl-l-alanine reduces but both act on the same substrate O-acetyl L-serine. The enzymatic activity of only β-isoxazoline-5-one-2yl-l-alanine synthase leads to formation of β-isoxazoline-5-one-2yl-l-alanine (BIA), precursor of β-ODAP.

CHAPTER-V

SUMMARY AND CONCLUSION

Pulses has an immense importance in Indian diet as the amino acids which is absent in the cereals like lysine were fulfilled by the consumption of pulses. Lathyrus being a leguminous pulse also provides with high energy (362.9K calories) and good nutrition. It is also considered as an 'insurance crop; to subsistence farmers as it can be grown with minimal agronomical inputs. Besides, it is tolerant to many biotic and abiotic factors like drought, waterlogging, cold and salinity conditions as compared to other pulse crops. Moreover, it can be grown in almost all types of soil including heavy clayey soil.

Despite being a hardy and wonderful crop, its consumption is limited in many countries due to the presence of an antinutritional factor β-N-oxalyl-L-α, β-diaminopropionic acid (β-ODAP) which causes paralysis of lower limbs when consumed for a prolonged period. Studies till now revealed the biosynthetic pathway of β-ODAP but the genes associated with it is still unknown. It is also suggested that there is some association between the β-ODAP biosynthesis pathway and sulfur and nitrogen metabolism (Jiao *et al*., 2011). Earlier studies by Nayak *et al.*, 2019 suggested the enzyme L-alanine synthase was present in 2 isomeric forms either -isoxazoline-5-one-2yl-l-alanine (BIA) or β-isoxazoline-5-one-4-yl-L-alanine formed from a commom precursor O-acetylserine. The formation of β-isoxazoline-5-one-4-yl-L-alanine might leads to formation of alanine while BIA leads to formation of β-ODAP. The homology sequence search of this enzyme revealed this to be a D-amino acid.

The current study attempts to characterize the putative genes associated with amino acid biosynthesis pathway and D-amino acid metabolism and to identify the transcripts corresponding to the enzymes involved in cyanide detoxification pathway in relation to ROS metabolism, metabolism of O-acetyl serine and cysteine amino acid pathways. The transcriptomes data of Mahateora (high ODAP genotype) and RLK-1950(low ODAP genotype) sequenced with the help of illumina sequencing (Banerjee *et al*., 2018) was functionally annotated with the help of pipeline MapMan Mercator. Mercator functionally described a total of 94,793 trancripts in among which 20,562 were functionally categorized in to 34 categories on the

basis of MapMan BIN ontology (May *et al*., 2014). While the ealier approached CLC genomics workbench by Nayak et al., 2019 for the functional annotation of *Lathyrus* transcripts functionally described only 28,132 ranscripts. The Mercator output when screened for genes related to alanine synthase, it didn't predicted for alanine synthase but accommodate the functional category of Alanine aminotransferase. Similarly when screened for transcripts associated with Cyanoalnaine synthase, Cysteine synthase, Asparagine metabolism, Ornithine cycloaminase, d-amino acid metabolism and ROS metabolism 1,3,6,1,1,and 1 number respectively of transcripts were found. The Cyanoalanine synthase pathway also leads to the formation of D-amino acid like d-alanine, d-arginine, d-glutamine, etc. also the enzyme D-aminoacyl-tRNAdeacylase or D-Tyr-tRNA (Tyr) deacylase involved in the cyanoalnine (cyanide detoxification pathway) metabolism. Consequently, all the enzymes screened were interrelated to each other.

CONCLUSION

- The MapMan Mercator functionally described 94,793 transcripts in which 20,562 were assigned with a functional category based on MapMan BIN ontology.
- To assess the function predicted by Mercator, homology sequence searches of selected transcripts was done with the help of NCBI BLAST. The Results predicted highest identity with the *Medicago truncatula* followed by *Cicer arienatum* with the similar functions as predicted by Mercator functional annotation tool.
- 17 transcripts were selected for the primer designing with contig ID's 202r_contig_6184, 202R_contig_5489, 202R_contig_13221, 202R_contig_18786, 202R_contig_20127, 202R_contig_17606, 202R_contig_3873, 202R_contig_3873, 202R_contig_2358, 202R_contig_15653, 202R_contig_17961and 202R_contig_31383, 202R_contig_427, 202R_contig_8260, 202R_contig_18458, 202R_contig_7854 and 202r_contig_5018.
- One enzyme PLPD was differentially correlated in different genotypes of *L.sativus*.

SUGGESTIONS FOR FUTURE WORK

- Validation of designed primer in the high and low ODAP genotype for evaluating the expression analysis.
- Further validating this primers in the population.

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