DEVELOPMENT OF GENETICALLY ENGINEERED MUSTARD (*BRASSICA JUNCEA*) FOR LOW VISCOSITY BIODIESEL PRODUCTION

BY

IJAZ NAEEM

A thesis submitted to The University of Agriculture Peshawar, in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY AND GENETIC ENGINEERING
DEVOLPEMENT OF GENETICALLY ENGINEERED MUSTARD (BRASSICA JUNCEA) FOR LOW VISCOSITY BIODIESEL PRODUCTION

BY

IJAZ NAEEM

A thesis submitted to The University of Agriculture Peshawar, in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY AND GENETIC ENGINEERING

Approved By:

_____________________
Chairman Supervisory Committee
Prof. Dr. Iqbal Munir

_____________________
Member (Major Field)
Dr. Aqib Iqbal

_____________________
Member (Minor Field)
Prof. Dr. Farhatullah

_____________________
Chairman & Convener Board of Studies
Prof. Dr. Safdar Hussain Shah

_____________________
Dean Faculty of Crop Production Sciences
Prof. Dr. Muhammad Afzal

_____________________
Director Advanced Studies & Research
Prof. Dr. Jamal Khattak

INSTITUTE OF BIOTECHNOLOGY AND GENETIC ENGINEERING
FACULTY OF CROP PRODUCTION SCIENCES
THE UNIVERSITY OF AGRICULTURE
PESHAWAR-PAKISTAN
JUNE, 2015
TABLE OF CONTENTS

Chapter No. Title Page No.

LIST OF TABLES ................................................................. i

LIST OF FIGURES .............................................................. ii

ACKNOLOGMENTS .............................................................. iii

ABSTRACT ........................................................................ iv

I. INTRODUCTION ................................................................. 1

II. REVIEW OF LITRATURE .................................................... 12

III. MATERIALS AND METHODS ................................................ 23

IV. RESULTS ........................................................................ 40

V. DISCUSSION ................................................................. 76

VI. SUMMARY ....................................................................... 85

VII. CONCLUSION ............................................................. 88

RECOMMENDATION .......................................................... 89

REFERENCES ................................................................. 90

LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Different Concentrations of PGRs for Callus Induction</td>
<td>22</td>
</tr>
<tr>
<td>3.2</td>
<td>Different Concentrations of PGRs used for root induction</td>
<td>23</td>
</tr>
</tbody>
</table>
3.3: The primers used for the PCR amplification of target *EaDACt* gene in the putative transformed *B. juncea* plants. ................................................................. 29

3.5: Plants Tagging Plan for Lipid Analysis of Developing Seeds. ................................. 32

3.6 Analytical conditions for Fatty acids analysis using GC-FID .................................. 34

4.1 Impact of different concentrations of Indole Acetic Acid (IAA) and Kinetin (Kin) on root number, length and rooting induction in *B. juncea*................................. 40

4.2 Percent Growth of Transgenic Plants with Different Media ................................. 45

4.3 Showing the summary of the *In planta* transformation using *Agrobacterium* strain *GV3101* harboring the binary vector pBinGlyBar4- *EaDACt* .......................... 52

4.4 Fatty acid compositions of *B. juncea* seed oils in wild ........................................ 54
cultivar and T2 transgenic in % Molar.

4.5 Individual fatty acid compositions of *B. juncea* seed oils in non-................................ 54
transgenic plant and T2 transgenic lines expressing EaDACt.
<table>
<thead>
<tr>
<th>Fig. No.</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Application of different PGRs concentrations and their impacts on callus formation frequency in <em>B. juncea</em></td>
<td>35</td>
</tr>
<tr>
<td>4.2</td>
<td>Impacts of BAP, NAA and GA3 on Organogenesis in <em>B. juncea</em></td>
<td>36</td>
</tr>
<tr>
<td>4.3</td>
<td>Impact of plant growth regulator (BAP GA3 and NAA) on shoot number in <em>B. juncea</em></td>
<td>37</td>
</tr>
<tr>
<td>4.4</td>
<td>Impacts of different PGRs on mean shoot length in <em>B. juncea</em></td>
<td>38</td>
</tr>
<tr>
<td>4.5</td>
<td>Invitro regeneration in <em>B. juncea</em></td>
<td>41</td>
</tr>
<tr>
<td>4.6</td>
<td>Transformed <em>E.coli</em> culture overnight growth on solid LB medium in the presence of Kanamycin at 37ºC</td>
<td>42</td>
</tr>
<tr>
<td>4.7</td>
<td>PCR amplification of <em>EaDAcT</em> gene. Lane 1 and 2 represents DNA Ladder and the positive control while Lane 3 to 9 are transformed <em>E.coli</em> colonies</td>
<td>43</td>
</tr>
<tr>
<td>4.8</td>
<td>Transformed <em>Agrobacterium</em> Colonies on YEP medium containing kanamycin (50 mg/L), Gentamycin (50 mg/L) and Rifampicin (25 mg/L)</td>
<td>44</td>
</tr>
<tr>
<td>4.9</td>
<td>Conformation of transformants. Lane C and 15, 16 represent1.5 kb ladder and negative control where Lane 1-14 indicate transformed colonies</td>
<td>44</td>
</tr>
<tr>
<td>4.10</td>
<td>Tissue culture transformation (A) Plants growth on ½ MS medium (B) Co-cultivation medium</td>
<td>46</td>
</tr>
<tr>
<td>4.11</td>
<td>(A) Plantlets on selection media having BAP 2mg/l and NAA .2mg/l with BASTA 20mg/l and Timintin (B) Plantlets without infection on selection media (Control)</td>
<td>46</td>
</tr>
<tr>
<td>4.12</td>
<td>Growth parameters of tissue culture transformed plants. (A) Plants on second selection media (only Basta level increased to 25mg/L) (B) Plants on rooting medium</td>
<td>46</td>
</tr>
<tr>
<td>4.13</td>
<td>Plants grown in different conditions.</td>
<td>47</td>
</tr>
<tr>
<td>4.14</td>
<td>The PCR confirmation analysis of the putative transgenic <em>B. juncea</em></td>
<td>48</td>
</tr>
<tr>
<td>4.15</td>
<td>(A) Transformation of <em>B. juncea</em> with <em>EaDAcT</em> gene using floral dip (A) With desiccator. (B) Without desiccator</td>
<td>48</td>
</tr>
<tr>
<td>4.16</td>
<td>The image illustrates the effects of 0.1, 0.2 and 0.3% of basta spray on wild <em>B. juncea</em> plants</td>
<td>49</td>
</tr>
<tr>
<td>4.17</td>
<td>Floral dip transformation</td>
<td>50</td>
</tr>
</tbody>
</table>
4.18. Representing the PCR confirmation of the plants ........................................... 51
4.19. TLC analysis of fatty acid extracted from both wild and transgenic plants. .......... 53
4.20. TLC profile of the triacylglyceride isolated from the seed at different days after flowering. ............................................................................................................................... 55
4.21. Quantification of total TAG, from seeds at different days after flowering. .......... 56
4.22: Palmatic acid (16:0) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 57
4.23: Stearic acid (18:0) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 58
4.24: Arachidic acid (20:0) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 58
4.25: Behenic acid (22:0) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 59
4.26: Oleic acid (18:1∆9) concentration estimated after 10 days of interval in the developing seed B. juncea ........................................................................................................... 60
4.27: Vaccenic acid (18:1∆11) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 60
4.28: Linoleic acid (18:2∆9,12) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 61
4.29: Linolenic acid (18:3∆9,12,15) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 62
4.30: Gondoic acid (20:1∆11) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 62
4.31: Paullinic acid (20:1∆13) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 63
4.32: Brassic acid (20:02) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 64
4.33: Erucic acid (22:1∆9) concentration estimated after 10 days of interval in the developing seed of B. juncea ........................................................................................................... 64

ACKNOWLEDGEMENTS
The successful completion of this journey is due to the combined efforts of many people. Ph.D is long and complicated journey. During this journey, I was helped by many and I would like to show my gratitude to all of them. This work would not have been finished without their help. Primarily, I would greatly appreciate my supervisor Dr. Iqbal Munir, Professor, Institute of Biotechnology and Genetic Engineering, the University of Agriculture Peshawar for his dynamic supervision. It is his confidence, imbibing attitude, splendid discussions and endless endeavors through which I have gained significant experience. My special thanks are due to Prof. Dr. Safdar Hussain Shah, Director, Institute of Biotechnology and Genetic Engineering for his immense concern throughout my Ph.D period. I wish to express my deep gratitude and respect to all my teachers specially Dr. Aqib Iqbal and Dr. Ihsan Ullha whose knowledge and guidance enabled me to attain this target. I feel pleasure in acknowledging the nice company of my lab fellows Wajid Ali khan, Dr. Ibad Ullha Jan, Bilal Mohammad Khan, Faiza Tawab, Rizwan Ullha Shah, Uzma Jamal, Rafiullah and Zeeshan Naseem. I will always remember the nice company of my friends Dr. Asif Ali and Engr. Ashraf Ali.

I must acknowledge my debt to Assistant Professor Dr. Timothy Patrick Durrett, Department of Biochemistry and Molecular Biophysics, Kansas State University USA for providing me an opportunity to carry out part of my research project in his laboratory. His constructive comments, guidance and great co-operation played a fundamental role for carrying out this epic work. I am also thankful to technical staff members Libin Yao, for her support and assistance during my stay in The Kansas State University. This project was supported by Higher Education Commission of Pakistan and I acknowledge this institution for providing me Indigenous and Overseas “IRSIP” scholarship for my research.

Deepest regards and gratitude to my brothers Engr, Sajjad Zamin and Dr. Amad Zamin for their support. My all five sisters prayed for my success, I am also thankful to them. My infinite thank goes to my wife for her moral support and tolerance in this long and hard journey. Lots of love to my kids Maryna Ijaz, Aimal Ijaz and Urwa Ijaz for making me smile, whenever I met them. I must say that all the happiness in my life is because of my beloved father and my ever caring and sweetest mother. Their constant prayers and appreciations always kept me satisfied.

*Ijaz Naeem*
DEVELOPMENT OF GENETICALLY ENGINEERED MUSTARD (*BRASSICA JUNCEA*) FOR LOW VISCOSITY BIODIESEL PRODUCTION

Ijaz Naeem and Iqbal Munir
Institute of Biotechnology and Genetic Engineering,
The University of Agriculture Peshawar

ABSTRACT

Indian mustard (*Brassica juncea* L.) is a member of the Brassicaceae family that is now becoming a global crop because of its drought tolerance, high oil content, short growing season and the ability to grow on low quality soils. The present study was aimed to reduce the viscosity of *Brassica juncea* oil by altering the fatty acid profile, via developing efficient and simple *Agrobacterium* mediated based tissue culture and floral dip methods of plant transformation. For optimization of regeneration protocol, cotyledon explants of the *Brassica juncea* CV- Raya Anmol were cultured on MS medium supplemented with different concentrations of 6-Benzylaminopurine (BAP), (2, 4, 6, 8, and 10 µM) alone and in combination with 1µM of Gibberellic acid (GA$_3$) and Naphthaleneacetic acid (NAA) separately. Maximum calli production (87%) was observed on MS medium containing 4 µM BAP only. The highest shoot induction efficiency (92%) was observed on medium supplemented with BAP (4 µM) and NAA (1 µM). For rooting, Indole-3-acetic acid (IAA) (6µM) and Kinetin (Kin) (1µM) was found as the best combination. A binary vector containing the *EaDACt* (*E. alatus* diacylglycerol acetyltransferase) gene under the transcriptional control of a glycinin promoter and with a basta selection marker, was introduced into *A. tumefaciens* strain GV3101 via electroporation. An efficient tissue culture and *In planta* transformation was developed for *B. juncea*. In tissue culture transformation, cotyledon explants were used while for floral dip, plants at early stage of flowering with and without vacuum desiccator were used. The basta resistant putative transgenic plants were selected and further confirmed by PCR. The vacuum infiltration was found more efficient (0.85%) as compared to the normal floral dip method (0.15%). The developed transgenic *B. juncea* seeds showed altered TAG fatty acid composition with enhanced level of oleic acid (from 41% to 63%) and reduced euric acid level (from 13.7% to 2.53%), which is an ideal composition of fatty acids in oil to be used as biodiesel. The developed protocols could be used to accumulate unusual acTAG in *B. juncea* seed, providing a direct way of biodiesel production from plant oil. Such results will be useful for the development of *B. juncea* as an alternative source of energy.
I. INTRODUCTION

Energy is considered to be the lifeline of economic development. For a developing economy with a high population growth rate, it is important to keep a balance between energy supply and emerging needs. The natural resources are in limited supply. While they do occur naturally, it can take hundreds of thousands of years to replenish the stores. The factors contributing to demand of energy are: overconsumption, overpopulation, poor infrastructure, unexplored renewable energy options, delay in commissioning of power plants, wastage of energy, poor distribution system, major accidents and natural calamities, wars and attacks and other miscellaneous factors. The best possible solution is to reduce the world’s dependence on non-renewable resources and to improve overall conservation efforts. In addition, much of the industry is compelled to utilize fuels, however, there are yet some technologies using other types of renewable energies such as steam, solar, wind and mainly biodiesel. The major concern isn’t so much that we will run out of gas or oil, but that the use of coal is going to pollute the atmosphere and destroy other natural resources in the process of coal mining and so it has to be replaced as an energy source. This isn’t easy as many of the leading industries use coal, not gas or oil, as their primary source of power for manufacturing. This is why production of the biofuel (biodiesel) becomes a hot choice of exploration for scientists.

Independence in the energy requirement is critical to the survival of any society in the present world. Availability of the energy and its resources in any country directly augment a proportional strength to its financial stability. The per-capita energy consumption in Pakistan is about thirty-eight hundred and ninety-four (3894kWh), far less than the average energy requirement of 17620 KWh according to international standards. Thus, on this basis, Pakistan is ranked 100 in the world (IES 2006, ESP 2004). Pakistan is a net importer of petroleum and petroleum products. An average of 64,950 bbl/day crude oil production was achieved in Pakistan in 2010. Because of the less production of oil, Pakistan depends on importing the same to meet its needs. The consumption of oil in Pakistan in the month of November 2010 was over 410,000 bbl/day, with the import accounting for over 80% of the total. Pakistan mainly imports oil from the Middle East, particularly Saudi Arabia (EIA, 2010, World Bank Report). Roughly 1/6 of Pakistan’s oil demand is met by internal oil production. Pakistan’s energy supply matrix consists of a variety of sources with natural gas and oil making up
75.30% (oil with 23.30%, gas with 51.60% and LPG with 0.40%). Coal with 6.20%, hydro-power with 11.30% and nuclear-power with 1.20% from other sources of energy in Pakistan (MPNR Pakistan 2005). A report of the State Bank of Pakistan showed that the country waged a total of $6.69 billion for the import of petroleum products and crude oil during the July-Nov 2014-15.

In a broader perspective, the increase in crude oil prices and the introduction of various subsidies, the production of biodiesel in both the euripi union (EU) and the USA has expanded dramatically during last decade. From 2002 to 2008, the production of biodiesel increased 15-fold in the USA, and almost fivefold in the EU. Despite the relatively large recent increase in production in the USA, European production is still at least six times greater than that in the USA: in 2006, Europe produced approximately 5.6 billion liters of biodiesel compared to 0.86 billion liters produced in the USA. Consequently, the consumption of canola, soybean, palm and other oil crops for biodiesel has grown too. In the USA, it is estimated that approximately 22% of domestic soybean oil production by 2016 will be devoted to biodiesel (United State Department of Agriculture, 2007). The rapid expansion of EU and US plant oil consumption as a feedstock for biodiesel production has played a major role in achieving record high prices for plant oils.

Biodiesel is a source of renewable energy, originating predominantly from biomass feed stock, processing of agriculture or food products or from reprocessing of vegetable oils. Biodiesel refers to the mono-alkyl ester of long chain fatty acid obtained from feedstock of a renewable lipid. It is produced by transesterification of plant oil or animal fats using catalyst and short chain alcohols, glycerol and soap are produced as a byproduct in significant quantities (Apstolakou et al., 2009). Among the most frequently utilized catalysts is potassium hydroxide (KOH) and sodium hydroxide (NaOH) which are inexpensive and can be easily handled for storage and transportation (Singh et al., 2006). The chemical components of oils and fats include triglyceride molecule of three long chain fatty acid that are ester bounded to a single glycerol molecule (Moreira et al., 2006). Plants oil consist of various triacylglycerols (TAGs) that composed of three fatty acid long chain usually 16 to18 carbon esterified to glycerol. These fatty acyl chains are similar to the aliphatic hydrocarbon found in diesel (10-12 carbons) and petrol 5-12 carbons). The mixing of this volatile fuel with air and
ignition with a spark runs a conventional engine. Ignition of diesel in an engine requires high compression than petrol.

In early stages engines were designed from diesel engine to run on oil derived from peanut indicating a similarity in structure of plant triacylglycerol’s and petroleum fuels. Plants triacylglycerol have a viscosity range 17.3-32.9 mm²s⁻¹ while conventional diesel have 1.9-4.1 mm²s⁻¹, so plant TAGs have much higher viscosity than conventional diesel (ASTM D975; Knothe and Steidley, 2005). Biodiesel produced nowadays is facing high viscosity problem, however, the problem can be overcome through transesterification. The alteration of a carboxylic acid ester into a different carboxylic acid ester is known as transesterification (Srivastava and Parsad, 2000). The high viscosity of triglycerides is reduced by this method roughly hundred kilogram of oil and ten Kilogram of methanol reacts to produce hundred Kilogram of biodiesel and ten Kilogram of glycerol. Biodiesel can be used as substitute of diesel because both have similar molecular structure and no modifications are needed in the diesel engine to run it on this alternative fuel. The bio-degradability, flash point, and aromatic and Sulphur content of biodiesel are far better than conventional diesel (Martini and Schell, 1997). The tremendous increase in the prices of petroleum products is due to their enhanced use which can be overcome by replacing these with alternative sources like biodiesel (Karme and Chadha, 2005). Furthermore, the ease of use, bio-degradability and nontoxic of biodiesel as well as its low sulfur and aromatic content make it highly desirable which lead to research initiation in developed as well as under developed countries like Pakistan.

Biodiesel, in fact, represents one of the hastily growing markets for agriculture related products worldwide (Mathews, 2007; Fairless, 2007). The obligatory feedstock crops for biodiesel production, sugarcane and oil palm, are mainly cultivated in tropical nations like Malaysia, Brazil and Indonesia (Johnston and Holloway, 2007; FAOSTAT, 2007; Dufey, 2006). Pakistan, which mainly relies on agriculture, possess diverse resources needed for biodiesel production (Ahmad et al., 2007). A large number of crops are cultivated for vegetable oil production and also numerous oil producing weeds are present in various localities of Pakistan. The varied edaphic and environmental conditions in the country are responsible for this assorted nature of flora
in Pakistan. As a result of deficient education, confidence and abated communications between research stations and industry no conclusive research has ever been designed to unfold the potential of biodiesel production in Pakistan. The maximum utilization of petroleum products has thus caused a tremendous increase in their prices worldwide. This has quested for alternative options, and has opted for replacement of fossil fuel with other substitutes.

The Governments and other donor agencies are giving extensive incentives to distributors and producers for the development renewable energy sources. Brassica is the second major source of edible oil production in Pakistan. However, because of the high concentrations of glucosinolates and erucic acid in the seed oil, it is generally not recommended to be used for food purpose. Brassica, particularly *B. juncea* oil is potentially an important source of biodiesel production in Pakistan that can resist extreme temperatures and requires lower moisture for its production. *B. juncea* has a higher yield potential under extreme conditions, therefore, this species will be used during the current research. Furthermore, there is huge piece of marginal land that could be used for large scale production of brassica for biodiesel, thus the promotion of brassica as a biodiesel crop will not hamper the food supply chain as well. The biodiesel produced from this oil has the disadvantage of being of high viscosity which is determined by the quantity and composition of fatty acids in it. The existence of long chain fatty acids in enormous amount makes biodiesel unfit for direct use in diesel engines and it must first be trans-esterefied to reduce its viscosity (Kulkarni and Dalai, 2006).

*Brassica juncea* belongs to the family Cruciferae (Brassicaceae), of the kingdom plantae. Brassicaceae family consists of 37 species, among which *B. rapa*, *B. napus*, *B. carinata* and *B. juncea* are commonly cultivated for production of vegetables and oil seed crops (Sovero, 1993). However, *B. Juncea* is among the most vital and cultivable varieties for the worldwide oil production. It is cultivated in numerous countries of the world i.e. Canada, Australia, China, India and Russia, however, above 6 million hectares of land is cultivated with this plant in the Indian subcontinent (Wood et al., 1991; Kumar, 1999; Rabbani et al., 1998). The enormous increase in *B. juncea* cultivation has made this plant the second largest contributor of vegetable oil
worldwide (Zhou, 2001). Statistical data evaluate the *B. carinata* as third most important oil seed crop, subsequent to plam and soybean oil. Moreover, it is the fifth most cost effective crop, following rice, cotton wheat and maize (Sovero, 1993; Cardoza and Stewart, 2003). In addition, the species also contain some medicinally important phytochemicals such as vitamins, minerals and anti-carcinogenic agents (Steinmetz and Potter, 1996). Moreover, it is used in salad and meat dressings and a condiment in pickles. For centuries mustard oil has been used for multiple purposes. Initially it was used as oil for lighting up the lamp, predominately in Asia and Europe. After it was revealed that it can be used as cooking oil a large part of the oil was then used by the people for cooking different food items (Reed, 1976). *B. juncea* thrives in regions with hot days and cool nights, is drought resistant, and grows best in sandy loamy soil. Its growing period is 60 to 90 days, its seeds contain around 38% oil, and seed yields range from 0.5 to 2.5 MT/ha depending on factors such as cultivar, soil type, irrigation, season, and fertilizer inputs (Bryan et al., 2015). *B. juncea* thrives in regions with hot days and cool nights, is drought resistant, and grows best in sandy loamy soil. Its growing period is 40 to 60 days, its seeds contain around 38% oil, and seed yields range from 0.5 to 2.5 MT/ha depending on factors such as cultivar, soil type, irrigation, season, and fertilizer inputs (Bryan et al., 2015).

Altering the composition of the fatty acid of TAGs will lead to the improvement of lower viscosity oils. As described previously, the higher viscosity of oils extracted from plant leads to week fuel atomization, which avoids the direct utilization of oil as fuel in modern diesel engines. The production of lower viscosity plant oils, which could be utilized directly in the engines, would eradicate the requirement of chemical modification, and hence reduction in the cost of biodiesel. Viscosity in the biodiesel increases with the number of acyl carbons, and is reduced by the existence of double bonds (Allen et al., 1999). Low molecular weight TAGs have lower viscosity then those present in conventional plant oils. Low molecular weight TAGs i.e., Tributyrin (4:0) and tricaprin (10:0) have presumably better fuel atomization features than conventional TAGs (Goodrum and Eiteman, 1996). Thus, there is a need for an increase in production of biodiesel feedstock and a need of higher quality biofuel feedstock before biodiesel can be used as an alternative for fossil fuel worldwide and in Pakistan. Less viscous plant oil can be produced by altering the fatty acid composition of TAGs because poor fuel atomization is resulted from highly viscous plant oil which prevents their uninterrupted usage as a fuel in most contemporary diesel engines.
Biodiesel, indeed, can be made cost effective by producing less viscous plant oils that can be directly used in engines, hence, eliminating the prerequisite chemical modification. The number of acyl carbons increase viscosity while it is decreased by the abundance of short-chain fatty acids (Allen et al., 1999). Traditional plant oils have high viscosity than the low molecular weight TAGs. This is the reason for the prediction of better fuel atomization of low molecular weight TAGs such as Tributyrin (4:0) uptotricaprin (10:0) in comparison with traditional TAGs (Goodrum and Eiteman, 1996).

Another approach to lower the viscosity of triacylglycerol’s is by acetylating the sn-3 position of the long chain of triacylglycerol’s and increases the likelihood of direct combustion of acTAGs (3-acetyl-1,2-diacyl-sn-glycerols ) rich vegetable oils, eliminating the requirement of transesterification. The seed of *Euonymus alatus* produces unusual triacylglycerol exclusively of 3-acetyl-1,2-diacyl-sn-glycerols (acTAGs), where the sn-3 position is esterified with acetate instead of the long-chain fatty acid found in the triacylglycerols (lcTAGs) of many plants. The sn-3 position acetyl group changes the physical and chemical property of acTAGs, reducing their viscosity to 30% less than the lcTAGs. For acTAG production a membrane-bound O-acyltransferase (MBOAT) family enzyme is responsible, which most abundantly is present in the endosperm but absent in the aril. This enzyme was sequenced and named as *EaDAcT* (*Euonymus alatus* diacylglycerol acetyltransferase.). Expression of this gene in yeast and *Arabidopsis* accumulated acTAGs successfully (Durrett et al., 2010). Thus, this activity of expressed oil producing crops would allow the assessment of these unusual acTAGs for biofuel and other applications. Altering the fatty acid composition of TAGs could also lead to the development of low-viscosity plant oils. The development of low-viscosity plant oils that could be used in engines directly, would eliminate the need for chemical modification, thus improving the cost-effectiveness of biofuels. *B. Juncea* will be engineered with the acetyltransferase gene *EaDAcT* which in turn would be able to produce high levels of (acTAGs) 3-acetyl-1,2-diacyl-sn-glycerol. Such results will be useful for the development of *B. juncea* as an alternative source of biodiesel fuel.
Objectives

- To standardize the regeneration and transformation protocol for *B. juncea* under local conditions.
- To modify the seed oil fatty acid composition of *B. juncea* by stably integrating *EaDAcT* gene from *Euonymus alatus* (Burning bush).
- To develop a transgenic technology for producing low viscous seed oil for application in other potentially important biodiesel plants.
- To compare lipid and fatty acid profiling of wild and transgenic *B. juncea*
- To study the free fatty profiling of developing seed of wild *B. juncea*

II. REVIEW OF LITERATURE

Liu et al. (2015) reported that seed oils have proved obstinate to change for the production of industrially useful lipids. Here, they determine the fruitful metabolic
engineering with the maximum accumulation of unusual oil achieved so far in transgenic plants. In the previous experiments, expression of the Euonymus alatus diacylglycerol acetyltransferase EaDaCT gene in wild-type Arabidopsis plants caused in the accumulation of 45 mol% of unusual lipids 3-acetyl-1,2-diacyl-sn-glycerols (acetyl-TAGs) in the seed. While in dgat1 mutants, the expression of EaDaCT increased acetyl-TAGs to 65 mol%. Soybean and camelina transformed with the EaDaCT gene accumulate acetyl-triacylglycerols acTAG at up to 70 mol% of seed oil. A similar approach of coexpression of Euonymus alatus diacylglycerol acetyltransferase (EaDaCT) gene together with RNAi suppression of DGAT1 improved acetyl-TAG levels to up to 85 mol% in field-grown transgenic Camelina. Analysis of the acTAG portion exposed a twofold decrease in very long chain fatty acids, consistent with their displacement from the sn-3 position by acetate. Germination of seed remained efficient, and seedlings were able to metabolize the stored acTAGs as quickly as regular TAG. Viscosity, caloric content and freezing point of the Camelina acetyl-TAG oils were reduced, enabling use of this oil in several food and nonfood uses.

Bangash et al. (2013) established a reproducible and effectual protocol for transformation through Agrobacterium with wasabi defensin gene in B. juncea variety NIFA RAYA. The gene was obtained from leaves of Wasabia japonica and cloned into the pEKH-WD plasmid, driven by the 35S promoter. In this study number of factors affecting transformation such as age of explants, the choice of explants, various proportions of growth hormones, different concentrations of growth hormones and media composition was optimized. It was observed that explants from cotyledons and hypocotyls from four to seven days old seedlings showed best transformation in terms of producing when used as explants in terms of producing transgenic calli and shoots. Best callus initiation of the explant was found on solidified MS medium added with growth regulators 2 mg/L BAP and 0.2 mg/L NAA. Hormone mixture of 3 mg/L BAP and 0.3 mg/L NAA was best for callus formation. High frequency of shoot induction was obtained on media supplemented with 3 mg/L BAP and 0.5 mg/L NAA and 20 µM AgNO3. The transgenic line was confirmed by using Wasabi defensin gene specific primers against their genomic DNA through PCR analysis.

Mollika et al. (2011) developed efficient in vitro regeneration protocol using MS media supplemented with various combinations and concentrations of plant growth hormones for two varieties of B. juncea, BARI Sarisha 11 and BARI Sarisha 16 and one variety of B. campestris, Tori 7. In this study, the highest percentage of
shoot regeneration was obtained on MS medium supplemented with 0.2 mg/L NAA, 2.0 mg/L BAP, and 0.5 mg/L Kn in case of BARI Sarisha 16 and BARI Sarisha 11. They observed that maximum number of shoots per explant of Tori 7 was obtained on MS media supplemented with 0.2 mg/L NAA and 3.0 mg/L BAP. Among the explants of three varieties, the explant of BARI Sarisha 11 showed high efficiency of shoot organogenesis as well as maximum number of shoot per explant with minimum days of shoot formation. They found that hormone free MS media was best for root induction in case of the two varieties of BARI Sarisha 11 and BARI Sarisha 16. The in vitro regenerated plantlets were effectively transplanted into soil after proper hardening procedure. They observed that the most interesting situation when in vitro regenerated shoots produced in vitro flowers on regeneration media as well as hormone free MS.

Das and Joshi (2011) made necessary changes in transformation protocol to evade the disparities related with in planta transformation by using four different inoculums (inoculum-1, inoculum-2, inoculum-3 and inoculum-4) to increase chances in transformation efficiency. Amongst four types of inoculums, the inoculum-3 showed the highest rate of transformation. Moreover, Tween-20 (0.07%) and silwet L-77 acts in same manner. In order to enhance the transformation efficiency, glucose rather than sucrose can be applied in inoculum to transform Arabidopsis. The vacuum infiltration of the Agrobacterium infected plants was followed by incubation at 28°C for seven to eight hours horizontally in dark condition and obtained maximum amount of transformed seeds. The modifications in the working protocol lead 12-14% increase in transformation efficiency. In this study they used kanamycin with soil in selection pots instead of MS agar plates supplemented Kanamycin and observed best results, as no MS medium and no aseptic conditions were required for selection of transgenic plants. At the end of experiment they found that transformation efficacy subsequently enhanced the percentage of single copy and homozygous integration in transgenic lines.

Bano et al. (2010) optimized the regeneration protocol by using three genotypes of B. juncea (UCD-635, NIFA RAYE and RL-18). They used two, two different combination of phyto-hormones, including auxins (Indole acetic acid and Naphthalene acetic acid) and cytokinins (Kinetin and Benzylaminopurine). They cultured the explants taken from three genotypes on MS-medium containing (NAA 0.2 mg/L; BAP 2.0 mg/L) (BAP 3.0 mg/L; NAA 0.3 mg/L), (IAA 0.1 mg/L; Kinetin 1.0 mg/L), (IAA
0.2 mg/L; Kinetin 2.0 mg/L) and (IAA 0.3 mg/L; Kinetin 3.0 mg/L). The highest callus production efficiency (65.55) was observed on MS medium supplemented with (NAA 0.2 mg/L; BAP 2.0 mg/L). They found that maximum organogenesis (22.31) was produced on MS media containing (NAA 0.3 mg/L; BAP 3.0 mg/L) and (IAA 0.3 mg/L; KIN 3.0 mg/L). In the above study they observed high regeneration efficiency (7.13) on media supplemented with (NAA 0.3 mg/L; BAP 3.0 mg/L). They concluded that shoot production and regeneration efficiency of the three genotypes of *B. juncea* were found significantly different from each other at *P* ≤0.05.

Durrett et al. (2010) studied the storage lipids in *Euonymus alatus* (Burning Bush) and observed that the endosperm and embryo of the seed accumulated an unusual triacylglycerol 3-acetyl-1,2-diacyl-sn-glycerols commonly known acTAG, while the arils accumulated long-chain TAGs (lcTAGs). The acetyl group at the sn-3 position changes the physicochemical property of the TAGs providing it with a reduction of viscosity such as 30%. They performed Pyrosequencing for isolation of the gene which is responsible for the production of acTAGs and named it *EaDAcT* (*E. alatus* diacylglycerol acetyltransferase). They transformed *EaDAcT* in Yeast, which showed production of acTAGs but no lcTAGs. Similarly *EaDAcT* was expressed in Arabidopsis plant which resulted in accumulation up to 40 mol% of acTAGs, of the total TAG in its seed oil. At the end of experiment they concluded that if this gene is transformed in the other oil accumulating plants they will produce acTAG and will alter fatty acid of TAG. In addition, by production of acTAG in the transgenic plants can be directly used as a biofuel and for other purposes.

Rashid et al. (2010) explored the value of *Jatropha curcas* seed oil for biodiesel production. They transmethylated Jatropha oil under optimized set of reaction conditions. They assessed the thermal stability of the JOMEs by thermogravimetric analysis (TGA) and produced biodiesel. They found biodiesel produced by trans-methylation was to be within the standards specifications of ASTM D 6751 and EN 14214. Moreover, they recommended that biodiesel produced by this method can be used as a biofuels.

Sexton et al. (2009) used a partial equilibrium model to stimulate biofuel impacts. They found biofuel production reduces gas prices benefiting gasoline consumers. Current biofuel creates a tradeoff between food and fuel. The need for agriculture to provide food and fuel creates the importance to improve agricultural
production. Biotechnology –based production of transgenic crops play an important role in this investment. Biotechnology will reduce land-use changes, greenhouse gases emission in atmosphere and provide a suitable biofuel energy source.

Chhokar et al. (2008) studied the lipid composition and fatty acid profile during seeds development of mustard \textit{B. juncea}. They observed that lipid contents in mustard seed increased gradually with the development of seeds and the fatty acid showed remarkable variation during seed development. In this study the polar and non-polar lipids linolenic acid, linoleic acid, stearic acid and palmitic acid showed the decreasing trend until maturity, while the concentration of oleic acid and erucic acid increased regularly at maturity.

Demirbas (2008) reported on the progress and recent trends in biofuels. Biodiesel fuel typically consists of lower alkyl fatty acid and it chain length is mostly from C_{14} to C_{22} esters of short-chain alcohols, primarily, ethanol or methanol. It was stated that various methods have been used for the production of biodiesel from plants oil, such as blending and direct use, pyrolysis, micro-emulsification, and transesterification. Among these, transesterification is the most widely used and accepted technique. Transesterification is used for the purpose to lower the viscosity of the plants oil. It was also found that most significant variables affecting methyl ester produce during the transesterification reaction are the molar ratio of alcohol to vegetable oil and the reaction temperature. The commonly used alcohol is methanol in this process, due to cost effectiveness. The outstanding advantages of methyl esters of vegetable oils over other non-renewable engine fuel was suggested. Biodiesel can be utilized in any mixture with petro-diesel fuel, because it has very matching properties with petro diesel.

Durrett et al. (2008) reported that the plant oil represents a logical alternate for energy source. The TAGs produced by plants are energy-rich compounds in nature. Various properties of fatty acids can be utilized in the modification of such lipids and enhancing their ability for consumption as biodiesel. A proper understanding of the production and regulation of \textit{TAG} in plants must be obtained to increase the limited supply of biodiesel feedstock. Alternation of fatty acid composition can solve the problem of biodiesel having low oxidative stability and poor cold temperature performance. Recombinant DNA technology in plants contributed to enhancement of biodiesel by production of transgenic soybean having high oleic acid, for example.
Rashid and Anwar (2010) optimized protocol for biodiesel production from safflower oil through base-catalyzed transesterification. Results of their study showed that methano-lysis is best for biodiesel production from safflower oil under a proper set of procedure. Fuel produced by this methods have quite fine properties comparable to those of conventional diesel and thus may be explored for likely applications in compression-ignition engines.

Verma et al. (2008) developed a floral dip method of genetic transformation in two different species of Brassica, namely, B. carinata cv, Pusa Gaurav and B. napus cv Elect. In this study they used binary vector containing B. carinata Zinc Finger transcription factor (BcZF1) gene under the transcriptional control of abiotic stress-inducible late Embryogenesis Abundant-1 (LEA1) promoter. This construct was transformed into Agrobacterium tumefaciens strain GV-3101. Brassica spp. was transformed with the modified strain of Agrobacterium via floral dip method. The transformed Agrobacterium cells cultured in Yeast Extract Mannitol (YEM) were harvested and resuspended in half MS media containing 0.05% Silwet L-77 and 5% sucrose. The transformation was done by dipping inflorescence at the initial blossom stage. They collected seeds from these plants and screened with kanamycin (25 mg/L) by germinating them on MS medium. For further confirmation, they evaluated the kanamycin resistant putative transgenic plants by PCR and RT-PCR using nptII gene-specific primers. The results indicated that the Agrobacterium mediated floral-dip transformation can be a successful strategy to get transgenic B. carinata and B. napus in a short time without undergoing cumbersome tissue culture steps or vacuum infiltration.

Owing to deficiency of energy resources in Pakistan, biodiesel could be an alternate choice for energy generation Ahmad et al. (2007). Pakistan has a plenty of agricultural lands, suitable climatic conditions and large feedstock production of biodiesel producing crops. They collected the data throughout the country to find plant species having seeds rich in oil for biodiesel production. Among all, most important are Pongamia pinnata, Ricinus communis and Brassica spices. They suggested that the usage of the biofuel will reduce pollutants and other greenhouse gas emissions because they are biodegradable and also the country economy will be raised by increasing demand and prices for agricultural products. The dependence on imported petroleum with associated economic and political susceptibility will be decreased.
Demirbas (2007) demonstrated the progress and recent trends in biofuel utilization. He reported that current biomass can be used for the production of heat and electricity. The most current biomass-based transportation fuels are biodiesel and bioethanol as well as diesel formed from biomass by Fischer-Tropsch synthesis. Bioethanol is a substitute of petrol additive. He further suggested that it is likely that straw, wood and even household wastes may be carefully converted to bioethanol. Recently crops producing sugar, starch or oil are the source for transport fuel production. Biodiesel is a renewable substituent of conventional diesel form fossil petroleum. Improvement in the use of plant oils in order to make the biodiesel is due to its renewable nature and lower pollution that is unlike the petroleum-based diesel fuel. Biomass energy conversion are utmost important for bio-fuel especially biodiesel production.

Hill et al. (2006) reported that as fossil fuel is getting short and using it has many disadvantages, therefore there must be an alternate source of energy to be used. This source of energy must be economic, environmentally friendly, can be reproduced in large quantities without affecting the production of food negatively. They used corn oil and soyabean for the production of bioethanol and biodiesel respectively. They observed increase of energy production from ethanol as 25% and biodiesel 93%. Compared to fossil fuel, biodiesel decreases greenhouse gas emissions by 41% and combustion of ethanol by 12%. Biodiesel release less pollutants in air compared to ethanol. They concluded that biodiesel produced from waste biomass or from agriculture feed stock are environmental friendly and biodegradable.

Peterson and Thompson (2005) verified biodiesel production from two varieties of mustard seeds: B. juncea (oriental spice mustard) and Sinapis alba (yellow mustard). Compared to 40-45% of canola species, Sinapis alba had about 24 to 27% and B. juncea has 34-38% oil content. Dodge pickup truck fueled with mustard biodiesel, driven up to 56,465 miles without any hurdles and difficulty on road trail with a 1999 cummins 5.9 L diesel engine. However, the problem came in the fuel filter, as the filter was being kept changing more often as compared to conventional diesel because in the biodiesel, the fuel filter material did not hold well. The study came to the conclusion that biodiesel produced from animal fats and/or tropical oils such as palm oils have greater cold point then Indian mustard.
Pimentel and Patzek (2005) studied the energy outputs from ethanol. The ethanol was produced from wood biomass, corn and switch grass. It was found that ethanol produced from each biomass was less than the respective fossil energy inputs. The same was right for the production of biodiesel from soybeans and sunflower; however, the price of generating soybean biodiesel was slightly higher than ethanol production. Moreover, energy inputs were matched and compared with the energy outputs. Corn grain ethanol production need 29% more fossil energy than the ethanol fuel. Production of ethanol from switch grass want 50% more energy compared to ethanol fuel. Ethanol production using wood biomass need 57% more fossil energy as compare to ethanol fuel and biodiesel produced from soybean need 27% more energy than the biodiesel fuel. Biodiesel from sunflower required 118% more fossil energy than the biodiesel fuel produced.

Lionneton et al. (2004) stated that for developing an efficient mustard (B. juncea) breeding program, a good knowledge of the genetic control and relationships of the key selected traits is required. In this study they evaluated over 2 years doubled haploid (DH) lines in the field for traits like plant height, days to flowering, fatty acid composition, thousand-seed weight, seed oil content, gluconapin, sinigrin, and total glucosinolate contents. They valued effect of seed coat color in double haploid lines. Results of their research activity indicated that significant difference was observed between brown and yellow seeds only for fatty acid and oil contents. Molecular analysis revealed that seed coat color is related with two Mendelian trait Bjc2 (on LG6) and loci: Bjc1 [on linkage group (LG) 3]. They reported the non-co-localization of quantitative trait loci and indicated genetic independence. The results of their study also indicated the independent breeding of the most important agronomic and quality traits of brown mustard.

Alternative Energy Development Board (AEDB) in 2003 reported that Pakistan is mainly dependent on conventional fuels for energy resources, which are usually imported. Furthermore, the country fuel price is directly proportional to the international market price. Due to this situation along with the rapid reduction of country’s natural gas reserves, the AEDB was established in 2003 to take necessary action in the country for the use of renewable energy and to facilitate, manage and boost the use of substitute / renewable energy technologies so as to get minimum 5% share of power generation through renewable energies by year 2030. This article offers
an overview that government of Pakistan needs to start various projects on renewable energy sources for its midterm and long term proposal.

Jaworski et al (2003) reported that many non-agronomic plant species have unusual fatty acids in the seed oil which is useful for industrial properties. To produce high levels of these fatty acids in the seeds of existing crop plants researcher are trying to use biotechnological tools. For this reason through the use of expressed sequence tags cDNAs for a wide variety of unusual fatty acid biosynthetic enzymes have been sequenced and identified. However to use these cDNA for large scale production of unusual fatty acids in seeds of transgenic plants it has not yet been possible. To solve this difficult issue there is need for a greater knowledge of fatty acid metabolism in oilseeds.

Thelen and Ohlrogge (2002) reported that fatty acids have various uses ranging from food to industrial feed stocks and are the richest form of reduced carbon chains available from living organism. Plants are major renewable source of fatty acids, as many species store the fatty acids in the form of TAG as major storage constituents in seeds. Metabolic engineering of seed fatty acids became possible with the plant transformation technology and the transgenic plant oils represent some of the first successes in design of altered plant products. Specific tailoring of common fatty acids have been enabled by the gene down-regulation strategies in several oilseed crops. Furthermore, the manipulation of important and novel fatty acid biosynthetic gene(s) from non-agronomic plants in oilseed crops has changed the oil compositions. In addition, the future and current aims for the production of seeds with desired characteristics, such as new oils that are more stable, as well as healthier for humans, richer in oil content can be served as a feed stock for industrial commodities. It was concluded that engineered plant oils are the perspective of large-scale new industrial uses but will require a better understanding of factors that limit the accumulation of unusual fatty acid structures in seeds.

Bechtold et al. (2000) reported that in planta transformation is now days the most commonly used method for transformation of Arabidopsis thaliana. They got the transformants via in planta transformation by inoculating them at various floral stages and determined the expression of beta-glucuronidase gene encoding GUS. Moreover, they observed that transformation succeeded where embryo sacs have approached to 3rd division stage. However, they could not get GUS expression in pollen of plants and/or
embryo sacs penetrated with an Agrobacterium strain containing a GUS gene under the control of a gametophyte-specific promoter. Insertion mutant was used to identify the genetic target, having a gene essential for male gametophytic. Whereas, only in the mutant female gazettes, the GUS marker was transmitted to the progeny. In addition, a hygromycin resistance marker was transformed in to plants to obtain double resistant plants. They studied one ninety three progeny of these transformants and revealed that twenty five plants in which the two resistance markers were linked in coupling and only one plant where they were linked in repulsion. Finally they concluded that the main target for the T-DNA as chromosome set of the female gametophyte.

Stoutjesdijk et al. (2000) reported that to change the fatty acid profile of elite Australian germplasm of B. napus and B. juncea different genetic engineering methods have been used successfully. These breeding lines transformed by co-suppression plasmids having oleate desaturase via Agrobacterium tumefaciens plant-transformation techniques. In this study they used intron-interrupted hygromycin-resistance gene as selectable marker by bringing modification in the existing brassica transformation protocols which resulted in an increased transformation efficiencies. They examined enhancement in oleic acid concentration, up to 89% in B. napus and 73% in B. juncea through silencing the endogenous oleate desaturase genes.

Velasco et al. (1998) analyzed fatty acid content of 21 species of Brassica through gas-liquid chromatography (GLC) and near-infrared reflectance spectroscopy (NIRS). NIRS was found out to be more efficient than GLC analysis. High results were found in linoleic oleic, linolenic and erucic acid that showed large taxonomic and chemical variability. High erucic acid levels were recognized in B. oleracea L, B. napus L, and Brassica rapa L. and in wild species B. incana Tenore, B. rupestris Raf., and B. villosa. Erucic acid levels were followed by polyunsaturated linoleic and linolenic acid in various Brassica species.

Barfield and Pua (1991) developed an efficient system for gene transfer into plants of Indian Mustard (B. juncea) by using Agrobacterium tumefaciens mediated transformation. They used hypocotyl explants for tissue culture transformation and got 100% shoot regeneration by using MS media supplemented with 3.3 mg/L AgNO3 ,0.9% agarose, and 0.5-2 mg/L BAP in combination with 0.1-1 mg/L NAA or 0.01-0.05 mg/L 2,4-D. They tested different types of Agrobacterium strains and found the Agrobacterium tumefaciens A208-SE strain most active for B. juncea transformation,
carrying the disarmed Ti plasmid and a binary vector pROA93 carries the coding sequences of the GUS and the NPTII genes, both driven by a common promoter CaMV 35S in two opposite directions. Infected explants were grown on the selection medium supplemented with 0.1 mg/L NAA and 0.5 mg/L BAP gave rise to transgenic shoots at the maximum frequency (9%). All T0 transgenic plants were phenotypically normal, but variation was noted in expression patterns of the GUS gene occurred among the transgenic plants. Both the GUS and the NPTII genes were transferred to the T1 seed progeny and displayed co-segregation.

Block et al. (1989) developed an efficient transformation method for *B. napus* and *B. oleracea* which is largely genotype-independent. As a selectable marker gene they used neo and bar genes. They infected hypocotyl explants of both species for transformation. *Agrobacterium* strains carrying chimeric bar genes and neo. To improve shoot regeneration, they used AgNO3 is necessary. In this research activity they escaped vitrification to improve the effectiveness of rooted transformed shoots, by decreasing the cytokinin concentration and also by reducing the water potential of the medium. They also decreased the relative humidity in the tissue culture vessel. They described that genetic analysis and Southern blotting of SI-progeny as average, the transformants have one and three copies of the chimeric genes. Among all these transformants, various range of expression levels of the chimeric genes were detected. Moreover, up to 25% of the transformants revealed no detectable expression of phosphinotricin acetyltransferase and/or neo-mycin phosphotransferase enzyme activities. The transformation of neo and bar genes were affirmed via Southern blotting.
III. MATERIALS AND METHODS

The present research work was undertaken to standardize the regeneration and transformation protocol for *B. juncea*. Further, the seed lipid and fatty acid profile was determined at different physiological stages of development. The experiment was conducted at the Institute of Biotechnology and Genetic Engineering, the University of Agriculture, Peshawar-Pakistan and department of Biochemistry and Molecular Biophysics, Kansas State University, USA during the year 2013-15.

1. STANDARDIZATION of *IN VITRO PROPAGATION OF B. JUNCEA*

1.1. Plant Material and Growth Condition

*Brassica juncea* CV- Raya Anmol seeds available and maintained at the Institute of Biotechnology and Genetic Engineering, were surface sterilized by washing thoroughly with tap water, followed by washing with 70% ethanol for two minutes. The seeds were then thoroughly cleaned by washing with distilled water and dipped in a bleach containing two drop of tween 20 for twenty minutes. Finally, the seeds were cleaned by washing with distilled water and cultured on ½ MS (Mursashige and Skoog, 1962), containing 20g/l sucrose. The seeds were germinated in growth chamber at continues light (1000 LUX) and temperature 24± 2 °C.

1.2. Equipment Used

The following equipment were used during this experiment. Laminar air flow hood (*ESCO* AC2-4A1), Electronic Balance (*SHIMADZU* BL-2200H), pH meter (*ino* Lab PH7110), Centrifuge machine (*Beckman coulter*, ALLEGRA-64R), Centrifuge Mini (Sigma 1-14), Shaking incubator (*Lab TECH SI-100R*), Incubators (*ESCO IFA-32-B*), Thermocycler (*BioRAD T100™*), Microwave oven (*National NN 7855*), Gel Electrophoresis and gel documentation machine (*Bio-RAD mini subcell GT and SGE 020-02*), Refrigerator 04°C (*Sanyo MPR 513*), R Tubes (*Pyrex, Germany*), Micropipettes (*Gilson France*) Eppendorf, Falcon tubes and Vacuum Desicator (*Bel-Art Scienceware*)
1.3. Explants Isolation and Callus Induction Medium

Cotyledonary explants were aseptically isolated from 4-7 days old seedling by cutting with sterilized sharp blade. The explants were cultured on MS medium supplemented with different plant growth regulators (PGR) in different concentration. Benzyl amino purine (BAP) was used in different concentration alone or in combination with 1 µM of naphthalene acetic acid (NAA) or gibberellic acid (GA3). Each treatment was replicated 5- times and callus induction was recorded as number of cotyledon producing calli (Table 3.1).

<table>
<thead>
<tr>
<th>Table 3.1 Different Concentrations of PGRs for Callus Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGR Concentration (µM)</td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>13</td>
</tr>
<tr>
<td>14</td>
</tr>
<tr>
<td>15</td>
</tr>
</tbody>
</table>
1.4. Shoot Induction Medium

After 35 to 40 days, the calli were aseptically transferred to sterilized petri dishes and were cut into small size with sterilized blade. The calli were cultured on fresh medium augmented with various concentrations of plant growth hormones, mentioned in Table 3.1. In each culture box one to five calli were cultured and sealed with micro pore tap. The culture box was kept in growth chamber at the photoperiod of 16 h at 24°C ± 2. The plantlets were then transferred to fresh media after three weeks of culturing. The experiment was planted in 5- replication and the data were recorded as % of shoot induction as well as number of shoot produced and mean shoot length (cm).

1.5. Root Induction Medium.

After six weeks, regenerated shoots were transferred to rooting medium containing different concentrations of Indole-3- Butyric acid (IBA) alone and or in combination with Kinetin as shown in the Table 3.2. The pH of MS medium having 20g/l sucrose and 8g/l agar was kept as 5.7. The experiments was planted in 5- replicate and data was recorded for percentage of rooting, number of root for plantlet and mean root length (cm).

Table 3.2 Different Concentrations of PGRs used for root induction.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IAA (µM)</th>
<th>Kin (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>6.0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>6.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7</td>
<td>8.0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>10.0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>10.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

2. IN VITRO TRANSFORMATION OF B. JUNCEA AND PLANT REGENERATION
2.1. Transformation of *E. coli* by Freeze/thaw method

For multiplication of the designed construct, (*P*Bin*G*ly4*EaDAcT) it was transformed to the *DH5α* strain of *E. coli* bacteria employing Freeze/thaw transformation method. Briefly, competent *DH5α* cells were prepared using method of (Froger and Hall, 2007). Then about 2 µl (100 ng/µl final concentration) of the plasmid DNA was added to about 100µl competent cells then incubated on ice for 5 min followed by incubation at -80°C for 10 min. A heat-shock was given to the incubated cells at 37°C for a period of 5 min. Afterward 1 ml LB medium was added to the transformed cells and incubated at 28°C for 2-3 hours at 120 rpm for its growth. After significant growth monitored at OD<sub>600</sub> (optical density) the culture was centrifuged at 7000 rpm for 3 min at 37°C. The supernatant was discarded and the pellet was re-suspended in the remaining LB medium and spread on LB plates containing kanamycin (50 mg/L) for the selection of transformed *E. coli* cells and incubated at 37°C. After the appearance of resistant colonies, the transformation was confirmed by the PCR analysis of the target *EaDAcT* gene.

2.2. *Agrobacterium* Competent Cells Preparation :

A colony of *Agrobacterium tumefaciens* strain GV3101 was cultured overnight in 2ml of YEP medium at 28 °C with continues shaking. Half of this culture was transferred to 100ml YEP in a 500ml flask and incubated at 28 °C with constant shaking until the OD<sub>600</sub> was 0.25~ 0.30.

The cell culture was chilled on ice for 15 minutes and harvested by centrifugation at 3300g for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was suspended in 30-40ml of 0.1M CaCl<sub>2</sub> containing 10 % cold glycerol and again chilled on ice for 30 minutes. The cell suspension was centrifuged again at 3300g for 10 minutes at 4 °C. The cells were obtained after discarding the supernatant, re-suspended in 6ml of 01M CaCl<sub>2</sub> supplemented with 15% glycerol. Aliquots of 40~ 50µl of the suspended cells were made in 1.5ml Eppendorf tubes, snap frozen in liquid nitrogen and stored at -80 °C.

2.3. *Agrobacterium* Transformation through Electroporation

For transformation of *Agrobacterium*, the frozen competent cells of *Agrobacterium tumefaciens* were thawed on ice and 100 µL competent cells with
100 ng of plasmid DNA were taken in an electroporation cuvette. The electroporation cuvette containing the competent cells/DNA mix was placed into the electroporator and an electric pulse of 2.4 kV was given for a brief period of 5 milliseconds. After electroporation, the transformed Agrobacterium cells were removed and mixed with ~1 ml YEP. The YEP added transformed cells were then grown by shaking at 28°C for about 3 hours. Then serial dilutions of the transformed cells were plated on YEP medium with triple antibiotics selection having kanamycin sulphate (50 mg/L), Gentamycin (50 mg/L) and Rifampicin (25 mg/L). The bacterial colonies that were found to be resistant to antibiotics used were confirmed through colony PCR analysis using the gene specific primers of EaDaCT gene.

2.4. Agrobacterium Tumefaciens strain and Binary vector

The *Agrobacterium tumefaciens* strain GV3101 harboring the binary vector plasmid *pBinGlyBar4-EADcT* was used for the transformation of *Brassica* plants. The plasmid, *pBinGlyBar4-EADcT*, harboring the chimeric *EaDacT* gene isolated from *Euonymus alatus* (burning bush) plant, encoding diacylglycerol acetyltransferase was kindly provided by Timothy Patrick Durrett, Department of Biochemistry and Molecular Biophysics, Kansas State University USA. Plasmid also contained gene for resistance to kanamycin and the basta for selection of bacterial and putatively transformed *Brassica* plants (De Block et al., 1987; Thompson et al., 1987) as shown in figure 3.1.

![Figure 3.1](image-url)  
*Figure 3.1: Representing EaDaCT gene Construct (T DNA region of *PBinGly4EaDaCT* harboring EaDaCT and bar genes. The gene for *EaDacT* are driven by Gly Promoter while for bar by Nos promoter. The double arrows represent PCR –amplified region used to confirm the existence of the*
introduced gene in transgenic plant. LB and RB Left and Right border sequence of T-DNA region respectively. nos-T, terminator, Kan and restriction enzymes are also shown.

2.5. Colony PCR to Confirm the Presence of Plasmid DNA in Agrobacterium

The transformation of Agrobacterium with plasmid DNA was confirmed with colony PCR using EaDacT specific primers amplifying 1092bp region of EaDacT gene. A single Agrobacterium colony was suspended in 5 µl of sterile distilled water. About 1µl of this suspension was then amplified in a reaction containing 2X PCR Master Mix 10 µl, 1.6 µl (conc.) of forward and reverse primer, 5µl ddH2O and 2 µl DNA polymerase. The PCR condition was an initial denaturation at 95°C for 1 min, 30 cycles at 94°C for 30 seconds, 52°C for 1 min and 72°C for 1 min and final extension at 72°C for 10 min.

2.6. Agrobacterium Inoculum Preparation

The PCR confirmed transformed Agrobacterium tumefaciens was cultured overnight at 28°C on a rotatory shaker with a speed of 225 rpm in YEP medium containing Kanamycin sulphate (50 mg/L) Gentamycin (50 mg/L) and Rifampicin (25mg/L). The overnight bacterial suspension was then centrifuged for 10 min at 3,000 rpm, the supernatant was decanted and bacterial pellet was re-suspended in plan MS medium. The resulting suspension was diluted to get an OD 600nm of 0.5 which was then used for the transformation experiments. The inoculum was used to transform the plants simply by dipping the B. juncea explants for 30 seconds in it.

2.7. Seed Germination and Explant Isolation and Inoculation

Seeds for transformation were germinated on half strength MS medium containing 20g/l sucrose, vitamins and iron solidified with 8.0g/l agar (Musrashige and Skoog., 1962). Seeds were sown on the germination medium at a density of 25 seeds per petri dish and the petri dishes were then sealed with microspore tape and transferred to incubator for incubation at 25°C under 16 hour day length for 4 days. After germination cotyledons were excised using a sharpe blade from 4 days old seedling by gentle holding the base of cotyledon with forceps just above the meristem region. Extreme care was taken, not to include any meristem region because meristem is hard to be transformed easily and escape shoots on selection medium. The excised cotyledons were then immediately placed on the cultivation medium in petri dishes in such a manner that 2 mm of the cut petiole is implanted into agar. About eight explants
were established on each petri dish. After completion of the explant isolation, they were inoculated by dipping one time in the transformed *Agrobacterium* suspension. Special care was taken to ensure that only the cut end of the petiole was dipped in the suspension medium. After infection, the cotyledons were then returned to cultivation plates that were sealed with micropore tape. Then the plates were transferred to the growth chamber and incubated at 21°C under 16 hour day length for three days.

### 2.8. Co-cultivation and Selection Media

The nutrient media used for the co-cultivation of infected *B. juncea* explants contained 4% MS media 4.43 g/L, carbon source (sucrose) 20 g/L, iron sources (20X), Myo-inositol 0.1g/L supplemented with PGRs i.e. BAP with a concentration of 2.5mg/L and NAA with a concentration of 0.2 mg/L.

The selection media for infected *B. juncea* explants contained 4% MS media 4.43g/L carbon source (sucrose) 20g/L iron sources (20X) with PGRs i.e. BAP + NAA (4.0 + 1.0 µM), basta, in a concentration of 20mg/L and timintin, having an overall concentration of 160mg/L for the selection of putative transgenic explants.

The putative transgenic explants were further subjected to higher concentration of basta (25 mg/L) along with timintin (160 mg/L) for confirmation of putative transgenic plants. Remaining ingredients of the media were the same as mentioned earlier.

After three days on the co-cultivation medium, the explants were transferred to the selection medium in a laminar flow hood. The plates were sealed with micropore tape and returned to growth chamber under 16 hours day length with 21°C. In each experiment a control plate was also included. The explants in the control plates were not dipped in the *Agrobacterium* culture and were transferred directly. After three weeks of incubation, the explants were then directly transferred to a fresh media in a 250ml flask with increased concentration of basta (25 mg/L).

### Shoot Isolation and Rooting

The transgenic shoots were then isolated and transferred to a 250 ml flask containing rooting medium with IAA (6.0µM)+Kn (1.0 µM). Shoots were maintained on rooting medium at 21°C under 16 h day length until roots were developed. After root elongation the plantlets were then transferred to pots for acclimatization.
2.9. Rooting media and Transplantation of Plantlet into Soil

The rooted plantlets were then shifted to plastic pots containing soil, horticulture and vermiculite at ratio 4:2:1 along with osmocote covered with transparent plastic pots and placed in acclimatization room at 21°C with 70-90% humidity. Temperature of the room was gradually increased up to 25°C. The transparent cover was removed for 4 to 6 hours/day for two weeks of and then completely removed after 3 weeks for proper hardening to obtain established plants. During the acclimatization of tissue culture grown plantlets, the gradual increase in temperature and use of polythene bags improved the survival rate.

2.10. Molecular Analyses of Plant Samples:

A polymerase chain reaction was carried out to confirm the putative transformed plants. Genomic DNA from basta-resistant plants and for control wild B. juncea plants was extracted using Cetyl trimethylammonium bromide (CTAB) method (Rogers and Bendich 1988). The PCR reaction was carried out in a mixture contained 50ng genomic DNA, 200 µM dNTPs, 0.4 µM of each EaDACT specific primers (Table.3.3), 2.5 mM MgCl₂, 2 µl Taq polymerase buffer (10X) and 2.5 units Taq polymerase enzyme (Thermoscientific, USA) with the volume adjusted to 20 µl using PCR grade water. The mixture was first heated at 95°C for the complete denaturation of genomic DNA and then amplified in a 30 cycled reaction with the thermal profile of 94°C for 30 sec, 57°C for 30 s and 72°C for 1 min. A final extension at 72°C was given for 5 min at the end of reaction.
Table 3.3: The primers used for the PCR amplification of target \(EaDAC\) gene in the putative transformed \(B.\ juncea\) plants.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>GC Contents</th>
<th>Tm (°C)</th>
<th>Expected size</th>
</tr>
</thead>
<tbody>
<tr>
<td>(EaDAC)-F</td>
<td>ACCCAATTGATGATGGATGCTCATCAAGAG</td>
<td>44%</td>
<td>58.9</td>
<td>1KB</td>
</tr>
<tr>
<td>(EaDAC)-R</td>
<td>AGACCTGCAGGTTAAGCGTAATCTGGAAATC</td>
<td>47%</td>
<td>68.9</td>
<td></td>
</tr>
</tbody>
</table>

3. **INPLANTA TRANSFORMATION OF \(B.\ juncea\)**

3.1. \(B.\ juncea\) Plant material and Growth Condition

A \(B.\ Juncea\) CV genotype “RAYA Anmol” was used for all experiments. For transformation, seed were directly germinated in pots filled with the mixture of soil, vermiculite and horticulture (Clinton, OK, USA) (4:2:1) along with ½ spoon osmocote in the greenhouse. The growth conditions in the greenhouse were 21°C (day/night) with natural lighting. The relative humidity was maintained at 60%.

3.2. **Agrobacterium** Inoculum Preparation and Vacuum Infiltration Transformation.

Bacterial cell growth was started 2–3 days before its transformation into the plants. A single bacterial colony was inoculated into 5 mL YEP medium (10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, pH 6.8) augmented with 50 mg/mL kanamycin and incubated overnight. Then 1.6-mL bacterial culture was transferred into a flask comprising 160 mL YEP medium and let the growth of bacteria to be continued overnight at 28°C. The culture was then centrifuged at 6,000 rpm for 20 min and the bacterial cells were harvested and suspended in the infiltration medium, comprising half- strength MS medium, [Silwet L77 (Lehle Seeds, Round Rock, TX, USA), 50 g/l sucrose and 0.05% (v/v)]. Plants at the flowering stage in the pots were kept a 310 mm-height vacuum desiccator (Bel-Art, Pequannock, NJ, USA) and the inflorescences were immersed into the inoculum in a 300 mL container. The vacuum was maintained for 5 min at 85 kPa pressure and the treated plants were enclosed by polythene bags and put at a dark place overnight for 24 h before returning to normal growth in greenhouse until maturity.

3.4. **Floral dip Transformation**
In this method the *Brassica* inflorescences were directly dipped in infiltration medium without using vacuum desiccator. *B. juncea* plants at the early flowering stages were used for transformation. The flower buds were submerged for 1–3 min in *Agrobacterium*-containing solutions without using vacuum desiccator.

### 3.5. Basta Screening

In order to standardize the efficiency of basta herbicide on *B. juncea* plants, three different basta concentrations, i.e. 0.1%, 0.2% and 0.3% were sprayed on wild *B. juncea* plants. For screening of putative transformed plants, the *B. juncea* seeds from floral dipped plants were germinated on soil and sprayed with 0.2% basta herbicide. Spraying was preformed on five days after germination and repeated five times at an interval of two days.
3.6. Molecular Analysis of $T_0$ Plants

A polymerase chain reaction was employed to confirm the putative transformed plants. Genomic DNA from Basta-resistant and control $B. \ juncea$ plants was extracted using CTAB method (Rogers and Bendich 1988).

4. LIPID AND FATTY ACID PROFILE OF TRANSGENIC AND WILD PLANT AS WELL AS THE DEVELOPING SEED OF $B. \ juncea$

4.1. Plants material and growth condition

Mustard $B. \ juncea$ were raised in earth ware pots filled with vermiculite, horticulture and soil along with osmocot in a green house. The plants were not exposed to any type of spray. After germination one plant per pot were retained. Water was given as and when required.

4.2. Sampling

Seed samples were collected at 10, 20, 30, 40, and 50 days after flowering (DAF) and at maturity. The age of the seeds was determined by tagging the flowers (Table. 3.5) at the age of anthesis. From the plants seeds pods were removed picked it in falcon tubes, the falcon tubes were label and kept it on dry ice and stored at -80°C.
### Table 3.5: Plants Tagging Plan for Lipid Analysis of Developing Seeds.

<table>
<thead>
<tr>
<th>Tagging date</th>
<th>Colors</th>
<th>Harvesting days after flower (DAF)</th>
<th>Harvesting Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/26/2014</td>
<td>Blue</td>
<td>20</td>
<td>4/15/2014</td>
</tr>
<tr>
<td>3/31/2014</td>
<td>Pink</td>
<td>40</td>
<td>5/10/2014</td>
</tr>
<tr>
<td>4/1/2014</td>
<td>Orange</td>
<td></td>
<td>5/11/2014</td>
</tr>
<tr>
<td>4/2/2014</td>
<td>Blue</td>
<td>50</td>
<td>5/22/2014</td>
</tr>
</tbody>
</table>

### 4.3. Lipid Extraction from Seeds

Two tubes were needed for each sample which were cleaned and dried prior to use. Isopropanol was then heated up to 90°C in a large glass tube. Seeds were immediately separated from the pods and weighted. About 15 to 20 mg of seeds were taken in each tube and 2 ml of hot isopropanol (90°C) was added to it. These tubes were then heated for ten minutes up to 85°C and ground with Polytran and 3 ml hexane was added to it. After grinding the Polytran was rinsed with 70% ethanol from each sample. Samples were then incubated for 5 minutes at room temperature. Then 50 µg internal standard of odd chain tri 15:0 was added to each sample. Then 2.5 ml 6.6% Na₂SO₄ was added followed by vortexing. After vortexing the samples were centrifuged at 25000 rpm for five minutes. The upper organic phase was transferred to a new tube and the sample was again extracted with 2 mL hexane, isopropanol 7:2. This extraction was added to the previously obtained organic phase and dried eluent under nitrogen stream. Finally the dried samples were dissolved in 500 µl toluene and were stored at 4°C.

### 4.4. Thin Layer Chromatography Analysis
In order to determine the contents of TAG, Thin layer chromatography (TLC) was performed. The sample was applied as fine spot, 2 cm above the bottom, on the line present on thin layer chromatography plate coated with silica gel G60. TAGs were separated on TLC plates developed once in hexane/ether/acetic acid (70/30/1, v/v/v). As the solvent reached near the top of the plates, the plates were removed and dried for 5 minutes. After drying, 0.1% (w/v) 2,7 dichlorofluorescein (in 85% methanol) was sprayed on plates for visualization of TAG spots.

4.5. Seed Fatty Acid Transmethylation

For transmethylation of fatty acid each band on TLC plates were scratched through blade. Before scratching added internal stranded 5µl of 10mg/ml C17:0 TAG to each band. For each sample a separate glass tube was used. To each samples 1ml of 5% (v/v) H₂SO₄ in MeOH, 250µl of 0.2% BHT and 300µl toluene were added. Samples were then vortexed for 30 second followed by heating for 90 minutes at 95°C. Samples were then allowed to cool at room temperature. After cooling, 1.5ml of 0.9% NaCl and 2ml hexane were added to each tube followed by centrifugation at 2000 rpm for 5 minute. The upper organic phase was separated into a new glass tube and repeated the extraction with 2ml hexane. The samples were then dried under nitrogen stream and 500µl hexane was added with vertexing. In last, the samples were added to GC vials.

5.6. Fatty acids Profile through GC-FID

Gas Chromatograph-Flame Ionization Detector (GC-FID) provides an appropriate approach to determine the fatty acid composition of plants oil. Through GC-FID analysis, one can physically isolate the fatty acids and identify them in a one go analysis. The GC-FID analysis is carried out by fatty acid derivatization in which the fatty acids are converted in to their methyl esters, reducing their polarity and lowering the boiling point of the target analytes. The methyl esterification can be carried out through sodium methoxide, diazomethane or BF₃-methanol solution.

In the present study, the fatty acid derivatization was performed through methylation solution before the GC-FID analysis on the analytical conditions (Table.3.6). The Fatty acid methyl esters (FAMEs) was recognized by similarity in their mass spectra to the mass spectra in the NIST mass spectral database. The mass spectral similarity was done by using the GC-FID solution software. The similarity search function enables a researcher to compare an unidentified mass spectrum with a huge number (more than one hundred thousand) of reference mass spectra in less than a
minute. Composition of fatty acid present in the oil was profiled through the area% of the FAMEs peaks. Furthermore, the individual peak was identified by comparison of their retention time with chromatogram of standard fatty acid.

Table 3. 6 Analytical conditions for Fatty acids analysis using GC-FID.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>6890 GC-FID Agilent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Polyethylene glycol HP- 88, 100m x 250 μ m x 0.20 μ m</td>
</tr>
<tr>
<td>Injection method</td>
<td>Split (ratio 1 μ l)</td>
</tr>
<tr>
<td>Injection temperature</td>
<td>275 °C</td>
</tr>
<tr>
<td>Column temperature</td>
<td>120°C(0min)180°C(10min)@5°C/min—200°C (4min)@5°C/min</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Helium, 99.9995% purity</td>
</tr>
</tbody>
</table>
IV. RESULTS

This chapter deals with the results obtained for different parameters during the course of experiments “Development of Genetically Engineered Mustard (Brassica juncea) with EaDaT gene for low viscosity biodiesel production” conducted at the Institute of Biotechnology and Genetic Engineering, the University of Agriculture Peshawar-Pakistan and Department of Biochemistry and Molecular Biophysics Kansas State University, USA during the year 2013-14.

1. REGENERATION OF B. JUNCEA.

1.1. Callogenesis

The supplementation of MS media with plant growth regulators had a significant effect on the calli induction of B. juncea (Fig 4.1). Maximum (87%) callus induction was noted when 4.0 µM BAP was used. Further increasing the concentration of BAP resulted in decrease in callus induction. Supplementation of BAP either with GA₃ or NAA resulted in an inhibition of callus induction. Consequently, maximum callus induction with GA₃ and NAA (58% and 68%) was noted at 2.0 µM BAP, respectively. However, no proliferation of explants was noted in MS medium without supplementation of PGRs.

![Figure 4.1:](image)

Figure 4.1: Application of different PGRs concentrations and their impacts on callus formation frequency in B. juncea. Values are mean of five replicates with ± SE and observations were recorded after 4 weeks of culture. Columns with common alphabets are not significantly different at \( p \leq 0.5 \).

1.2. Organogenesis

1.2.1. Shoot Induction
The calli were subcultured on shoot induction media with variable concentration (2-10 µM) of BAP alone or in combination with constant concentration of GA₃ or NAA (1.0 µM each). Shoot induction was observed after 4 weeks of inoculation (Fig 4.2). Maximum shooting was observed in medium supplemented with 4.0 µM BAP and 1.0 µM NAA (92%), followed by medium supplemented with 4.0 µM BAP alone (88%). Supplementing the medium with BAP and GA₃ negatively affected the shoot induction and consequently maximum shooting (70%) was noted at combination of 2.0 0 µM BAP and 1.00 µM GA₃ concentration.

Figure 4.2: Impacts of BAP, NAA and GA₃ on organogenesis in B. juncea. Values are mean of five replicates with ± SE and observations were recorded after 4 weeks of sub-culture. Columns with common alphabets are not significantly different at p≤0.5.
1.2.1.1. Shoot Number

Similar to shoot induction media supplementation of BAP alone or in combination with GA$_3$ or NAA had a significant effect on the number of shoots per explant (Fig 4.3). Maximum number of shoots were observed with supplementation of 4.0 µM BAP and with 1.0 µM NAA (19 shoots/explant), followed by 2.00 µM BAP and 1.0 0 µM NAA (17 shoots/explant). Maximum shoot number (14 shoots/explant) was observed at 4.0 0 µM of BAP and further increase in BAP concentration resulted in inhibition of shooting. Supplementation of GA$_3$ with BAP (1.00 + 2.0 0 µM) resulted in maximum shooting (8.5 shoot/explant) and further increasing the concentration of BAP negatively affected the shooting. It was observed that addition of BAP with GA$_3$ had inhibitory effect on shooting.

Figure 4.3. Impact of plant growth regulator (BAP GA$_3$ and NAA) on shoot number in B. juncea. Values are mean of five replicates with ± SE and observations were recorded after 4 weeks of sub-culture. Columns with common alphabets are not significantly different at $p \leq 0.5$. 
1.2.1.2. Shoot Length

Supplementation of BAP alone or in combination with GA$_3$ or NAA had significant effect on shoot length (Fig 4.4). Maximum shoot lengths were observed with supplementation of 4.0 µM BAP in combination with 1.0 µM NAA (6.5 cm). Among the different treatments combinations after supplementation of BAP alone maximum shoot length (5.8 cm) was observed at 4.0 µM. Consequently supplementation of BAP with GA$_3$ resulted in maximum shoot length of (4.5 cm) at 2.0 + 1.0 µM concentration, respectively and further increasing the concentration of BAP negatively affected the shoot length. It was also observed that addition of BAP with GA$_3$ had inhibitory effect on shoot length.

![Figure 4.4](image)

**Figure 4.4.** Impacts of different PGRs on mean shoot length in *B. juncea*. Values are mean of five replicates with ± SE and observations were recorded after 4 weeks of sub-culture. Columns with common alphabets are significantly different at $p \leq 0.5$. 
1.2.2. Root Induction

Elongated shoots were transferred to root induction medium with variable concentration of IAA (2-10 µM) alone or in combination with constant concentration of Kin (Table 4.1). After four weeks of inoculation maximum roots were observed in medium supplemented with 6.0 µM IAA and 1.0 µM Kin (82%) followed by medium supplemented with 8.0 µM IAA and 1.0 µM Kin. Among the different treatment combinations after supplementation of IAA alone maximum root induction (58%) was observed at 6.0 µM. Supplementing the medium with IAA and Kin positively affected the root induction.

1.2.2.1. Root Number

Similar to root induction media, supplementation of IAA alone or in combination with Kin had a significant effect on the number of roots (Table 4.1). Maximum number of roots per explant (5±1.93) were observed with supplementation of 6.0 µM IAA and with 1.0 µM Kin followed by a combination of 8.0 µM IAA and 1.0 0 µM Kin (4.8±1.33). Among the different treatment combinations, after supplementation of IAA alone, maximum root number (3.1±0.83) was observed at 6.0 µM and further increase in IAA concentration resulted in inhibition of root numbers. Consequently supplementation of IAA with Kin positively affected the rooting.

1.2.2.2. Root Length

Similar to root induction, supplementation of IAA in combination with Kin showed higher root lengths as compared to IAA alone (Table 4.1). Among the different treatments combinations after supplementation of IAA alone, maximum root length (6.2± 1.23 cm) was observed at 6.0 µM. Overall, the maximum root length was observed with combination of 6.0 µM IAA and 1.0 Kin (8.2±1.23 cm).

<table>
<thead>
<tr>
<th>Treatments (Conc. in µM)</th>
<th>Rooting (%)</th>
<th>No. of roots/shoot</th>
<th>Root-length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 Impact of different concentrations of Indole Acetic Acid (IAA) and Kinetin (Kin) on root number, length and rooting induction in *B. juncea*. 
<table>
<thead>
<tr>
<th>IAA only</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0</td>
<td>4.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>32±2.43</td>
<td>47±2.55</td>
<td>58±1.93</td>
</tr>
<tr>
<td>4.0</td>
<td>1.9±0.43</td>
<td>2.1±0.73</td>
<td>3.1±0.83</td>
</tr>
<tr>
<td>6.0</td>
<td>3.8±0.93</td>
<td>4.2±1.12</td>
<td>6.2±1.23</td>
</tr>
<tr>
<td>8.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IAA + Kin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0 + 1.0</td>
<td>4.0 + 1.0</td>
<td>6.0 + 1.0</td>
</tr>
<tr>
<td>2.0 + 1.0</td>
<td>55±1.93</td>
<td>65±2.13</td>
<td>82±3.43</td>
</tr>
<tr>
<td>4.0 + 1.0</td>
<td>2.7±0.63</td>
<td>3.8±1.93</td>
<td>5±1.93</td>
</tr>
<tr>
<td>6.0 + 1.0</td>
<td>3.1±0.23</td>
<td>5.2±2.13</td>
<td>8.2±1.23</td>
</tr>
</tbody>
</table>

Note: Values are mean of five replicates and observations were recorded after 4 weeks of culture.
1.3. Transplantation of the Plantlets into the Soil

Once the roots were induced, plantlets were moved to the soil containing pots for acclimatization. All the plantlets were transferred to plastic pots containing soil, vermiculite and horticulture at 4:2:1 ratio along with osmo-coat. All the pots were properly protected with transparent covers and placed in acclimatization room at 20°C in 70-90% humidity, followed by gradual increase in temperature to 25°C. The transparent covers were removed for 4 to 6 h/day for two weeks and eventually removed completely in 3rd week. The acclimatization of plantlets by gradual increase in temperature and the use of polythene bags to control humidity significantly improved the survival rate during hardening process (Fig 4.5).

![Image](image_url)

**Figure 4.5.** *In vitro* regeneration in *B. juncea*. (A) Callus formation from cotyledon explants after three weeks of culture (bar=1.5 mm). (B) Shoot organogenesis in terms of mean shoot number (bar=2.5mm). (C) Shoot organogenesis in terms of mean shoot length (bar=1mm). (D, E) Root organogenesis (bar=200µm).

2. PRODUCTION OF TRANSGENIC LINES

2.1. Cloning, Transformation and Confirmation of *EaDAcT* gene in DH5α
We utilized already prepared kanamycin resistant recombinant construct *PB*in*Gly4EaDAcT* that contained *EaDAcT* gene. To achieve multiple copies the recombinant plasmid was transferred to *E.coli* strain DH5α (freshly prepared) and cultured at 37 °C overnight on solid agar medium containing 50 mg/L kanamycin (Fig. 4.6). Individual colonies were grown in 15 ml kanamycin containing LB and pellets were collected by centrifugation at 5000×rpm.

**Figure 4.6.** Transformed *E.coli* culture overnight growth on solid LB medium in the presence of Kanamycin at 37°C.
The extracted pellet was lysed through alkaline lysis method and DNA was isolated for PCR analysis. The *EaDAcT* specific primers were used against isolated DNA to confirm gene of interest. The PCR product was resolved on 2% agarose gel and the expected product of about 1 *Kb* was successfully amplified (Fig. 4.7).

![Image](image_url)

**Figure 4.7.** PCR amplification of *EaDAcT* gene. Confirmation of transformants. Lane 2 is positive control (amplified from DNA construct) and 10, 11 represent negative controls. Lane 1 is 1 kb ladder (Quick-load 1kb DNA ladder, NEB) and lanes 3-9 indicate transformed colonies. The size of construct is 1092bp.

### 2.2. Cloning, Transformation and confirmation of *EaDAcT* gene in *GV3101*

The confirmed gene of interest in *PBinGly4EaDAcT* plasmid was further transferred to *Agrobacterium* strain *GV3101* and subsequently cultured for screening on YEP medium that contained 50 mg/L kanamycin, 50 mg/L gentamycin and 25 mg/L rifampicin at 28°C. The transformed colonies of *Agrobacterium* appeared on YEP medium within 24 hours of culturing (Fig. 4.8).

Positive transformants were again confirmed by colony PCR analysis using *EaDAcT* specific primers. The PCR products electrophoresed on 2% agarose gel with appropriate positive controls is shown in Fig. 4.9.
Figure 4.8. Transformed Agrobacterium colonies on YEP medium containing kanamycin (50 mg/L), Gentamycin (50 mg/L) and Rifampicin (25 mg/L).

Figure 4.9. Confirmation of transformants. Lane C is positive control (amplified from DNA construct) and 15, 16 represent negative controls. Lane M is 1 kb ladder (Quick-load 1kb DNA ladder, NEB) and lanes 1-14 indicate transformed colonies. The size of construct is 1092bp.

3. AGROBACTERIUM MEDIATED TRANSFORMATION OF B. JUNCEA WITH EaDAcT GENE
To obtain valuable results, we used the standard tissue culture method as well as floral dip method for *Agrobacterium* mediated transformation of *B. juncea* with the target gene.

### 3.1. STANDARD TISSUE CULTURE TRANSFORMATION METHOD

#### 3.1.1. Inoculation, Selection and Regeneration

Total 200 cotyledons were infected with *Agrobacterium* suspension, followed by incubation for three days on co-cultivation medium and transferred to selection medium. We used two different mediums designated as selection medium I and II (SM I and SM II), supplemented with different basta concentrations of 20 mg/L and 25 mg/L, respectively (Tab. 4.2). After 10-15 days of inoculation on SM I, creamish-green callus formation at the cut end and shoot induction was observed with growth efficiency of 50%. Non-transgenic explants became yellow and started dying. After three weeks the plants were transferred to SM II that contained high basta level giving a growth efficiency of 25%. Multiple shoot regeneration was observed from the callus on SM II. It is noteworthy, that all the white color escaped roots were removed to make the transformation more effective.

Next single transgenic shoot was isolated and transferred to rooting medium consisted of PGRs IAA (6.0µM) and Kin (1.0 µM) with 25 mg/L of basta concentration. We observed small creamy callus on the cut end of the shoot, followed by root formation. After 5-6 weeks well firmed roots were formed (Figs. 4.10AB, 4.11AB and 4.12AB).

<table>
<thead>
<tr>
<th>Explants</th>
<th>PCM</th>
<th>SM-I</th>
<th>SM-II</th>
<th>PRM</th>
<th>TE</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>200</td>
<td>50</td>
<td>25</td>
<td>14</td>
<td>07</td>
</tr>
</tbody>
</table>

(PMC, Plant Cultivation Medium; SM, Selection Medium; PRM, Plant on Rooting Medium; TE, Transgenic Efficiency)
Figure 4.10. Tissue culture transformation (A) Plants growth on ½ MS medium (B) co-cultivation medium

Figure 4.11. (A) Plantlets on selection media having BAP 2mg/l and NAA .2mg/l with BASTA 20mg/l and Timintin (B) Plantlets without infection on selection media (Control)

Figure 4.12. Growth parameters of tissue culture transformed plants. (A) Plants on second selection media (only Basta level increased to 25mg/L) (B) Plants on rooting medium.

3.1.2. Transplantation of the Plantlets into the Soil
Root induced plantlets were shifted to soil containing covered plastic pots provided soil vermiculite, horticulture (4:2:1) with osmo-coat and kept at 20 ± 1°C in acclimatization room with 70-90% level of humidity. The gradual alteration in temperature was brought up to 25°C with the removal therapy of transparent covers for 4 to 6 hours for 2 weeks. After 3 weeks’ time all the covers were completely removed to assist proper hardening. About 50% plants were successfully acclimatized in the soil (Fig 4.13).

![Figure 4.13. Plants grown in different conditions. (A) Plant grown in covered pot. (B) Plant grown in open pot (C) plant grown in open field.](image)

3.1.3. Molecular Analysis of DNA Extracted from Transgenic Plants

Genomic DNA was extracted from transgenic plants and subjected to PCR with appropriate controls to confirm the presence of EaDAcT gene (Fig. 4.14). Wild type genomic DNA was used a negative control. Positive transformants of the Brassica plants were confirmed by the successful amplification against the positive control and the final transformation efficiency was found as 7%.

![Figure 4.14. The PCR confirmation analysis of the putative transgenic B. juncea plants. Lane PC is positive control (amplified from DNA construct) and NC represent negative controls. Lane M is 1 kb ladder (Quick-](image)
load 1kb DNA ladder, NEB) and lanes 1-6 indicate transgenic plants. The size of construct is 1092bp.

3.2. FLORAL DIP METHOD OF TRANSFORMATION

Small and large pots were used to germinate *B. juncea* in a screen house for vacuum infiltration and simple dip method respectively.

3.2.1. Vacuum infiltration transformation

The florescence of 40-50 days old germinated plants were immersed in a suspension of *Agrobacterium* GV3101 harboring the binary vector *pBinGlyBar4-EaDaT*.

To perform infiltration transformation the inflorescences were immersed into the inoculum of 1 L and vacuum was applied for 5 min at a pressure of 85 kPa. The treated plants were recovered and subjected overnight to a dark period before returning to normal growth in greenhouse (Fig. 4.15, A).

![Figure 4.15](image)

Figure 4.15. (A) Transformation of *B. juncea* with *EaDaT* gene using floral dip (A) with desiccator. (B) without desiccator.

3.2.2. Basta Screening of Wild and Transgenic Plants

Wild plants were screened with three different concentrations. 0.1%, 0.2% and 0.3% of basta herbicide to optimize the condition for basta selection. All the concentrations were found effective against wild plants in different durations. However, we selected 0.2% basta concentration for our screening analysis (Fig. 4.16).

Transgenic *B. juncea* plants were also screened with 0.2% basta herbicide outside the growth chamber after five days of germination and repeated five times at
the interval of two days. The transgenic plants were able to resist basta and continued to grow while wild plants became white, remained small and died after 15 days of selection. A total number of 40 plants were found alive after basta spraying (Fig. 4.17).

**Figure 4.16.** The image illustrates the effects of 0.1, 0.2 and 0.3% of basta spray on wild *B. juncea* plants.
Figure 4.17. Floral dip transformation. (A) Plants grown in green house for floral dip transformation. (B) T₁ plants grown from the seeds obtained. (C) Application of basta spray on the grown T₁ plants. (D) Showing the mature T₁ plants confirmed through PCR analysis.
3.2.3. PCR Confirmation of Basta Resistant Plants

Genomic DNA was extracted from basta resistant plants and used as template in PCR analysis. Total of 20 basta resistant plants were confirmed through PCR analysis for the gene introduced (Fig. 4.18).

Figure 4.18. (A) Figure: Representing the PCR confirmation of the plants that were transformed using floral dip method of *Agrobacterium* mediated transformation without vacuum infiltration. Lane C1 and C2 is positive control (amplified from DNA construct) and NC represent negative controls. Lane M is 1 kb ladder (Quick-load 1kb DNA ladder, NEB) and lanes 1-13 indicate transgenic plants. The size of construct is 1092bp (B) The PCR confirmation of basta resistant plants. Plants were transformed using vacuum infiltration method of *Agrobacterium* mediated transformation. Lane C1 and C2 is positive control (amplified from DNA construct) and NC represent negative controls and Lane 13 represent wild plant. Lane M is 1 kb
ladder (Quick-load 1kb DNA ladder, NEB) and lanes 1-12 indicate transgenic plants. The size of construct is 1092bp

3.2.4. Transformation Efficiency

The transformation efficiencies of simple floral dip and vacuum infiltration were calculated. In our experiments the vacuum infiltration was found more efficient and produced more positive transformants (43%) as compared to normal floral dip that produced 12% (Table 4.3).

**Table 4.3** Showing the summary of the *In planta* transformation using *Agrobacterium* strain GV3101 harboring the binary vector *pBinGlyBar4- EaDaCt*.

<table>
<thead>
<tr>
<th>Method</th>
<th>No of plants infected</th>
<th>No of Seeds recovered</th>
<th>Germination frequency</th>
<th>No of plants resistant to basta</th>
<th>No of PCR confirmed plants</th>
<th>Transformation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floral Dip</td>
<td>30</td>
<td>~2000</td>
<td>99%</td>
<td>25</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Vacuum infiltration</td>
<td>30</td>
<td>~2000</td>
<td>99%</td>
<td>40</td>
<td>17</td>
<td>43</td>
</tr>
</tbody>
</table>

3.2.5. Progeny Analysis of Transgenic Plants of *Brassica juncea*

The basta resistant plants were able to germinate and produced enough seeds for further analysis and displayed a normal morphology compared to wild type plants i.e., plant height and leaf size. In T₂ generation transgenic line was obtained and further analyzed for fatty acid composition (Table 4.4).

4. NEUTRAL LIPIDS ANALYSIS AND FATTY ACID PROFILE OF THE TAGS FROM WILD TYPE AND TRANSGENIC PLANTS.

Total lipids were isolated from transgenic and wild type plants of *B. juncea*. The fatty acid profile of developing seeds of *B. juncea* was determined for further analysis
to develop a strategy for the production of acTAGs in the *B. juncea* plants. Significant variation was observed in fatty acid composition of TAGs between wild type and transgenic lines (Table 4.4). The mono-unsaturated fatty acids contents of the transgenic line increased to 68.80 mol % compared to 53.7 mol % in wild plants where, as poly-unsaturated fatty acids concentration decreased to 19.50% in the transgenic line, as compared to 22.5% in wild plants. However, saturated fatty acids showed non-significant reduction in transgenic line. Similarly, transgenic plants showed significant increase in oleic acid concentration to (63.02%) compared to 39.21% in its wild type line (Table 4.5). Concentration level of gondoic and erucic acids in TAGs transgenic plants was significantly lowered to 8.22% and 11.18%, from respectively from 10.1% and 13.71% as (Table 4.5). Total of twelve fatty acids were detected in the TAGs of wild plants among which, Pallinic acid and Brassic acid were found absent in transgenic plants.

![TLC analysis of fatty acid extracted from both wild and transgenic plants. Lanes 1, 14, 17, 18, 26 indicate transgenic lines and Ea indicates *Eunymous alatus*, as a positive control.](image)

**Figure 4.19.** TLC analysis of fatty acid extracted from both wild and transgenic plants. Lanes 1, 14, 17, 18, 26 indicate transgenic lines and Ea indicates *Eunymous alatus*, as a positive control.

**Table 4.4.** Fatty acid compositions of *B. juncea* seed oils in wild cultivar and T2 transgenic in % Molar.
<table>
<thead>
<tr>
<th>Brassica Juncea</th>
<th>Saturated Fatty acid in Molar%</th>
<th>Mono unsaturated fatty acid molar%</th>
<th>Poly unsaturated fatty acid Molar%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.1</td>
<td>53.7</td>
<td>22.5</td>
</tr>
<tr>
<td>Transgenic</td>
<td>9.6</td>
<td>68.8</td>
<td>19.5</td>
</tr>
</tbody>
</table>

Table 4.5. Individual fatty acid compositions of *B. juncea* seed oils in non-transgenic plant and T2 transgenic lines expressing *EaDAcT*.

<table>
<thead>
<tr>
<th>Line No</th>
<th>Fatty acid (% mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:00</td>
</tr>
<tr>
<td>Wild type</td>
<td>5.59</td>
</tr>
<tr>
<td>Transgenic</td>
<td>6.13</td>
</tr>
</tbody>
</table>

Nomenclature of Fatty acid. Palmitic acid (16:0), Stearic acid (18:0), Arachidic acid (20:0), Behenic acid (22:0), Oleic acid (18:1Δ⁹), Vaccenic acid (18:1Δ¹¹), Linoleic acid (18:2Δ⁹,12), Linolenic acid (18:3Δ⁹,12,15), Gondoic acid (20:1Δ¹¹), Paullinic acid (20:1Δ¹³), Brassic acid (20:02), Erucic acid (22:01)

4.1. TLC Separation of Natural Lipids of *B. juncea* of developing seed of wild plants

Lipids were isolated from the seeds of *B. juncea* at the interval of 10, 20, 30, 40, and 50 days of flowering and mature seeds. The lipid fraction with standard (17:0 with 10mg ml⁻¹) of TAG was run on TLC plate utilizing hexane:ether:acetic acid (70:30:1) solvent system. TLC plate was dried and stained with di-chloro-fluorescien (0.1% (w/v) in 85% methanol. The TLC profile indicated relatively different quantities of triacyl glyceride at different developmental stages and maturity (Fig. 4.20).
**Figure 4.20.** TLC profile of the triacylglycerides isolated from the seed at different days after flowering. The spot size represents the relative quantity of triacylglycerides.
5. LIPID ANALYSIS OF THE DEVELOPING SEED OF Wild *B. JUNCEA*

5.1. Total lipid concentration of *B. juncea* during seed development

Total lipid extraction in developing *B. Juncea* seed at different intervals showed a significant difference in the TAG concentration. Increase in TAG accumulation was observed with the passage of time after flowering that tend to decrease with maturity. Slow accumulation of lipid up to 20 DAF was observed. Increased concentration of TAG was recorded (0.341911 μmol/mg) at 50 days after flowering whereas minimum (0.003554 μmol/mg) was detected at 10 DAF (Fig. 4.21).

![Graph showing total lipid concentration over days after flowering](image)

**Figure 4.21.** Oil content of developing *Brassica juncea* seeds.
5.2. Individual Fatty acid Compositions of *B. juncea* Seed Oil at Different Days after Flowering

5.2.1. Palmitic acid (16:0)

Palmitic acid concentration was high in the early stages of seed development followed by a gradual decrease with the passage of time. The higher concentration of palmitic acid (10.77%) was found in the *B. juncea* seeds at 20<sup>th</sup> day after flowering while minimum content of palmitic acid (4.01%) was noted at 40<sup>th</sup> day (Fig. 4.22).

![Figure 4.22](image-url) Palmitic acid (16:0) concentration estimated at 10 day intervals in the developing seed of *B. juncea*.

5.2.2. Stearic acid (18:0)

Stearic acids are found in the pool of free fatty acids of the lipid in wild *B. juncea* seeds at different days after flowering (DAF). Maximum concentration (5.04%) of stearic acid was found at 20 DAF that decreased with the passage of time. The minimum concentration of steric acid (1.68%) was observed in 40 DAF in the seed of *B. juncea* (Fig. 4.23).
5.2.3. Arachidic acid (20:0)

Significant concentration variability was recorded for arachidic acids with a maximum of 1.99% and 1.36% in the seeds early at 20 and 30 DAF, respectively that decreased till maturity up to 0.88% (Fig. 4.24).

5.2.4. Behenic acid (22:0)

Behenic acid was found in lowest amount in the free fatty acid poll of *B. juncea*. The maximum concentration (1.5%) of behenic acid was observed in the seeds at 20 DAF whereas it decreased to 0.32% at 50 DAF (Fig. 4.25).
5.2.5. **Oleic acid (18:1Δ⁹)**

Contrary to other acids, oleic acid concentration was lower at the early stage of seed formation that accounted for 1.78% of free fatty acids at 20 DAF and increased up to 41.6% at 50 DAF. Interestingly, at mature seed its concentration decreased to 26.2% (Fig. 4.26). Previous studies show that it concentration have no effect on the accumulation of acetyl-TAGs. As 70 mol% of seed TAG in field-grown high-oleic Camelina had minor or no effect on oil content, seed weight, harvest index and seed yield (Liu et al., 2015).

**Figure 4.25.** Behenic acid (22:0) concentration estimated at 10 day intervals in the developing seed of *B. juncea*.

**Figure 4.26.** Oleic acid (18:1Δ⁹) concentration estimated at 10 day intervals in the developing seed *B. juncea*. 
5.2.6. Vaccenic acid (18:1Δ11)

The vaccenic acid followed an alternating pattern of concentration management during the seed development. Vaccenic acid was 0% at 10 DAF and then reached to its maximum value of 4.09% at 20 DAF, followed by gradual decrease to 2.33% at maturity stage (Fig. 4.27).

![Figure 4.27. Vaccenic acid (18:1Δ11) concentration estimated at 10 day intervals in the developing seed of B. juncea.](image)

5.2.7. Linoleic acid (18:2Δ9,12)

Significant differences were found in the amount of linoleic acid at different DAF. At 20 DAF, the content of linoleic acid was maximum (25.97%) that decreased to 12.52% at 30 DAF that remain constant at maturity with a little increase at 50 DAF to 18.14% (Fig. 4.28).
Linoleic acid (18:2Δ^9,12)

Estimates were made at 10 day intervals in the developing seed of *B. juncea*. No linolenic acid contents were found at 10 DAF, however, its concentration was found to be 7.09% of the total free fatty acids at 20 DAF. At 30, 40 and 50 DAF the concentration remained almost constant while at mature stage, it reached to its highest value (8.62%).

Gondoic acid (20:1Δ^11)

From free fatty acid pools of *B. juncea* variable concentrations of gondoic acids were observed at different DAF. Maximum content (22.49%) was noted at 10 DAF.
however, a conspicuous decrease was noted in later stages. The minimum concentration (12.7%) was noted at 40 DAF (Fig. 4.30).

**Figure 4.30.** Gondoic acid (20:1Δ₁₁) concentration estimated at 10 day intervals in the developing seed of *B. juncea.*

5.2.10. Paullinic acid (20:1Δ₁₃)

The maximum concentration of paullinic acid 3.3% was observed at 20 DAF that started decreasing at 30, 40 and 50 DAF, respectively. The minimum concentration (0.57%) was observed in 40 DAF in the seed of *B. juncea* (Fig. 4.31).

**Figure 4.31.** Paullinic acid (20:1Δ₁₃) concentration estimated at 10 day intervals in the developing seed of *B. juncea.*

5.2.11. Brassic acid (20:0₂)
Various contents of brassic acid were noted at different DAF. Figure 4.32 revealed that brassic acid is one of the minor fatty acid found in the seeds of *B. juncea*. Maximum content was noted 3.3% at 10 DAF however, a conspicuous decrease up to 0.31% was found at 50 DAF.

![Brassic acid concentration over time](image)

**Figure 4.32.** Brassic acid (20:02) concentration estimated at 10 day intervals in the developing seed of *B. juncea*.

**5.2.12. Erucic acid (22:1Δ⁹)**

Different concentrations of erucic acid in the free fatty acid pool of *B. juncea* were observed at different levels of seed development. A high erucic acid content was observed in the seed at 10 DAF that tend to decrease with maturity. The Maximum erucic acid (22.49%) was recorded at 10 DAF in *B. juncea* seed which dropped to a minimum of 14.66% at mature seed stage (Fig. 4.33).
In the list of oil producing crops, *Brassica* holds a vital place and oil extracted from *Brassica juncea* is an alternative option for bio-diesel (Zhou, 2001). As an

V. DISCUSSION

...
alternative source of biodiesel, researchers from different groups have shown keen interest to investigate related problems of high viscosity, poor cold temperature properties and low oxidative stability and volatility in the oil of *B. juncea* (Moser et al., 2015). An acetyltransferase of *Euonymus alatus EaDAcT* acetylates di-acyl-glycerol (DAG) to produce (acTAG) that reduces the viscosity (Durrett et al., 2010). Transgenic plants that harbor *EaDAcT* gene under a seed specific promoter have yielded up to 45% of acTAGs in the seed of wild type *Arabidopsis* (Durrett et al., 2010). Inspired from the above results, we proposed and designed this study that acTAG production and the stable integration of this gene in *B. juncea* may reduce its oil viscosity that would be used as a potential biodiesel crop. To investigate our hypothesis, both the tissue culture and *in planta* transformation methods were applied on *B. jancea* plants.

For tissue culture transformation, the growth of explants (cotyledons) was optimized with different concentrations of three plant growth regulators (6-Benzylationpurine, 1-Naphthaleneacetic acid and Gibberellin A3) for callus and shoot induction. Two plant growth regulators (Indole-3-acetic acid and Kinetin) were used at different concentrations and combinations to optimize the root growth. Varying frequencies of callus induction were observed with different concentrations of Plant Growth Regulator. Explants initiated callus formation with brown mass was observed that cover the upper surface and showed highest growth and regeneration frequency of 87% with BAP (4 µM) (Fig. 4.1). In our study BAP alone gave the highest response of 87% in callus induction which is consistent with previous results (Shahzad et al., 2006; Parveen and Shahzad, 2011) where formation of high organogenic calli from cotyledonary explants of *Acacia sinuate* has reported with 4.0 and 1.0 µM BAP, respectively. These results suggest that BAP alone is able to effectively induce callogenetic response in cultured explants. It has also been reported that BAP alone harbors an explant-dependent significant role in the regeneration frequency, developmental phase of leaves, age of plantlet and type of cultivar (Sanikhani et al., 2006). Explants from different species were found to respond differently to BAP with different impact on cell division pathway at different hormonal levels (Steward and Caplin, 1951).

Previously different groups have used combination of PGRs to achieve maximum callogenetic frequency. Callogenetic frequency of 75.3% was achieved with 1.0 mg/L of NAA and 1.0 mg/L GA3 from cotyledonary explants of *Sinapisalba* (Jain et
Enhanced callus induction in *B. napus* by using 2,4-D in combination with GA$_3$ and AgNO$_3$ was also observed (Ali et al., 2007). In the current experiment we observed 60% callus induction frequency using the combination of BAP and NAA in concentrations of 4 and 1.0 µM, respectively. A slightly lower frequency (55%) was observed using BAP (6 µM) in combination with GA$_3$ (1.0) (Fig. 4.1).

For plantlets regeneration, shoot culture system is considered more attractive than the cell suspension or callus cultures (Khan et al., 2014). We found that the efficiency of BAP in shoot proliferation is better than other PGRs employed for *in vitro* regeneration and were found to be in accordance with the results of Canli and Tian (2008). This study reports 88% shooting response with BAP (4 µM), 92% with BAP + NAA (4 µM + 1.0 µM) and 68% with BAP + GA$_3$ (2 µM + 1.0 µM) (Fig. 4.2). Previously, similar results were obtained by using mature cotyledons of soybean that achieved 68% shooting frequency on BAP (4.54 µM) while 50% response on GA$_3$ (13.3µM) (Franklin et al., 2004; Abbasi et al., 2011). The role of BAP inducing morphogenesis is associated with endogenous metabolism of growth regulators i.e. cytokinins, auxins, GA$_3$ and ethylene (Murthy et al., 1998). It is suggested that BAP molecules enhance the susceptibility of plant tissues for exogenous and endogenous factors which are involved in differentiation and de-differentiation of the plant cells *in vitro* (Khan et al., 2015). Bano et al. (2010) has proposed that BAP in combination with NAA could be a better choice for shoot proliferation. Besides, in different *Brassica* spp., NAA has also been indicated as an effective PGR for rooting (Teo et al., 1997; Khan et al., 2009; Cogbill et al., 2010). The above published results are in harmony with our results that indicate that shooting frequency is significantly enhanced by different PGRs alone or in combined concentrations (Fig. 4.2). Contrary, to the impact of PGRs concentration, our results further suggest that shooting number per explant is greatly affected by PGRs combination. We observed high shoot number per explant with BAP (2 µM and 4 µM) in combination with 1.0 µM GA$_3$ (Fig. 4.3). Interestingly, mean shoot length was found high in response to BAP alone and in combination with NAA (Fig. 4.4). Moreover, we found that rooting can be induced by IAA alone and in combination with Kin, however, the combination of IAA and Kin showed enhanced rooting in comparison to IAA alone. We further found that root length and number of roots per shoot are greatly affected by IAA and Kin combination. But beyond the threshold level of IAA (i.e.6.0 µM) with constant Kin concentrations increased
inhibitory effects on root length and roots number per shoot and (Table 4.1) as reported earlier by (Shabbir et al., 2012)

Number of protocols for tissue culture transformation and in vitro regeneration for Brassica species has been reported varying in regeneration responses from different explants (Sanjida et al., 2011). In this study, 05 days old cotyledonary plants were co-cultivated with Agrobacterium tumefaciens on medium containing BAP and NAA at 25°C for 72 h (8 h dark/16 h light). Initially, 25 mg/L was found minimum basta concentration lethal to the non-transgenic explants. Shoot were induced in transgenic calli when cultured in SIM II medium containing 25mg.L\(^{-1}\) Basta, whereas the non-transgenic calli could not survive. Khan et al (2003) reported that explant co-cultivated for more than 72 h could cause necrosis due to excessive growth of bacteria. Further, swelling and shoot induction was observed on explants cultured on selection medium containing Basta 20 mg/L, Timintin 25 mg/L and Kan 50 mg/mL. It is noteworthy that the callus response was better than the callus derived from used Agrobacterium-mediated B. juncea (Barfield and Pua, 1991; Pental et al., 1993; Dutta et al., 2005; Das et al., 2006).

Different studies have reported vitrification in many Brassica species that is a variety-dependent process and can potentially cause problems in tissue culture transformation due to reduced acclimatization ability in greenhouse (De Block et al., 1989; Radke et al., 1998). Vitrification can potentially be reduced by adding 0.5% agar in the rooting medium. As the meristematic cells in the hypocotyl explants are resistant to Agrobacterium infection (Block et al., 1989), we used young cotyledons (4-5 days old) without meristems that gave excellent response to transformation and regeneration. We used external selection marker (basta resistance) i.e. bar gene which expresses PAT (protein acetylate transferase) enzyme and activates herbicide phosphinotricin (glufosinate) a glutamate analog that inhibits glutamine synthetase and assists the accumulation of poisonous Ammonia in plants (Murakam et al., 1986).

The floral dip method of Agrobacterium mediated transformation has been successfully used in Arabidopsis and B. napus to avoid regeneration and tissue culture of target plants (Bechtold et al., 1993; Wang et al., 2003). This method is cheap, fast and effective whereas in classical tissue culture method the explants are sensitive to Agrobacterium infection and shoot regeneration is highly genotype dependent that takes about 7–8 months for the production of transgenic B. juncea seeds (Seol et al.,
The transformation frequency of floral dip method in the harvested seeds mainly varied from $1 \times 10^{-4}$ to $3 \times 10^{-4}$ over several years (Rani et al., 2013). The transformation frequency of both normal floral dip ($1.5 \times 10^{-3} = 0.15\%$) and vacuum infiltration method ($8.5 \times 10^{-3} = 0.85\%$) was found higher than previous reported studies on *Brassica rapa* ($1 \times 10^{-4}$ to $3 \times 10^{-4}$) in the harvested seeds (Rani et al., 2013). Overall, vacuum infiltration was found more efficient than simple floral dip method which is similar to the findings of (Lin et al., 2009; Clough and Bent, 1998).

The plants selection marker basta remains active in desiccated mature seeds and hence the putative T₁ transgenic seedling can easily be screened. Basta selection allows us to screen young transgenic plants conveniently out of a huge number of untransformed plants and quickly assesses the effect of transgene expression on fatty acid accumulation. The optimized floral dip transformation method of our study can facilitate transformation of *B. juncea* for functional genomics and crop improvement.

Greater interest has been shown for plants harboring compounds of different industrial applications e.g., TGAs, that has an immense global demand being a potential alternative to fossil fuel (Napier, 2007; Cahoon et al., 2007; Durrett et al., 2008; Jaworski et al., 2003). Seed of oil producing plants are promising basis of renewable resource of fuel and energy and vital efforts have been made to improve the industrial value of such seeds by introducing uncommon fatty acids with augmented functionality. A number of oil producing plants has been engineered for the ability to synthesize unusual fatty acids (Badami and Patil, 1981; Jaworski and Cahoon, 2003). No acTAG accumulation in the transgenic plants occurred in the current study (Figure 4.19). It has been demonstrated that certain fatty acids can influence the distribution of other fatty acid in the TAGs synthesis (Ohlrogge J., and John B. 1995). By knocking down the expression of two protein i.e. DAGT1 and PDAT using in situ RNAi by stimulating the expression EaDAcT we may be able to divert the DAG pools to acTAG production (Jose Aznar-Moreno and Timothy P. Durrett, 2015).

New constructs with DGAT1 and PDAT RNAi silencers may result in accumulation of acTAG. Coexpression of *EaDAcT* together with RNAi suppression of DGAT1 increased acetyl-TAG levels to up to 85 mol% in field-grown transgenic Camelina (Liu et al., 2015). Also perious studied of (Sunil Bansal and Timothy P. Durrett, 2015) shows that DGAT1 Knockdown alone produced maximum number of acTAG in transgenic Camelina. RNA and protein expression of *EaDAcT* could not be
confirmed because of lack of facilities. Such studies, however, are set as future objectives.

Contrary to acTAG production, we observed structurally modified TAGs, suggesting the interdependency interplay of DGAT1, PDAT1 and *EaDAcT* in production of TAG and acTAG. In future, structural studies of these enzymes in complex will shed light on its detail interaction mechanism with each other.

We quantitatively analyzed twelve different fatty acids in the pool of transgenic and wild plants and further compared for major alterations between the two genetically different plants. Overall, we did not observe obvious changes in saturated fatty acids e.g., stearic acid, benic acid and arachidonic acid. On the other hand paullinic and brassic acids (unsaturated) completely disappeared while oleic acid increased tremendously in the transgenic lines. Besides, gondoic and erucic acids (unsaturated) showed significant and obvious reduction in transgenic plants (Table. 4.5). This study suggests that all such changes are due to the newly modified functions of DGAT1, PDAT1 and *EaDAcT*. Previously, transgenic expression of individual gene(s) involved in denovo fatty acid synthesis has not stimulated fatty acid production (Dehesh et al., 2001; Thellen et al., 2002). Ideally biodiesel production needs increased mono-unsaturated fatty acids such as oleic and palmitoleic acids, that effectively reduces the presence of polyunsaturated acids and control the contents of saturated acids, thus increasing oxidative stability and decrease viscosity (Drexler et al., 2003; Moser and Vaughn. 2012). Genetically modified crop plants can synthesize and accumulate unusual fatty acids efficiently (Pinzi et al., 2009). Different groups have proposed strategies to overcome evolved metabolic mechanisms in seed that naturally accumulate large amounts of unusual fatty acids in transgenic plants (Broun and Som-erville 1997; Smith et al., 2003). Moreover, basic understanding of the seed lipid metabolism and how it leads to the production of novel fatty acids is required (Jaworski and Cahoon, 2003). Techniques like EST sequencing can provide quick progress towards the understanding of unusual fatty acids pathways (Durrett et al., 2010).

The primary substrates of triacylglycerol are glycerol-3-phosphate and fatty acyl-coenzyme A, which takes place in seed endoplasmic reticulum. The acylation of glycerol backbone is a stepwise process catalyzed by the three acyltransferases and a phosphohydrolase, namely glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15), lyso-phosphatidic acid acyltransferase (LPAT, EC 2.3.1.51), phosphatidate
phosphohydrolase (PAPase, EC 3.1.3.4) and diacylglycerol acyltransferase (DGAT, EC 2.3.1.20). The sole enzyme which is entirely devoted to TAG synthesis in the customary Kennedy pathway is DGAT which catalyzes the acyl-CoA dependent acylation of sn-1,2-DAG. The chief rate-limiting step, in addition to others, in triacylglycerides synthesis in seed is the availability of fatty acids (Bao and Ohlrogge, 1999). The objective of producing improved vegetable oil can be achieved through modification of oil seeds which require deep knowledge of the enzymology of oil accumulation. High selectivity was shown to different fatty acids by the Acetyltransferases from plants (Bajor and Stymne, 1992; Vogel and Browse, 1996). In a similar fashion, the quality and fatty acid composition of oils was determined by the preferential accumulation of some fatty acids into the TAGs by the DGAT (Cahoon et al., 2007). Therefore, in the present experiment the fatty acids content and composition at different developmental stages were determined to design an appropriate strategy for biodiesel production through genetic transformation.

Mustard oil is very important from nutritional and industrial point of view. The quality of oil depends on the presence and proportion of individual fatty acids. This part of research was focused on quantifying TAGs, various lipid classes and the composition of fatty acid during seed development in B. juncea, which is essential for any oil quality improvement program. There is no literature available on the composition of fatty acid in different lipid classes during seed development. In the current study we observed the variation in TAG concentration and fatty acid profile of both saturated and unsaturated fatty acids during seed development. At the early stage of seed development, we observed low concentration of TAG, reached to maximum concentration at 50 days after flowering, followed by a slight decrease at the mature seed stage. Our results are similar with findings of Rondanini et al. (2003) and Dong et al. (2007) proposing that accumulation of oil starts in the early stages of development of sunflower seeds and increase rapidly after 15-20 days, finally reaching the highest levels at 30-35 days post anthesis, thereafter being to decline. Fatty acid profile of TAG showed maximum concentration of saturated fatty acid at 20 days after flowering while minimum concentration was observed at 40 days after flowering. In saturated fatty acid, palmitic was present in higher proportion at 20 days after flowering, however, declined rapidly with seed age and with minimum concentration at 40 DAF. Stearic acid was found at maximum concentration at 20 days after flowering while minimum content of the same fatty acid was noted at 40 days after flowering. Similarly,
the other saturated fatty acids like behenic acid and arachidic acid attained maximum concentration at 20 days after flowering and decrease with maturity. Higher concentration of saturated fatty acids at 20DAF suggests that there were comparatively higher proportions of membrane components in immature seeds than in mature seed. Similar results were also reported by (Chhoka et al., 2008). Unsaturated fatty acids were present at low concentration. The high concentration of linoleic and linolenic fatty acids were observed at the early stage of seed development because these fatty acids are considered to be component of membrane lipids of chloroplast in seed (Gurr et al., 1972; Gupta et al., 1991). At maturity of seed, the content of these fatty acids decreased due to gradual stimulation of triacylglycerides (Gurr et al., 1972). Erucic acid was found in high concentration early after flowering while minimum concentration was observed at 40 days after flowering. Furthermore, erucic acid showed an inverse relation with each of linoleic and linolenic acidsahuja, and this is because the synthesis of linoleic, linoleic and erucic acids depends on a common metabolite, i.e. oleoyl CoA s (Kumar et al., 1978; Ahuja et al., 1984),
In context of the depleting fossil fuel reservoirs and growing environmental concerns, finding environmental friendly alternative fuel options is a gaining attention of researchers across the globe. The bottleneck of trans-esterification in making *Brassica juncea* as a preferred biofuel choice can be overcome by using the transformation procedures optimized in this study. If properly employed, findings of the current study have the potential to manipulate the natural oil production pathways for producing ready to use environmental friendly biodiesel.
VI. SUMMARY

Brassica oil is potentially an important source of biodiesel production in Pakistan. *B. juncea* is the most used species for biodiesel production. *B. juncea* along with *B. carinata* can resist extreme temperatures and requires lower moisture for their cultivation. *B. juncea* belongs to plant family Cruciferae (Brassicaceae) commonly known as the mustard family. This family contains about 350 genera and 3500 species. It is one of the ten most economically important families. Indian mustered is the most vital specie in genus *Brassica*. Moreover, it is the major oil producing crop plant throughout the world. In Pakistan, *B. juncea* is largely grown as an oil seed crops on arid and semi-arid region. *B. juncea* has a higher yield potential under extreme conditions, therefore, this species was used during the current research. Furthermore, there are large arable lands with good climatic conditions for large scale production of *Brassica* for biodiesel, thus the promotion of *Brassica* as a biodiesel crop will not hamper the food supply chain as well. However, the biodiesel produced from this oil has the disadvantage of being highly viscous. The amount and type of fatty acids in the biodiesel determines its viscosity, one of the most important characteristics of biodiesel. Due to the presence of high amount of long chain fatty acids, they cannot be used directly in diesel engines and must first be transestereified to reduce its viscosity (Kulkarni and Dalai, 2006; Barnwal and Sharma, 2005). But transesterification is a laborious, expensive and time consuming process. Lately, a novel approach has been devised to lower the viscosity of triacylglycerols by acetylating the sn-3 position of the long chain of triacylglycerols. The resulting acTAGs rich vegetable oil can directly be used without the need for transesterification. For this purpose, the acetyltransferase gene *EaDACt* isolated from *Euonymus alatus* have been introduced to different plant species which synthesized high levels of 3-acetyl-1,2-diacyl-sn-glycerols (acTAGs). In this study, acetyltransferase gene *EaDACt* was introduced in *B. juncea* for altering its fatty acid profile by producing acTAGs. We have optimized *in vitro* regeneration and transformation protocols in this study. For optimizing *in vitro* regeneration protocol, the cotyledon explants were cultured on MS media supplement BAP alone or in combination with NAA and GA3. Variation in different parameters i.e. percent of callus induction, days to callus formation, % of shoots induction from callus, mean shoot numbers, mean shoot length (cm), number of explants forming roots and there numbers, roots lengths (cm) was observed. Highest callus formation frequency was obtained on MS medium containing 4.0µM BAP, followed by media supplemented with combination of BAP (4 µM) and NAA (1.0 µM). Shoot organogenesis with
highest frequency of 92% was obtained at BAP+ NAA (4.0 + 1.0 µM) which was followed by 88% of shooting produced on media supplemented with BA (4.0 µM) alone. BAP individually produced feasible shoots per explant at 4µM concentration. However, at the concentration of 4 µMBAP + 1 µM NAA maximum shoots per explant was obtained, followed by 2 µMBAP + 1µM NAA. Highest shoot length (6cm) was recorded at BAP4 µM plus NAA 1.0 µM. For rooting IAA (6.0 µM) in combination with Kin (1.0 µM) was found best. The root induction frequency of 82% with 5 roots/shoot and 8.2 cm average root length was observed in this study.

Tissue culture transformation of *B. juncea* was performed using *Agrobacterium* strain GV3101 harboring binary plasmid vector carrying *EaDAct* under glycine promoter, neomycin phosphotransferase-II gene (nptII) that confer resistance against kanamycin and a herbicide resistant gene (bar gene). Transformation efficiency of 7% was obtained using this method. Other two non-tissue culture techniques of transformations were also used in this study; the normal floral dip and the modified vacuum infiltration method. The transformation frequency of vacuum infiltration was greater than normal floral dip method, and hence we recommend vacuum infiltration for transformation of *B. juncea* for future research programs. Furthermore, floral dip method of transformation is more effective as compared to tissue culture method of transformation because of their easy handling and cost effectiveness. The presence of *EaDAct* gene in plant tissue was confirmed through PCR analysis using specific primer of same gene.

Transgenic and wild plant seeds were assayed for lipid analysis during this study. We did not observe actAg formation in transgenic *B. juncea*. However, the analysis of TAG bands through GC showed fatty acid modification. Difference in fatty acid composition was detected between transgenic and wild seeds of *B. juncea*. In this study we observed that the concentration of saturated and polyunsaturated fatty acid in transgenic seed was decreased while mono-saturated fatty acid increased. However, paullinic and brassic acids which was present in wild seed not detected in GC analysis of transgenic seed.

Quality of oil depends on the presence and proportion of individual fatty acids. The knowledge of fatty acid composition of various lipids class during seed development in *B. juncea* is essential for oil quality improvement program. For this purpose, the current study was conducted to observe the variation in TAG concentration and fatty acid profile of saturated and unsaturated fatty acids during seed
development. At the early stage of seed development, we observed low concentration of TAG, reached to maximum concentration at 50 days after flowering followed by a slight decrease in mature seeds. Fatty acid profile of TAG showed maximum concentration of saturated fatty acid at 20 days after flowering while minimum concentration was observed at 40 days after flowering. In saturated fatty acid, palmitic, stearic acid, behenic acid and arachidic acid was present in higher proportion at 20 days after flowering, which declined rapidly with seed age and maturity. In unsaturated fatty acid profile most of them were present at high concentration in early stage of seed development with few exceptions. In unsaturated fatty acid profile, brassic acid, paullinic acid, gondoic acid, linolenic acid, linoleic acid, erucic acid and vaccenic acid were present at high concentration during the early stage of seed development and started decreasing with seed maturity. However, oleic acids were present at low concentration at early stage of seed formation and started increasing with seed age.

VI. CONCLUSION

The present study comes to the conclusion with the results in which, highest callus formation frequency (87%) was observed with 4.0µM BAP, followed by combination of BAP 4 µM + NAA 1.0 µM with 68% frequency. Shoot organogenesis maximum shooting was observed in medium supplemented with 4.0 µM BAP and 1.0 µM NAA (92%), followed by medium supplemented with 4.0 µM BAP alone (88%). However, at the concentration of 4 µM BAP + 1µM NAA maximum shoots per explant (19 shots/explant) were observed, followed by 2µM BAP + 1µM NAA. Highest shoot length (6 cm) was recorded at BAP4 µM plus NAA 1.0 µM. For rooting IAA (6.0µM)
in combination with Kin (1.0 μM) was found best. The root induction frequency was 82%, with an average root length of 8.2 cm and mean number of 5 roots/shoot.

The transformation efficacy was up to 7.0% using tissue culture based transformation method. We have successfully generated *B. juncea* transformants within 3–4 months with a transformation 0.15% by using simple floral dip and 0.85% while using desiccator. It was found that floral dip transformation using desiccator is best for *Agrobacterium* transformation. While there are some problems in tissue culture transformation such as the time consuming *in vitro* regeneration step, labor intensive, genotype dependent response to tissue culture practices and the requirement of highly skilled personnel. Similarly the regenerated plants might exhibit some undesirable somaclonal variation.

Oleic acid substantially increased in the transgenic seeds at the cost of the poly unsaturated acid that is a desirable character for increasing oxidative stability while maintain low-viscosity. For biodiesel the most suitable fatty acid should consist of high percentage of mono unsaturated fatty acid and minimum amount of polyunsaturated fatty acid and saturated fatty acids.
RECOMMENDATION

- High amount of acTAG production in the transgenic plants of *B. juncea* is the upcoming challenge in seed-oil research, which could be a milestone for the energy production.

- High level of research and skills are demanded to overcome the complexity and control of fatty-acid flux between the phosphatidylcholine and the triacylglycerol biosynthetic pathways in the developing seeds.

- Furthermore, manipulation of fatty acid elongation through genetic transformation will also increase the availability of acetyl-CoA for incorporation and production of acTAG.

- The transcription-profiling data from developing *Brassica* seeds obtained in this research could be used to design new *Brassica* specific promoters that can optimize the timing of transgene expression to produce high amounts of unusual fatty acids in *B. juncea* seeds.
REFERENCES


Bao, X and J. Ohlrogge. 1999. Supply of Fatty Acid is One Limiting Factor in the Accumulation of Triacylglycerol in Developing Embryos1, Plant Physiology, 4:1057-1062


Food and Agriculture Organization of the United Nations, FAOSTAT database


Reed, C.F. 1976. Information summaries on 1000 economic plants. Typescripts submitted to the USDA


APPENDICES

Appendix 1: Gas chromatographic analysis of FAMEs prepared from single mature seed of wild *B. juncea* (A) and transformed lines (B)