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A REVIEW ON USE OF BIOCHEMICAL METHOD LIKE ISOZYMES IN PLANT SYSTEMATICS

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ABSTRACT

Plant taxonomy and systematics are two sides of the same coin, which are used to identify and describe organisms. Biochemical methods are of the most important tool on which forms the basis for the development of field like the molecular biology. Isozyme are the different molecular forms of enzymes showing the same substrate specificity. The isozyme finds its application not only in the early colonizing plants viz., algae, fungi, bryophytes, pteridophytes and gymnosperms but also in the highly evolved flowering plants. However the use of Isozymes in plant systematics is highly appreciable. The Isozymes helps in plant systematics by suggesting the circumscription of the taxon namely, genus, species, varieties, etc.

Keywords*: Isozymes, Plant systematics, Biochemical, Angiosperms.*

I. INTRODUCTION

Plant systematics is a science which not only includes traditional taxonomy but also to establish the evolutionary history of plants. The classical difference between plant taxonomy and systematics is that plant taxonomy is involved in the classification and naming of plants whereas systematics is involved in the determination of evolutionary relationships of plants (Naik, 1984). Plant systematics is considered as one of the most active areas of biology because of the progress in molecular phylogenetics during recent times (Savolainen and Chase, 2003). Thus the one of the primary goals of plant systematics is to understand the phylogeny through cladistics, phenetics and phyletics (Haider, 2018). Taxonomy has an essential role to play in conservation efforts since the global biodiversity crisis demands quick and effective measures of conservation. However empirical approach in taxonomy or alpha taxonomy needs to re-invent itself to manage the crisis. The essential step to conserve and safeguard biodiversity is to know individually the species, enumerate their unique combination of characters, analyze their cladistic relationships, describe, name and classify them. Since the advent of plant classification morphological characters have been the backbone of taxonomy (Labhane, 2011). The use of various characters for plant systematics is very well accepted by the APG (2009). Earlier the study of plant systematics was restricted to use of various morphological, anatomical, embryological, palynological, biochemistry, cytology, etc. However now a days due to the development in the field of science and technology the dimensions of study of plant systematics is changing very fast. The use of biochemical methods like Isozymes is now being explored in many details.

Markert and Moller (1959) invented the term Isozyme to describe diverse molecular forms of enzymes showing the same substrate specificity. The discovery and standardization of the protocols in Isozymes led to sudden spurt in the use of Isozymes in various disciplines of biological sciences. The use of biochemical method like Isozymes was not restricted to plant sciences but it also became very popular in animal sciences, microbiology, human biology, veterinary sciences, agricultural and horticultural sciences etc. Even within these broader science streams the utility of Isozymes was exploited in plant conservation, biodiversity, genetics and plant breeding, origin, evolution and plant systematics etc (Labhane & Dongarwar, 2013, Soltis & Soltis, 1989). Thus looking at the enormous and manifold use of Isozymes, the present review will be addressed to its utility in the field of plant systematics.

History of Isozyme

There have been several early evidences for multiple forms of the same enzymes, even before the discovery of isozyme. Mallette and Dawson (1949) working on common mushroom, *Psalliota campestris* isolated five purified tyrosinase enzyme, can be considered as one of the initial studies regarding multiple forms of enzymes. The

controversy continued throughout 1950's over whether the multiple forms of enzymes reported were due to the artifacts produced during preparation or whether the enzymes were distinct forms. Several refinements in the electrophoresis technique over a long period of time led to the conclusive evidence that multiple forms of enzymes did exist. Gillespie et al. (1952) compared the resolution obtained by paper chromatography to paper electrophoresis and found that there were multiple forms of esterase, amylase, glucosidase, sucrase, cellulase, protease and alkaline phosphatase in *Aspergillus oryzae* and in horse-radish. Boroughs (1954) studied phosphatases in higher plants namely sugar beet, tobacco and spinach.The first major contribution towards the discovery of Isozymes can be attributed to the developed the starch gel electrophoresis by Smithies (1955). The second major milestone in the discovery of isozyme was the demonstration that enzymes could be visualized directly on starch gel when stained with a specific histochemical stain by Hunter and Markert (1957).Finally the multiple forms of same enzyme catalyzing same substrate was christened as Isozyme by Markert and Moller (1959).

II. METHODOLOGY

The classical way for studying Isozymes was starch gel electrophoresis, however after the advent of the electrophoresis the starch gel electrophoresis was gradually replaced by the Polyacrylamide gel electrophoresis (PAGE). There are two types of PAGE, namely SDS-PAGE and native-PAGE (Sadasivan, & Manickam, 1996). However for studying Isozymes, the enzymes in active form the native-PAGE is used. The native polyacrylamide gel is made by copolymerizing acrylamide (CH_2= $CHCONH_2$) with an appropriate quantity of cross – linking reagent, N – N methylene–bis acrylamide (CH_2 $(NHCOCH=CH_2)_2$). In this mixture of acrylamide and bis acrylamide, free radical catalyst and initiator is added to form a gel whose properties are very effective for the electrophoretic separation of proteins. Specifically, this gel is stable and inert in the presence of an electric field, and exhibits uniform porosity. The resulting gel is also very transparent as compared to starch gel. Catalyst used for formation of gel is Ammonium Persulphate (APS) whereas the TEMED (Tetramethylene diamine) acts as an initiator(Thimmaiah, 1999).

Sample preparation
The samples from the leaves, seeds or any other plant part is used for extraction of the enzymes. Samples are prepared in chilled mortar and pestle using the extraction buffer. The extraction buffer is prepared with 50 mM Tris HCL buffer, pH 7.2 containing 5% sucrose and 14 mM mercaptoethanol. The extraction buffer also varies with the type of Isozymes to be isolated.1 gm of sample is homogenized in extraction buffer and centrifuged at 4^0 at 15000 rpm for 20 minutes. A clear supernatant is collected in a fresh vial for loading. For loading the samples 250 µl of the extract is added to 125 µl of 50% v/v glycerol and 0.5 mg/ml bromophenol blue. 20-30 µl of this is loaded for gel electrophoresis (Weeden & Wendel, 1989).

Gel Preparation
The most accepted concentration of acrylamide was 8% for better resolution, however higher or lower concentration of acrylamide may be used depending upon the requirement. Separating and stalking gels are prepared separately. 1.3 ml acrylamide, 1.3 ml Lower Tris (pH 8.8), 2.3 ml distilled water, 6 µl APS and 8 µl TEMED is used for the preparation of the separating gel. While for the preparation of the stalking gel 1.2 ml of acrylamide, 1.8 ml of Upper Tris, 2.88 ml of distilled water, 48 µl APS and 10 µl of TEMED is mixed together. The amount of separating or stalking gel required depends upon the size of the glass plates used. The comb is inserted into the upper stalking layer before the start of polymerization of the gel.

Electrode Buffer
For preparation of 1000 ml of electrode buffer 3 g Tris with 14.4 g Glycine is added and the pH is adjusted to 8.3. However there are different types of buffers which varies according to the types of Isozymes to be investigated.

Electrophoresis
Isozymes electrophoresis separates native proteins (enzymes) in an electric field because of difference in there mobility. The mobility of the native protein depends on the size, net electric charge as well as shape determine the speed of mobility. Gel was run approximately at 60 V for 3-5 hours or until the tracking dye has migrated to the

bottom of the gel. Once the tracking dye reaches the bottom of the gel, the electrophoresis unit is shut down and the gel is carefully separated from the glass plates and immersed in the gel staining solution.

Staining of Gel
It contains the substrate for the enzymes which are under investigation. The enzymes reacts with the substrate present in the solution to produce a colored band or sometimes achromatic bands. The colored or achromatic bands appear on the gel wherever enzyme reacts with the substrates on the gel. The success of the staining depends mainly on the pH of the staining solution, incubation condition and the reaction mixture. Each band represents an Isozymes, which are recorded for further interpretation.

Post staining treatment and interpretation
The reactions are stopped by immersing the gel in 7.5% acetic acid solution for storing the gel for some days. In some Isozymes the bands tends to fade very quickly, hence it is highly advisable to photograph the gel as soon as the bands appears using gel doc instrument or using simple camera. The banding pattern varies greatly in its complexity, depending on numerous factors including the organisms, tissue and enzymes assayed (Wendel and Weeden, 1989). Many computer aided softwares are available which can also be used for the interpretation of the gel, like Popgene, statastica, NTSYS-PC, etc. The population of taxa showing one band for any enzyme are considered as monomorphic, while those showing more than one band for any enzyme is considered as polymorphism.

Isozymes in relation to plant systematics
Isozymes are frequently used for taxonomic purposes. More importantly, Isozymes becomes very important when a taxon is morphologically varied or flexible. The time when Isozymes were exploited for systematic purpose, this technique, it is commonly used to make recommendation on the combination or separation of a taxon. The taxa can be distinguished by simple banding method, although cladistic and phylogenetic information can be derived from the allelic frequencies and ratio derived from a genetic interpretation of the data (Krishnamurthy et al., 2004). Since there were no review on the aspect of utility of Isozymes in relation to plant systematics in the last 10-20 years, this review is aimed to fill this void for the benefit of all the student researchers.

One of the pioneer review was written by Gottlieb (1977) where he tried to correlate the use of electrophoresis evidences in the plant systematics, which can be considered as the stepping stone for the exploitation of the isozyme technique. Crawford (1979) reported the allozyme variation in two species of *Chenopodium incanum* and *Chenopodium fremontii*. Later Crawford (1989) had written a detailed review on use of enzyme electrophoresis in relation to plant systematics. He enumerated that the major contribution of enzyme electrophoresis in the field of plant systematics that it allows the quantification of genetic similarity and differences amongst population and taxa. He also found the Isozymes very useful to ascertain if the plant is diploid or a polyploidy genetically, since polyploidy will exhibit duplicate gene expression for many enzymes where as a diploid will exhibit minimal conserved number for most enzymes. An extra gene for one or two enzymes can be viewed as the result of gene duplication in a diploid species. Thus any report of new species can be ascertained for being the result of gene duplication through polyploidy or not. Elisens and Crawford (1988) worked on the systematics, genetic variation and differentiation of the genus *Mabrya* belonging to family Scrophulariaceae.

The work of Soltis and Soltis (1989) is very profound can be illustrated by large number of papers published till date. However, the most stupendous was the publication of book “Isozyme in Plant Biology”, where both the editors have covered almost all the aspects of plant biology in which Isozymes can be used. Thus it tells the importance of Isozymes in plant biology. Soltis, et al (1995) studied the Genetic variation in *Tragopogon* species with respect to the origins of the allotetraploids *T. mirus* and *T. miscellus* (Compositae), and concluded that the polyploidy may be one of the reason for genetic diversity in the population. Purdy and Bayer (1995) stuided the Genetic diversity in the endemic *Deschampsia mackenzieana* and its widespread diploid progenitor *D. cespitosa* (Poaceae), where they reported the low levels of genetic diversity in *D. mackenzieana* suggest a restricted origin with limited gene flow from the progenitor since speciation.

Das & Mukherjee (1997) while working on 12 species of *Ipomoea*which is the largest genus of family Convolvulaceae compared the seedling morphology with the biochemical-Isozymes data, and found that both the data generated from the seedling morphology and Isozymes were more or less the same while preparing the dendrogram amongst the 12 *Ipomoea* species. Apavatjrut et al., (1999) used isozymes as a molecular marker in the identification of some early flowering *Curcuma* L. (Zingiberaceae) species. *Curcuma* species is traditionally identified using morphological characters, which exhibit intra-specific and inter- specific morphological variations, which may be misleading and sometimes confusing. However the Isozymes technique has proved to be fruitful in supporting the taxonomical identification, and also revealing their relationship within the early-flowering groupin *Curcuma* species.

Akashi et al (2002) worked on the *Cucumis melo* L. based on the analysis of five isozymes to study the Genetic variation and phylogenetic relationships in East and South Asian melons. They observed that the gene diversity calculated for the nine loci indicated that Indian melon was rich in genetic variation, which decreased from India towards the east. Mateu-Andres and Segarra-Moragues (2003) studied eight taxa belonging to six species of *Antirrhinum* using Isozymes and found it very useful for the identification and segregation of various species. Krishnamurthy et al., (2004) also defined the role of Isozymes in biodiversity studies which is an important aspect of plant systematics. Britto (2004) also emphasised the use of Isozymes as one of the most important molecular tool in studying various aspect of plant systematics. Rosenbaumová etal (2004)studied the *Lamium* subg. Galeobdolon in Central Europe to evaluate karyological, morphological, and isozyme variations and found that the Isozymes were important in the delimitation of certain species. Jaaska & Leht. (2007) investigated based on the isozymes the Phylogenetic relationships between and within sections Hypechusa, Narbonensis and Peregrinae of genus *Vicia* (Fabaceae), in which Isozyme variation in *V. mollis, V. noeana, V. sericocarpa and V. ciliatula* of section *Hypechusa* is described for the first time. Sarma & Rathi (2009) studied the diversity of rice using the Isozymes and found to be very encouraging.

Labhane (2011) studied the *Justicia- Rungia* complex coupling the embryology and Isozymes. He found that the Isozymes were quite useful in the circumscription of the species of *Rungia repens* into species of *Justicia.* The dendrogram developed using embryological data (Labhane& Dongarwar, 2014) and dendrogram developed using Isozymes both validated the presence of a complex of *Justicia- Rungia.* Bharathi (2017) using isozymes tried to give the first report regarding the Identification and characterization of five species of *Memecylon.* Esawi et al (2017) used Isozymes to investigate the genetic variability, population structure, and relationships of *Lactuca* germplasm, isozymes results confirms the hypothesis of the polyphyletic origin of *L. sativa.*Sammour et al (2019) investigated genetic variability and population structure of *Lathyrus sativus L.* germplasm using Isozyme.Cluster analysis based on isozyme data suggested that the environment had no influence on the genetic diversity and confirmed that *Lathyrus sativus L.* had a polyphyletic origin.

III. FUTURE OF ISOZYMES IN RELATION TO STUDY OF PLANT SYSTEMATICS AND CONCLUSION

Crawford (1989) concluded that the increasing popularity of using the DNA based molecular markers will not dimish the value of enzymes electrophoresis. On the contrary both the DNA based molecular approach and Biochemical based isozyme approaches are complementary in many instances. Zeidler, (2000) compared the DNA based molecular markers with that of Isozymes and concluded that Isozymes being more cheaper and large number of samples using the more established statistical software gives it an advantage over the DNA based molecular marker. However he also said that DNA based molecular markers can supplement where Isozymes techniques become limiting. But looking at the popularity of Isozymes since the discovery and its sustenance even after the popularization of DNA based molecular marker, justifies that Isozyme studies will continue to challenge researchers who are mostly interested in post translational modification leading to the formation of the enzymes which represents the final form of gene expression.

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