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STUDIES ON THE FRACTIONS OF PROTEINS DURING SEED DEVELOPMENT OF CALOPHYLLUM APETALUM WILLD., AN ECONOMICALLY IMPORTANT ENDEMIC TREE SPECIES OF WESTERN GHATS.

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ABSTRACT Calophyllum apetalum Willd., an endemic medicinal tree species of Southern Western Ghats belonging to the family Calophyllaceae (Clusiaceae). The seeds are recalcitrant nature and lose their viability within few days after detachment from the mother plant. The mature fruits of C. apetalum were collected from the different locations of Alappuzha district, Kerala. Moisture content was noted from seven consecutive developmental stages. October to January (0, 15, 30, 45, 60, 75, 90 days after anthesis) and in each developmental stage the seeds were subjected to moisture content and other bimolecular analysis. Proteins are vital for seed development, acting as structural components. Fractions of proteins during the development of Calophyllum apetalum seeds depicted in this article.

KEYWORDS :

INTRODUCTION

Calophyllum apetalum Willd., is a species under the genus Calophyllum belonging to the Clusiaceae family. According to the IUCN Red List, C. apetalum is classified as vulnerable. The genus Calophyllum L. (Clusiaceae) includes about 187 species. Of these,179 are present in the Old World (mainly in the Indo-Malaysian region) and about 8 are in the New World, from Mexico and the Caribbean to Argentina (Chinthu et al., 2023). Among the species of genus Calophyllum occurring in Western Ghats, only three species have been evaluated, while other species remain unevaluated and data-deficient (Chinthu et al., 2023).

More than 70% of the proteins consumed by humans are derived from storage proteins of legumes and cereal seeds. Seed storage proteins accumulate in the cotyledon and embryo of dicotyledonous plants and in the endosperm of monocotyledonous plants. These proteins are deposited in specialized membrane – bound organelles called protein bodies. The predominant legume storage proteins are salt soluble globulins and are grouped under two classes, 7S and 11S, while the major storage proteins of cereals are the alcohol soluble prolamins. Exception is oats and rice, in which major storage proteins are globulin like. Because of the abundance of these proteins, they are mainly responsible for the nutritional quality of human diet. The scientific study of cereal grain proteins extends back for over 250 years, with the isolation of wheat gluten first being described in 1745 (Beccari, 1745). Since then, more systematic studies have been carried out, notably by T B Osborne (1924) who can be regarded as the father of plant protein chemistry. Osborne developed a classification of plant proteins based on their solubility in a series of solvents, for example, albumins in water, globulins in dilute saline. Although 'Osborne fractionation' is still widely used, it is more usual today to classify seed proteins into three groups: storage proteins, structural and metabolic proteins and protective proteins. Seed storage proteins fall into three different Osborne fractions and occur in three different tissues of the grain.

The embryo and outer aleurone layer of the endosperm contain globulin storage proteins. These proteins are readily soluble in dilute salt solution. Furthermore, although the aleurone and embryo are rich in proteins compared with the starchy endosperm, the globulins in these tissues have limited impact on the end use properties of the grain. It is now known that these proteins are related to the widely distributed 'legumin' type globulins which occur in most dicotyledonous species (Casey, 1999). The rice proteins are not readily soluble in dilute salt solutions and hence are classically defined as glutelins. Proteins related to legumins, called 'triticins', are present in starchy endosperm of wheat, although they account only for about 5% of the total seed protein (Singh et al., 1988). The high content of globulin storage proteins in oat grain may contribute to the high nutritional value when compared with other cereals, such as barley and wheat, an important factor in view of the widespread use of oats for livestock feed (Lockhard and Hurt, 1986; Cuddeford, 1995). With the exceptions of oats and rice, the major endosperm storage proteins of all cereal grains are prolamins. This name was originally based on the observation that they are generally rich in proline and amide nitrogen derived from glutamine, but it is now known that the combined proportions of these amino acids actually vary from about 30 - 70% of the total among different cereals and protein groups. Similarly, although prolamins were originally defined as soluble in alcohol water mixtures (e.g. 60–70% ethanol, 50–55% (propan-1-ol or propan-2-ol), some occur in alcohol-insoluble polymers. Nevertheless, all individual prolamin polypeptides are alcohol-soluble in the reduced state.

But in the case of recalcitrant seeds, maturation drying is absent and remain desiccation sensitive both during development and after they are shed. Mechanisms of plant drought/desiccation tolerance have been studied by numerous groups, and a broad range of molecules have been identified to play some roles. Examples are proline, oligosaccharide and late embryogenesis abundant (LEA) proteins and so on. However, most recalcitrant seeds accumulated LEA proteins (the current LEA proteins identified from recalcitrant seeds all belonged to LEA II proteins) and responded to ABA, drought, or temperature stimulation. In addition, oligosaccharides, which existed in orthodox seeds, accumulated in some recalcitrant seeds. In summary, LEA proteins and oligosaccharides accumulated in some recalcitrant seeds, and bio glasses might form during dehydration. However, before these protection mechanisms operate, slowly dried recalcitrant seeds already lost viability at water contents far above the essential condition these mechanisms need (Berjak and Pammenter, 2008). Seed physiological studies are a key to conservation and regeneration of endemic species. Seed physiological studies include the development, desiccation and germination in the case of recalcitrant species. Here Calophyllum apetalum seeds are recalcitrant in nature so their developmental studies are important for the conservation of the species. Therefore, the study of proteins involved in development, the total protein content and their fractions will give an idea that what mechanisms happen inside the seed during development.

MATERIALS AND METHODS

The fruit is an indehiscent drupe, ovoid or globose in shape. It contains one seed, with large cotyledons, exalbuminous and rich in oil content. Following pollination and fruit set, fruit initiation occurs. The fruit matures within 2 to 3 months (November to January). In C. apetalum, peak fruiting is observed during January. Sometimes fruiting may be extended until March or April (Chinthu et al., (2025)

Inflorescence

Mature Fruit

Seeds

Collection Of Seeds

Mature fruits of Calophyllum apetalum were collected randomly from Alappuzha district.C. apetalum is the dominant species in some sacred groves as in the case of Aikkarakavu, Kalavoor, Illathukavu and Kanjikkuzhi (Raveendran et al., (2024). The fruits initiation starts during the month of October. But maturity attain during January-February. The seeds are considered as mature, when the seeds attained maximum dry weight during which they start shedding. Collected seeds were scientifically sterilized and used for further studies.

Moisture Content Analysis

Moisture content was determined by the difference between fresh and dry weight. For dry weight determination, the seed material was taken in a pre - weighed bottle and weighed in an electronic balance, dried in a hot air oven at Low Constant Air Oven Method (130^{0}C for 1 hr) until the constant weight was obtained. ISTA (1985).

Extraction Of Total Protein And Estimation Of Fractions Of Protein During Development

Extraction Of Protein Fractions

The fractionation of proteins, based on solubility characteristics, was carried out according to the method of Osborne (1924).

A) Water Extraction

From the randomized samples of the seed,1 gm dry tissue was ground in a motor with pinch of sand and distilled water, the extract was centrifuged at 3000 rpm at room temperature for 15 minutes in a tabletop centrifuge. The supernatant was collected and kept at 4^{0}C. The residue was re-extracted twice with distilled water and the combined supernatant was referred as the 'water soluble' fraction of protein.

B) Sodium Chloride Extraction

The residue left behind after extraction with water was stirred well with a glass rod with addition of 5.0 ml of 5% (w/v) NaCl solution. After stirring well, the slurry was centrifuged at 3000 rpm at room temperature for 15 minutes and the supernatant was collected. The residue was re-extracted twice with 5.0 ml of NaCl and the combined supernatant is served as the source of NaCl soluble fraction of protein

C) Ethanol Extraction

The residue after NaCl fraction was extracted thrice with 80% (v/v) ethanol as detailed earlier and the combined supernatant referred as the 'alcohol soluble' fraction.

D) Sodium Hydroxide Extraction

After extraction of ethanol fraction, the residue was extracted with NaOH. Each time, the residue was extracted three times and the pooled extract obtained on extraction with NaOH was referred as 'alkali soluble' fraction.

All the extracted fractions were pooled and kept in ice cold condition till the entire operations were over. Fractional proteins were determined in suitable aliquots of the extracts.

Estimation Of Protein Fractions

Appropriate aliquots in triplicate of various fractions obtained from the Osborne extraction method were mixed with sufficient quantity of Trichloro Acetic Acid (TCA). Stock (100% w/v) to bring the final TCA concentration to 5% (w/v) in 15 ml centrifuge tubes. After mixing thoroughly they were kept in an ice bath for 30 minutes for the flocculation of proteins. These precipitated proteins were centrifuged for 15 minutes at 3000 rpm and the supernatant was discarded. This precipitate was washed 3 times with 2% (w/v) TCA. The residue was re-suspended in appropriate volume (5-10ml) of 0.2 N NaOH solutions and heated for 5 minutes in a boiling water bath to dissolve proteins. After cooling and centrifugation at 3000 rpm for 5 minutes, the clear supernatant was used for the determination of proteins as per the Lowry et al. (1951) method.

Statistical Analysis

The data from different experiments were analysed following one way Analysis of Variance (ANOVA) and the ratio obtained were checked for significance at 1 and 5 % probability (P) level. From this calculated ANOVA the means of each treatment were separated following the least significance Difference (LSD) by Duncan's multiple range test (DMRT) at 1 and 5% level.

RESULTS

At harvest, the moisture content of fresh Calophyllum apetalum seeds was 74.81%. The moisture content was high at first, but it decreased 15 days after anthesis as development went on. The moisture level progressively rises after 30 days of anthesis. When it gets to for 45 days after anthesis, it starts to decline.

The increased rate of dry matter is the reason for the progressive decrease in moisture content that was noticed after 45 days. Throughout development, the fruit's fresh weight, length and width progressively increase. The syntheses of various storage proteins of a seed initiated at similar but not identical during development and proteins are accumulated at about the same rate.

Protein Fraction During Development

a) Albumin (Water Soluble Fractions)

Water soluble protein (albumin) content of C. apetalum seeds during the development showed a gradual increase from 0th day to 90 days after anthesis.

The initial amount of albumin content was 76.01±0.49 (mg gm^{-1}) dwt whereas on 90th day it became 291.59±0.87 (mg gm^{-1}) dwt. A gradual increase of albumin was observed throughout the development.

b) Globulin (Salt soluble)

Sodium chloride (NaCl) soluble fraction of proteins (Globulin) in C. apetalum seeds were recorded maximum at 90th day. Initially the globulin content was 79.70±0.24 (mg gm^{-1}) dwt. After 90 days of anthesis the globulin fraction was increased as 425.85±0.55 (mg gm^{-1}) dwt.

c) Prolamin (Soluble In Alcohol)

Ethanol fractions of protein (prolamin) in seeds of C. apetalum was initially minimum ie, 81.27±0.71 (mg gm^{-1}) dwt. When the development progresses, the fractions were gradually increases and reaches its maximum of 173.75±0.97 (mg gm^{-1}) dwt.

d) Glutelins (Alkali soluble)

Alkali fractions of proteins in C. apetalum were initially 81.70±1.02 (mg gm^{-1}) dwt.

After 90 days of anthesis it became 428.86±0.21 (mg gm^{-1}) dwt. A gradual increase in the amount of alkali fractions were observed throughout the development.

Total Protein Content During Development

Total protein content initially 460.37±0.01 (mg gm^{-1}) dwt. Then after each developmental stages the total protein content was rises and reaches maximum to 980.64±0.01 (mg gm^{-1}) dwt. after 90 days of anthesis. One of the main categories of storage products found in seeds are proteins.

As a result, a steady rise in both the fractional protein (Fig:1) and total protein (Fig:2) content was noted during the growth of C. apetalum seeds. (Table:1)

Table 1: Total Proteins And Fractions Of Proteins During Development Of C. Apetalum Seeds.

Development (days)	Albumin (mg gm^{-1}) dwt ± SE	Globulin (mg gm^{-1}) dwt ± SE	Prolamin (mg gm^{-1}) dwt ± SE	Glutelin (mg gm^{-1}) dwt ± SE	Total Protein (mg gm^{-1}) dwt ± SE
0	76.01±0.49^{g}	79.70±0.24^{g}	81.27±0.71^{g}	81.70±1.02^{g}	460.37±0.01^{g}
15	82.54±0.45^{f}	83.37±0.65^{f}	83.83±0.15^{f}	196.70±0.32^{f}	517.75±0.02^{f}
30	98.65±0.24^{e}	94.43±0.33^{e}	96.38±0.69^{e}	228.88±0.12^{e}	615.44±1.01^{c}
45	113.41±0.73^{d}	113.54±0.86^{d}	78.48±0.64^{d}	277.99±0.90^{d}	779.15±0.02^{d}
60	126.45±0.84^{c}	127.21±0.67^{c}	113.24±0.81^{c}	282.30±0.62^{c}	858.65±0.02^{c}
75	168.76±0.34^{b}	142.71±0.49^{b}	156.87±1.39^{b}	417.72±0.37^{b}	915.14±0.01^{b}
90	291.59±0.87^{a}	425.85±0.55^{a}	173.75±0.97^{a}	428.86±0.21^{a}	980.64±0.01^{a}
Main effect; D f (n-1) = 6 F value	15009.56***	45792.90***	2114.20***	41409.74***	284784.40***

± SE: Standard Error of the Mean, Significance level *

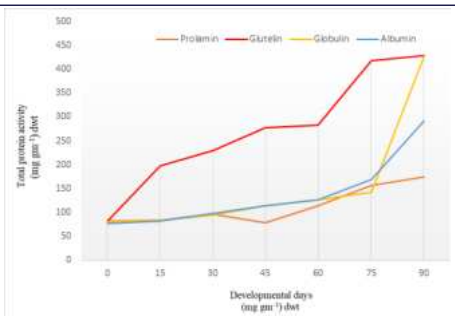

Fig: 1 Fractions of proteins during development of C. apetalum seeds.

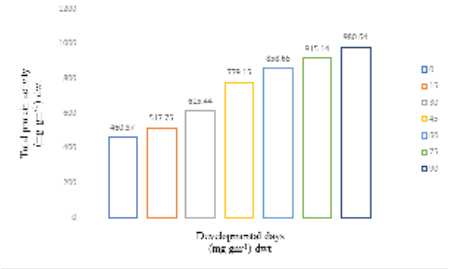

Fig: 2 Total protein content during development of C. apetalum seeds.

DISCUSSION

Seed storage proteins are proteins that accumulate significantly in the developing seed, whose main function is to act as a storage reserve for nitrogen, carbon and sulphur. These proteins are rapidly mobilized during seed germination and serve as the major source of reduced nitrogen for the growing seedlings. In general, seed storage proteins do not carry out any enzymatic functions. Even though storage proteins from diverse plants are structurally different, they all share some common characteristics. One of the main characteristics of storage proteins is that they accumulate in high levels in specific tissues at a specific stage of development (H.B. Krishnan, E.H. CoeJr, 2001).

Calophyllum apetalum seeds exhibited a gradual increase in protein fractions during development. The moisture content analysis of C. apetalum shows an increase initially, after 45 DAA a gradual decline was noted. The chemical composition of the cotyledon, being rich in oils (Bringi 1987), appears to be responsible for difference in initial moisture content as reported in recalcitrant Quercus rubra (Pritchard 1991) and Q. robur (Finch-Savage 1992). The total protein content was likely increasing throughout the development. The storage proteins majorly albumin, globulin, glutelin and prolamin were present in the developing seeds.

All fractions of proteins found minimally at initial stages while during the course of development the fractions were increases and reaches its maximum. When the fruit attains its maturity, all proteins attained their maximum level to carry out the metabolism. Out of four fractions the glutelin and globulin content were found to be high in C. apetalum seeds. It is concomitant with the data of, globulins are the storage proteins of cereal embryos, but as such they represent only very a small proportion of the total reserve protein of the whole grain (Beweley and Black,1994) and the Moringa oleifera is mainly composed of proteins type globulins and albumins which represent 53% and 44% of total proteins of the seed respectively (Aline Takaoka Alves Baptista et al., 2017).

As a result, during the early stages the protein fractions albumin, globulin, glutelin and prolamin were all present in small amounts. However, as the seeds matured all of the protein fractions and the total protein showed a rising tendency. The moisture content of seed decreasing when the seed attain its maturity.

CONCLUSION

The biochemical analysis of protein fractions on the seeds of Calophyllum apetalum extracts revealed that there is a gradual increase of fractions of proteins throughout the development. The moisture content during development initially high then it declines due to the dry matter accumulation. Ethanol extract results showed protein concentrations ranged from 81.27 mg gm^{-1} dwt. to 173.75 mg gm^{-1} dwt., NaOH extract results showed protein concentrations varied from 81.70 mg gm^{-1} dwt.to 428.86 mg gm^{-1}dwt., NaCl extract results showed protein concentrations ranged from 79.70 mg gm^{-1} dwt.to 425.85 mg gm^{-1}dwt.,Water extracts results showed protein concentrations varies from 76.01 mg gm^{-1} dwt. to 291.59 mg gm^{-1} dwt. and the total protein concentration varies from 460.37 mg gm^{-1} dwt. to 980.64 mg gm^{-1} dwt.

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