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Physiological and Biochemical Changes during Seed Development in *Hopea ponga* (Dennst.) Mabberley: An Endemic Endangered Tree Species of Western Ghats

Research Article

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Abstract

Hopea ponga (Dennst.) Mabberley is an endemic and endangered tree belonging to family Dipterocarpaceae, is economically important as timber and reported with several active compounds that have effective as antioxidant and antimicrobial activities. Due to its high economic and medicinal value, the species is over exploited and has become rare and endangered and need high conservation protocols. The present study includes physiological (fresh and dry weight, moisture content and germination) and biochemical changes (total soluble sugars, total soluble protein, lipids, total phenols) during seed development in *Hopea ponga*. Mass flowering of *H. ponga* occurred during the month of February and extended upto April followed by mass seed set, that took two months for its maturation. The majority of individuals have developed their seeds in the month of April to June and dispersal occurs with the help of wind. Changes in fresh and dry weights, % MC showed a sigmoidal pattern during seed development and maturation. Total soluble sugar and protein recorded an increase with the seed development while starch and phenols were followed the sigmoidal pattern and were significant as per calculated r^2.

Introduction

Hopea ponga (Dennst). Mabberley is an endemic and endangered tree belonging to family Dipterocarpaceae, found in the tropical evergreen forests distributed all along the Western Ghats of Tamil Nadu, Kerala, Maharashtra and Karnataka [1]. The tree is economically important as timber, the bark is also a good tanning material and astringent with slow speed of diffusion [1]. The wood obtained from the *H. ponga* is used for building construction, wooden article and furniture preparation [2]. The effect of desiccation on germination and vigour of seeds of the *Hopea parviflora* and *Hopea ponga* was carried out [4]. The influence of wing loading and viability of seeds of *Hopea ponga* was investigated [5]. Phytochemical evaluation, antioxidant and antibacterial activity of seed wings of *Hopea ponga* has been evaluated [6]. The entire plant can be used to cure piles [7]. The methanolic extracts of the leaf of *H. ponga* revealed, excessive scavenging ability towards specific free radicals. Further, the tree reported with several active compounds that have effective as antioxidant and antimicrobial activities [8]. Due to its commercial value, economic and medicinal importance, this tree has been illegally cut down for various commercial uses in the past few decades. The seeds of Dipterocarpaceae species are generally short lived and are incapable of overcoming desiccation [9]. Knowledge on seed development is essential in case of endemic and endangered species for successful application of conservation strategies. There has been no report on the seed development and related biochemical changes in recalcitrant seeds of endemic and endangered Dipterocarpaceae members of Western Ghats. Therefore the present study was

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undertaken to evaluate the seed development from fertilization to maturity. Data includes physiological (fresh and dry weight, moisture content and germination) and biochemical changes (total soluble sugars, total soluble protein, lipids, total phenols) during seed development in Hopea *ponga*.

Materials and Methods

Healthy and disease free *Hopea ponga* trees (15 numbers) naturally grown in the campus of KSCSTE-Malabar Botanical Garden and Institute for Plant Sciences (MBGIPS), Kozhikode Kerala India was selected and observed regularly for the detailed studies on phenology and seed development. Frequent visits have been made to each tree to observe flower initiation, anthesis, seed development etc. For ascertaining the pollen-ovule ratio, the number of pollen grains has been calculated using a Haemocytometer. The number of ovules in an ovary has been calculated by taking section of the ovary at young stage. Total number of flowers in a tree was calculated by counting the number of flowers per inflorescence and then number of inflorescences per branch. By counting the number of branches per tree, average number of flowers has been calculated. Stigma maturity has been recognized by observing the stigma at the time of flowering. Average number of buds/flowers produced in an inflorescence and branches are assessed, along with the number of seeds produced in each inflorescence also counted. This will give an idea about the seed production and thereby reproductive efficiency of the plants.

For the experimental studies flowers were tagged on the day of opening and the developing seeds were collected periodically at an interval 4 days, from 4 DAA to 60 DAA for *H. ponga*. For the pollination and related studies both ex situ and in situ observations were made. For the laboratory studies the flowering twigs were taken and kept in a moist environment till the observations. The collected developing seeds were sealed in polythene bags to avoid moisture loss. The diameter and length of the fruits were measured using Vernier callipers and their weight by an electronic balance (ANAMED, Model Z-400). External features of the seeds like texture, colour and hairiness etc. were also noted and this helped to identify the approximate age of the developing seeds. Percent Moisture Content (MC) of the collected seeds were analyzed by Low Constant Air Owen method (103 °C for 17 hours) as stipulated by ISTA.

Sample materials after taking dry weights at different periods were used as a source material for estimating metabolites like total soluble sugar, phenols, total soluble proteins and starch. Three samples of each stage were sampled for biochemical analysis. Tissue samples was ground in known volume of 80% ethanol (v/v) in distilled water and centrifuged at 4000 rpm for 10 minutes. The residue was washed thrice and part of the combined supernatant used for the estimation of total sugar, phenol and amino acids. The rest of the supernatant was kept in a china dish and evaporated in a hot air owen at 60 °C and the residue dissolved in distilled water, centrifuged and served as the source for soluble sugar. The left over residue was ground in 30% perchloric acid centrifuged, re- extracted and the combined supernatant is used for starch estimation. Total soluble sugar was estimated using phenol sulphuric acid method [10], total phenols by Swain and Hillis [11], Protein content by Lowry et al. [12], starch by Mc Cready et al. [13], amino acid by Sadasivam and Manickam [14].

Statistical analysis

The data obtained on total soluble sugar and total protein were analysed by linear regression analysis and the regression coefficient were calculated and significance tested at 0.05% as per Sokal and Rohlf [15]. Data for starch and phenols were anlysed by polynomial regression at 3rd level and the trend was drawn and correlation coefficient calculated and significance tested at 0.05 or 0.01% P as the case may be.

Results

Phenology and Seed development

The observation on the phenological studies of *H. ponga* showed that it is an evergreen tree growing in the lowland forest area to mid elevation up to 900 msl. Healthy mature individuals of *H. ponga* selected for phenological studies showed thatleaf shedding occurred as a periodical event once after the maturation of old leaves.The tree periodically renewed their leaves by mass leaf flushing in the month of October to December. Mass flowering of *H. ponga* occurred during the month of February and sometimes extended upto April. The peak flowering of the tree was followed by mass seed set, that took two to three months for its maturation. The majority of individuals have developed their seeds in the month of April to June and the fruit dispersal occurs with the help of wind. The mass seed set was followed by mass seed germination through rainfall during June with the aid of southwest monsoon.

Hopea ponga produces axillary raceme/ panicle in every leaf axis as well as in the nodes of side branches. The tree produced an average of 12,536 ± 3106 flowers per tree during the peak flowering period. The continuous observation on the matured floral buds confirmed the diurnal flowering habit of the tree. Anthesis of flowers started with the opening of floral parts in the morning hours between 6 AM and 11 AM (Table 1). Flowers were pale pink to white in color with a mild fragrance and the petals twisted at the tip with a pointed end. The flowers were remained fresh for 3 days from the day of anthesis and the petals were withered. This floral mechanism showed that the flowers had a short lifespan of 3±1 days. Each flower consists of 13±2 epipetalous anthers.The unopened flowers observed for anther dehiscence showed that the anthers were dehisced one day before anthesis which in turn confirmed the protandrous nature of the flowers. The stigma of the ovary was projected out to receive cross pollen grains. The calculated flower fruit ratio and ovule seed ratio were 6:1 and 25776:1432 respectively. Flowers are pale pink to white in color with a mild fragrance and the petals twisted at the tip with a pointed end. The flowers were remained fresh for 3±1 days from

Table 1: Reproductive Efficiency of *H.ponga*

Sl. No	Character	Observation
1	No. of flowers per inflorescence	88.50±27.30
2	Life span of the flower	3±1 days
3	Flower opening time	6AM to 10.30AM
4	Ovules/flower	17±1
5	Anther/flower	13±2
6	Flower-Fruit ratio	6:01
7	Ovule-Seed ratio	25776:1432

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the day of anthesis and the petals were withered along with the epipetalous anthers. The reproductive efficiency was calculated and depicted in (Table 1).

On anthesis (identified with the mid fragrance) the flowers were tagged as 0 day (DAA) and the data on seed development was carried out by measuring the length, width of the developing seeds at an interval of 4 days starting from 4th day after anthesis (DAA) till maturity at 60 days together with fresh and dry weight and % MC (Table 2). Seed length and width increased from 3.14±0.15 mm and 0.94±0.01 mm at 4 DAA to 10.34±0.46mm and 7.11±0.36 at 60 DAA respectively. Parallel with the increase in length and width there was a linear and significant increase in Fresh Weight (FW) and Dry Weight (DW) during seed development. FW has increased from 5.36±1.12 mg per seed at 4 DAA to 232.82 mg per seed at 60 DAA which is about 4343.65%. Similarly the final DW increase was about 2621.08% from the 4DAA. The initial %MC was 34.15% at 4DAA which slowly increased and reached 81.37% at 36DAA and then declined slowly on maturity, 60.43 ± 1.31 % at 60DAA (Table 2).

Biochemical changes

Because of the low amount of samples during the initial phases, changes in Total Soluble Sugars (TSS), Total protein, starch and phenol contents were evaluated from 12 DAA. Gradual increase in TSS (Figure 1A), was recorded with the seed development in *H.ponga*. TSS recorded 3.41±0.11 mg-1 g.dwt at 12 DAA to 41.33 ± 0.24 mg^{-1} gd.wt at 60 DAA, a 1212.02% increase and was significant at 0.05% P level. Changes in total protein also sowed the same pattern as that of TSS and the changes were significant at 0.05% P level (Figure 1B). O the other hand, starch and phenol content recorded a linear increase up to 40 DAA which furthers reduced and stable and later a slight decrease was noted. The data were analysed using polynomial curve as per the formula ($y = -0.0032x3 + 0.2998x2 - 4.1142x + 240.65$ $R^2 = 0.993$ for starch and $y = -0.0014x3 + 0.132x2 - 1.4609x + 50.666$, $R^2 = 0.9964$ for phenol) and were significant in both cases at 0.05% P level (Figure 1C and 1D).

Discussion

Healthy mature individuals of *H. ponga* selected for phenological studies from KSCSTE MBGIPS campus showed that leaf shedding occurred as a periodical event once after the maturation of old leaves. However, leaf flushing was observed during the month of October to December though the tree canopy appears to be evergreen throughout the year. The majority of individuals that produced their floral buds in the month of February were attained their peak flowering in the month of March and April. After flowering it took two months for the complete development and maturity of the seeds. The majority of individuals have developed their seeds in the month of April to June (Table 1). Accordingly that many dipterocarps species has the phenomena of mass flowering and fruiting and the seeds mature by the onset of South – East monsoon as the seeds are short lived and sensitive to desiccation (1 & 16). Supporting to this the seeds of *H.ponga* matured at 60 DAA with a high % moisture content of 60.43 ± 1.31% indicate the recalcitrant nature and lose viability within short period of 8 days.

Analysis of seed measurements started at 4 DAA in case of seed length, width, fresh and dry weight and % moisture content as the seeds were not large enough for analysis till 4DAA. Similarly, biochemical and enzyme analysis started at 12 DAA before which sufficient samples could not be obtained due low size and weight. Seed length, width and fresh and dry weights followed the typical sigmoid growth pattern of development and reaches maximum at maturity (Table 2).

The deposition of storage substances is one of the key process of zygotic embryogenesis providing compounds that will be used from the early stages of the embryonic development until autotrophy, after germination [17]. As sugars may be a major component as biochemical precursors, an understanding on the changes in sugar content during maturation is important. In the present study sugar content tended to increase throughout the maturation and the increase was significant at 1 % P level (Figure 1A). Sucrose synthesised in green (photosynthetically active) tissues is transported through the phloem to support growth and maturation of heterotrophic tissues such as seeds [18]. Thus, seed filling depends above all on the rate of photo assimilate supply and on metabolic regulation of transport [19]. Starch normally considered a carbohydrate reserve in seeds recorded accumulation during seed development in *H. ponga* till 50 DAA and later it stabilised and reduced at 60 DAA (Figure 1C). The polynomial curve fitted was significant at 0.01%P level and showed a sigmoid pattern. The possible role of starch reserve is to provide sugar

Table 2: Morpho-physiological changes during seed development in *H.ponga*.

Days after Anthesis (DAA)	Fruit Length (mm)	Fruit Width (mm)	Fresh weight (mg)	Dry weight (mg)	Moisture Content (%)
4	3.14±0.15	0.94±0.01	5.36±1.13	3.51±0.93	34.03±1.83
8	3.94±0.15	1.23±0.01	9.52±1.09	5.46±0.74	42.58±1.94
12	4.32±0.23	1.93±0.02	16.49±1.08	8.37±1.03	49.24±1.48
16	5.61±0.26	2.64±0.02	20.54±1.03	8.61±1.14	58.06±1.96
20	5.97±0.18	2.84±0.04	32.36±1.73	11.82±1.32	63.48±1.34
24	6.32±0.23	3.17±0.11	58.26±1.19	17.74±1.21	69.64±1.87
28	6.94±0.13	4.03±0.14	79.38±1.24	20.15±1.14	74.61±2.07
32	7.36±0.25	4.64±0.25	93.72±1.97	22.13±1.98	76.38±2.38
36	7.94±0.25	5.12±0.19	118.59±2.18	22.09±1.67	81.37±2.53
40	8.78±0.32	5.34±0.28	142.67±3.03	30.69±1.48	78.49±2.03
44	8.91±0.29	5.43±0.27	173.24±3.16	42.84±1.87	75. 28±1.40
48	8.93±0.31	6.04±0.18	204.37±2.87	56.63±2.03	72.29±1.86
60	9.01±0.46	6.11±0.36	232.82±3.05 (4343.65%)	92.00±2.48 (2621.08%)	60.43±1.31

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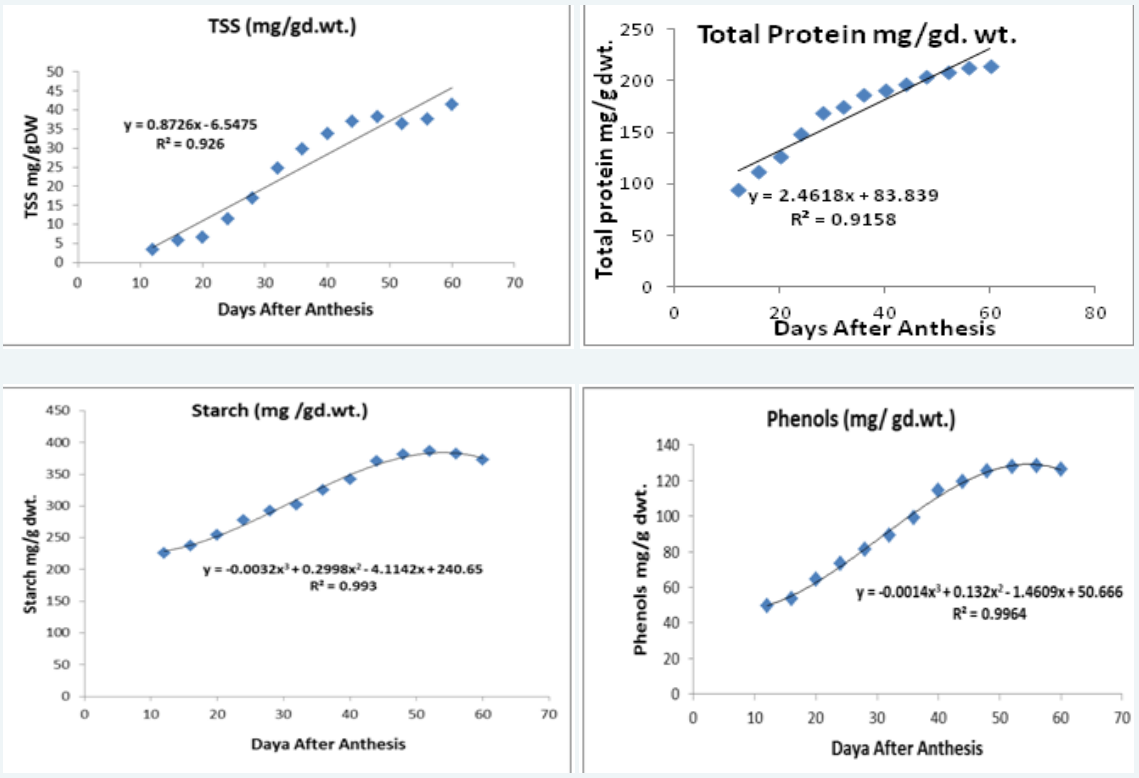

Figure 1: Changes in Total soluble sugar (A), total soluble protein(B) , starch(C) and total phenols (D) contents with the best fit curves and R^2 during seed development in *H.ponga*.

source during germination. During seed development in *H. ponga* total protein content increased linearly till 50DAA andlater the rate was reduced but the changes were significant at 0.05% P level (Figure 1B). Phenol content recorded a typical sigmoidal pattern during seed development in *H.ponga*. Weidnera et al. reported that both phenolic acids and total phenolic compounds reach the highest levels at the initial stage of development of cereal caryopses and decrease considerably at the final stage of grain maturation [20]. Most higher plants responded to various environmental stimuli by activating secondary metabolic pathways such as the phenylpropanoid metabolism. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is the first and one of the key regulatory enzymes of this pathway [21].

The increase in DM (dry weight) during seed development is a result of the synthesis and deposition of storage substances (Table 2). In our work the total soluble sugars, proteins and starch constituted 4.13%, 21.51% and 37.32% respectively per gram dry weight of mature seed. The accumulation of storage proteins in conifer somatic embryos is influenced by ABA and water stress, by addition of an osmoticum into the culture medium [22]. It has been suggested that the effects of ABA and osmoticum are additive, where the synthesis of storage protein is initiated by ABA and regulated at the post-transcriptional level by the osmoticum [22].

Many of the physiological and biochemical effects brought about in developing embryos by ABA can also be induced by low osmotic potentials [23]. The continuous increase in starch content and increase in dry matter may result in the proportional reduction of osmotic potential (though in the present work not evaluated). Probably, the increase in starch content during the seed development was due to triacylglycerols breakdown during seed development. Stone and Gilford in P. taeda reported changes in starch accumulation [24], during early seedling growth and role of triacylglycerols breakdown and sub- sequent carbohydrate metabolism. It has been suggested that endogenous ABA plays a role in the stimulation of specific storage proteins, in the dehydration at the end of development and in the prevention of precocious germination [25].

In conclusion, our results represent a snapshot of activities of various morphological, physiological and biochemical changes during the seed development of *H. ponga* an endangered and endemic tree species from Western Ghats. This may useful for further studies on accumulation patterns of different metabolites like individual sugars, protein fractions and specific phenols and their role in the recalcitrant nature of the seeds of *H. ponga* which may help in the conservation and sustainable utilization of this tree species.

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