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PHYSIO-CHEMICAL CHANGES DURING REPRODUCTIVE PHASE IN *AZADIRACHTA INDICA* A. JUSS. (NEEM) AFFECTING YIELD POTENTIAL

SUHANI SABHERWAL AND K. K. DHIR
Department of Botany, Panjab University, Chandigarh 160 014.

The *in vivo* morpho-physiological responses in Neem (*A. indica*) were studied during the annual reproductive flush from bud development onwards to seed set. Number of buds and flowers per inflorescence, and physiological responses were observed. Sample protein, total sugar and reducing sugar were measured. Low contents of the above compounds resulted in poor flowering response and longer juvenility. The enzyme assay measuring the activities of invertase, protease, acid- and alkaline phosphatases was consistent with the biochemical response. In effect, a shorter regeneration phase seems to lead to lower productivity in terms of floral inflorescence and fruit set. Therefore, it is suggested that well formed regeneration initials may regulate the reproductive phase resulting in improved organ / biomolecule production.

Key Words : *Azadirachta indica*, biochemical, physiological, reproductive phase.

Neem, a prodigious multipurpose tree of the tropics, has immense potential to benefit mankind and to protect the environment (Kraus 2002, Koudal *et al.* 2003). Often called 'Gift of the Gods' or 'Nature's Pharmacy', the tree is exploited as a commercial medicine and antibiotic. Extracts from its extremely bitter seeds and fruit may, infact, be the source of a new generation of chemicals for use in Integrated Pest Management practices (Koul and Wahab 2004, Nathan *et al.* 2006).

Most of the information is restricted to the vegetative phase of a plant and there is little information with respect to reproductive phase, especially on fruit. The present study investigates the reproductive growth of Neem in morpho-physiological units using some biochemical parameters. The principle aims of this study are: (i) to determine floral anthesis and fruit development, (ii) to assess the role of biochemical substances in the primary metabolic pathway during reproduction and (iii) to exploit the maximum potential of the tree in increasing the yield.

MATERIALS AND METHODS

The experiment was conducted in the campus of Panjab University, Chandigarh (Lat. 30.5 N Long. 77.0 E). Healthy post-juvenile plantations (reproductively mature trees of 5-6 years age) were selected in an ecological niche with uniform climatic and edaphic conditions. The experimental trees representing the study were designated as tree I , tree II, tree III and tree IV. There was a deliberate selection of trees varying in canopy as well as locales. Tree I represented an interactive ecotype associated with perennials and shrubs. The tree canopy was bushy with evenly spreading branches bearing conducive foliage. Tree II was associated with vegetation as *Mangifera indica* L. and grassland pasture. The canopy was dense with extended woody branches. However, the foliage had a tendency to wilt and etiolate even in seasonal prime. Tree III bore a roadside situation, subjected to indiscriminate pruning by the local residents. The experimental canopy was medium with

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Table 1: Biochemical evaluations during reproduction – Bud Sprout (Pre-anthesis)

Metabolites	Tree I	Tree II	Tree III	Tree IV	C.D. (at 5%)
Proteins mg/g fr.wt.	260.0 ± 0.61	253.0 ± 0.15	243.0 ± 0.17	237.0 ± 0.25	0.65
Total Sugars mg/g fr.wt.	619.0 ± 0.14	605.0 ± 0.58	591.0 ± 0.15	573.0 ± 0.25	0.20
Reducing Sugars mg/g fr.wt.	293.0 ± 0.15	283.0 ± 0.58	274.0 ± 0.40	267.0 ± 0.23	0.15
DNA μg/g dry wt.	160.0 ± 0.23	153.0 ± 0.20	143.0 ± 0.20	137.0 ± 0.30	0.27
RNA μg/g dry wt.	204.0 ± 0.20	196.0 ± 0.58	188.0 ± 0.20	181.0 ± 0.20	0.13
Invertase μg/mg prot.	255.0 ± 0.29	247.0 ± 0.45	242.0 ± 0.11	234.0 ± 0.17	0.26
Protease μg/mg prot.	103.0 ± 0.20	99.0 ± 0.20	96.0 ± 0.17	93.0 ± 0.25	0.25
Acid Phosphatase μg/mg prot.	454.0 ± 0.23	444.0 ± 0.25	434.0 ± 0.51	422.0 ± 0.42	0.16
Alkaline Phosphatase μg/mg prot.	353.0 ± 0.93	344.0 ± 0.17	337.0 ± 0.40	329.0 ± 0.45	0.16
No. of buds/panicle	112.0 ± 1.46	103.0 ± 1.97	98.0 ± 2.21	89.0 ± 3.09	10.89

frequent leaf shed. Tree IV was deprived of direct sun light by the surrounding thick foliage. It was also associated with thorn and scrub vegetation. The canopy was sparse with weak branches.

In vivo regeneration was keenly monitored in the experimental trees. Just prior to onset of the regeneration phase in March, apices of the test branches were marked and tagged for study. The tagged reproductive branches were periodically harvested for laboratory analyses.

Preliminary experimentation: In accordance to previous test analyses and studies (Sokal and Rohlf 1973, Devkumar and Sukhdev 1993), certain parameters such as number of reproductive structures per panicle, their size, weight and quality were recorded and the best correlations were standardized for biochemical survey (Sabherwal 2000).

Main experimentation: The biochemical parameters selected for final study were conducted selectively in the test branches (Trees I-IV). Observations were recorded separately at various stages of reproductive development, such as bud sprout (pre-anthesis), flower bloom (anthesis), fruit set (post-anthesis) and seed (senescence). Extraction and estimation of total sugars and reducing sugars were carried out by

Table 2: Biochemical evaluations during reproduction – Flower Bloom (Anthesis)

Metabolites	Tree I	Tree II	Tree III	Tree IV	C.D. (at 5%)
Proteins mg/g fr.wt.	237.0 ± 0.15	229.0 ± 0.20	220.0 ± 0.11	213.0 ± 0.11	0.26
Total Sugars mg/g fr.wt.	506.0 ± 0.17	494.0 ± 0.58	473.0 ± 0.20	453.0 ± 0.17	0.17
Reducing Sugars mg/g fr.wt.	264.0 ± 0.58	256.0 ± 0.22	246.0 ± 0.25	237.0 ± 0.23	0.27
DNA μg/g dry wt.	137.0 ± 0.13	129.0 ± 0.58	120.0 ± 0.58	113.0 ± 0.40	0.26
RNA μg/g dry wt.	184.0 ± 0.26	175.0 ± 0.40	168.0 ± 0.32	160.0 ± 0.36	0.13
Invertase μg/mg prot.	240.0 ± 0.11	233.0 ± 0.20	227.0 ± 0.26	220.0 ± 0.25	0.13
Protease μg/mg prot.	89.0 ± 0.20	84.0 ± 0.20	81.0 ± 0.11	78.0 ± 0.55	0.16
Acid Phosphatase μg/mg prot.	414.0 ± 0.29	401.0 ± 0.26	392.0 ± 0.36	382.0 ± 0.36	0.18
Alkaline Phosphatase μg/mg prot.	348.0 ± 0.58	341.0 ± 0.40	331.0 ± 0.70	323.0 ± 0.58	0.17
No. of flowers/panicle	125.0 ± 1.19	112.0 ± 1.44	105.0 ± 1.00	96.0 ± 2.06	11.43

Table 3: Biochemical evaluations during reproduction – Fruit Set (Post-anthesis)

Metabolites	Tree I	Tree II	Tree III	Tree IV	C.D. (at 5%)
Proteins mg/g fr.wt.	303.0 ± 0.15	296.0 ± 0.17	286.0 ± 0.23	280.0 ± 0.20	0.15
Total Sugars mg/g fr.wt.	534.0 ± 0.10	519.0 ± 0.20	502.0 ± 0.58	488.0 ± 0.69	0.15
Reducing Sugars mg/g fr.wt.	404.0 ± 0.58	395.0 ± 0.20	385.0 ± 0.25	376.0 ± 0.46	0.15
DNA μg/g dry wt.	203.0 ± 0.17	196.0 ± 0.18	186.0 ± 0.29	180.0 ± 0.11	0.21
RNA μg/g dry wt.	227.0 ± 0.31	218.0 ± 0.20	210.0 ± 0.23	202.0 ± 0.42	0.15
Invertase μg/mg prot.	276.0 ± 0.20	268.0 ± 0.20	260.0 ± 0.11	250.0 ± 0.40	0.18
Protease μg/mg prot.	109.0 ± 0.29	106.0 ± 0.20	103.0 ± 0.23	99.0 ± 0.40	0.35
Acid Phosphatase μg/mg prot.	506.0 ± 0.11	494.0 ± 0.14	484.0 ± 0.21	476.0 ± 0.11	0.38
Alkaline Phosphatase μg/mg prot.	386.0 ± 0.11	378.0 ± 0.58	368.0 ± 0.26	358.0 ± 0.60	0.34
No. of fruit set/panicle	120.0 ± 1.45	107.0 ± 1.37	92.0 ± 2.08	85.0 ± 1.17	18.70

adopting the classical procedures of Dubois *et al.* (1956) and Somogyi (1952). Soluble protein content was estimated following the protein-dye binding method (Bradford 1976). RNA content was determined by the modified orcinol method at a wavelength of 610.0 nm on Shimadzu double beam spectrophotometer (Nieman and Poulson 1963). The *in vivo* invertases and proteases were assayed by the methods of Jaynes and Nelson (1971) and Basha and Beevers (1975), respectively. The statistical analysis of data was done using ANOVA as outlined by Sokal and Rohlf (1973).

RESULTS AND DISCUSSION

Azadirachta indica A. Juss. (family Meliaceae) has a single annual growth flush with corresponding four periods, pre-anthesis (bud sprout), anthesis (flower bloom), post-anthesis (fruit set) and senescence (seed). The annual regenerative phase initiates in March marked with vegetative rejuvenation; and terminates with fruit drop in July, which coincides with the monsoons and strong breeze while the ripe fruit is subject to microorganism and insect activity, feeding birds and human harvest.

Reproductive development : It was observed that inflorescence size, number of reproductive structures per panicle was high at initiation (Table 1). Pre-anthesis bud sprouts were healthy in all the experimental trees, while leading in tree I with an average 112.0 buds per panicle followed by 103.0, 98.0 and 89.0 mean buds per panicle in tree II, tree III and tree IV, respectively. The mean average values peaked at anthesis (Table 2) displaying 125.0, 12.0, 105.0 and 96.0 flowers per panicle in the representative trees I-IV, respectively. Though tree I had the maximum fruit set at post-anthesis (Table 3), the number had declined distinctly from the flower bloom (yield per panicle: 107.0, tree II; 92.0, tree III; 85.0, tree IV). As trees III and IV maintained their previous trends of low harvest, flowering was considered important in determining fruit produce. Poor fruit set was related to absence of pollination or, fertilisation or, perhaps, abnormality in development as advocated by Kaska (1989). The possible evolutionary reason for investment in surplus flowers and in fertilising them, may be to obtain selective advantage of a larger floral display, which would reduce uncertainties in pollinator visits. A climacteric and sharp decrease in reproductive structures, as at fruit ripening (Table 4) resulted in senescence (average per

panicle: 97.0, tree I; 89.0, tree II; 75.0, tree III; 56.0, tree IV). This was suggested to be the result of multi-causal factors (Coleman *et al.* 2010) like endogenous growth-retarding substances, nutritional status of tree, fruit decay on plant itself, insects' activities, local weather conditions with strong breeze, monsoons, and so on.

Maximum fruit growth potential was related to budding initiation (as inherent growth/number) attained under optimal environmental conditions in the presence of a non-limiting supply of nutrients. Hence, bud admission and floral development may restrict fruit yield in size as well as in number.

Total Sugars : Results (Table 1) indicated that starting with low counts at pre-anthesis (total sugars/reducing sugars mg g^{-1} fresh weight: 619/293, tree I; 605/283, tree II; 591/274, tree III; 573/267, tree IV, total sugars rose at flower bloom (Table 2) and post-anthesis ovary development (Table 3). The estimated range for total sugars/reducing sugars for anthesis (mg g^{-1} fresh weight) elaborated as: 510/265, tree I; 490/255, tree II; 470/245, tree III; 450/235, tree IV. At post-anthesis stage, sugars registered resolutions as 540, tree I; 520, tree II; 500, tree III; 480, tree IV. However, the reducing sugar optima showed considerable fluctuations. While dropping between 237.0-264.0 at flower bloom, the titres recorded as 376-400 at post-anthesis. Hence, about two-third of total sugars contributed towards fruit set was by reducing sugars.

The developing fruit form a major sink organ where the photo-assimilates are efficiently translocated (Rolland *et al.* 2006). Therefore, utilisation of the carbohydrate reserves is probably important for rapid flowering and setting fruit/seeds. Exhaustion of this storage pool during extremely rapid flower and fruit development constitute an essential part of the plants' adaptation to a short growth flush to produce quality seeds (Li *et al.* 2009). Solhaug and Aares (1994) working with other plant materials formulated a source-sink equation for efficient regeneration potential. Relatively excess sugars were maintained during senescence (mg g^{-1} fresh weight: 392.0, tree I; 374.0, tree II; 359.0, tree III; 339.0, tree IV) (Table 4) with the reducing sugar titres recording proportionate losses (mg g^{-1} fresh weight: 203.0, tree I; 194.0, tree II; 184.0, tree III; 176.0, tree IV).

Soluble sugars form the major source-carbon translocations to sink tissues (Sung *et al*. 1988). The total sink strength of the

Table 4: Biochemical evaluations during reproduction - Seed Set (Senescence)

Metabolites	Tree I	Tree II	Tree III	Tree IV	C.D. (at 5%)
Proteins mg/g fr.wt.	221.0 ± 0.50	213.0 ± 0.25	202.0 ± 0.26	197.0 ± 0.17	0.41
Total Sugars mg/g fr.wt.	392.0 ± 0.17	374.0 ± 0.38	359.0 ± 0.15	339.0 ± 0.11	0.16
Reducing Sugars mg/g fr.wt.	203.0 ± 0.17	194.0 ± 0.25	184.0 ± 0.25	176.0 ± 0.29	0.14
DNA μg/g dry wt.	121.0 ± 0.17	113.0 ± 0.20	102.0 ± 0.36	97.0 ± 0.36	0.25
RNA μg/g dry wt.	144.0 ± 0.17	138.0 ± 0.11	130.0 ± 0.20	122.0 ± 0.58	0.19
Invertase μg/mg prot.	207.0 ± 0.35	200.0 ± 0.58	193.0 ± 0.26	190.0 ± 0.46	0.15
Protease μg/mg prot.	95.0 ± 0.52	90.0 ± 0.60	87.0 ± 0.35	85.0 ± 0.58	0.19
Acid Phosphatase μg/mg prot.	370.0 ± 0.84	357.0 ± 0.23	349.0 ± 0.15	335.0 ± 0.62	0.17
Alkaline Phosphatase μg/mg prot.	187.0 ± 0.31	176.0 ± 0.17	168.0 ± 0.51	158.0 ± 0.66	0.17
No. of seed set/panicle	97.0 ± 1.00	89.0 ± 2.00	75.0 ± 1.37	56.0 ± 1.07	18.89

regeneration meristems, as measured by assimilates, determine yield produce. Assimilates in sink tissue as total sugars were observed to be regulated by ABA (Hein *et al.* 1984, Kojima *et al.* 1995). Accordingly it was suggested that storage carbohydrates were broken down resulting in fruit rot, decay and abscission.

Invertase : Invertase activity μg mg^{-1} protein peaked at pre-anthesis, ranging between 234 to 255 μg mg^{-1} protein. This coincided with rapid growth and metabolism contributing to flower bloom (anthesis) (Table 1). Relatively, a constant invertase level was maintained throughout the period of flower bloom (Table 2) and fruit set (Table 3). With onset of senescence, invertase activity declined (Table 4). During seed set, enzyme activity ranged between 190-207 μg mg^{-1} protein.

Soluble Proteins : Large variations were observed in the proteins levels recorded in our study . We began with the stage of rapid protein syntheses (Table 1), mg g^{-1} fresh weight: 260.0, tree I; 253.0, tree II; 243.0, tree III; 237.0, tree IV, which were ultimately catabolised (Table 4) ranging between 197.0-221.0 mg g^{-1} fresh weight at senescence. Variable protein titres (mg g^{-1} fresh weight) were observed during floral anthesis (213.0-237.0) as well as post-anthesis fruit set (Table 3) at 280.0-303.0. Protein turnover is an active component of the dynamic metabolic pathway during reproduction (Lalonde *et al.* 2004). Brady and Spiers (1991) suggested that proteolytic pathway constitute an important part for selective catabolism during regeneration.

Protease : Matching the protein turnover during fruit development, protease had a definite role in the process. Throughout our analyses, low activities of the proteolytic enzymes were detected relative to certain other key enzymes involved in reproduction (Tables 1-4). However, these may be sufficient for the basic requirement in fruit/seed development. The initial higher levels of protease at pre-anthesis (93.0-103.0 μg mg^{-1} protein) were rejuvenated at post-anthesis with enzyme activity ranging between 99.0 to 109.0 μg mg^{-1} protein. The active protein metabolism, indeed, coincided with the temporarily enhanced protease titre. This may be explained by the concept of protein cycle (Muntz 1996). During dynamic metabolic reactions, proteases act as nitrogen sources while protein reserves are re-instated, with proteolytic cleavage having a functional role in protein deposition and re-activation. *In vivo* protease activity showed a concentration-dependent response for the substrate proteins. At senescence, it showed decreased sensitivity. Accordingly during flower bloom (Table 2) the active range being 96-125 μg mg^{-1} protein, at fruit-drop (Table 4) the effective content of protease was restricted to 56-97 μg mg^{-1}.

Phosphatases : High enzyme activity was recorded at pre-anthesis budding (acid phosphatase 422-454 μg mg^{-1} protein, alkaline phosphatase 329-353 μg mg^{-1} protein) (Table 1) as well as fruit set (acid phosphatase 476-506 μg mg^{-1} protein, alkaline phosphatase 358-386 μg mg^{-1} protein) (Table 3). At the end of the season during senescence, the enzymes lost their activity (Table 4). Acid phosphatase as well as alkaline phosphatase had values ranging 382-414 μg g^{-1} protein and 323-348 μg g^{-1} protein, respectively at floral anthesis (Table 2). At senescence, we had difficulty in enzyme detection and therefore, a large sample had to be test analysed for satisfactory results (acid phosphatase 335-370 μg mg^{-1} protein, alkaline phosphatase 158-187 μg mg^{-1} protein).

The major role of phosphatases seem to be in phosphorus metabolism during early periods of reproductive phase (Park *et al.* 2010). Enzymatic activity during rapid growth and development suggested a positive correlation with floral organogenesis as well as post-anthesis modifications. It was also proposed

that phosphatases may assist carbohydrate metabolism in developing seed (Strother *et al.* 1985).

CONCLUSIONS

The above study indicated that a fairly well established primary metabolic pathway operates during the reproductive phase in *Azadirachta indica* A. Juss., with characteristic physiological and biochemical events leading to fruit/seed development, as also, the commercially important secondary metabolites abundant in reproductive tissues (Sabherwal 2000). The regeneration meristems form an important sink organ where assimilates are efficiently translocated for rapid flowering and fruit set. This constitutes an essential part of the plants' adaptation to a short growth flush to produce seeds and develop future progenies.

Differential nutrient syntheses/ metabolism was demonstrated in the present study with a period of very rapid pool reserves, followed later by an equally rapid loss of assimilates in the ephemeral reproductive parts. Moreover, the decline in carbon and nitrate reserves coincided temporarily with the loss in activity of the associated enzymes, as during floral organs' senescence at post pollination stage. It was also observed that the enhanced metabolic status of trees I and II were retained throughout the various biochemical titres, while trees III and IV showed low levels of metabolites in the different stages throughout the period. Hence, a developmental perspective provides a rather simple sequence of events, each exclusive, but yet interwoven by inherent endogenous factors. These processes may be employed to describe patterns of floral initiation upto fruit development in Neem.

Apparently, tree I yielded the most favourable harvest in terms of morphology as well as endogenous nutrients and metabolic potential. Biochemical titres for all the experimental trees generally developed similar trends during the growth stages.

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