HISTOCHEMICAL AND PHYSIOLOGICAL STUDIES OF SEED (NUT) AND PSEUDOCARP (APPLE) OF *ANACARDIUM OCCIDENTALE* L.

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ABSTRACT

The growth rate, structure, development, time-course of deposition and distribution of major storage components, minerals and path of transport of assimilate in seed (nut) and pseudocarp (apple) of *Anacardium occidentale* L. were investigated. Both nut and apple growth starts at the same time, however, nut grows faster than the apple and reaches the maximum length and breadth by about 40 days after fertilization (DAF). The pseudocarp growth is slower at the beginning and after about 30 DAF the rate of growth increases and the apple reaches maximum length and breadth by about 55 DAF and full maturity by about 60 DAF. Histochemical studies revealed the pattern of distribution of lipids, protein, starch, iron, ascorbic acid and phenols in cotyledons. The study indicated that the pattern of distribution of these compounds is same in all the varieties examined. Phloem specific fluorochrome 5(6) carboxyfluorescein (CF) used to study the path of movement of assimilates in pseudocarp, placenta and cotyledons showed that CF movement was much faster in pseudocarp than in cotyledons. The study further revealed that vascular bundles of pseudocarp and placenta are important transport tissues and nutrients supply from pseudocarp to developing cotyledons occurred only through placenta.

INTRODUCTION

Cashew (*Anacardium occidentale* L.), a member of the family Anacardiaceae, is an evergreen tree. It is a native of the Tropical American country of Brazil (Paul, 1936). In India, cashew is predominantly found in Southern, Central and few States of North Eastern India. At present Malabar and South Kanara contribute maximum cashew production (Bhaskara Rao *et al.*, 1998). Cashew is now widely cultivated throughout the tropics for its nuts and pseudocarp. Cashew apple is mostly wasted in many places, but many preparations like juices, jams, candies, pickles, chutneys and alcoholic beverages can be prepared from the apple. "Feni" and cashew apple juice are said to have medicinal value and are very popular in Goa, India. Cashew nut shell liquid is a by-product of the cashew processing, which has applications in industries (Nambiar *et al.*, 1996). In this paper, we are presenting our finding on growth rate of seed (nut) and pseudocarp (apple) and structure and development, time-course of deposition and distribution of proteins, carbohydrates, lipids and minerals (iron) and other compounds (ascorbic acid, tannins and phenols) and path of transport of assimilates in seed and pseudocarp of cashew.

MATERIALS AND METHODS

Plant materials

The different stages of seed and pseudocarp of *Anacardium occidentale* were collected from various locations in the State of Goa. Mature seeds of varieties Goa-1, Balli-5, Vengurla–1 and Vengurla–4 were obtained from the Indian Council of Agricultural Research (ICAR) Complex, Ela, Old Goa, Goa, India for comparative study.

Growth measurements

To determine the growth rate of seed and pseudocarp, the fertilized ovules were tagged in the field and measured every 24 h. The size of the cotyledons and embryos were measured after removing the shell. Embryo size was determined under stereomicroscope.

Histochemistry and microscopy

Free-hand and cryo-microtome sections were stained with various fluorescent and non-fluorescent dyes using standard histochemical procedures (Krishnamurthy, 1998; Krishnan *et al.*, 2001, 2009; Pearse, 1972, 1980). The path of transport of assimilates was determined by using phloem specific fluorochrome 5(6)-carboxyfluorescein (CF) (Krishnan & Dayanandan, 2003). The inflorescence branches were cut under water to avoid embolism in the transport tissue. The cut end was placed in CF 0.01% dye solution. Movement of CF through pseudo-carp, placenta and cotyledons were observed by taking sections at different intervals after placing the cut ends in dye solution. Specimens were examined and photographed with a Nikon E800 microscope provided with bright-field and fluorescence attachments connected to Coolpix995 digital photographic system.

RESULTS AND DISCUSSION

Cashew (*Anacardium occidentale* L.) flowers at three to five years of age. In South India and West Coast flowering starts from the middle of October and continues till the end of February, the main season being November-December. The staminate flowers open earlier than the hermaphrodite flowers. The majority of staminate flowers open between 7 and 9 am and hermaphrodite flowers open between 8 a.m. and 12 noon. Stigma is receptive throughout the day after anthesis. However, the optimum period for receptivity was found to be immediately after anthesis. Anther dehiscence generally commences after 10:30 a.m. The pollen grains of cashew are sticky which emphasize the importance of insect pollination. The study of cross-compatibility and fruit-set in cashew showed variation in fruit yield, the maximum value of 55% fruit set was recorded in cross pollination. The significant correlation between cross-compatibility and variation in fruit yield suggests the important role of parental compatibility in selection of planting materials for the establishment of cashew plantation (Aliyu, 2007).

Growth and Development of Fruit

Measurements were made at regular intervals to understand the rate of growth of seed (nut) and pseudocarp (Figure 1). Both nut and pseudocarp growth starts at the same time. However, nut grows faster and reaches the maximum length and breath by about 40 DAF. Pseudocarp growth is slower at the beginning and after about 30 DAF the rate of growth increases. The pseudocarp reaches the maximum length and breadth by about 55 DAF. It was observed that the nut reached maximum size in 30 days, hardened in the next 10 days and declined in size by 10% at harvest (Rao *et al.*, 1962). From the fifth week onwards, when the growth of the nut ceases completely, the peduncle starts growing rapidly and outgrows the nut. The fruit ripens in about 60 days. As the season advances, the number of days required for the fruit to mature is reduced from 60 to 45 days. Sigmoid growth pattern and development of fruit was observed in guava and mango (Datta & Mukherjee, 1980; Pandey *et al.*, 1973). However, in cashew different patterns have been observed for different components (Chattopadhyay *et al.*, 1983). Further, a sigmoid pattern of growth takes place in cashew true fruit, whereas linear growth occurs in cashew apple (Thamburaj *et al.*, 1980).

Anatomy of pseudocarp, placenta and cotyledons

In order to understand structure, development and distribution of storage components it is necessary to identify the broad stages of development of pseudocarp and nut. The changes in size, shape, colour of the pseudocarp and nut from first day after fertilization (DAF) to maturity (60 DAF) are summarized in Figure 2 a, b. It was observed that each variety has specific shape and size of pseudocarp and nut. The study revealed that the ovary is unilocular and it contains a single anatropous ovule. Immediately after fertilization, the ovary enlarges considerably whereas the ovule growth is slow at the beginning, with the result that the kernel does not fill the entire loculus. The early growth of the ovule consists largely of the extension and curving upward of the chalazal end.

Pseudocarp is a fleshy peduncle. The pseudocarp has a single-layered epidermis with prominent single-celled hairs. Very thin cuticle is present over the epidermis. The cortex consists of juicy parenchyma cells and canals (Figure 2 c). Juicy parenchyma cells are rich source of vitamin C and sugar. Vascular bundles are scattered throughout the pseudocarp. During early stages of development the size of vascular bundle is much smaller and as the size of the pseudocarp increases the vascular bundle size also increases proportionally (Figure 2 d). However, in the mature pseudocarp the vascular bundle shrinks and xylem vessels also decreased in size, this is due to the early maturation of true fruit, which does not require any further transport of water or nutrients.

Transverse section of young ovary shows pericarp (cotyledonary shell or testa). In young ovary, the testa consists of 10-15 cell layers and the vascular bundles are embedded within the tissue. Transverse sections of mature pericarp revealed the presence of large number of canals and vascular bundles. It was observed that the canals primarily store tannins and phenols. Further, the pericarp contains 24-26% of tannins, which are predominantly used in leather industries (Nagaraja, 2000). Besides tannins the pericarp also contains considerable amount of proteins and starch, which could be a good source for developing feed additives.

The placenta is a small tube like structure, which connects the pseudocarp and nut through which nutrients transported to the cotyledons. Transverse section of placenta shows single layered epidermis with large cortex consisting of parenchyma cells and the vascular bundles are in concentric rings.

Cotyledons and embryo develop within the enclosed pericarp. Transverse section of mature cotyledon show clear inner and outer epidermis, storage parenchyma cells and vascular bundles. The size of the inner epidermal cells are much smaller when compared with outer epidermal cells. The cotyledons possess vascular bundles at the peripheral region. Cotyledons are filled with storage parenchyma cells, which store all the major storage components, minerals and vitamins (Figure 2 e).

Histochemical localization of major storage compounds

The young developing ovarian tissues do not posses any lipids. Transverse section of cotyledons 10 DAF stained with Nile blue showed the presence of lipids (Figure 2 h). It was observed that the intensity of fluorescence varies in different regions in young and mature stages of cotyledons. Both inner and outer epidermis and sub-epidermal regions show higher amount of lipids. Lipid concentration decreases towards the center of the cotyledon. Decreased amount of lipids at the center of the cotyledons may be due to the presence of high amount of starch in that region (Table 1).

Table 1. Distribution of major storage compounds in mature cotyledons.

Cotyledons	Lipids	Proteins	Starch
Inner epidermis	++	+++	+
Outer epidermis	++	+++	++
Centre of cotyledon	+	+	+++

+++maximum; ++moderate; +minimum

Cashew has a unique combination of fat, proteins, carbohydrates, minerals and vitamins. Cashew contains 47% fat, among which 82% are unsaturated fatty acids. The unsaturated fat content of cashew reduces the cholesterol level (Nambiar *et al.*, 1996).

Proteins were localized in the mature cotyledons of variety Balli-5 and Goa–1. Transverse sections of mature cotyledons were stained with the fluorescent dye barbituric acid for proteins. The yellow fluorescence indicates the presence of protein (Figure 2 f). Protein concentrations also show variation in different regions of cotyledons. The outer epidermis and sub-epidermal regions of 6 to 8 layers shows the presence of high concentration of proteins. An inner epidermis and sub-epidermal regions show comparatively low concentrations of protein. Towards the center of the cotyledon protein concentration is low, when compared to starch. The pattern of distribution of proteins was compared among the varieties. Among the varieties studied, the general pattern of distribution of protein was same in all the varieties. Protein content of the cashew kernel increases steadily up to 40 days after fruit set and remains high till harvesting stage (Rao *et al.*, 1962). The reducing and non-reducing sugars also increase up to 40 days, but decline sharply at harvest, while polysaccharide level continues to rise.

Cashew contains 21% proteins and 22% carbohydrates and a right combination of amino acids, minerals and vitamins and, therefore, nutritionally it stands on par with milk, eggs and meat (Nambiar *et al.*, 1996). Cashew has as low as 1% of soluble sugar. A person who is eating cashew does not have to worry about excess calories. The kernels supply about 6,000 calories energy/kg as against 3,600 by cereals, 1,800 by meat and 650 by fresh fruit. As the nut fats are complete, easily digestible, that could be used by both older people and infants. Eating cashew nuts do not lead to obesity, and instead helps to control diabetes. In general, it is a good appetizer, an excellent nerve tonic, a steady stimulant and a body builder (Nambiar *et al.*, 1996).

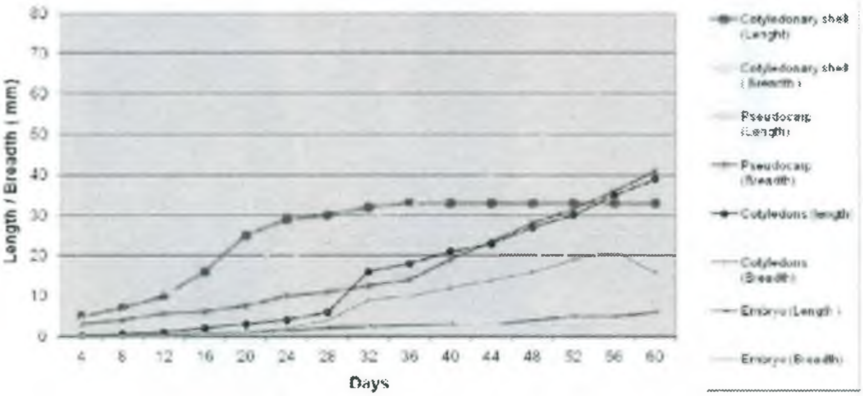

Figure 1. Growth rate of cotyledonary shell (pericarp), pseudocarp (apple), cotyledons (nut) and embryo of *Anacardium occidentale* L. from 4 days after fertilization (DAF) to maturity.

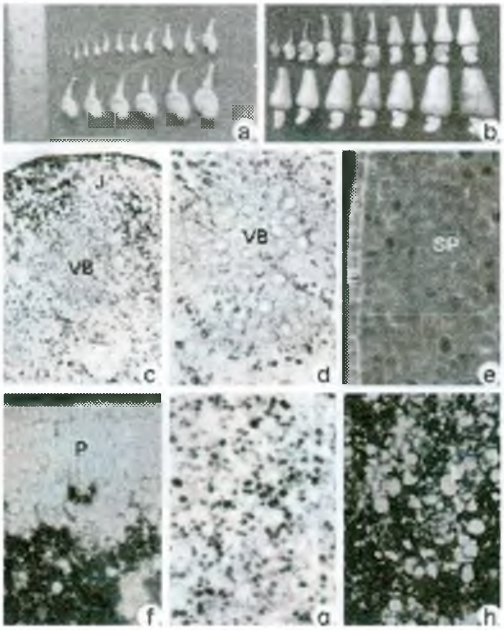

Figure 2. (a and b) : Developmental stages of pseudocarp and nut of *Anacardium occidentale* (a) From anthesis (upper left) to 12 days after fertilization (DAF) (lower right), (b) From 13 days after fertilization (upper left) to maturity (lower right); (c and d) Transverse section of pseudocarp (20 DAF) stained with Coomassie Brilliant blue, (c) X80; (d) Enlarged view of vascular tissue (X400); (e) Transverse section of mature cotyledon stained with a fluorescent dye calcofluor white, UV excitation (X400); (f) Transverse section of mature cotyledon stained with barbituric acid. Proteins fluoresce yellow in blue excitation (X400); (g) Transverse section of mature cotyledon stained with I_2KI. Starch appears black (X400); (h) Transverse section of mature cotyledon stained with Nile blue. Lipids fluoresces yellow in blue excitation (X400); E-epidermis; J-juice cells; L-lipids; P-proteins; SP-storage parenchyma cells; VB-vascular bundle.

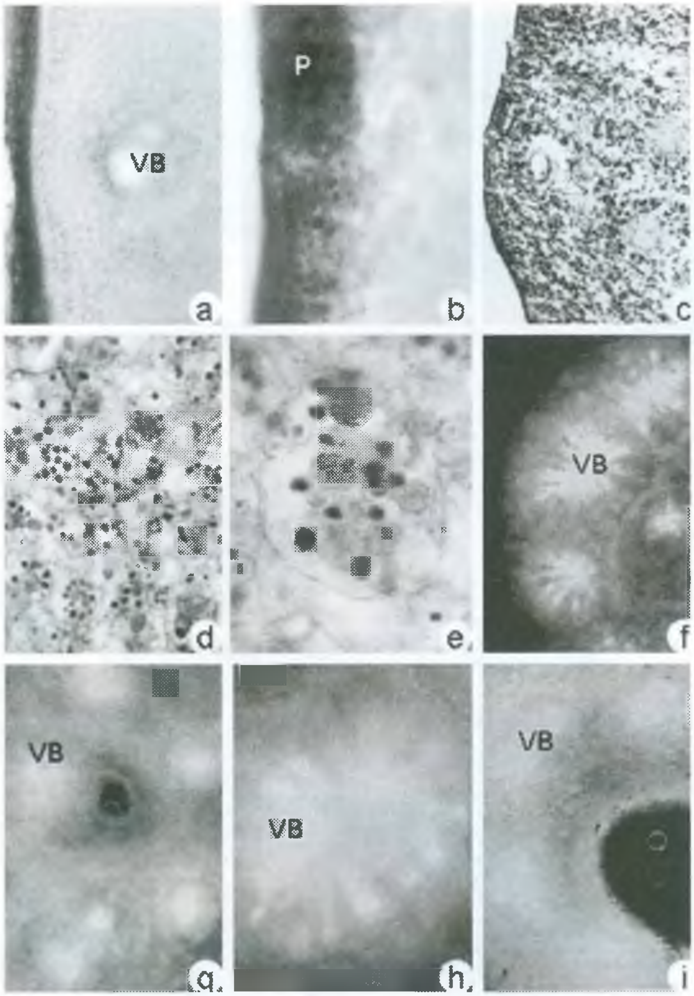

Figure 3. (a) Transverse section of cotyledon stained for iron using Prussian blue technique. Blue colour indicates the presence of iron. Iron is closely associated with vascular bundle (X200); (b) Transverse section of mature cotyledon stained for phenol using nitrozo reaction. Phenol appears dark brown (X200); (c) Transverse section of mature pseudocarp stained with ferric chloride. Tannins appear black (X200); (d and e) Transverse section of cotyledon stained with silver nitrate. Black colour deposits indicate the presence of ascorbic acid; (d) X200; (e) Enlarged view of single cell showing the deposition of ascorbic acid (X1200); (f, g, h and i) The phloem specific fluorochrome 5(6)-carboxyfluorescein (CF) used for dye movement studies to trace the path of assimilate transport in pseudocarp, placenta and young ovary. CF fluoresce yellow under blue excitation; (f and g) Transverse section of pseudocarp after fifteen minutes of CF movement. Note the presence of CF in the vascular bundles; (f) Peripheral regions of pseduocarp (X200); (g) Central portion of pseudocarp (X200); (h) Transverse section of isolated placenta after 30 minutes of CF movement showing the presence of CF in their vascular bundle (X400); (i) Transverse section of young developing ovary after CF movement. Note the presence of CF in vascular bundles of ovary wall. (X400). P-phenol; VB-vascular bundle.

Using I_2KI staining reaction starch was localized in mature cotyledons. Starch appeared black (Figure 2 g). The distribution of starch varies in different regions of cotyledon (Table 1). Outer epidermis and sub-epidermal regions of cotyledon show high concentration of starch. Inner epidermis and sub-epidermal regions reveal low concentration of starch. Starch concentration is very high in the central portion of the cotyledon. Decreased amount of starch in the inner epidermal regions may be due to the presence of high concentration of protein and lipids in that region.

Histochemical localization of minerals and other compounds

Ascorbic acid in pseudocarp and cotyledons was localized using silver nitrate staining reaction. Most of the juicy cells in the pseudocarp contain ascorbic acid. In the cotyledons ascorbic acid is localized as clear black-brown deposits (Figure 3 d, e). It was reported that ascorbic acid content in cashew apple varies among trees, different localities and during storage. Ascorbic acid content of about 230 mg/100 ml of cashew juice was recorded (Attri & Singh, 1997).

Iron was localized in the cotyledons of cashew. Prussian blue staining reaction revealed the presence of iron in cotyledons. Iron was localized only in the cells closely associated with vascular bundles (Figure 3 a). Tannins in the pseudocarp were localized using ferric chloride staining reaction. Tannins stained black (Figure 3 c). Phenols in the cotyledons were localized using nitrozo reaction. Phenols stained brown to black. Most of the phenols were found in epidermal and sub-epidermal regions of cotyledons (Figure 3 b). It was found that different high yielding varieties have different concentration of tannins and phenols (Nagaraja, 2000).

Path of transport of assimilates in pseudocarp, placenta and nut

The path of transport of assimilates in pseuodcarp, placenta and nut (cotyledons) was carried out using the phloem specific fluorochrome carboxyfluorescein (CF). When cut ends of inflorescence branches were placed in dye solution, CF enters into phloem tissue, once it reaches phloem, the rate of movement of CF increases. It was estimated that CF moves at the rate ranges from 20 to 25 cm per hour. The pseudocarp, placenta and cotyledons were periodically sectioned. The transverse sections observed under the fluorescence microscope under blue excitation showed the presence of CF in phloem tissues of pseudocarp, placenta and young ovary (Figures 3 f-i). The movement of CF within the pseudocarp, placenta and cotyledons showed that movement of CF was much faster in pseudocarp than in cotyledons. The study further revealed that vascular bundles of pseudocarp and placenta are important transport tissues and the nutrients supply to the ovule and developing cotyledons occurs only through placenta.

CONCLUSIONS

This study has established the basic pattern of growth and development of cashew nuts and pseudocarps and has demonstrated the specific locations of various nutrients. The path of transport of assimilates in pseudocarp, placenta and nut have also been studied. Such knowledge will be useful in improving the nutritional quality of cashew and in the utilization of cashew as a source of food. Further, the histochemical procedures standardized for localization of various chemical components in cashew may be useful for large scale screening of available cashew varieties, which may be useful for cashew breeders.

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