ISSN : 0974 - 7508 Volume 9 Issue 9

Natural Products

An Indian Journal

Full Paper

NPAIJ, 9(9), 2013 [355-362]

Histological characterization of annatto seed coat and variations of bixin drift during physiological maturation

Renata Vianna Lima[1]*, José Carlos Lopes[2], Renata Maria Strozi Alves Meira[3]

[1]Centro de Ciências Agrárias da Universidade Federal do Espírito Santo (CCA-UFES). Alto Universitário, s/n. Caixa Postal 16. Brasil. Alegre-ES. Cep: 29500-000, (BRASIL)

[2]Centro de Ciências Agrárias da Universidade Federal do Espírito Santo (CCA-UFES), (BRASIL)

[3]Universidade Federal de Viçosa (UFV), (BRASIL)

E-mail: renataviannalima2005@yahoo.com.br

ABSTRACT

The objective of this study was to investigate the maturation process of *Bixa orellana* (Bixaceae) seeds. Each inflorescence was identified considering the day of its respective anthesis. Diameter, weight, color, moisture content, and dry weight were determined for each fruit. Seeds were extracted and color, moisture content, dry weight, weight of one thousand seeds, speed germination, percentage of germination and bixin extraction were determined. The conclusions was: the physiological maturity point of the seeds occurred 77 days after anthesis; moisture content and dry weight were the best parameters for describing seed physiological maturity; better germination percentage and better bixin content were certified for seeds extracted 77 days after anthesis; seed vigor increased up to 77 days after anthesis; size and color of the fruits and seeds were also found to be efficient maturation parameters; the macrosclereids present high impermeability with initial fruit dehiscence occurring after 77 days after anthesis.

KEYWORDS

Bixa orellana;
Bixaceae;
Maturation;
Pigment;
Dormancy.

INTRODUCTION

The pigment extracted from the pericarp of annatto seeds, a shrub native Brazil and other tropical regions of the planet, gets calleding international annatto is widely used in various parts of the world on an industrial scale, for checking attractive coloration to a wide range of products. The annatto is a mixture of coloring pigments yellow-orange by the presence of various carotenoids absolute predominance of an atypical, known as bixin. The producers of annatto are Peru, Brazil and Quênia[6].

The annatto seed pericarp has a rich bixin which is soluble in organic solvents and being the predominant pigment in the seeds. The norbixin is found in small amounts in seeds and can be obtained by saponification of bixin in alkaline medium[17].

Propagation of this species is generally accomplished through seeds. It can be done through direct sowing in seed beds or plastic bags, with the fruits being harvested at their most adequate maturation stage and the seeds dried under the shade to allow them a high germination.

During maturation phase, after egg fertilization, some changes become more prominent, especially changes in size, water content, fresh and dry mass, germinative capacity and vigor, besides other visible alterations in the external aspect of the fruits and seeds, such as: shape, color, senescence and stoppage of translocation of reserve substances from the plant to the seed. However, besides alterations in physiological quality, changes may also occur in the colorant contents of annatto seeds, and according to Mendes et al.[13], harvesting must be conducted at 76 days after the opening of the first inflorescence flower.

When a viable seed is maintained under favorable conditions for its germination and it is unable to germinate, it is known to be in a dormant state[15]. Dormancy can be divided into two categories: extra embryonic, in which the structures involving the embryo such as the coat, pericarp, endosperm and floral organ remainders exert some physical resistance the embryo is unable to overcome; and, the embryonic, due to the presence of germination inhibitors[3]. The factors determining this phenomenon are: immature or rudimentary embryo, impermeability to oxygen, impermeability to water, mechanical restrictions, dormant embryo and the presence of inhibiting substances.

This work aimed to determine the physiological maturation point of the annatto seeds, cultivar Casca Verde, by characterizing *in situ* the histology of the coat of the seeds and quantifying their bixin content.

EXPERIMENTAL

To study the physiological maturation of the seeds, the flowers were labeled and the fruit harvested weekly.

The fruits formed from the labeled flowers were collected and immediate evaluation. The seeds were extracted manually and the following determinations were conducted: diameter, length, weight and fruit color – by using a paquimeter and electronic scale; water content (%) and dry mass of the fruit (g) – determined by the oven method at 105 ± 3^0C, during 24 h, according to Seed Analysis Rules (Brasil, 2009); seed color – visually determined, at the moment the seeds were extracted from the fruits; water content (%) and seed dry mass (g) – obtained by the oven method at 105 ± 3^0C, during 24 h, according to Brasil (2009), with two subsamples of 50 seeds, weighed in an electronic scale; germination– carried out using four sub-samples of 50 seeds sown in germination paper, moistened in distilled water equivalent to 2.5 times its dry substrate weight. After placing the seeds, the paper was rolled and the rolls placed in germinator at 30°C. Evaluations were conducted daily after setting the test and the percentages of normal seedlings, abnormal seedlings, dormant seeds and dead seeds were computerized[4]; germination velocity index – determined by means of daily count of the number of germinated seeds, i.e., those presenting protrusion of major root longer or equal to two millimeters and calculation performed according to Maguire[12]; colorant content quantification – the colorant was extracted from the annatto seeds under different maturation stages, according to Stringheta et al.[22]. This work applied the KOH methods, through which norbixin is extracted from the annatto seed pulp and is converted into bixin through ebullition. Following pigment extraction, the samples were analyzed in a spectrophotometer and the results converted into bixin percentages by applying the following correction factor: DO x 6.97 = % Bixin. Optical Density (DO) is determined with sample registration in 480 nm; histological lamina ware acquisition – seeds were used from the third maturation stage on, removed from five fruits at the same maturation stage and stored in aluminum capsules kept in the freezer, aiming to interrupt cellular activities. For the anatomic studies, the seeds were fixed in FAA_{50} (50 ml of formaldehyde, 50 ml of glacial acetic acid, 900 ml of ethanol 50%) for 24 h, maintained in a desiccator under vacuum[10] and stored in 70% ethanol. To obtain the cuts, the seeds were included in metacrylate (Historesin Leica). The samples were dehydrated in ethylic series and until included in methacrylate, the flasks were kept in a desiccator under vacuum. The blocks were submitted to automatic advance rotatory microtome, to obtain longitudinal cuts, 6 micrometer wide, being distended in recipients containing distilled water, placed on histological laminas and browned with toluidine blue at acid pH for 13 minutes at ambient temperature to detect anionic radicals and metachromasy[14]. The laminas were mounted with synthetic resin. Photographic analysis and documentation were conducted in a microscope equipped with a photographic system.

The experiment was arranged in a completely randomized design, with four repetitions of 20 fruits and 50 seeds. The physiological maturation data were analyzed, with the polynomial regression model being established as the level of significance of the tests ($p < 0.05$). Germination percentage, bixin content and germination velocity index were obtained through histograms.

RESULTS AND DISCUSSION

In this work, flowering, corresponding to the period from initial to complete formation of the floral buds, started mid March until early June. Fruit formation occurred only at 14 days after anthesis.

TABLE 1 lists the physical and morphological characteristics of *Bixa orellana* for fruits and seeds in different harvest times. Considering the fruits harvested at 14, 21, 28, 35, 42, 49, 56, 63, 70, 77 and 84 days after anthesis, the effect of fruit age on length and diameter was verified, reaching a larger dimension at 49 days after anthesis, with these characteristics remaining practically unchanged after this period.

TABLE 1 : Physical and morphological parameters of the fruits and seeds of annatto (*Bixa orellana* L.) Casca Verde cultivar, collected at 84 days after anthesis

Days after anthesis	Fruit length (cm)	Fruit diameter (cm)	Fruit coloration	Seed coloration
14	1.0	0.6	Green	Peach
21	3.0	2.0	Green	Rose
28	3.8	2.7	Green	Red
35	3.8	2.5	Green	Red
42	3.7	2.5	Green	Red
49	3.9	2.9	Green	Red
56	3.7	2.5	Green	Red
63	3.8	2.8	Green	Red
70	4.1	2.8	Green	Red
77	3.3	2.	Brownish-green	Dark-red
84	3.4	2.7	Dark-brown	Dark-red

The fruits harvested up to 70 days after anthesis presented a green coloration. From this stage on up to 84 days, the shades started to change, acquiring slightly darker shades, changing into brownish-green, becoming darker (dark-brown), and acquiring a drier aspect up to 77 days, in agreement with Franco et al.[8]. At 84 days, dehiscence was observed to initiate due to an opening through which the seeds are liberated, although some fruits remained closed (Figure 1). Similarly, the seeds presented a continuous coloration changing process, turning from peachy, at 14 days after anthesis to rosy coloration after 21 days, remaining so up to 77 days after anthesis, acquiring a black coloration at 84 days.

Using characteristics such as fruit and seed coloration to determine the physiological maturation of annatto seeds has been found to be quite efficient, since these characteristics are relatively easy to be correlated with physiological maturity, in agreement with observations made on calendula by Silveira et al.[20].

The variables: water content and seed dry mass; and water content and fruit dry mass, fresh mass, and diameter fitted the polynomial regression model (Figure 2).

Seed dry mass had a behavior inversely proportional to seed water content (Figures 2A and 2B). The seeds extracted from fruits harvested 14 days after anthesis, i.e., during growth phase, showed mean water content of 83.39%. Such content ranged from 85.1 to 81.2% of water in the assessments carried out 21 and 42 days after anthesis, as characterized by the curve obtained from the regression equation ($R^2 = 0,86$).

Dry mass of the seeds reached a maximum value (1.26 mg $seed^{-1}$) at 77 days after anthesis ($R^2 = 0.85$), while moisture reached values of 34.35% ($R^2 = 0.86$), during the same period. Fruit water content (Figure 2C) remained more or less constant until the 9th collection, 70 days after anthesis (74, 69%), reducing progressively from that point on. At 77 days after an-

Figure 1 : Annatto tree fruits at different stages of maturation

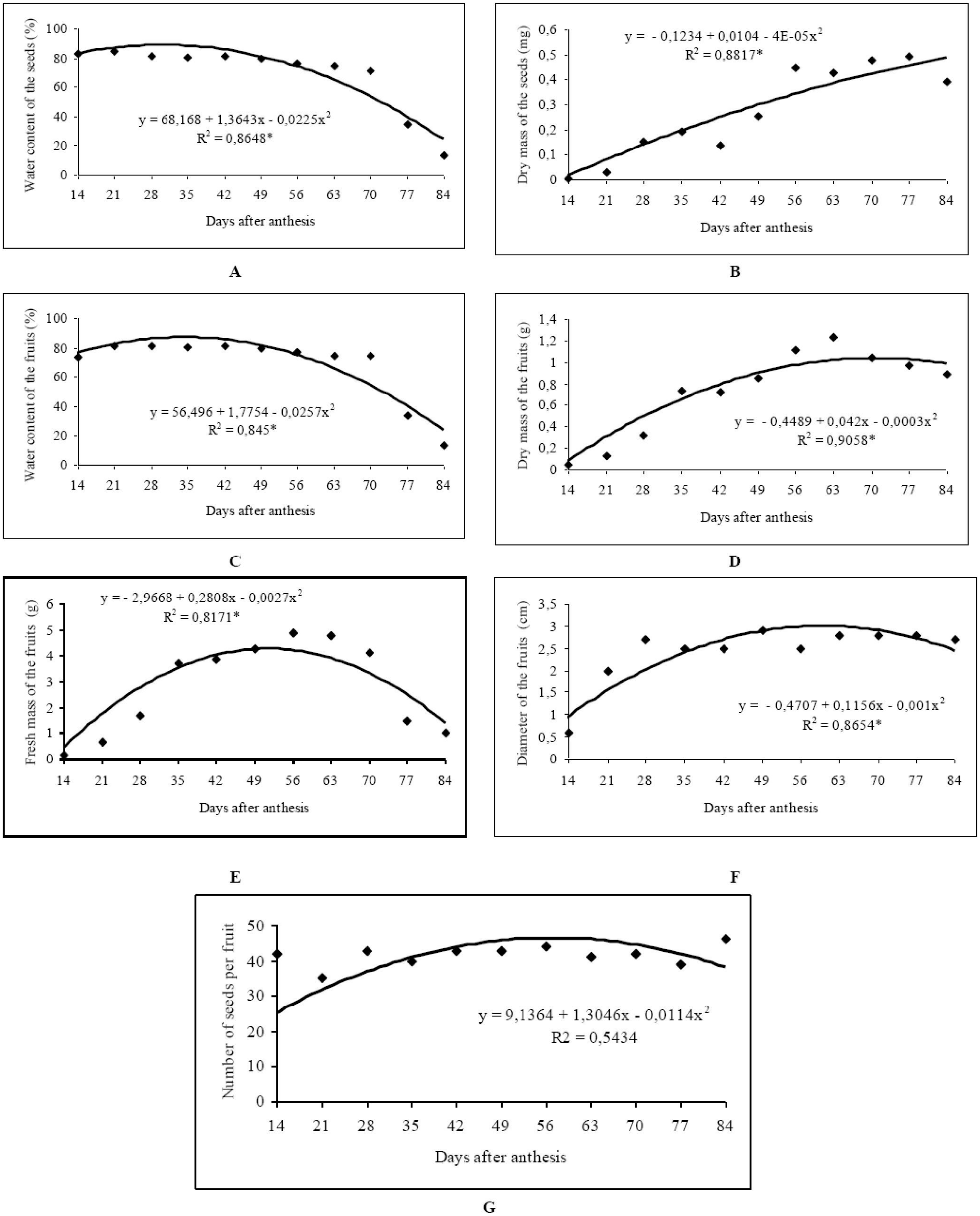

Figure 2 : Water content (A) and dry mass (B) of the seeds; water content (C), dry mass (D),fresh mass (E) and diameter (F) of the fruits and number of seeds per fruit (G) of annatto (*Bixa orellana* L.), Casca Verde cultivar, in function of days after anthesis

Full Paper

thesis, the fruits had a marked drop in humidity content (33. 8%), and continued dropping until 84 days after anthesis (11^{th} collection with the fruits then presenting a water content of 13.73%, as shown by the regression curve ($R^2 = 0.84$) and opaque, dark brown coloration, with initial dehiscence and seeds with very intense red coloration, initiating a process of oxidation, turning to black coloration, easily coming off from the interior of the fruits.

Fruit dry mass (Figure 2D) presented a value of 0.04 g fruit^{-1} 14 days after anthesis, reaching 0.89 g fruit^{-1} after 84 days ($R^2 = 0.90$). During this period, the fruits presented a dark-brown coloration. Fruit dry mass (Figure 2E) increased up to 35 days after anthesis, remaining more or less constant up to 70 days, and markedly dropping from this point on ($R^2 = 0.81$). Fruit diameter (Figure 2F) showed progressive growth until 28 days after anthesis, remaining unchanged after this period, while fruit mass increased up to 56 days, reducing after 70 days, reaching values of 1.03 g after 84 days.

The number of seeds per fruit (Figure 2G) did not show significant alteration during maturation phase, suggesting that the number of seeds formed in the fruit may be more related with pollination and fertilization rate than with age of fruit. Mean seed number per fruit is 42, with the fruit with least weight (0.17 g) displaying 42 seeds and the fruit with most weight (4.87 g) displaying 44 seeds.

Absence of germination by the seeds extracted from the fruits collected from 14 to 49 days indicates their physiological maturity. Germination (protrusion of main root of first seed) started only 56 days after anthesis (Figure 3A), without, however, originating a normal seedling, according to Brasil[4]. Higher germination percentage was obtained 77 days after anthesis (22.5%), with higher vigor being also verified during this time (Figure 3B).

Figure 4 shows variations in bixin content (%) and production (kg ha^{-1}). During the first maturation stages (35, 42, 49 and 56 days after anthesis), pigment content remained more or less constant (0.64%, 0.65%, 0.68%, 0.68%, respectively), likely due to the fact the seeds did not accumulate a substantial amount of pigment, as it can be seen in Figure 5. These results disagree with those obtained by Kato et al [11], who, study-

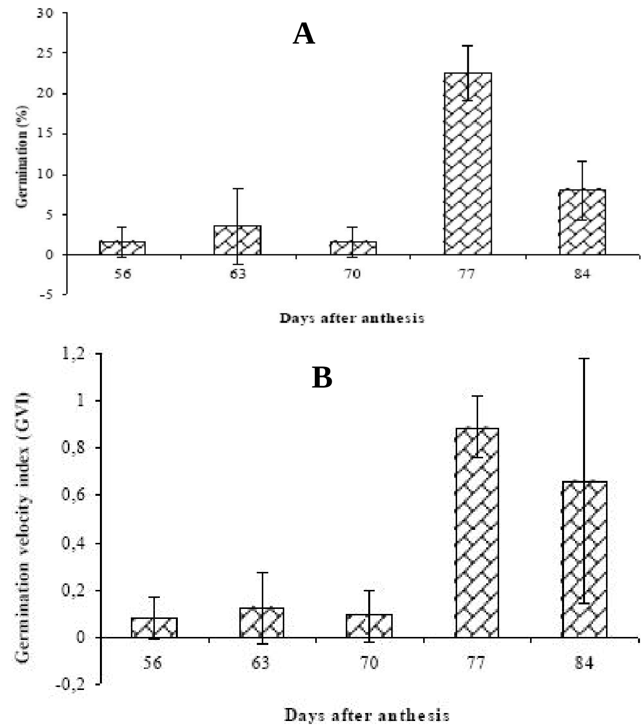

Figure 3 : Germination and germination velocity index of seeds of annatto (*Bixa orellana* L.), Casca Verde cultivar, at different stages of maturation

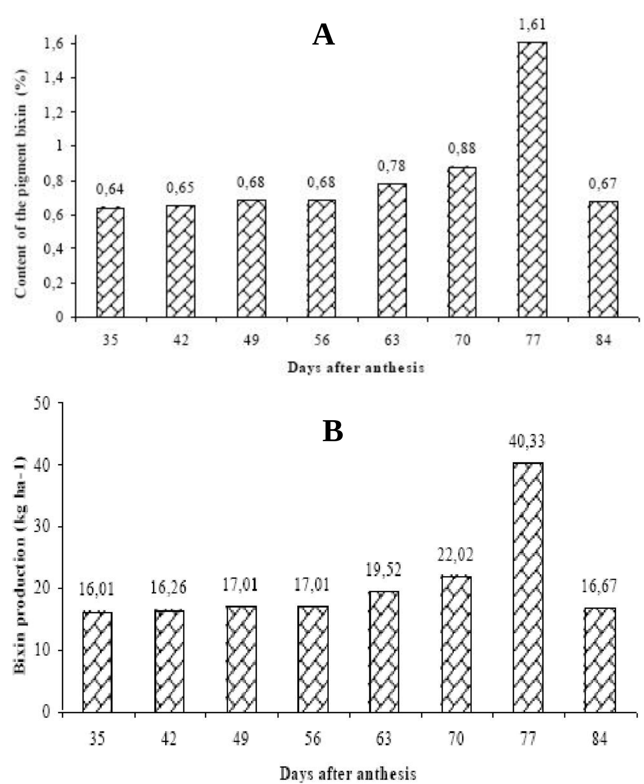

Figure 4 : Variation in the content of the pigment bixin and bixin production (kg ha^{-1}) from seeds of annatto (*Bixa orellana* L.), Casca Verde cultivar, at different stages of maturation

ing annatto seed harvest time, verified that only 30 to 51 days after the opening of the first inflorescence flower, the seeds showed acceptable bixin contents. If it takes too long for the seeds to be harvested, they undergo an oxidation process, acquiring a black coloration.

At 84 days after anthesis, a decrease in bixin content was verified (0.67%), as visualized in Figure 4F. The teguments were highly permeable and lignified at the last developmental stage of the annatto seeds, decreasing the imbibition's rate, which is one of the causes of dormancy in seeds of such species[2,7]. Lignification generally occurs in cells of the vascular tissue, found in almost all organs, and being most abundant in stems and roots[16]. Lignin is one of the greatest plant polymers, playing an important role in plant adaptation to the environment, and thus, suggesting, that, besides its contribution to plant adaptation in the evolution pro-

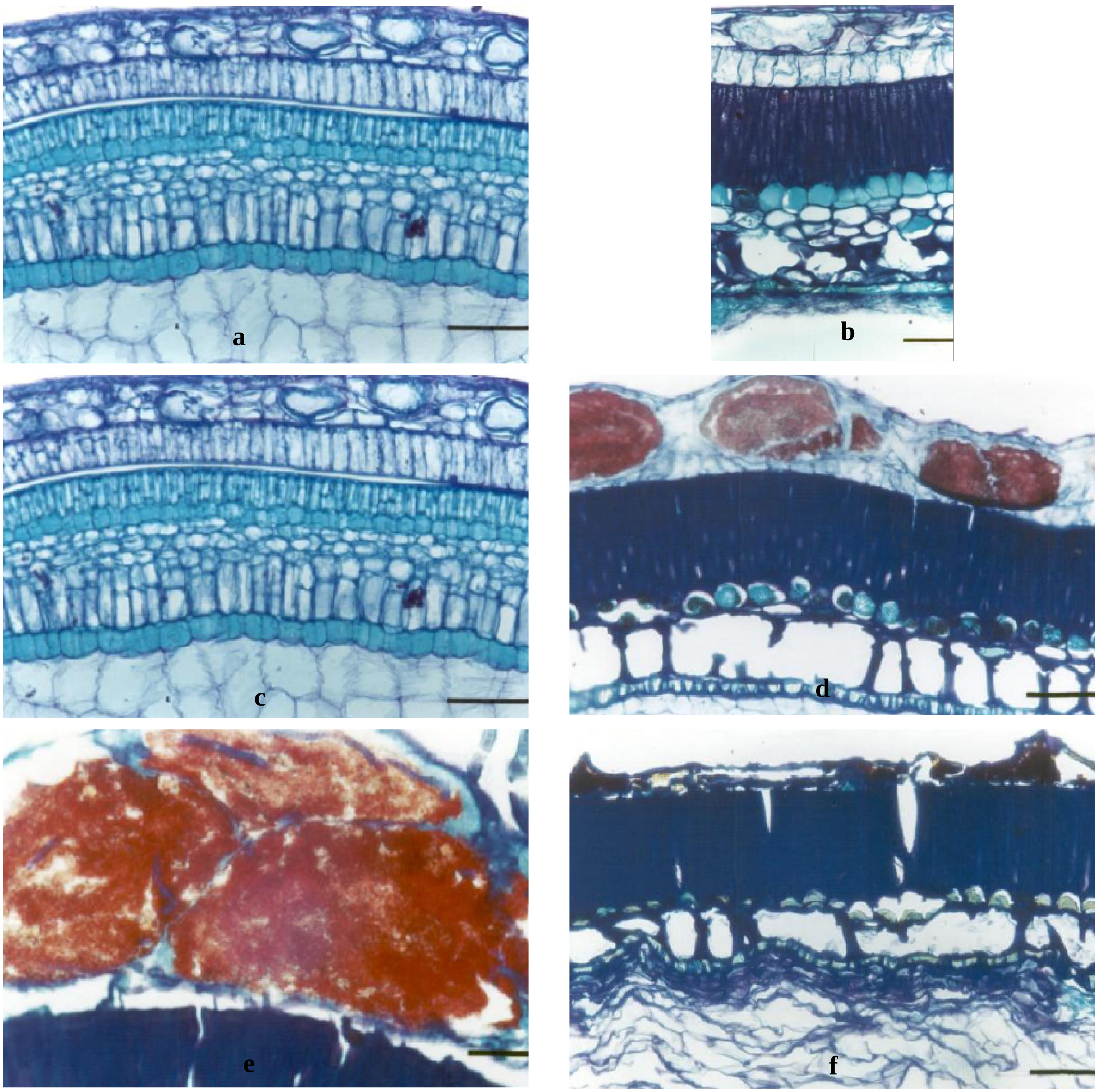

Figure 5 : Histological tegument cuts showing bixin secretory channels in seeds of annatto at 21 days after anthesis (A), 35 days after anthesis (B), 56 days after anthesis (C); 70 days after anthesis (D), 77 days after anthesis (E) and 84 days after anthesis (F). Scales: A (50 µm), B (50 µm), C (50 µm), D (50 µm), E (20 µm), and F (100 µm).

cess, it also makes cellular wall impermeable, ensuring water transportation and vascular system solutions.

Besides fruit dehiscence, bixin oxidation was also found to occur 84 days after anthesis, leading to a decrease in its content, what can be confirmed by the channel contents coloration (Figures 4A and 5F). At this stage of maturation, the oxidation process was found to promote a darkening of the seeds, giving them a dark red coloration, with blackened spots.

As the seed starts to form (Figure 5A), after 21 days, the external coat and internal coat cells remain distinct, though not completely differentiated. In the external coat, secretory channels (Figure 5A) can be found where, at more advanced stages, the pigment bixin will be formed (Figures 5D, 5E).

The differentiation of the idioblasts and the cellular layer occurring below these can be observed 35 days after anthesis (Figure 5B). The macrosclereids show a high level of lignification on their walls, made evident by the bluish coloration of toluidine blue. At more advanced maturation stages, such as 56 days after anthesis, an increase in lignification can be observed (Figure 5C), with more accentuated metachromasy.

At 70 days after anthesis, formation of the pigment bixin is observed in the secretory channels (Figure 5A). At this stage, the coat content showed an increase (0.88%), reaching 1.61% at 77 days after anthesis, a maturation phase with a higher colorant concentration (Figures 5D and 5E).

Rolston[18] also showed that the internal tegument becomes quite thick during the development of *Bixa orellana* seeds, showing, when mature, a pattern similar to that of the arboreous leguminous plants, which are water-impermeable, a type of dormancy very frequent in this family as well as in *Bauhinia ungulata*[1]; *Mimosa caesalpiniaefolia*[5]; *Bowdichia virgilioides*[19,21]; *Lotus subbflorus*[9].

The results obtained in this work allowed concluding that: physiological maturation of the seeds is reached at 77 days after anthesis; fruit dehiscence occurs at 84 days after anthesis; the seeds show dark red coloration at 84 days after anthesis; the indices water content and dry matter weight best characterize seed maturation and harvest time; a higher germination percentage and higher bixin contents are obtained 77 days after anthesis, when harvest must be carried out; macrosclereids at 84 days after anthesis are rather lignified, with high metachromasy evidenced *in situ*, by toluidine blue.

ACKNOWLEDGMENTS

We thank CNPq, for granting a scholarship to the first author and the students and technicians of the Laboratory of Pigments and Drying of the Department of Food Technology of the UFV, for the assistance provided during colorant extraction.

REFERENCES

[1] M.C.S.Alves, S.Medeiros-Filho, M.Andrade-Neto, E.M. Teófilo; Rev.Bras.Sem., **22**, 2 **(2000)**.

[2] L.I.V.Amaral, M.F.D.A.Pereira, A.L.Cortelazzo; Rev.Bras.Fisiol.Veg., **12**, 3 **(2000)**.

[3] J.D.Bewley, M.Black; Seeds: physiology of development and germination. 2ndEdition., Plenum Press, New York and London, **(1994)**.

[4] Brasil. Ministério da Agricultura e Reforma Agrária. Regras para análise de sementes, SNDA/DNDV/ CLAV, Brasília, **(2009)**.

[5] R.L.A.Bruno, E.U.Alves, A.P.Oliveira, R.C.Paula; Rev.Bras.Sem., **23**, 2 **(2001)**.

[6] C.L.S.Costa, M.H.Chaves; Quim.Nov., **28**, 1 **(2005)**.

[7] C.C.Custódio, N.B.Machado-Neto, R.F.Caseiro, M.Ikeda, D.C.Bomfim; Rev.Bras.Sem., **24**, 1 **(2002)**.

[8] C.O.F.Franco, F.C.P.Silva, J.Cazé-Filho, M.Barreiro-Neto, A.R.São José, T.N.H.Rebouças, I.S.C.Fontinélli; Manejo da cultura do urucum (Bixa orellana L.), EMEPA, Paraíba, **(2001)**.

[9] E.A.Jacob-Júnior, G.E.Meneghello, P.T.B.S.Melo, M.S.Maia; Rev.Bras.Sem., **26**, 2 **(2004)**.

[10] D.A.Johansen; Plant microtechnique, Mcgaw, New York, **(1940)**.

[11] O.R.Kato, F.J.C.Figueirêdo, A.J.Belfort, O.L.Nogueira, W.C.Barbosa; Pesq.Agro.Bras., **27**, 9 **(1992)**.

[12] J.B.Maguire; Crop Sci., **2**, 2 **(1962)**

[13] A.M.S.Mendes, A.F.Figueiredo, J.F.Silva; Rev.Bras.Sem., **28**, 1 **(2006)**.

[14] T.P.O'Brien, M.E.McCully; The study of plant structure: principles and selected methods, Termarcarphi Pty, Melbourne, **(1981)**.

[15] F.Popinigis; Fisiologia da semente, AGIPLAN, Brasília, **(1985)**.

[16] J.Raes, A.Rohde, J.H.Christensen, Y.V.Peer, W.Boerjan; Plan.Phys., 133 **(2003)**.

[17] A.O.Rios, A.Z.Mercadante; Alim.Nutr., **15**, 3 **(2004)**.

[18] M.P.Rolston; Bot.Rev., 44 **(1978)**.

[19] L.S.V.Sampaio, C.P.Peixoto, M.F.S.P.Peixoto, J.A.Costa, M.S.Garrido, L.N.Mendes; Rev.Bras.Sem., **23**, 1 **(2001)**.

[20] M.A.Silveira, F.A.Villela, M.A.A.Tillmann; Rev.Bras.Sem., **24**, 2 **(2002)**.

[21] O.J.Smiderle, R.C.P.Souza; Rev.Bras.Sem., **25**, 12 **(2003)**.

[22] P.C.Stringheta, J.M.O.Mascarenhas, L.O.Pontes, J.N.Silva, F.P.Reis; Rev.Bras.Cor.Nat., 3 **(1999)**.