Morphological Development of the North American Pawpaw during Germination and Seedling Emergence

Cynthia H. Finneseth and Desmond R. Layne¹

Land-Grant Program, Kentucky State University, Frankfort, KY 40601

Robert L. Geneve

Department of Horticulture and Landscape Architecture, University of Kentucky, Lexington, KY 40547

Additional index words. *Asimina triloba*, biomass allocation, dormancy, haustorial cotyledons, Annonaceae, cryptocotylar

Abstract. The North American pawpaw [*Asimina triloba* (L.) Dunal], a temperate member of the Annonaceae, is a deciduous woody tree with ornamental value and has merit as a fruit crop. Anatomical studies of pawpaw seed revealed a small, linear embryo that does not change in length during cold or warm stratification. Radicle and cotyledon growth from planting until radicle protrusion was concurrent and at about the same rate. Cotyledons grew through a specialized channel of cells extending above the cotyledon tips, but never emerged from the seed. The extended period of time required for the development of the cotyledons delayed seedling emergence more than 50 days. The cotyledons appear to be haustorial and translocate storage material from the endosperm to the growing embryo. At the time of epicotyl elongation, the radicle and developing root system were well developed and comprised 81% of the seedling biomass. Seedling development could be divided into four distinct stages, including radicle protrusion, hypocotyl emergence, epicotyl elongation, and seedcoat abscission.

The North American pawpaw, a temperate member of the tropical Annonaceae or custard apple family (Darrow, 1975) is a deciduous woody tree with value as an ornamental and fruit crop (Layne, 1996). The native range of the pawpaw is primarily Atlantic North America, from Florida to southern Ontario, but trees have been reported as far west as eastern Nebraska and Oklahoma (Darrow, 1975). The fruit is an oval to oblong berry weighing up to 1 kg, making it the largest tree fruit native to the United States (Darrow, 1975; Peterson, 1991). In addition to its potential as a landscape tree and fruit crop, pawpaw is also a potential source of botanical and medicinal extracts (Zhao et al., 1994).

Seed anatomy and seedling development have been outlined for many dicotyledons, including a limited number in the Annonaceae

(Corner, 1948, 1976; De Vogel, 1980; Hayat, 1963; Hayat and Canright, 1965, 1968; Mohana Rao, 1982; Periasamy and Swamy, 1961). However, little information has been published concerning North American pawpaw. Studies have described reproductive biology (Willson and Schemske, 1980), floral morphology (Lampton, 1957), seed and fruit anatomy (Mohana Rao, 1982), and embryo formation (Hermes, 1907), but no data are

known on the morphological changes that occur during seed germination and seedling emergence in pawpaw.

Pawpaw has a characteristic large seed that averages 3 cm in length and 1.5 cm in width. The dark-brown seed shape is oblong, rounded, and flat (Corner, 1976; Vines, 1960), and contains a white endosperm with thick and irregular ruminations (Corner, 1976; Mohana Rao, 1982). Embedded within the persistent endosperm is a small embryo that is dicotyledonous and apical (Mohana Rao, 1982). The testa is fibrous and 16 cell layers thick (Corner, 1976; Mohana Rao, 1982).

Pawpaw seeds are recalcitrant and germination percentages were reduced when seed moisture was <25% (Finneseth et al., 1998). Some seed lots of pawpaw also required at least 80 d of chilling stratification at 5 °C to relieve endogenous physiological dormancy (Finneseth et al., 1998). The embryo of pawpaw is very small relative to the large endosperm and can be described as morphologically dormant. Whether the embryo develops during or after moist chilling stratification is not known. Seed germination and seedling emergence require an extended period of time (>40 d) and can be sporadic (Bonner and Halls, 1974).

In the commercial nursery trade, pawpaw is clonally propagated by budding and grafting named cultivars onto seedling rootstocks. Stem cutting and micropropagation techniques to clonally propagate pawpaw have met with limited success. Until alternative clonal propagation systems are developed, pawpaw seedlings will be valuable to commercial nurseries as rootstocks and as species trees.

The objective of this study was to observe changes in morphological development of the embryo and surrounding tissues in pawpaw seeds during germination to better understand the relatively long time required for seedling emergence.

Received for publication 15 Oct. 1997. Accepted for publication 3 Feb. 1998. This research was supported by USDA 1890 Institution Capacity Building grant no. 95-38814-1721 to DRL and is Univ. of Kentucky Experiment Station Publication no. 97-11-150. The authors gratefully acknowledge R. Neal Peterson of the Pawpaw Foundation for seed used in this study. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

¹Former principal investigator of horticulture and curator, USDA National Clonal Germplasm Repository for *Asimina* spp. Current address: Assistant Professor of Horticulture, Dept. of Horticulture, Box 340375, Clemson Univ., Clemson SC 29634. To whom reprint requests should be addressed.

Fig. 1. Developmental stages of North American pawpaw (*Asimina triloba*) during germination and seedling emergence. Pairs of seeds, from left to right, illustrate radicle protrusion (day 12), hypocotyl emergence (day 27), epicotyl elongation (day 40), seed abscission (day 50), and seedling (day 70). Scale bar represents 2.5 cm.

Materials and Methods

In Fall 1994, seeds were extracted from ripe fruit from trees in the pawpaw orchard at the Western Maryland Research Experiment Station, Keedysville. These trees were grown from seeds collected from native stands along the C&O Canal, Md., and Prince George's County, Md. After macerating the pulp and floating off the fruit flesh, seeds were washed and surface disinfested using 10% bleach solution for 10 min, followed by three rinses with sterile deionized water. Cleaned seeds were mixed with moist peatmoss and stored in fastened plastic bags at 5 °C for 12 months.

Cold-stratified pawpaw seeds were sown 3 cm deep in two Styrofoam coolers (39 × 26.5 × 31 cm) filled with 227 cm³ of vermiculite and placed in a growth chamber (1-60LLVL; Percival Mfg. Co., Boone, Iowa). About 100 seeds were planted in each container. Air temperature was 25 °C, and photoperiod was 16 h of 35 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ light photosynthetically active radiation (PAR) supplied by cool-white fluorescent lamps. Every 2 d, 500 mL of water was applied to the substrate surface and excess water was allowed to escape through drainage holes.

Ten seedlings were destructively harvested at 3-day intervals prior to radicle protrusion and at 5-day intervals thereafter. Fresh and dry mass were determined for the entire seed and for individual components, including the endosperm, radicle, hypocotyl, cotyledons, and epicotyl, but not the seedcoat. Total embryo or seedling length was measured as well as individual lengths for radicle, hypocotyl, epicotyl, and the longest cotyledon.

To record lengths prior to radicle protrusion, seeds were sectioned and stained or embryos were excised from germinating seeds. For sectioned seeds, the seedcoat was removed and a small portion (4 × 4 × 2 mm) of endosperm containing the embryo was removed from the hilar end of the germinating seed. Tissue samples were fixed in formalin-acetic-alcohol (FAA) and vacuum-infiltrated for 24 h. Tissue was rinsed twice with 50% ethyl alcohol and dehydrated using a tertiary butyl alcohol dehydration series. Tissue was then embedded in paraffin blocks, attached to a rotary microtome (model 820; American Optical, Buffalo, N.Y.) and sectioned at 12 to 15 μm . Tissue sections were affixed to glass slides (8 × 3 cm) and stained with safranin-fast green.

Embryos were excised by removing the seedcoats and placing the decoated seeds in a glass beaker with 100 mL of boiling water. After 2 min in boiling water, seeds were removed, and the embryo was separated from the endosperm using a scalpel and placed on a glass slide. Initial radicle and cotyledon growth rate were compared using slopes from linear regression of data transformed to common logs.

Slides of excised embryos and tissue sections were placed under a compound microscope (model BX-40; Olympus Corp., Japan) equipped with a CCD camera (8210 Cohu Inc., San Diego) and digital images were cap-

tured using a MacIntosh Quadra 700 computer (Apple Computer, Cupertino, Calif.) and MediaGrabber software (RasterOps Corporation, Santa Clara, Calif.). Embryo lengths were measured using IP Lab Spectrum software (Vienna, Va.). At 0 and 3 d, radicle length was measured from the radicle tip to the base of the cotyledons and cotyledon length was measured from the base of the petiole to the tip of the longest cotyledon. At days 6 and 9, radicle length was measured from the growing tip to the hypocotyl base, distinguishable by a darkened ridge and enlargement of the radicle. Hypocotyl length was measured from the radicle-hypocotyl juncture to the cotyledonary node and cotyledon length as previously described.

After radicle protrusion, 10 seedlings were randomly chosen and destructively harvested at 5-day intervals for seedling length and fresh-mass determinations. In addition to previously described measurements, epicotyl length was measured from the cotyledonary node to the

tip of the unfolded leaf covering the growing meristem.

Fresh mass of each seed was recorded before removing the seedcoat and dissecting the seed into respective components. Seedling components were separated and fresh mass was determined for the radicle, hypocotyl, cotyledons, epicotyl, and endosperm. Tissue was dried in paper envelopes at 50 °C for 5 d to determine dry mass for the seed and seedling components.

Results

Seed germination and seedling development consisted of four distinct developmental stages (Fig. 1), including radicle protrusion, hypocotylemergence, epicotyl elongation, and seedcoat abscission.

A small embryo with a distinguishable radicle and cotyledons was at the hilar end of the seed, surrounded by a ruminant endosperm (Fig. 2). Extending from the tips of the cotyle-

Fig. 2. (A) Photomicrograph of a longitudinal section of a pawpaw seed at 9 d after planting, revealing dark-stained embryo (Em), ruminant endosperm (En), and specialized endosperm channel cells (arrows). Scale bar represents 1 cm. (B) Close-up of endosperm (En) and specialized channel cells (arrows). Scale is 5× that of Fig. 2A.

Fig. 3. Changes in total seedling and seedling constituents during germination and emergence of pawpaw. (A) Fresh mass; (B) dry mass; (C) length. Bars represent $\pm$ SE.

dons were two parallel channels of cells that stained differently from the embryo or endosperm tissues (Fig. 2 A and B).

Embryo length did not significantly increase during cold (12 months at 5 °C) or warm stratification (12 months at 25 °C). Following stratification, mean radicle and cotyledon lengths were 0.70 and 0.36 mm, respectively. Seedling length, and seed and embryo fresh and dry mass increased after planting, but endosperm fresh and dry mass decreased as embryo development progressed (Fig. 3). Initial embryo dry mass was <1 mg and <1% of the total dry mass of the seed with the seedcoat removed. Hypocotyl and epicotyl were not visible at this stage of development.

At radicle protrusion (day 12), the hypocotyl was visible, and radicle, hypocotyl, and cotyledon lengths were 3.7, 2.8, and 3.4 mm, respectively. Cotyledons had become spatulate and had expanded through the channel

cells to about half the length of the seed. The endosperm still comprised 99.2% of the total dry mass at this time. Radicle and cotyledon growth began at the same time and growth rates for the two organs were the same until radicle emergence (comparison of linear regression line slopes of log transformed data $P < 0.05$; Fig. 4).

Hypocotyl emergence occurred at day 27 and average lengths of radicle, hypocotyl, and cotyledons were 48.3, 18.9, and 13.3 mm, respectively. Cotyledons had expanded to fill nearly three-fourths of the length of the seed at this stage, and the embryo comprised 32.7% of the total dry mass.

At day 45, cotyledons had expanded to fill the length of the seed as the epicotyl began to elongate. The embryo (seedling) was 70.3% of the total dry mass and 76.9% of this mass was in radicle and root tissue.

On day 50, cotyledons spanned the length

of the seed and were shed with the seedcoat and remaining endosperm. Average radicle, hypocotyl, and epicotyl lengths were 183.6, 91.6, and 14.2 mm, respectively. Endosperm had decreased to <30% of the total dry mass, while the radicle and lateral root system represented 81% of the seedling dry mass.

Discussion

Pawpaw seeds are recalcitrant and showed combinational dormancy that required stratification treatment (>80 d at 5 °C) to satisfy an endogenous physiological dormancy and the development of a morphologically underdeveloped embryo when the seeds were exposed to 25 °C (Finneseth et al., 1998). There were no aboveground signs of germination until 45 d after planting (Fig. 1).

The embryo in freshly harvested seed was only $\approx$ 1 mm long, but the radicle and cotyledons were easily distinguished at the hilar end of the seed. However, during the stratification treatment, embryo length did not change significantly and was <1% of the dry mass of the seed excluding the seedcoat (Fig. 3).

The ruminant endosperm and small embryo have been documented for pawpaw and other Annonaceous species (Hayat, 1963). Morphological development of embryos differs among species in the Annonaceae. Rizzini (1973) described *Annona squamosa* as having a small (2–3 mm long), well-developed embryo consisting of half cotyledons and half hypocotyl. Embryos in *A. crassiflora* are also small (2 mm long), but organized into a poorly differentiated cylindrical, hyaline mass. Embryos in *A. reticulata* are soft and hyaline (1 mm long), similar to *A. crassiflora*, but are differentiated into visible hypocotyl and cotyledons (Rizzini, 1973). Pawpaw embryos are similar to those found in *A. squamosa* and *A. reticulata* in being small, but having easily distinguishable features.

Cotyledons were observed growing through two specialized channels of cells extending above the cotyledon tips (Fig. 2). The channels were easily distinguished from endosperm tissue by the difference in staining pattern. During elongation of the cotyledons through the channels, the specialized endosperm cells may aid in nutrient absorption and translocation to the growing embryo or the cells may be passage cells facilitating cotyledon growth.

Cotyledon and radicle growth occurred simultaneously in the germinating seed until radicle emergence (Fig. 4). In seeds with morphological dormancy, the cotyledons often develop within the seed prior to significant radicle growth (Nikolaeva, 1977; Selvaratnam, 1952; Steinbauer, 1937). After penetrating the soil surface, the hypocotyl hook elongated. During this developmental stage, the seedcoat containing the cotyledons sometimes remained subterranean, but most often was raised above the soil surface by the elongating hypocotyl. However, the cotyledons never emerged from the seed. When the seedcoat containing the cotyledons and residual endosperm was shed from the plant, the cotyledons were pale yellow. The change in dry mass and moisture

Fig. 4. Early development of the cotyledon and radicle of pawpaw. Lines represent regression of length vs. time and slopes of transformed data did not differ significantly at $P < 0.05$. Data presented are not transformed. Cotyledon regression is $y = 0.269 + 0.053x + 0.011x^2$ and radicle regression is $y = 0.74 + 0.051x + 0.013x^2$.

content of the endosperm during germination supports the idea that the cotyledons were needed only as long as the endosperm was being metabolized. At the time of cotyledon abscission, the reduced dry mass of the endosperm indicated that the storage reserves had been metabolized and translocated to the developing embryo. When cotyledons were no longer needed for absorption and translocation of carbohydrates and other nutrients, the organs still contained within the seedcoat abscised.

Pawpaw exhibited a unique pattern of epigeal germination as the cotyledons were shed within the remaining parts of the seed. De Vogel (1980) classified this process as cryptocotylar. Hayat (1963) documented a germination pattern in *Annona squamosa*, where the seed parts remained subterranean. However, *Annona cherimola* cotyledons may be retained on the seedling when the seedcoat is shed (Hayat, 1963). Hayat (1963) suggested that the shedding of the cotyledons in some Annonaceae was a consequence of the ruminant endosperm. Differences between *A. squamosa* (cotyledons shed) and *A. cherimola* (cotyledons retained) may be related to the shallower ruminations in *A. cherimola* (Hayat, 1963). The current study indicated that pawpaw was similar to *A. squamosa* in seed anatomy and seedling development.

Callaway (1993) reported seedling emergence in pawpaw between 45 and 90 d after planting and Peterson (1991) indicated that radicle and epicotyl emergence occurred at ≈ 18 and 64 d, respectively. In this study, the first true leaves on the seedling occurred at 50

d. The extended period of time required for emergence was due to the need for development of haustorial cotyledons within the seed to transfer stored reserves from the endosperm to the radicle and developing root system (Figs. 1 and 3). The seedling did not display true leaves until 50 d after planting. At that time, the root system was well established (Fig. 1) and represented 81% of the seedling biomass. Although this represents a delay for the propagator, the establishment of a significant root system may present some ecological advantage. Seedling emergence may be hastened by increasing the temperature of the growing medium. Callaway (1993) reported that the rate and percentage of pawpaw seed germination was greater at 27 °C, while Peterson (1991) noted that germination and radicle elongation occurred most rapidly at 30 °C compared to cooler temperatures. Increasing substrate temperature from 24 to 32 °C decreased the time to seedling emergence by 10 d (Layne and Kwantes, 1995).

Literature Cited

- Bonner, F.T. and L.K. Halls. 1974. *Asimina*. Seeds of woody plants in the United States. For. Serv., USDA Agr. Hdbk. 450, Washington, D.C.
- Callaway, M.B. 1993. Pawpaw (*Asimina triloba*): A "tropical" fruit for temperate climates, p. 505–515. In: J. Janick and J.E. Simon (eds.). *New crops*. Wiley, New York.
- Corner, E.H.J. 1948. The Annonaceous seed and its four integuments. *New Phytol.* 48:332–364.
- Corner, E.H.J. 1976. Annonaceae, p. 68–86. In: *The seeds of dicotyledons*. v. 1. Cambridge Univ. Press, Cambridge, U.K.
- Darrow, G.M. 1975. Minor temperate fruits, p. 269–284. In: J. Janick and J.N. Moore (eds.). *Advances in fruit breeding*. Purdue Univ. Press, Lafayette, Ind.
- De Vogel, E.F. 1980. Annonaceae, p. 168–181. In: *Seedlings of dicotyledons*. Centre for Agr. Publishing and Documentation, Pudoc, Wageningen, The Netherlands.
- Finneseth, C.H., D.R. Layne, and R.L. Geneve. 1998. Requirements for seed germination in North American pawpaw [*Asimina triloba* (L.) Dunal]. *Seed Sci. Technol.* (In press.)
- Hayat, M.A. 1963. Morphology of seed germination and seedling in *Annona squamosa*. *Bot. Gaz.* 124:360–362.
- Hayat, M.A. and J.E. Canright. 1965. The developmental anatomy of the Annonaceae. I: Embryo and early seedling structure. *Amer. J. Bot.* 52:228–237.
- Hayat, M.A. and J.E. Canright. 1968. The developmental anatomy of the Annonaceae. II. Well-developed seedling structure. *Bot. Gaz.* 129:193–205.
- Hermes, W.B. 1907. Contribution to the life history of *Asimina triloba*. *Ohio Nat.* 8:211–217.
- Lampton, R.K. 1957. Floral morphology in *Asimina triloba* Dunal. I. Development of ovule and embryo sac. *Bul. Torrey Bot. Club* 84:151–165.
- Layne, D.R. 1996. The pawpaw [*Asimina triloba* (L.) Dunal]: A new fruit crop for Kentucky and the United States. *HortScience* 31:777–784.
- Layne, D.R. and M.G. Kwantes. 1995. The influence of root-zone temperature and fertilization regime on early growth and development of pawpaw (*Asimina triloba* Dunal) seedlings. *HortScience* 30:870. (Abstr.)
- Mohana Rao, P.R. 1982. Seed and fruit anatomy in *Asimina triloba*, with a discussion of the affinities of Annonaceae. *Bot. Jahrb. Syst.* 103:47–57.
- Nikolaeva, M.G. 1977. Factors controlling the seed dormancy pattern, p. 51–74. In: A.A. Khan (ed.). *The physiology and biochemistry of seed dormancy and germination*. Elsevier, Amsterdam, The Netherlands.
- Periasamy, K. and B.G.L. Swamy. 1961. Studies in the Annonaceae. II. The development of ovule and seed in *Canaga odorata* and *Milusa wightiana*. *J. Indian Bot. Soc.* 40:206–216.
- Peterson, R.N. 1991. Pawpaw (*Asimina*). *Acta Hort.* 290:567–600.
- Rizzini, C.T. 1973. Dormancy in seeds of *Anona crassiflora* Mart. *J. Expt. Bot.* 24:117–183.
- Selvaratnam, E.M. 1952. Embryo of the coconut. *Nature* 169:714–715.
- Steinbauer, G.P. 1937. Dormancy and germination of *Fraxinus* seeds. *Plant Physiol.* 12:813–824.
- Vines, R.A. 1960. Common pawpaw: *Asimina triloba* L. (Dunal), p. 289–290. In: *Trees, shrubs and woody vines of the southwest*. Univ. of Texas Press, Austin.
- Willson, M.F. and D.W. Schemske. 1980. Pollinator limitation, fruit production, and floral display in pawpaw (*Asimina triloba*). *Bul. Torrey Bot. Club* 107: 401–408.
- Zhao, G., L.R. Miesbauer, D.L. Smith, and J.L. McLaughlin. 1994. Asimin, asimiacin and asiminecin: Novel highly cytotoxic asimicin isomers from *Asimina triloba*. *J. Med. Chem.* 37:1971–1976.