Propagation of Pawpaw—A Review

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Additional index words. *Asimina triloba*, **cuttings, grafting, budding, seeds, recalcitrant, dormancy, layering, micropropagation**

Summary. **Pawpaw (*Asimina triloba*) is an under-exploited small tree with commercial potential as a fruit crop, ornamental tree, and source of secondary products with insecticidal and medicinal properties. It is most often propagated from seeds that are recalcitrant and must be stored moist at a chilling temperature. Seeds display combinational (morphophysiological) dormancy. Endogenous, physiological dormancy is broken by about 100 days of chilling stratification followed by a period of warm moist conditions where the small embryo develops prior to seedling emergence about 45 days after the warm period begins. Pawpaw cultivars with superior fruit characteristics are propagated by grafting onto seedling understocks. The most common practice is chip budding. Other methods of clonal propagation have proven problematic. Pawpaw can be propagated from cuttings, but only in very young seedling stock plants. Micropropagation from mature sources is not yet possible, but shoot proliferation has been accomplished from seedling explants and explants rejuvenated by induction of shoots from root cuttings of mature plants. However, rooting of microcuttings and subsequent acclimatization has not been successful.**

The pawpaw is a temperate member of the mostly tropical Annonaceae or custard apple family. Pawpaw has commercial value both as a small landscape tree, an orchard fruit crop, and as a source for novel secondary products (Layne, 1996). Research in pawpaw as a commercial crop began in 1990 (Callaway, 1992) at Kentucky State University (KSU) and became a collaborative effort between KSU and the University of Kentucky in 1995. In 1994, United States Department of Agriculture approved the initiation of an *Asimina* clonal germplasm repository located and supervised by KSU. In addition to maintaining an extensive orchard collection of pawpaw germplasm, one of the long-term objectives of our research program is to improve propagation in pawpaw to make it more available for commercial production. Pawpaw has some unique characteristics for seed germination and clonal propagation can prove challenging. The objective of this review is to provide an overview of propagation methods for pawpaw and highlight the research efforts from our program over the past 7 years.

Univ. Ky. Expt. Sta. Publ. 02-11-22. This research was funded through grants from U.S. Dept. of Agriculture Capacity Building grants no. 95-38814-1721 to DRL and no. 00-38814-9539 to KWP. Rgeneve@uky.edu.

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Seed propagation

Pawpaw produces a relatively large seed that averages 2.8 ± 0.1 cm (1.10 ± 0.04 inch) in length and 1.5 ± 0.1 cm (0.59 ± 0.04 inch) in width. It is a flat, spatulate seed with a dark brown seed coat that is fibrous and 16 cell layers thick (Corner, 1948; Mohana Rao, 1982). The ruminant endosperm occupies most of the seed cavity and is living at seed maturity. A small [about 1 mm (0.04 inch) long] rudimentary embryo is located at the hilar end of the seed and although small, it has easily distinguishable cotyledons and radicle (Finneseth et al., 1998a) (Fig. 1). Ruminant endosperm surrounding a small embryo is characteristic of seeds from annonaceous species (Hayat, 1963; Rizzini, 1973).

Seeds are usually extracted from the fruit after several days of fermentation in water (Hartmann et al., 2002). Care must be exercised in handling pawpaw seeds before germination because they are recalcitrant and sensitive to drying in storage (Bonner and Halls, 1974). As little as 5 d under open air conditions can reduce the moisture content of pawpaw seeds to 5% and result in total loss of viability (Finneseth et al., 1998a). Recalcitrant seeds can be classified as either highly, moderately or minimally recalcitrant based on their ability to withstand desiccation (Farrant et al., 1988). Pawpaw seeds display a moderate form of recalcitrance (Finneseth et al., 1998b). Seed moisture in mature seeds immediately removed from fleshy fruits is about 37%. This value does not rise significantly if seeds are imbibed. Seeds lose 50% viability when dried from their initial 37% to 25% moisture. Total loss in viability occurs between 15 and 5% moisture.

Pawpaw seeds must be stored moist at chilling [5 °C (41.0 °F)] temperature to retain viability in long term storage (Finneseth et al., 1998b). Under these conditions, seeds retained between 52% and 74% viability depending on the seed lot. However, germination was reduced to less than 25% after a third season of storage. In contrast, seeds stored moist at warm [25 °C (77.0 °F)] temperature lost between 40 and 65% viability after only 28 weeks in storage and no seeds remained alive after 52 weeks of storage.

In addition to being recalcitrant, pawpaw seeds display combinational (morphophysiological) dormancy (Nikolaeva, 1977) requiring a period of chilling stratification to satisfy intermediate physiological endogenous dormancy followed by a moist warm period to satisfy morphological dormancy (Finneseth et al., 1998b). Several references suggest that pawpaw seeds need to be stratified between 60 and 120 d (Dirr and Heuser, 1987; Reich, 1991; Young and Young, 1992). Pawpaw has a wide geographic distribution (Florida to Maine) and the varied times cited for stratification could reflect inherent variation found between provenances native to different locations. Using pawpaw seeds collected from six locations within Kentucky, Finneseth et al. (1998b) determined that about 7 weeks of chilling (5 °C) stratification was required to reach 50% germination and that the greatest germination percentage (84% to 90%) occurred after about 100 d of stratification.

Once endogenous dormancy is relieved, pawpaw seeds still exhibit morphological dormancy. Morphological dormancy is described as seeds containing either a rudimentary or linear embryo that is not fully developed at the time the seed is mature and occupies less than one-half of the seed cavity (Baskin and Baskin, 1998; Nikolaeva, 1977). Pawpaw has a small linear embryo that is about 1 mm in length at maturity (Finneseth et al., 1998a). The size of the embryo does not increase during chilling stratification. However, when stratified seeds are moved to warm conditions the cotyledons and radicle begin simultaneous growth at comparable rates (Finneseth et al., 1998a). The cotyledons grow through two specialized channels that are distinct from the rest of the ruminant endosperm (Fig. 1B), while the hypocotyl and radicle emerge from the seed coat. The expanding cotyledons eventually extend to the tip of the seed and it is postulated that the cotyledons act as haustorial structures transferring digested materials from the endosperm to the developing radicle (Finneseth et al., 1998a). The hypocotyl and radical continues to thicken to form a tap root.

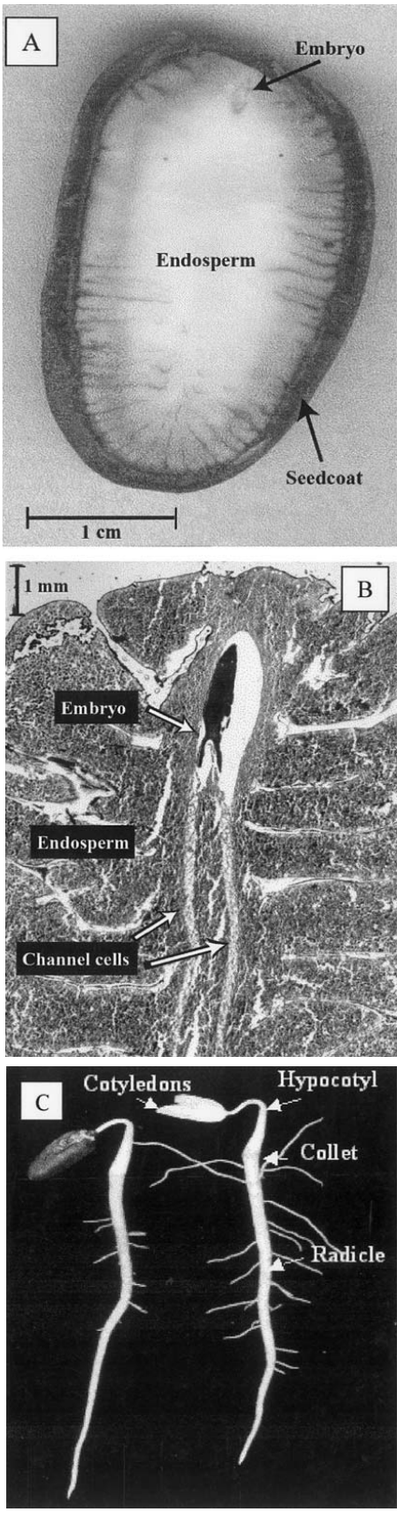

Fig. 1. Seed and seedling morphology in pawpaw. (A) Free hand longitudinal section through the center of the seed. (B) Photomicrograph of the micropylar end of seed showing embryo and specialized channel cells that will contain the developing haustorial cotyledons as the seed germinates. (C) Pawpaw seedlings about 45-d-old showing the extensive growth of the hypocotyl and radicle prior to any epicotyl expansion. The seedling on the right has had the testa and endosperm removed to show the extent of haustorial cotyledon development.

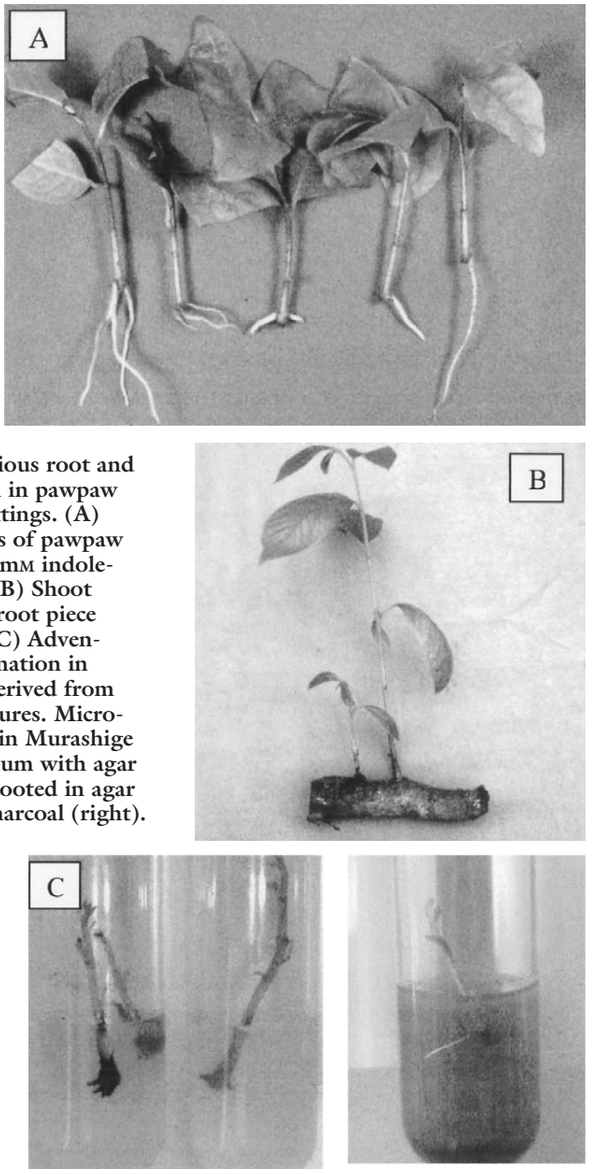

Fig. 2. Adventitious root and shoot formation in pawpaw stem or root cuttings. (A) seedling cuttings of pawpaw treated with 50 mM indole-3-butyric acid. (B) Shoot formation on a root piece from pawpaw. (C) Adventitious root formation in microcuttings derived from RVT 10-11 cultures. Microcuttings rooted in Murashige and Skoog medium with agar alone (left), or rooted in agar plus activated charcoal (right).

Forty-five days after sowing the epicotyl emerges from the growing medium and the tap root averages 15 cm (5.9 inch) in length and represents about 75% of the dry mass of the seedling (Fig. 1C). Seedling emergence is via a hypocotyl hook, but the seed coat containing the exhausted endosperm and haustorial cotyledons may or may not emerge from the medium. In either case, the cotyledons never emerge from the seed coat and are shed along with the remnants of the seed soon after the epicotyl begins to elongate. This type of seed germination where the cotyledons emerge above the soil but remain inside the seed is described as Durian germination (Ng, 1973). de Vogel (1980) has further subdivided Durian germination in to several subcategories of which pawpaw represents the Blumeodendrom type. Pawpaw is the first non-tropical species reported with Durian germination (Baskin and Baskin, 1998).

Cutting propagation

STEM CUTTINGS. Propagation from cuttings is difficult in pawpaw and is not currently a viable commercial practice. The ability to propagate pawpaw cultivars from cuttings would present several advantages over using seed or grafting. Plants from cuttings would flower sooner than seedling produced plants and cuttings would eliminate problems with understock suckering in grafted plants. Unfortunately, in a systematic study using over 1200 stem cuttings taken from mature flowering trees only one cutting produced an adventitious root (Finneseth, 1997). In that study, cuttings were taken from flowering trees about 5 years old. Cuttings were taken at 7-d intervals from 17 June to 5 Aug. from trees growing at the KSU research farm. In addition, stem cuttings were collected on 27 July from 26 genetically different mature trees from the Western Maryland Research Experiment Station in Keedysville, Md. Cuttings were treated with indole-3-butyric acid (IBA) as a quick dip solution [0, 25, or 50 mM (0, 5000, or 10,000 ppm)], stuck in flats containing Pro-Mix (Premier Horticulture, Inc., Quakertown, Pa.) and perlite (1:1 vol/vol) and placed under intermittent mist with bottom heat.

Experiments using cuttings taken from seedlings of various ages showed a significant impact of juvenility on rooting in pawpaw. Seedlings up to 2 months old showed a high capacity to root. Cuttings treated with 50 mM (10,000 ppm) IBA rooted at 75% and averaged two roots per cutting (Fig. 2A). Seedlings beyond 2 months showed a reduced capacity to form roots. Cuttings taken from 7-month-old seedlings rooted at less than 10% regardless of auxin treatment (Table 1). These data suggest that strategies to revert stock plants to a more juvenile state (i.e., tissue culture or mound layering) will be required before a reliable method for cutting propagation can be obtained.

ROOT CUTTINGS. Pawpaw is a naturally suckering species, readily forming adventitious shoots from roots in native stands as well as field orchard conditions. Propagation from root cuttings can be an alternative method for multiplication of difficult-to-root species. In addition, shoots derived adventitiously from roots retain a juvenile character and could serve as a source for stem cuttings or explants for tissue culture (Hackett, 1985). Finneseth (1997) studied the impact of root diameter on shoot production in pawpaw. Root pieces were collected from a single pawpaw patch in Greenup County, Ky., during December. The root system was cut into 10 to 12 cm (3.9 to 4.7 inches) long pieces and separated into two groups [less than or greater than 5 mm (0.20 inch)]. Cuttings were dusted with Captan fungicide and planted horizontally, about 5 cm (2.0 inches) deep, in flats filled with perlite and vermiculite (1:1 vol/vol). Flats were placed out of

Table 1. Adventitious root formation in stem cuttings from 7-month-old pawpaw seedling stock plants after 12 weeks.

Indole butyric acid concentration [mM]	Rooting (%)[z]	Roots/ rooted cutting
0	3.0	1.0 ± 0
5	7.0	1.7 ± 0.6
25	5	2.2 ± 1.0
50	10	3.0 ± 1.5
100	7	1.3 ± 0.5

[z]Rooting percentages were not significantly different across indole-3-butyric acid concentration (analysis of variance $P \leq 0.05$); n = 100 cuttings per treatment.

direct sunlight in a 22 °C (71.6 °F) greenhouse and watered only after the medium dried. No shoots formed on root pieces that were less than 5 mm in diameter, however 56% of root pieces greater than 5 mm in diameter produced one or more shoots. On average, responding roots produced 2.5 buds and 1.1 elongating shoots. Buds were visible on root pieces 12 weeks after planting and shoot elongation was evident after 16 weeks (Fig. 2B).

MICROCUTTINGS. Preliminary experiments to root microcuttings of pawpaw have met with little success (S.T. Kester and R.L. Geneve, unpublished). In these experiments, microshoots were developed from pawpaw cultures maintained for over 2 years. The original explants were shoots developed on root pieces from the A10-11 understock. Initial treatments placing explants on one-half strength MS (Murashige and Skoog, 1962) salts medium containing IBA (0.49 to 4.9 μM) resulted in a small percentage (3.0%) of microcuttings developing one to two roots per cutting at the 4.9 μM IBA level (Fig. 2C). However, these rooted shoots did not thrive during the acclimatization stage and failed to develop further. Several other protocols used for other difficult-to-root species were tried on a limited basis for pawpaw. Similar to what was done with redbud, pawpaw microshoots were exposed to high levels of IBA (100 μM) for 1 or 2 weeks followed by transfer to in vitro (without auxin) or ex vitro conditions (Yusnita et al., 1990). Similar to what was done with atemoya cultures, cuttings of etiolated pawpaw microshoots in culture were subjected to either solid or liquid MS medium moved to a liquid medium containing 245 μM IBA for 3 d before moving to MS medium with 0.25% activated charcoal (Rasai et al., 1994). These treatments were not more effective than the original in vitro rooting experiments with pawpaw.

Tissue culture propagation

Initial experiments using seedling explants of pawpaw demonstrated that nodal explants responded more favorably than apical sections for establishment of cultures (Finneseth, 1997). When nodal explants were treated with a range of benzyladenine (BA) concentrations (0, 5, 10, or 15 μM) on MS medium, fewer than 0.5 shoots developed per explant. However, when seedling explants were treated with 1.0 μM thidiazuron (TDZ) plus 10 μM BA all cultures produced over 1.0 shoot per culture. Therefore, explant establishment from seedling, mature, or rejuvenated sources was attempted using MS medium supplemented with 10 μM BA plus 0.1 μM TDZ (Finneseth et al., 2000). Seedling explants were about 12 weeks from seeding and had six to ten nodes. Mature explants were taken from new growth forced from excised dormant stems held in a growth chamber. They included stems collected from 26 genetically different mature trees. Rejuvenated explants were from shoots developing on root pieces from mature plants as previously described for root cuttings. Seedling explants established at 100% and developed faster than other explants (Table 2). Explants from rejuvenated stems established at a slower rate but produced shoots in over 40% of the cultures after 8 weeks. Of the 551 mature explants cultured, 72% were successfully disinfested, but only 4% survived in culture and none produced shoots after 8 weeks in culture. Most mature source explants turned black and lost tissue integrity. Significant browning of the medium was evident in all cultures (Fig. 3). A small number of mature explants continued to survive and produced a limited number of shoot buds after 7 months in culture. However, these never stabilized and no mature cultures survived for more than 12 months in culture.

One accession (A10-11) developed from rejuvenated explant sources showed continued growth and shoot production during subculturing. It has been maintained in culture for over 3 years (Fig. 3D). Tissue culture conditions established for efficient shoot multiplication were MS or Woody Plant Medium (Lloyd and McCown, 1980) with 8.9 μM BA and 2.7 μM naphthalene acetic acid (NAA), 3.0 % sucrose, and 0.7% agar. Cultures were maintained at a photoperiod of 16 h at 20 $\mu mol \cdot s^{-1} \cdot m^{-2}$ of light provided by cool white fluorescent bulbs. Culture room temperature was 25 °C.

The ability for single stem explants from 3-year-old cultures of A10-11 to produce shoots on shoot multiplication was investigated using 9.8 μM IBA plus 5.4 μM NAA in combination with BA (0 to 20 μM) (Table 2). Initial explants elongated but did not form additional shoots after 8 weeks in culture (J.N. Egilla, unpublished). These were subcultured to the same medium and after 9 weeks cultures treated with 15 or 20 μM BA had the greatest number of shoots per culture and 15 μM BA had the most vigorous shoot growth (Table 2). These data indicate that cultures of pawpaw can retain morphogenetic potential for a considerable time in culture.

Table 2. Shoot production from single node explants derived from 3-year-old A10-11 cultures treated with various concentrations of benzyladenine (BA)[z].

BA [μM]	Survival (%)	Shoot no.	Fresh wt (mg)[y]	Growth rate[w]
0	70	1	130 ± 114	–
5.0	100	≈2	466 ± 166	++
10.0	73	≈2	524 ± 450	+
15.0	85	Cluster (>3)	629 ± 220	+++
20.0	80	Cluster (>3)	460 ± 380	+

[z]Woody plant medium with 9.8 μM indole butyric acid plus 5.4 μM naphthalene acetic acid.
[y]28,350 mg = 1.0 oz.
[w]Growth rate represented as poor (–), or with increasing relative vigor (+).

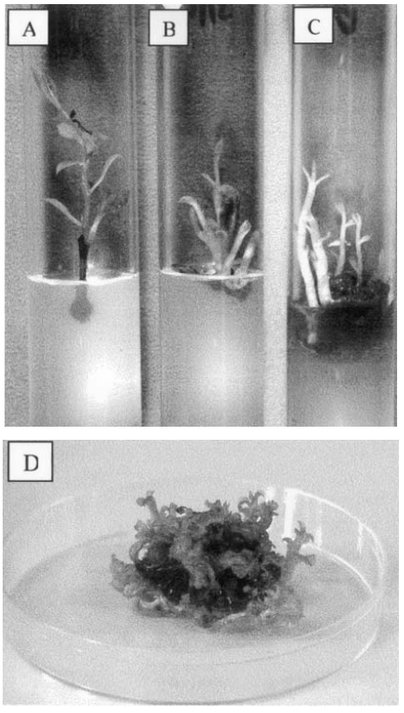

Fig. 3. Tissue cultures of seedling pawpaw. (A) Single nodal explant showing initial shoot elongation. (B) Seedling explant with multiple shoot formation. (C) Pawpaw after several subcultures in the multiplication stage showing shoot clusters and apparent oxidation of phenolics in the basal callus and medium. (D) Multiple shoot formation in A10-11 cultures. Most of these shoots fail to elongate satisfactorily to produce microcuttings.

Grafting

Grafting is the only current clonal propagation method used commercially and chip budding is usually the method of choice. Layne (1996) provided a detailed description of chip budding techniques used at the Pawpaw Germplasm Repository at KSU. He described spring, chip budding onto container-grown seedling understocks using dormant scion buds from wood stored under refrigeration. Larger buds showed a higher degree of grafting success compared to small buds. Successful buds began to grow within two weeks of grafting. He found that immediate removal of the understock just above the bud inhibited subsequent scion growth. The recommendation was to cut the understock back gradually. Initially the understock should be reduced to 30 to 60 cm (11.8 to 23.6 inches) in height retaining about 6 leaves. Once the scion has reached about 30 cm, all leaves from the stock should be removed and the stem cut back to 20 cm (7.9 inches). When the scion becomes woody, the remaining understock above the scion can be completely removed. Greenhouse produced plants from budding can reach 1.5 m (5 ft) after the first season.

There are numerous named cultivars under evaluation in a multistate pawpaw regional variety trial initiated by the Pawpaw Foundation (Frankfort, Ky.) and KSU (Layne 1996). These and other potential cultivars have been budded onto seedling understocks. There are currently no clonal understocks available for pawpaw propagation.

Layering

Juvenility is an important factor for cutting and tissue culture propagation. Developing shoots on roots of mature plants appears to be a viable rejuvenation method. However, cultivars are maintained on understocks making roots unavailable for clonal propagation. Layering is currently being investigated as a method to establish cultivars on their own roots (S. Crabtree, unpublished). There may be several important outcomes if layering is successful in propagating pawpaw. First, layering could prove to be a commercially viable option for clonal propagation of cultivars or understocks similar to other fruit crops (Hartmann et al., 2002). In addition, layering would establish cultivars on their own roots providing a source for roots in a rejuvenation strategy for tissue culture as has been successful for establishing shoot cultures (Finneseth et al., 2000). Finally, cultivars on their own roots would be important for maintenance of clonal germplasm at the Germplasm Repository for pawpaw established at KSU (Layne, 1996). Currently clonal germplasm is maintained in a field orchard as budded trees. Since pawpaw can regenerate from suckers developed from the root system, it would be prudent to have all accessions on their own roots. This would be a precaution against damage to the above-ground portion of trees (winter kill) that might still permit the root system to survive and produce clonal shoots. This could prove extremely important to those southern accessions in the germplasm collection that might be susceptible to winter injury.

Conclusions

Pawpaw is currently commercially propagated by seeds or budding. Seeds must be stored moist and require about 100 d of chilling stratification to overcome dormancy. Seed stocks for propagation are limited to yearly production cycles because seed storage life is short and supply is limited. Therefore, seeds are usually submitted to refrigerator stratification and greenhouse sown rather than field sown in the fall as is usually for many nursery crops. Seeds are an appropriate source for propagation of understocks or liners intended for ornamental use. Fruit quality from seed is variable and usually inferior to selected cultivars for orchard use. Liners intended for fruit production should be budded onto seedling understocks. Dormant, vegetative buds are chip budded on over wintered seedlings using standard budding methods. A propagation method to produce pawpaw on its own roots would be advantageous for orchard production because understock suckering can be a problem. However, clonal propagation of pawpaw from methods other than grafting (i.e., cutting and micropropagation) have proven difficult and remain to be developed for routine propagation of pawpaw.

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