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Ovule, embryo sac, embryo and endosperm development in leafy spurge (*Euphorbia esula* L.)[1]

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Abstract:

Leafy spurge (*Euphorbia esula*) is a noxious, invasive weed that dominates many agriculturally important regions. While many research efforts are currently aimed at controlling the spread of this plant, relatively little is known about its sexual reproductive biology, especially from a structural perspective. This report describes key features of ovule development, embryogenesis, and endosperm formation in leafy spurge. Ovules are anatropous, bitegmic, and form a zigzag micropyle. A distinct elaisome (caruncle) and hypostase are formed as ovules mature. Obturators are present and are derived from placental tissue. The embryo sac conforms to the *Polygonum* type. A single embryo is formed in each seed and stores nutrients primarily as globoid protein bodies. Endosperm is persistent and also contains protein bodies as its primary nutrient reserve. Preliminary structural evidence is presented that indicates the potential for apomixis.

Keywords:

Leafy spurge, Euphorbiaceae, *Euphorbia,* ovule, endosperm, embryo.

Introduction

Leafy spurge (*Euphorbia esula* L.) is an herbaceous perennial that has flourished as a noxious weed of economic and ecological significance (Lajeunesse *et al*. 1995; Lym and Messersmith 1983; Messersmith 1983; Messersmith and Lym 1983*a, b*). This plant has a

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remarkable ability to invade pastures and farmland which renders those areas unfit for crops and livestock (Lacey *et al*. 1984). Leafy spurge was introduced to North America from continental Europe and Asia in the early nineteenth century as a contaminant in seed grain (Hanson and Rudd 1933; Messersmith 1983). Since that time, it has become widespread in the Great Plains of the United States and the Prairie Provinces of Canada (Hanson and Rudd 1933; Lajeunesse *et al*. 1995). It is thought that several Old World spurges hybridized to produce the leafy spurge commonly found in the Northern Great Plains (Moore 1958). This hybridization has resulted in genetically diverse populations throughout the world, which unfortunately, present a barrier to the use of native European pests as control agents on the leafy spurge found in North America (Messersmith 1983; Rowe *et al*. 1997).

Leafy spurge possesses many qualities of an ideal weed and, thus, has proven difficult to eradicate (Galitz and Davis 1983). For example, leafy spurge produces large numbers of seed that, when mature, are ejected out of the ovary away from the parental plant at a distance of up to four and one-half meters. Leafy spurge also persists if cut back and can resprout through buds located on underground roots. The root system is extensive, enables plants to reappear year after year, and represents the primary means of survival and vegetative reproduction in leafy spurge (Bowes and Thomas 1978; Best *et al*. 1980). This plant's superior competitive abilities, due primarily to extensive root systems, have led to population reductions of native grasses and dramatic ecosystem changes throughout North America. Indeed, over two million hectares are infested by leafy spurge in the Northern Great Plains and Canada (Brinkman *et al*. 1997).

In light of the ecological and economic impact of leafy spurge, most of the current research is aimed at curbing the growth and spread of this invasive weed. Although burning, mowing, and herbicides have been used to minimize leafy spurge infestations, biological control strategies that use natural pathogens and herbivorous insects have become a major focus of control methodologies (Carlson and Littlefield 1983; Lym *et al*. 1996).

Despite the recent emphasis on biological control of leafy spurge, very little is known about the sexual reproductive biology of this invasive plant. Interestingly, except for basic information on flower structure and seed germination (Hanson and Rudd 1933; Galitz and Davis 1983), asexual regrowth through root systems (Raju *et al*. 1963, 1964; Morrow 1979) and tentative aspects of breeding strategies (Selleck *et al*. 1962), relatively few published results have addressed the sexual reproductive structures and characteristics of this plant. A single report published over a half century ago includes basic aspects of embryogenesis in leafy spurge but provides no information on endosperm development or any other significant sexual reproductive characteristics. Indeed, it has not been known whether leafy spurge exhibits evidence of apomixis and the potential for self-incompatibility has been poorly understood (Selleck 1962). Considering the clear impact that reproductive strategies have on a plant's ability to invade and dominate certain areas, a documentation of sexual reproductive features of leafy spurge seems merited.

The objective of this study was to examine various aspects of ovule and early seed development in leafy spurge from a structural perspective. This report is the first to provide conclusive documentation of the major events involved in ovule development, embryogenesis, and endosperm maturation. These results are compared with those of other

members of the Euphorbiaceae and also serve as a basis for future studies aimed at understanding the breeding system of this invasive weed.

Materials and methods

Plant materials

Populations of leafy spurge (*Euphorbia esula*) growing in Grand Forks, ND (U.S.A.) were sampled for this study. Flowers were collected from plants during the months of June and July 1997. Flowers were collected at various stages prior to anthesis through early stages of seed maturation. Pollinations were not controlled in any way. Care was taken to sample plants from several distant locations to minimize the chances of sampling from genetically identical shoot systems.

Bright-field microscopy

Ovaries collected for observation were immediately trimmed prior to chemical fixation. Trimmed ovaries were fixed in 4% acrolein dissolved in 0.05 M TRIZMA buffer, (pH 7.2), for 24 hours at room temperature. For plastic-embedded specimens, the chemically fixed ovaries were rinsed two times in TRIZMA buffer, dehydrated through an ethanol series (10%, 20%, 30%, 50%, 75%, 95%, 100%, 1 hour per step), and infiltrated with glycol methacrylate (JB-4 Embedding Kit, Polysciences, Inc., Warrington, PA). The samples were infiltrated over a seven-day period to insure complete displacement of ethanol with glycol methacrylate. Ovules were then embedded and the embedding medium was polymerized in an oxygen-free environment by flushing nitrogen gas through a closed chamber. Embedded samples were serially sectioned on a RMC (Tucson, AZ) 920 MT rotary microtome into ribbons of serial sections at thicknesses of 3-5 μm with a glass knife made from a microscope slide. Ribbons were mounted on microscope slides, stained with toluidine blue (O'Brien and McCully 1981), and preserved with mounting medium.

Fluorescence Microscopy of DAPI (4',6-diamidino-2-phenylindole) stained sections

Ovules used for fluorescence microscopy were collected as described previously. Fixation was conducted at room temperature for 24 hours in 3:1 (v/v), ethanol-glacial acetic acid. Ovules were then transferred to 75% ethanol for storage at 4ºC. At a later date, samples were dehydrated through an ethanol series, embedded in glycol methacrylate, and serially sectioned as previously described. The resulting slides were flooded with a solution containing 0.5 mg/mL DAPI and 0.1 mg/mL *p*-phenylenediamine (added to prevent fading) in 0.05 M TRIZMA buffer (pH 7.2) for 5 minutes at room temperature. Excess DAPI was then removed and cover slips were added. The slides were kept in a dark, humid environment to prevent desiccation. The stained sections were observed and photographed on an Olympus BX 60 microscope equipped with UV epifluorescence (HBO 100 W burner, Olympus wide band UV filter cube).

Histochemistry

Carbohydrates, proteins, and lipids were assayed using a variety of histochemical techniques on fresh tissue and serial sections through endosperm and embryo tissues. Carbohydrates were localized using the PAS (periodic acid – Schiff) reaction (Jensen 1962). Additionally, starch was observed by flooding slides with potassium iodide. Proteins were localized by the napthol blue-black method (Yeung 1984). The presence of lipids was ascertained by using osmium tetroxide (Yeung 1984), and Sudan III and Sudan IV (Jensen 1962). All tests for lipids were negative. For histochemical tests on sectioned material, samples were fixed in 4% acrolein dissolved in 0.05 M TRIZMA buffer (pH 7.2) for 24 hours at room temperature and were embedded and sectioned as previously described for bright-field microscopy.

Image Analysis

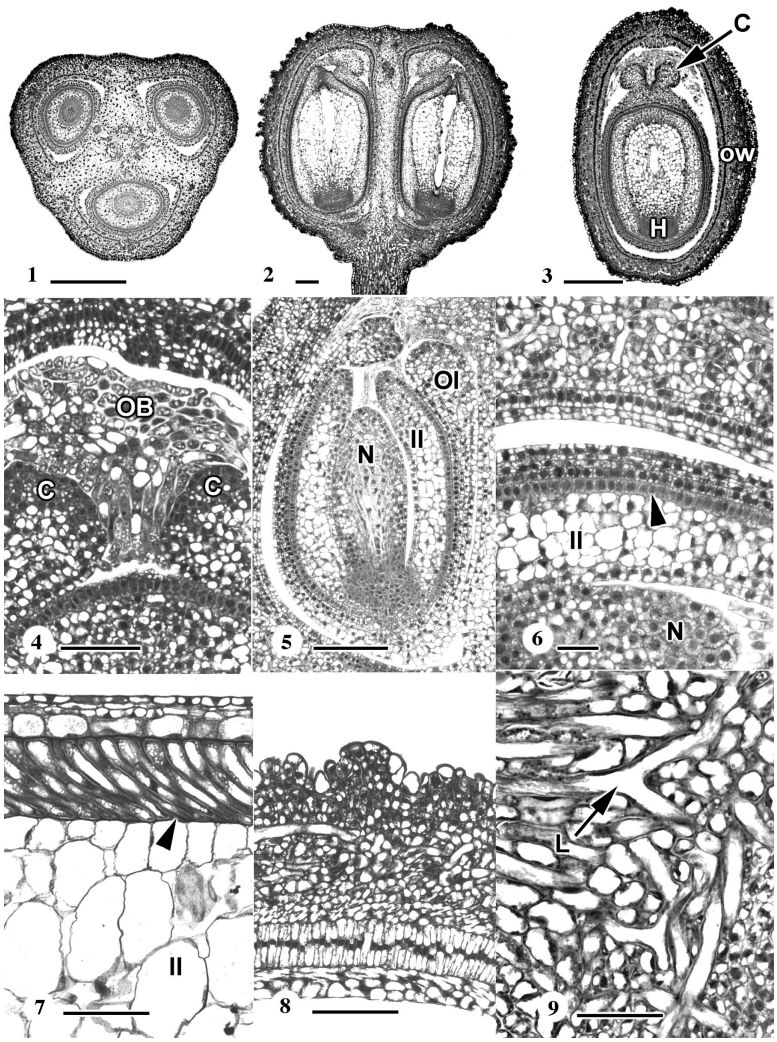
Computer-assisted image analysis was used to quantify cell sizes in various ovular tissues. Brightfield images were visualized with video microscopy (Panasonic color CCTV camera, model WV-CP410, 470 lines of resolution). The images were then digitized at a resolution of 640 x 480 pixels with Snappy Video Snapshot (Play Incorporated, Rancho Cordova, CA). Images were then analyzed on an IBM-compatible clone using Image Tool image analysis software, version 1.28 (developed at the University of Texas Health Science Center, San Antonio, available via anonymous FTP at ftp://maxrad6.uthscsa.edu). Cell size was measured as the length of the major axis of cells and was reported as cell diameter.

Results

Ovule development

Each ovary of leafy spurge (*Euphorbia esula*) consists of three carpels with a single ovule formed per carpel (Fig. 1). Placentation is centrally axile (Fig. 1). The three ovules display relatively synchronous development, are initiated very early during flower development, and occupy the majority of each locular chamber (Fig. 2). Ovules have a relatively short funicle, are anatropous at inception and remain in that orientation throughout seed maturation (Fig. 2).

Ovules are bitegmic, with the outer integument possessing a specialized elaisome (caruncle) (Fig. 3). The caruncle is located at the extreme micropylar end of each ovule and retains a small depression that is likely necessary for pollen tube growth into the embryo sac. An area containing densely cytoplasmic cells is evident in the most chalazal region of the nucellus (where the nucellus and the inner integument converge) (Fig. 3). This region of cells represents a hypostase and becomes increasingly distinct as ovules mature. The nucellus is extensive and occupies the majority of each young ovule. A distinct region of glandular, hair-like cells is found just above the caruncle and likely represents an obturator (Fig. 4). This structure arises from placental tissue and grows through the micropyle toward the nucellus.



Figs. 1-9. Ovary and ovule development in leafy spurge (*Euphorbia esula*). Fig. 1. Cross section through an ovary. Note the three ovules and centrally axile placentation. Bar = 350 µm. Fig. 2. Longitudinal section through an ovary showing two of three ovules. The third ovule is out of the plane of section. Ovules are anatropous and exhibit synchronous development within an ovary. Bar = 150 µm. Fig. 3. Longitudinal section through a single locular chamber. The immature ovule displays a distinct caruncle (elaisome) at the micropylar end of the outer integument. A hypostase is also evident at the extreme chalazal end of the nucellus. Bar = 300 µm. Fig. 4. High magnification image of the obturator tissue, which completely fills the micropyle and is surrounded by the caruncle. Bar = 50 µm. Fig. 5. Longitudinal section through a young ovule which shows the inner and outer integument and "zigzag" nature of the micropyle. Bar = 150 µm. Fig. 6. Section through a young ovule showing the multilayered outer and inner integuments. The arrow designates the outermost layer of the inner integument. Bar = 50 µm. Fig. 7. Section through inner and outer integuments at late stage of development. Most of the cells of the inner integument have become enlarged and highly vacuolate. The arrow designates the outermost layer of inner integument cells, which have become elongated and lignified. Bar = 50 µm. Fig. 8. Section through ovary wall showing several distinct cell layers and laticifers. Note the distinct layer of palisade cells. Bar = 50 µm. Fig. 9. High magnification image of ovary wall showing the branched, nonarticulated laticifers. Laticifers permeate most of the plant body and are first evident during embryogenesis. Bar = 50 µm. C, caruncle; H, hypostase; II, inner integument; L, laticifer; N, nucellus; OB, obturator; OI, outer integument; OW, ovary wall.

Ovules display several distinct features from the earliest stages of development. The micropyle consists of both the inner and outer integuments, with the outer integument completely surrounding the inner integument (Fig. 5). The inner and outer integuments also do not co-align, and thus a "zigzag" micropyle is formed (Fig. 5). The hypostase can be distinguished even before the embryo sac reaches maturity (Fig. 5). Although megasporogenesis was not conclusively documented, the earliest cells of the embryo sac are found several layers beneath the nucellar epidermis and thus likely represent the crassinucellate condition (Fig. 5).

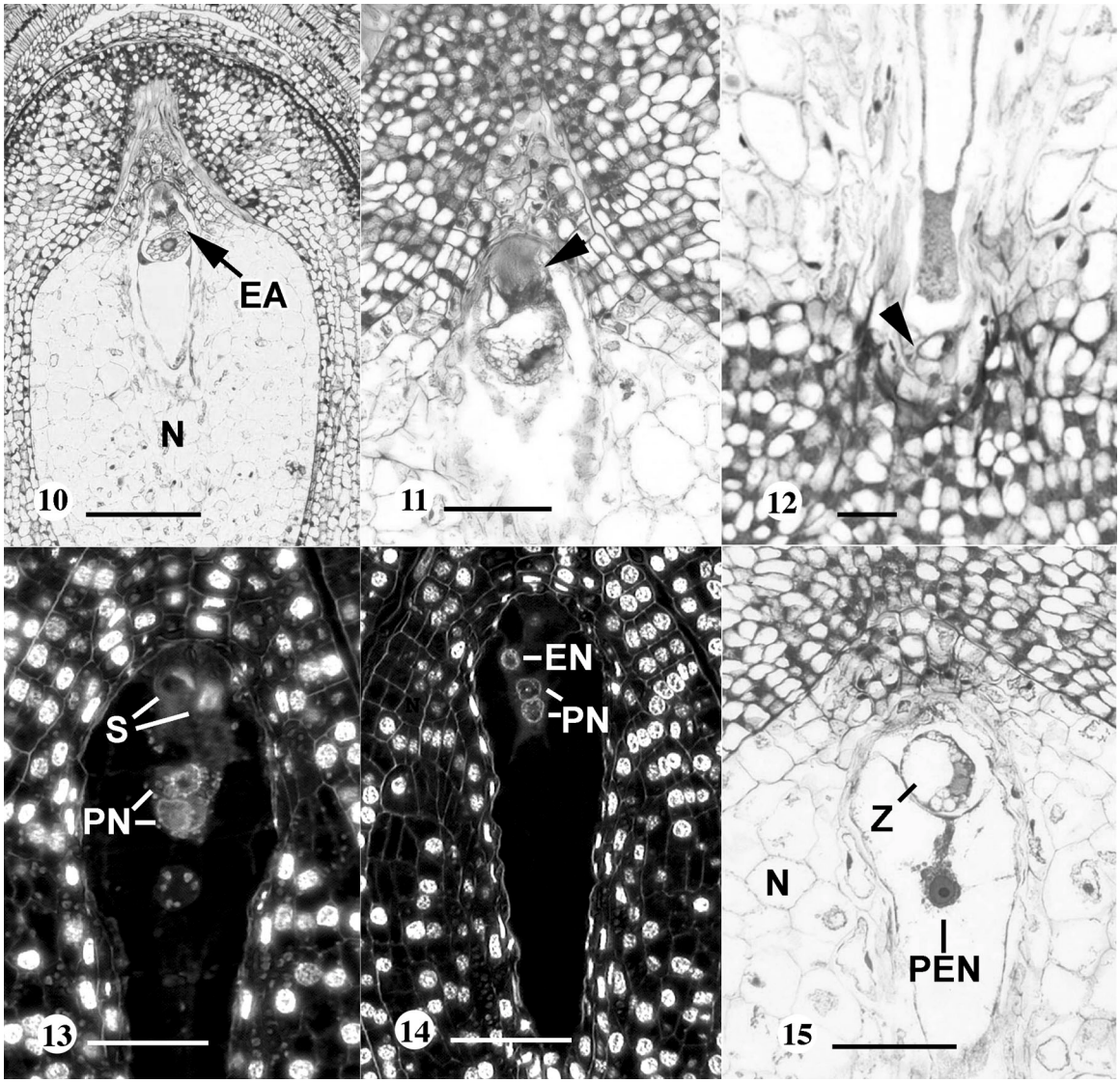
The tissues of the inner integument are slightly more extensive than the outer integument and are characterized by approximately six to nine layers of highly vacuolate parenchymatous cells (Fig. 6). The cells of the outermost layer of the inner integument are tightly compact and densely cytoplasmic (Fig. 6). These cells ultimately become elongate, highly lignified, and contribute to the hardened seed coat (Fig. 7). The inner integument expands significantly as the ovules mature.

However, the number of cell layers remains relatively constant (Figs. 6, 7) and the increase in size is due to a marked increase in size of individual cells (Table 1). The number of cell layers in the outer integument also remains relatively constant (three to four) as the integument matures, but cell diameters do not increase to any significant extent (Table 1). An inner layer of cells in the ovary wall also becomes distinctly elongate and likely contributes to the structural integrity of the mature ovary (Fig. 8). Branched, nonarticulated laticifers permeate the ovary (Fig. 9). These specialized secretory cells occur throughout the plant body and produce a milky latex that is characteristic of the Euphorbiaceae.

Table 1. Cell sizes of various ovular tissues in *Euphorbia esula* L. (leafy spurge).

CELL TYPE	DIAMETER (μm)*	NUMBER MEASURED
Inner Integument		
Embryo sac stage	19.01 ± 3.3	25
Globular embryogenesis	71.47 ± 13	25
Outer Integument		
Embryo sac stage	25.96 ± 6.3	20
Globular embryogenesis	28.26 ± 5.5	20
Mature Endosperm		
Vacuolate cells	42.20 ± 8.3	30
Densely cytoplasmic cells	43.45 ± 6.2	30
Nucellus		
Embryo sac stage	32.84 ± 6.1	20
Globular embryogenesis	76.89 ± 16	25

*Values are mean ± SD.



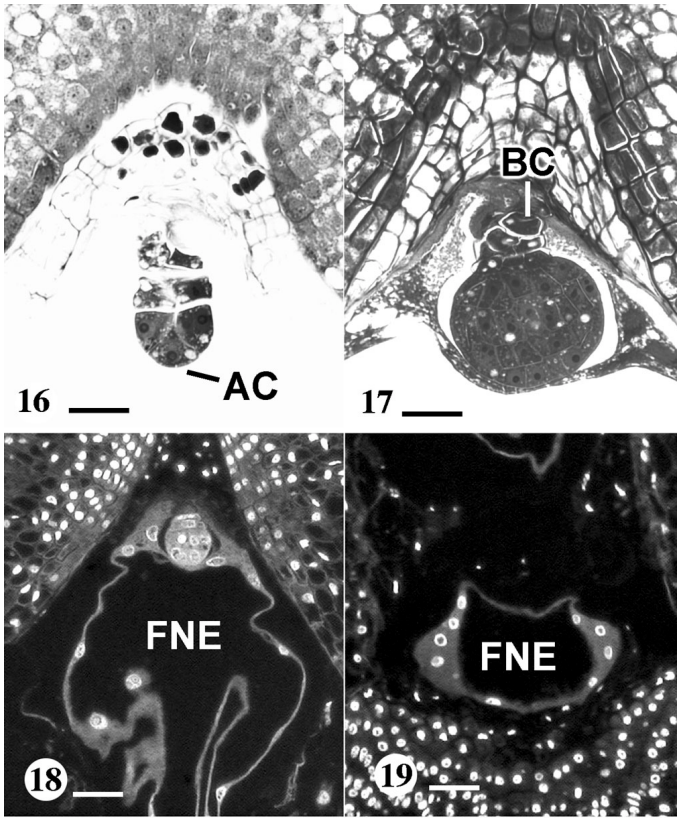
Figs. 10-15. Embryo sac of leafy spurge. Fig. 10. Longitudinal section through ovule showing embryo sac embedded within relatively large nucellar tissue. The egg cell is distinct and contains numerous inclusion bodies (most likely plastids). Bar = 100 µm. Fig. 11. A filiform apparatus (arrowhead) is present within the synergids. Bar = 50 µm. Fig. 12. Chalazal region of the embryo sac. Arrowhead denotes putative short-lived antipodals. Bar = 50 µm. Fig. 13. DAPI-stained, longitudinal section through ovule prior to embryogenesis. Note the synergid nuclei and two distinct, separate polar nuclei. Bar = 50 µm. Fig. 14. Similar to Fig. 13, except egg nucleus is clearly distinct. Note the absence of antipodals. Bar = 50 µm. Fig. 15. Zygote and primary endosperm nucleus after putative double fertilization. No pollen tubes, nor remnants of them, were observed in the micropyle or near the embryo sac. Bar = 50 µm. EA, egg apparatus; EN, egg nucleus; N, nucellus; PEN, primary endosperm nucleus; PN, polar nuclei; S, synergids; Z, zygote.

Embryo sac development

Embryo sac formation conforms to the *Polygonum* type (monosporic, seven-celled, eight-nucleate embryo sac consisting of three antipodal cells, a large, binucleate central cell, and an egg cell adjacent to two synergids) (Maheshwari, 1950). The mature embryo sac consists of two polar nuclei and a well-defined egg cell located between two synergids (Fig. 10). Prior to fertilization, the egg cell is densely cytoplasmic and contains numerous organelles (putative plastids) (Fig. 10). The micropylar region of the nucellus extends into the micropyle and thus represents a nucellar "beak" (Figs. 10, 11). Synergids are characterized by a filiform apparatus (Fig. 11), although it is not always easily discernible. Although antipodals are formed (Fig. 12), they are short lived and appear to degenerate prior to embryo sac maturation. Plastids of unknown nature (potentially small proplastids) are prevalent within the embryo sac (Fig. 13). The two polar nuclei remain separate and distinct throughout embryo sac maturation (Fig. 14) and appear to fuse only after formation of a zygote (Fig. 15).

Embryogenesis

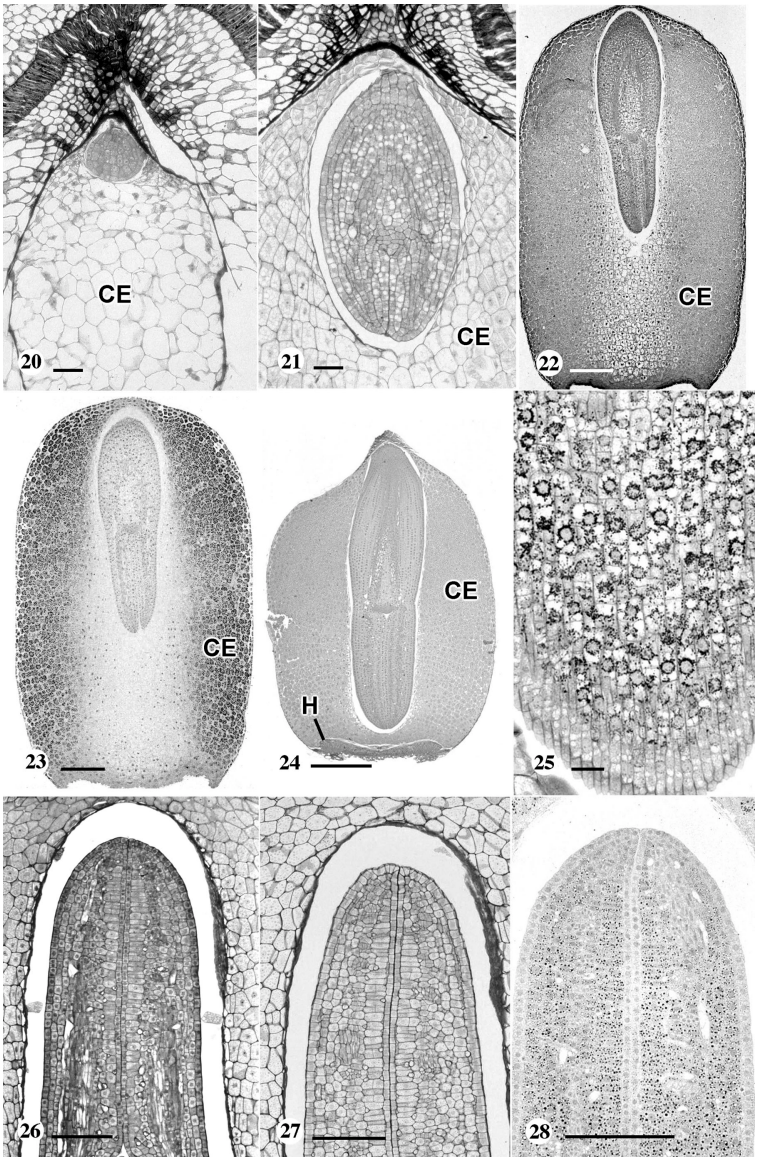
Early embryogenesis conforms to the Onagrad type, but also expresses features of the Asterad type (Figs. 16, 17). The basal cell is highly reduced and barely detectable in sectioned ovules (Fig. 17). An angular apical cell is differentiated after just the first few mitotic divisions (Fig. 16). The embryo soon reaches a globular stage of development and becomes roughly spherical in shape (Fig. 17). During early stages of embryogenesis, the endosperm is of the free nuclear type and undergoes precocious development with free nuclei positioned in a parietal band of cytoplasm (Figs. 18, 19).



Figs. 16-19. Early embryogenesis. Fig. 16. Very young embryo showing angular apical cell. Bar = 20 μm. Fig. 17. Globular proembryo with highly reduced basal cell. Bar = 20 μm. Fig. 18. DAPI-stained section showing proliferation of free nuclear endosperm during globular stage of proembryogenesis. Bar = 20 μm. Fig. 19. Chalazal region of section shown in Fig. 18 showing thin band of cytoplasm with free endosperm nuclei. Bar = 20 μm. AC, apical cell; BC, basal cell; FNE, free nuclear endosperm.

Endosperm becomes cellular and completely fills the former embryo sac while the embryo remains in the globular stage of development, (Fig. 20). Two cotyledons soon emerge, elongate, and grow through the cellular endosperm (Fig. 21). The endosperm is almost entirely cellular throughout most of the later stages of embryogenesis (Fig. 22). Most of the nutrients are found at the periphery of the endosperm tissue (Fig. 23). However, this pattern may reflect the metabolism of nutrients in close proximity to the developing embryo prior to those located at the periphery. The mature embryo consists of distinct cotyledons, shoot and root apical meristems, and laticifer initials located throughout the embryo proper (Fig. 24). The hypostase remains clearly evident at these later stages of development.

Embryo cells remain vacuolate and devoid of significant nutrient reserves throughout early stages of development. It is during this time that active cell division takes place. However, embryonic cells of the hypocotyl eventually begin to store nutrients in the form of carbohydrates (starch grains) that are positioned in thin bands of cytoplasm around each nucleus (Fig. 25). Within cotyledons, laticifer initials become clearly evident (Fig. 26) and globoid protein is the predominant nutrient reserve (Figs. 26, 27, 28). Cells of the protoderm remain free of protein reserves (Fig. 28).



Figs. 20-28. Embryo development. Fig. 20. Section showing globular proembryo and cellular endosperm. Note the embryo is at a similar stage of development as that shown in Fig. 17, but the endosperm has become completely cellularized. Bar = 50 μm. Fig. 21. Initiation of cotyledons. Note the endosperm cells are still highly vacuolate at this stage. Bar = 50 μm. Fig. 22. Section of ovule with seed coat removed. Endosperm cells are densely cytoplasmic at this stage of embryogenesis. Bar = 100 μm. Fig. 23. Section adjacent to that shown in Fig. 22 except stained with napthol blue-black for protein assay. Note the central region of endosperm contains vacuolate cells and is devoid of protein reserves. Bar = 100 μm. Fig. 24. Section through mature seed stained with periodic acid Schiff (PAS) reaction. Only the hypostase and cell walls of embryo and endosperm tested positive for carbohydrates. Bar = 300 μm. Fig. 25. High magnification image of hypocotyl stained with PAS reaction. Note the amyloplasts in bands of cytoplasm surrounding the nucleus in each cell. Bar = 25 μm. Fig. 26-28. Adjacent sections through cotyledons stained with toluidine blue, PAS, and napthol blue-black respectively. Note the protein globules in Fig. 28. Bars = 150 μm. CE, cellular endosperm; H, hypostase.

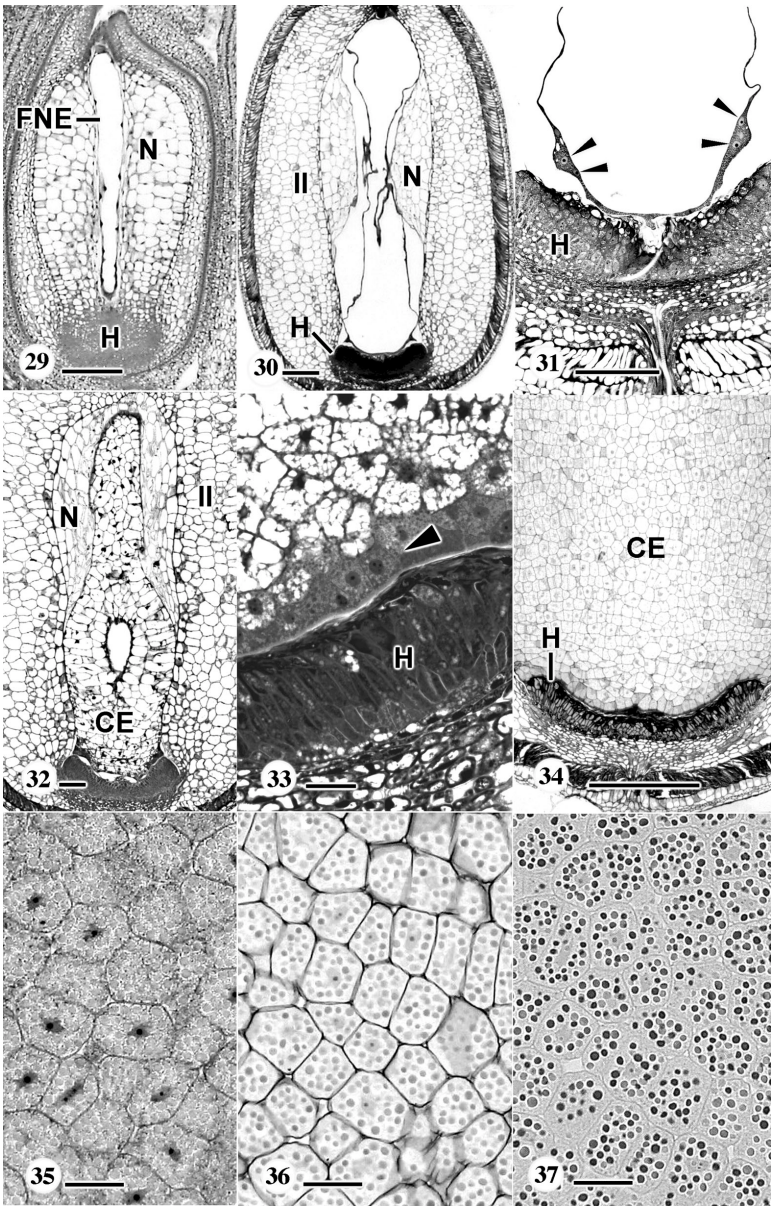
Although a single embryo was commonly found in each of the ovules sampled in this study, observations of over 100 serially sectioned ovules revealed the absence of pollen tubes near embryo sacs, nucellar tissues, and ovules in general. It is therefore possible that leafy spurge displays apomixis with embryos and seeds being formed without fertilization taking place. This phenomenon was not conclusively documented in this study but merits future investigation.

Endosperm development

Beyond the few stages mentioned briefly above, endosperm of leafy spurge exhibits some very distinct developmental stages. Repeated divisions of the primary endosperm nucleus yields an elongated embryo sac with free endosperm nuclei lining the periphery of a thin band of cytoplasm (Fig. 29). Mitotic figures were not observed and thus it is not clear if mitotic divisions are synchronous. While still in the free nuclear state, the endosperm begins to enlarge laterally and vertically and begins to crush the nucellus (Fig. 30). Concurrently, the inner integument enlarges, primarily by cell enlargement and not cell divisions (Fig. 30, Table 1). As the nucellus remains around the central region, the endosperm exhibits a distinct micropylar region (near the proembryo) and chalazal region (located superior to the hypostase). The free nuclei are clearly evident both in the micropylar region and the chalazal region (Fig. 31).

Cellularization of the free nuclear endosperm is initiated around the periphery and continues in a centripetal manner toward the center (Fig. 32). No signs of distinct alveoli were observed and, thus, it is not known whether these specialized cells occur in leafy spurge. Although cellularization of endosperm is centripetal, a small region of free nuclei persists in the chalazal region (Fig. 33). These free nuclei, and nearby endosperm cells as well, are likely meristematic and contribute to continued growth of the endosperm tissue.

Ultimately, the endosperm occupies the majority of the developing seed and persists in the mature seed. The hypostase persists throughout seed maturation and stains intensely with the PAS reaction (Fig. 34). Mature endosperm is characterized by densely cytoplasmic cells containing numerous storage bodies (Figs. 35, 36, 37). Prior to nutrient provisioning, the endosperm cells expand to their maximum size (Table 1). The walls of endosperm cells contain carbohydrates, but probably very little cellulose (Figs. 35, 36). Staining with napthol blue-black reveals that globoid protein is the major storage component in mature endosperm (Fig. 37). All histochemical tests for lipids were negative.



Figs. 29-37. Endosperm development. Fig. 29. Longitudinal section through ovule showing elongated embryo sac lined with peripheral band of free nuclear endosperm. Bar = 150 µm. Fig. 30. Slightly later stage of development than in Fig. 29. The nucellus has become crushed by the expanding embryo sac, which contains free nuclear endosperm. The hypostase has become well distinguished, inner integument has expanded, and seed coat has begun to mature. Bar = 300 µm. Fig. 31. Section showing close relationship between chalazal region of endosperm and hypostase. Arrowheads denote endosperm nuclei. Bar = 200 µm. Fig. 32. Section showing centripetal cellularization of endosperm. Bar = 100 µm. Fig. 33. High magnification image showing small region of endosperm (arrow) that remains free nuclear near the hypostase. Bar = 25 µm. Fig. 34. Section through central region of endosperm and hypostase stained with PAS. Bar = 300 µm. Fig. 35-37. Adjacent sections through mature endosperm stained with toluidine blue, PAS, and napthol blue-black, respectively. Note the distinct protein globules in Fig. 37. The dark appearance of the globules in Fig. 36 is due to light refraction and not a positive PAS test. Bars = 25 µm. CE, cellular endosperm; FNE, free nuclear endosperm; H, hypostase; II, inner integument; N, nucellus.

Discussion

This report is the first to provide a detailed account of ovule, embryo, and endosperm development in leafy spurge. The results presented indicate that many sexual reproductive features expressed in leafy spurge are similar to those of other members of the Euphorbiaceae. However, several unique structures are evident whose exact functional significance has yet to be determined.

Ovary and Ovule

Ovaries of leafy spurge exhibit three-locular carpels with highly organized and distinct cell layers (Figs. 1 - 3). Most euphorbs have three-locular ovaries except for *Crotonopsis* species which form a one-seeded utricle (Davis 1966). Ovaries of leafy spurge also possess latex-bearing branched, nonarticulated laticifers (Fig. 9). Laticifers represent an intriguing cell type, which are found across a broad range of plants. These cells are capable of intrusive growth and commonly produce a milky latex characteristic of many taxa (Metcalfe 1967). Laticifer structure is of particular interest because the Euphorbiaceae family includes members that possess both nonarticulated and articulated laticifers (Mahlberg 1961; Fahn 1982). Nevertheless, branched, nonarticulated laticifers appear to be the most common type in the Euphorbiaceae and, thus, this character is likely of taxonomic importance at the genus level (Mahlberg 1959; Mahlberg and Sabharwal 1967).

Based on the shape of the ovule and orientation of the micropyle relative to the funiculus, at least five major types of ovules have been traditionally recognized in angiosperms with anatropous being the most common form (Bouman 1984; Maheshwari 1950). Ovules of leafy spurge, as well as other euphorbs (Lyon 1898; Landes 1946; Davis 1966), are anatropous throughout all stages of development. However, orthotropous and even hemianatropous ovules have been reported in some members of the Euphorbiaceae (Davis 1966). The crasinucellate and bitegmic nature of leafy spurge ovules appear to be universal features of Euphorbiaceae (Davis 1966).

Most members of the Euphorbiaceae express growth of apical nucellar tissue in the form of a nucellar "beak." In most taxa, the beak is fairly extensive and even protrudes through the micropyle into the locular chamber (Lyon 1898; Landes 1946). The nucellar beak has been described as having a distinct central layer of cells that appear to allow passage of pollen tubes into the embryo sac. In leafy spurge, however, these specialized cells were not evident. In fact, pollen tubes were never observed anywhere near the nucellus or within synergids or embryo sacs during this study.

The obturator (Fig. 4), hypostase, (Figs. 30, 32, 34) and caruncle (Fig. 3) represent some of the most distinct reproductive structures found in leafy spurge ovules. Caruncles are commonly found in the Euphorbiaceae family (Landes 1946; Johri and Kapil 1953; Singh 1954; Kapil 1961; Morrow 1979; Bianchini and Pacini 1996), but are not representative of all members of this taxon (Park and Elisens 1997). The caruncle develops from the outer integument at the micropylar end of the ovule. This elaisome structure appears to aid in attracting insects for seed dispersal (Bouman 1984; Bianchini and Pacini 1996).

However, it has also been observed to be hygroscopic in some species and, by absorbing water from the soil, may aid in germination of the embryo (Bianchini and Pacini 1996). By virtue of its location at the micropylar region, the young caruncle may play a vital role in enabling passage of the pollen tube into the ovule. However, the exact functional significance of this structure has not been conclusively demonstrated in leafy spurge.

The hypostase represents another enigmatic structure expressed in leafy spurge ovules. This structure develops as a mass of thick-walled cells positioned at the base of the nucellus near the junction of the nucellus and inner integument (Fig. 3). It has been hypothesized that the hypostase mediates the translocation of nutrients into the megagametophyte and embryo because it forms a barrier between the ovular vascular strand and the embryo sac, thereby inhibiting growth of the embryo sac into the chalaza (Maheshwari 1950). The hypostase may also function in various aspects of seed physiological development, including production of hormones and regulation of water balance (Bouman 1984). In the mature seed of *Euphorbia millii* Desmoul., the hypostase becomes compressed and its contents are reabsorbed by the developing endosperm and embryo (Bor and Bouman 1974). However, the hypostase in leafy spurge is persistent throughout all stages of seed maturation and may have multiple roles including development of the embryo sac, embryo, and endosperm and maturation of the seed in general.

Leafy spurge, along with other members of the Euphorbiaceae, possesses a distinct obturator (Fig. 4). This hairlike structure consists of distinct glandular cells that form as an outgrowth of the placental tissue. Obturators are prevalent in Euphorbiaceae, Rosaceae, and Liliaceae, but are also found in many diverse taxa (Lyon 1898; Weniger 1917; Landes 1946; Gopinath and Gopalkrishnan 1949; Johri and Kapil 1953; Singh 1954; Kapil 1960; Bor and Bouman 1974; Tilton and Horner 1980). In leafy spurge, obturator cells arch over the caruncle, penetrate into the micropyle, and ultimately terminate at the apex of the nucellus. This extensive pattern of growth is similar to that in members of the Euphorbiaceae in which the micropyle and even the entire locular chamber become completely filled with obturator cells (Landes 1946; Gopinath and Gopalkrishnan 1949; Singh 1954). Leafy spurge also exhibits a zigzag type micropyle which the obturator completely fills. The growth and development of the integuments ultimately crushes the obturator, a phenomenon observed previously in other members of the Euphorbiaceae (Kapil 1960; Weniger 1917).

Obturators occupy a crucial position within ovaries and some produce an exudate (Gopinath and Gopalkrishnan 1949; Tilton and Horner 1980). As a result, the primary function of obturators has been attributed to directing the growth of pollen tubes into the ovule and they are considered to represent a type of transmitting tissue. While this is likely their function in many species, pollen tubes were never observed on or near the obturators in the current study. Therefore, the role of obturators in the reproductive cycle of leafy spurge is unclear and merits further investigation.

Embryo sac development

Maheshwari (1950) presented a scheme for classifying the multitude of embryo sac types found among angiosperms based on the number and pattern of mitotic divisions. Although this scheme may not hold any phylogenetic significance, it is still useful for

embryo sac descriptions and the standard *Polygonum* type (monosporic, seven celled, eight-nucleate embryo sac consisting of three antipodal cells, a large, binucleate central cell, and an egg cell adjacent to two synergids) is considered to be the most common and most primitive (Stebbins 1974). Several types of developmental patterns are evident within the Euphorbiaceae family, with the *Polygonum* type found most common in the genus *Euphorbia* (Lyon 1898; Landes 1946; Gopinath and Gopalkrishnan 1949; Srivastava 1952; Johri and Kapil 1953). Indeed, the embryo sac of leafy spurge conforms to the *Polygonum* type. Our observations show that antipodals, although formed, are ephemeral and appear to degenerate prior to embryogenesis. Ephemeral antipodals have been reported in other members of the Euphorbiaceae as well (Lyon 1898; Gopinath and Gopalkrishnan 1949; Weniger 1917). However, in *Euphorbia preslii*, antipodals persist after their formation (Weniger 1917).

The two polar nuclei found in embryo sacs of leafy spurge remain separate and distinct until the time at which a zygote is distinguished. Our observations are similar to those of Weniger (1917) who reported that after embryo sac maturation is completed in certain euphorbs, the polar nuclei remain separate and only come in contact with each other just at the time of fertilization. Similar observations were reported for other members of the Euphorbiaceae by Gopinath and Gopalkrishnan (1949) and Srivastava (1952).

Embryogenesis

Observations of several early stages reveal that leafy spurge embryogenesis conforms to a pattern similar to the Onagrad and Asterad type (Natesh and Rau 1984). Indeed, the Onagrad type is common within the Euphorbiaceae (Davis 1966) although the Solanad type occurs in *Phyllanthus* and the Piperad type is found in *Euphorbia preslii* and *E. rothiana* (Johansen 1950; Johri and Kapil 1953; Davis 1966). A report by Soueges (1924) represents one of the few studies on sexual reproductive development in leafy spurge. Unfortunately, that manuscript consists only of camera-lucida drawings of a few early stages of embryogenesis. Our results confirm some of Soueges' findings, namely, the presence of a reduced basal cell and suspensor. The nature of the basal cell varies among members of the Euphorbiaceae as *Acalypha rhomboidea* Raf. exhibits a conspicuous basal cell and suspensor (Landes 1946). Soueges (1924) described only very early stages of embryogenesis and did not provide evidence of when laticifer initials first appear. Our observations indicate that laticifer initials are not evident until cotyledons become well distinguished. These initial embryonic cells give rise to all of the laticifers found in the adult plant body (Rosowski 1968).

A variety of nutrient storage compounds, including carbohydrates, proteins, and lipids, have been identified in the embryos of angiosperms (Natesh and Rau 1984; Lopes and Larkins 1993). Our observations indicate that carbohydrates (starch grains) are present in the hypocotyl region. Besides carbohydrates, globoid protein bodies serve as the other major nutrient reserve in the embryo of leafy spurge and are most prevalent in the cotyledons. These protein bodies are similar to the aleurone grains of *Ricinus* (Frey-Wyssling 1948) except that leafy spurge appears to lack crystalloid proteins.

No more than one embryo per ovule was ever observed in leafy spurge. Thus, although polyembryony is characteristic of some members of the Euphorbiaceae (Kapil

1961), there was no evidence of this phenomenon in leafy spurge. Leafy spurge embryos are presumably the result of sexual reproduction, but pollen tubes were never observed within the micropyle, nucellar beak, or embryo sac. Thus, the potential exists for apomictic seed development in leafy spurge. If apomixis does occur, it is most likely of the pseudogamous type as pollination appears to be required for seed set (J.S. Carmichael and S.M. Selbo, unpublished). This phenomenon has been documented in *Euphorbia dulcis* L. (Kapil 1961) and thus merits further investigation in leafy spurge.

Endosperm development

Free nuclear endosperm, with subsequent cellularization, is the most common type found among all angiosperms (Lopes and Larkins 1993). Indeed, free nuclear endosperm is expressed in leafy spurge and is also characteristic of most members of the Euphorbiaceae (Landes 1946; Gopinath and Gopalkrishnan 1949; Srivastava 1952; Johri and Kapil 1953; Singh 1954; Davis 1966). The endosperm of leafy spurge is presumably triploid, being formed by the fusion of two polar nuclei and one sperm nucleus. However, this is equivocal since double fertilization was never observed and pentaploid endosperm has been reported in other members of the Euphorbiaceae (Kapil 1960). In leafy spurge, centripetal cellularization of the free nuclear endosperm takes place during the globular stage of proembryogenesis, a pattern reported previously in other members of the Euphorbiaceae (Johri and Kapil 1953; Kapil 1961). In *Ricinus communis* L., however, the embryo undergoes significant development and cotyledons are differentiated before cellularization of endosperm tissue commences (Singh 1954). During the free nuclear period of development, the entire embryo sac expands longitudinally thereby crushing the nucellar tissue, a developmental pattern also described by Kapil (1961).

Integument growth, maturation, and lignification take place concurrently with endosperm cellularization and development. It is also during this time that the hypostase develops into a well-distinguished structure whose cells consist predominantly of carbohydrates (Fig. 34). The hypostase of some euphorbs eventually becomes compressed and its contents reabsorbed by the developing endosperm and embryo (Bor and Bouman 1974). However, the hypostase of leafy spurge persists throughout all stages of endosperm development, and the precise relationship between endosperm and hypostase is unclear.

The formation of endosperm and its role in the provisioning of nutrients for developing embryos represent defining features of angiosperms. There is significant variation in the types of nutrients typically sequestered in endosperm cells of angiosperms, with most species storing some form of carbohydrates, proteins, or fats (Vijayaraghavan and Prabhakar 1984; Lopes and Larkins 1993). Our histochemical tests show positive results for protein (globoid bodies) and carbohydrate (but predominantly as cell wall material) in the endosperm. In leafy spurge, protein is stored throughout the entire endosperm tissue, except in the central region which consists primarily of highly vacuolate cells (Fig. 23). The lack of protein reserves in the central endosperm region may be due to the metabolism of these nutrients by the developing embryo, which ultimately occupies a large portion of the seed. Several members of Euphorbiaceae sequester fat reserves within their endosperm. We did not observe lipids to any significant extent within the endosperm cells

of leafy spurge. These results were unexpected since the genus *Euphorbia* is characterized by members that contain up to 49% of their total endosperm weight as oil or fat (Bewley and Black 1978).

Concluding remarks

Vegetative and sexual reproduction in leafy spurge will likely receive increased attention as this plant continues to spread and invade land that would otherwise be used for cattle, crops, and other agriculturally important purposes. Control of leafy spurge, especially the extensive root systems that are primarily responsible for large-scale infestations of this plant, represents an important step at increasing the world's food production throughout the next few decades. This report describes many intriguing reproductive features of leafy spurge, such as the caruncle, obturator, preliminary hints at the potential for apomictic seed development, and also serves as a basis for future breeding system studies. Collectively, an understanding of sexual reproductive strategies and vegetative growth patterns may enhance future biological control efforts.

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