MISSISSIPPI SOYBEAN PROMOTION BOARD
PROJECT 08-2017 (YEAR 1)
2017 ANNUAL REPORT

Title of project: Using weeds as a resource to develop herbivore-resistant soybean

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BACKGROUND AND OBJECTIVES

Among the current solutions to manage herbivores, fencing is expensive, labor intensive, and requires weekly inspection to ensure effective operation, while repellents decrease effectiveness after rainfall. Weeds, because of their vast genetic and phenotypic diversity, are a good resource for anti-herbivore traits. Studies have shown that sicklepod weed seeds and plants contain high amounts of anthraquinone, which may be the most effective chemical anti-herbivory strategy of any plant on earth against both insects and mammals.

The objectives of the project are:

1. Prepare plant extracts of potential weed species and develop a liquid formulation for application on soybean crop;
2. Conduct field trials using unmanned aerial vehicles (UAVs), plant surveys, and trail cameras to quantify herbivore use and damage to soybeans in treatment and control plantings;
3. Conduct chromatography and mass spectrometry analysis to identify target anti-herbivore compounds in weeds; and,
4. Conduct quantitative trait loci analysis to identify molecular markers associated with anti-herbivory compounds in weeds.

REPORT OF PROGRESS/ACTIVITY

Objective 1.

As chrysophanol, emodin, and physcion were listed as main anthraquinone components in sicklepod seeds and roots, they were selected as standards to estimate anthraquinone derivative concentrations.

Two extractions were adopted: *Extraction 1* was used for qualitative analysis, and *Extraction 2* was used for quantitative analysis; all quantitative analyses were conducted in three replicates.

Extraction 1: Plant part powder of 0.10 g was weighted into a 1.5 mL tube, followed by the addition of 1 mL methanol and 200 µL chloroform. The tube was mixed for 20 min and centrifuged at 13,200 rpm for 10 min. The resulting supernatant was filtered (0.2 µm pore size) and air evaporated overnight for HPLC and TLC analyses.

Extraction 2: The sicklepod leaf powder of 1.0 g was weighted into a 50 mL tube, followed by the addition of 20 mL methanol, 5 mL double deionized water, and 5 mL chloroform. The tube was mixed for 4 hours and vacuum filtered. Ten ml of methanol was used to rinse the plant powder in the Buchner

Funnel. The combined filtrate was evaporated to dry in a water bath at 60 °C, and then 3 mL methanol was added to dissolve for HPLC and TLC analyses.

Objective 2.

Field plots were established at the R.R. Foil Plant Science Research Center (North Farm) on June 2017 to determine the efficacy of different deer repellent treatments on deer. The various deer repellent treatments were: sicklepod root extract, three commercially available deer repellents, and water to serve as control.

Aerial imagery by UAVs and plant responses such as % injury, % stunting, chlorophyll content, and % browsing are being evaluated/recorded on weekly basis. In our Captive Deer Facility trials, soybean plants without sicklepod extract were consumed completely, while plants with sicklepod extract were entirely avoided (Figure 2, Figure 3). In field trials excluding all herbivores, application of sicklepod extracts did not cause any soybean yield reduction compared to untreated plants, thus indicating the sicklepod extract has no adverse effect on overall soybean yield (Figure 4).

We used imagery from 2016 to determine where to establish our field trials by targeting high-herbivory areas, but our treatments resulted in a shift in deer herbivory to another part of the field (Figure 5). Thus, we did not observe a difference between controls and treatments because deer avoided the entire area. The field experiment is currently being repeated at Andrew's Forestry and Wildlife Experiment station which has high deer density.

We will also repeat the field experiment next year, at no cost, by conducting whole field treatments to quantify the change in deer herbivory on entire field production. Insect damage was apparent in our treatment area, and the data suggest that soybean plants treated with sicklepod extract had less insect damage than our control or two other formulations on the market (Figure 6).

Results from the captive deer experiment demonstrated that anthraquinone extracts from sicklepod applied on soybean is able to repel deer and prevent insect feeding. Furthermore, in the field experiment, when all herbivores were excluded, we demonstrated that sicklepod extract application did not affect soybean yield.

Objective 3.

High pressure liquid chromatography (HPLC) analysis. An Agilent 1100 series HPLC (Agilent, Santa Clara, CA) was used to analyze anthraquinone derivatives in the extracts. The HPLC consisted of a tandem diode array (DAD) and fluorescence (FLD) detectors, an online vacuum degasser, a quaternary pump, an autosampler, and a thermostatted column compartment.

The Agilent Chemstation A.10.02 software with a spectral module (Agilent Technologies Inc., Wilmington, DE, USA) was used to process the data. Separation was achieved on an Alltech Adsorbsphere reverse phase C18 column (150 mm x 4.6 mm, Dr. A. Maisch High Performance LC GmbH, Germany) with particle size 3 µm. Anthraquinone derivatives were detected at 254 nm (Dionex, 2009) with a flow rate of 1 mL/min and column temperature of 30ºC. Peaks were identified using standard compounds (chrysophanol and emodin). The injection volume of 5 µL was used.

A gradient elution program was developed as follows: Eluent was mixed by acetonitrile with water. Acetonitrile keeps 40% for the first 8 minutes, followed by increasing to 70% from 8 minutes to 21 minutes, then keeps at 70% to 25 minutes. Sicklepod plant part extracts (stem, root, fruit and leaf) from *Extraction 2* were analyzed for chrysophanol and emodin.

UV Fluorescence of Anthraquinone derivatives. A fluorescence microscope (Olympus BX51) equipped with a cube consisting of filters D360/40x, 400dclp, and ET560lp, was used to observe the fluorescence of the anthraquinone derivatives. The latter filter completely blocks the UV excitation (350 nm) and allows only signals from approximately 560 nm and longer to be passed. The UV excitation of the microscope was 350 nm. This setting allowed the microscope to observe fluorescence of anthraquinone derivatives around 580-600 nm (yellow). Fresh plant tissues (sicklepod root, stem and soybean stem) were cut free-hand using a razor blade into thick sections and immediately examined under the microscope. All observations were in three replicates.

Conclusion. The UV fluorescence of anthraquinone derivatives can be used to observe anthraquinone distribution in sicklepod plant using fluorescence microscope. Cell types with anthraquinone levels listed in their descending order are: endosperm cell > podshell cell > root cell > stem pith cell (Figure 7). They all have high anthraquinone contents, but endosperm has less cellulose and high galactomannans, while other cells are rich in cellulose.

Cell cultures of endosperm or podshell (J) may be an alternate method to prepare sicklepod extracts rich in anthraquinone derivatives (Figure 8). Sicklepod fruit contains high anthraquinone concentration with anthraquinone derivatives primarily concentrated in the endosperm. The arrangement of the membrane structures rich in anthraquinone derivatives, open structures, and irregular shape, suggest they may not be cell membrane but instead organelle, although they are similar to cell size. The size and shape of the hollow membrane structures in endosperm (K, L & M) are similar to those of the bright yellow “cells” in podshell (J), thus indicating they may be the same structures (Figure 9).

Objective 4.

Conduct quantitative trait loci analysis to identify molecular markers associated with anti-herbivory compounds in weeds.

This objective was modified to more precisely target the genes related to anti-herbivory. Instead of screening the weed (sicklepod) for markers related to anti-herbivory, we plan to screen a large germplasm of wild soybean varieties for high anthraquinone production using HPLC analysis. Soybean varieties having high anthraquinone production will then be used in quantitative trait loci analysis to associate molecular markers to anthraquinone production. This objective will be continued throughout the second year of funding at no additional cost to MSPB.

IMPACTS AND BENEFITS TO MISSISSIPPI SOYBEAN PRODUCERS

The primary beneficiaries of the project will be all soybean growers in Mississippi, who represent over 2.3 million acres across the state. The estimated average yield for soybean in Mississippi is about 46 bushels per acre, and the soybean production in 2016 is estimated at 112 million bushels or $900 million in production value. Considering up to 26% and 41% yield reduction caused by deer and insect herbivory, respectively, the estimated economic loss could be $234 and $369 million annually in

Mississippi.

Developing a low cost spray is the first step in solving this problem. The field studies confirmed sicklepod as having anti-herbivore potential. The HPLC analysis identified anthraquinone derivaties and glycosides responsible for the anti-herbivore property, particularly emodin, chrysophanol, and physcion. These compounds identified to be responsible for anti-herbivore property will allow us to locate the biochemical pathway and genes related to the production of the particular compound.

Molecular markers can then be developed associated with this pathway/genes, which is in turn linked to the anti-herbivore compound. Using these anti-herbivore markers, we can screen row crops and vegetables for the anti-herbivore trait, or use it in molecular breeding to breed the anti-herbivore trait into crops. Crops with significant anti-herbivore property will prevent yield losses incurred due to herbivores such as deer. Soybean with significant anti-herbivore property will prevent yield losses incurred due to herbivores especially deer and insects.

END PRODUCTS

Publications

- Yue, Z., Tseng, T.M. and Lashley, M., 2018. Characterization and Deer-Repellent Property of Chrysophanol and Emodin from Sicklepod Weed. *American Journal of Plant Sciences*, *9*(02), p.266.
- Yue, Z., and Tseng, T.M., 2018. Tissue distribution and subcellular localization of anthraquinone derivatives in sicklepod plants. (In preparation for submission to *Frontiers of Plant Science*).
- Tseng, T. M., 2018. From Foes to Friends: Exploiting the Agricultural Potential of Weeds. (Accepted in *Scientia*).

Abstracts

- Yue Z., and **T. M. Tseng** (2018) Study of anthraquinone biosynthesis, transport and storage in sicklepod weed using fluorescence imaging. In *Proceedings of Southern Weed Science Society*, vol. 71.
- Yue Z., M. Lashley, S. Shrestha, G. Caputo, and **T. M. Tseng** (2018) Field testing of sicklepod extract as effective deer repellent to protect soybean. In *Proceedings of Southern Weed Science Society*, vol. 71.
- Yue, Z., and **T.M. Tseng** (2017) Tissue-specific distribution of anthraquinone compounds in sicklepod plants using fluorescence microscopy. In *Proceedings of Southern Weed Science Society*, vol. 70, p. 17.
- Yue, Z., and **T.M. Tseng** (2017) Characterization of anthraquinones: A potential anti-herbivory compound in sicklepod. In *Proceedings of Southern Weed Science Society*, vol. 70, p. 43.
- **Tseng, T.M.**, Z. Yue, and M. Lashley (2016) Characterization of Anti-Herbivore Property of Sicklepod Weed. In *Proceedings of ASA, CSSA and SSSA International Annual Meetings*, vol. 151-1005.

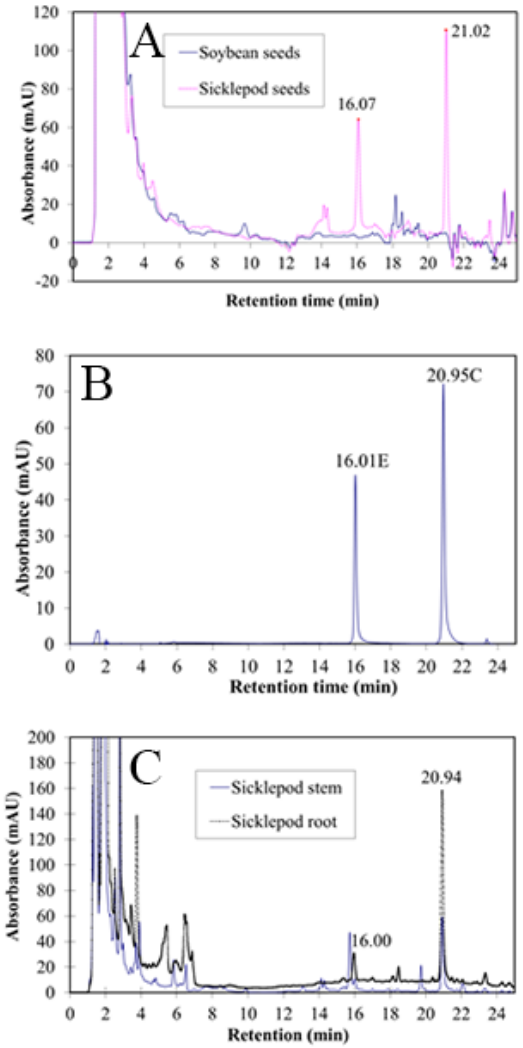

Figure 1 (a) Chromatogram comparison between sicklepod and soybean leaves; the most pronounced differential peaks were found at retention time (RT) 16.07 min and 21.02 min. (b) Chromatogram of mixed standards of chrysophanol and emodin, their RT 16.01 min and 20.95 min match with the differential peaks in (a). (c) Chromatogram comparison between sicklepod stem and root. The root showed higher concentrations of chrysophanol and emodin.

Figure 2. Comparison of deer browsing of soybean plants applied with sicklepod extract (A), and applied with water as control (B). About 95% of the vegetation of soybean plants applied with water was browsed by deer, while none of the vegetation of soybean plants applied with sicklepod extract was browsed.

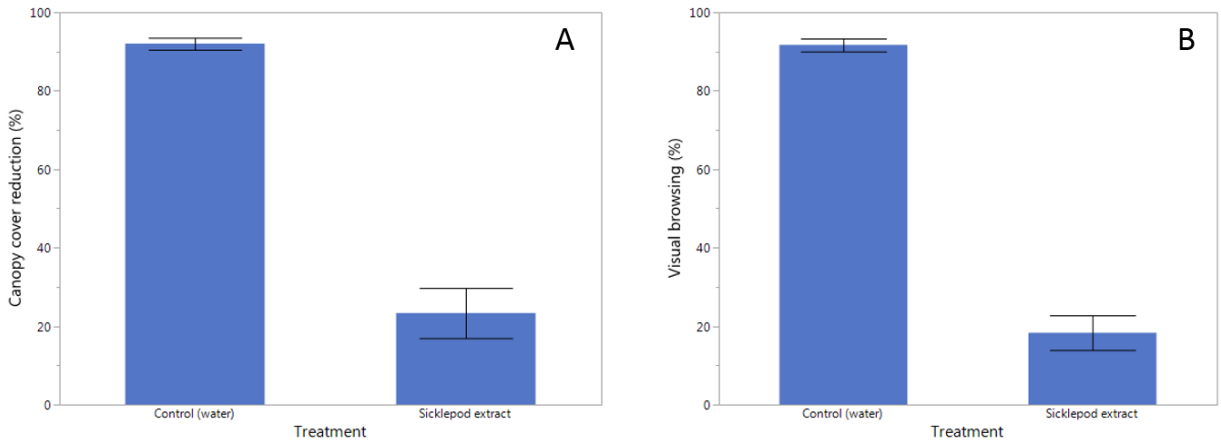

Figure 3. Canopy cover reduction (%) (a), and visual browsing (%) (b) of soybean seedlings treated with water (control), and sicklepod seed extract, after 4 hr of exposure to captive deer at the Captive Deer Facility at Mississippi State University. Images of soybean canopy were captured before and after deer exposure and analyzed using image analysis software Image J to determine percent canopy cover reduction caused by deer browsing. Visual browsing was recorded on a scale of 0 to 100% where 0 = no browsing, and 100 = completely browsed.

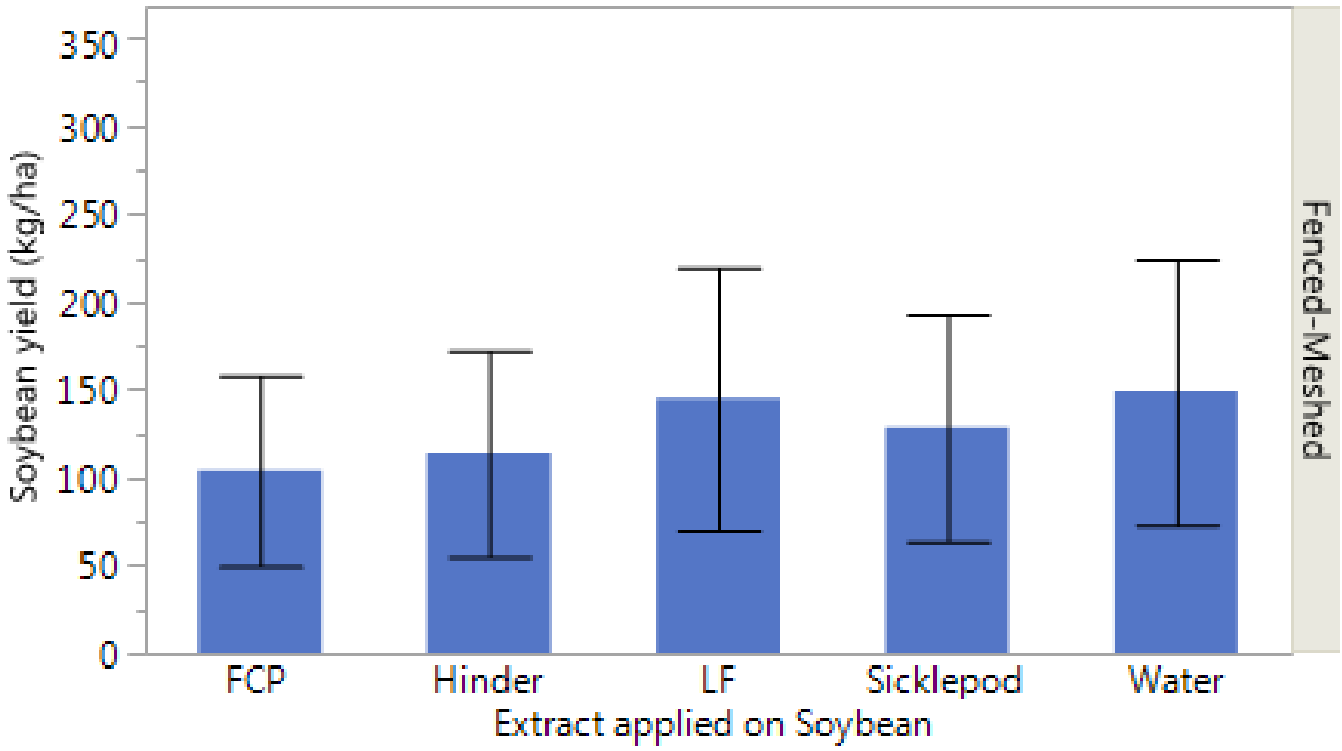

Figure 4. Effect of application of deer-repelling extracts on soybean yield. All treatments were under electric fencing and covered using nylon net (10 mesh/in) to protect plants from deer and insect damage, respectively. FCP (Flight Control Plus), Hinder, and LF (Liquid Fence) are commercially available herbivore-repellents (primarily deer), sicklepod is the extract of sicklepod plants, and water serves as an untreated control.

Figure 5. Aerial photograph of white-tailed deer herbivory damage from UAV flights. We targeted our treatment area (black solid line) in the high deer herbivory area from 2016 (red dotted line) and deer herbivory shifted to avoid our treatments entirely in 2017 (red solid line).

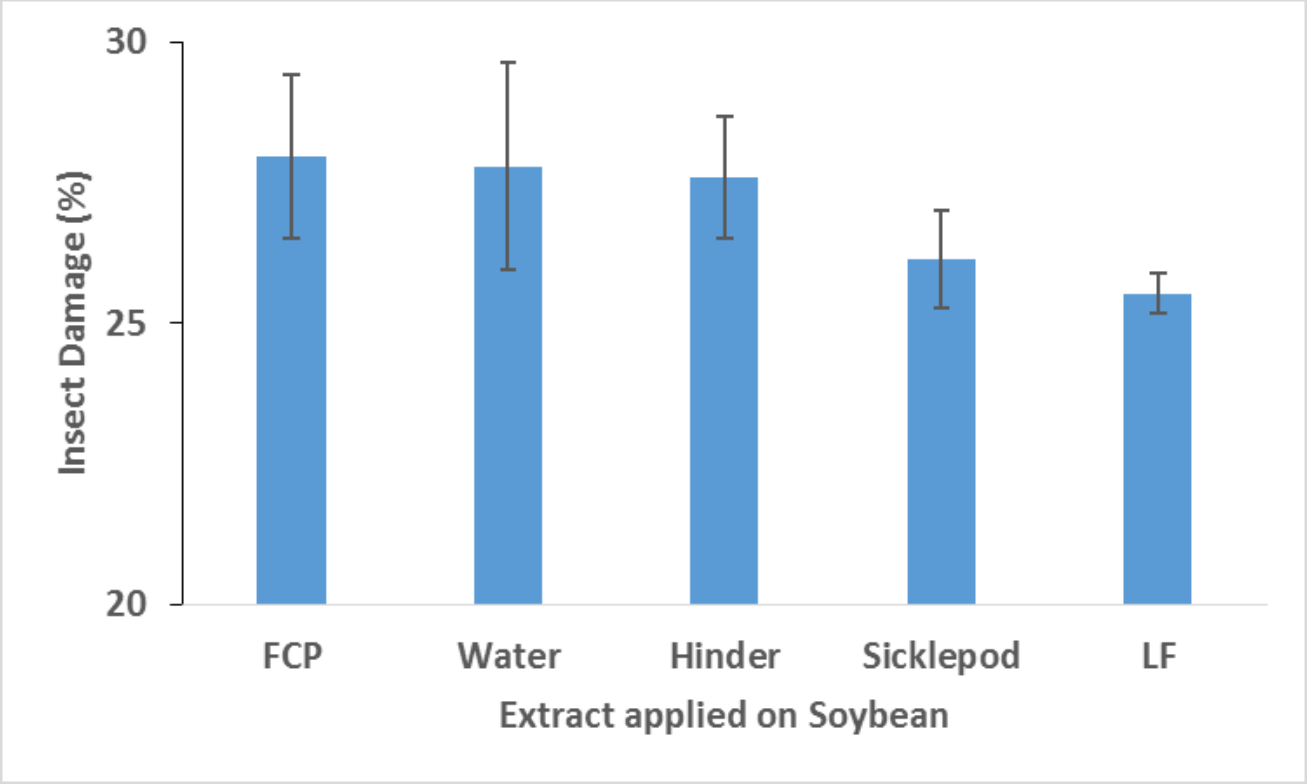

Figure 6. Effect of application of deer-repelling extracts on soybean insect damage. FCP (Flight Control Plus), Hinder, and LF (Liquid Fence) are commercially available herbivore-repellents (primarily deer), sicklepod is the extract of sicklepod plants, and water serves as an untreated control.

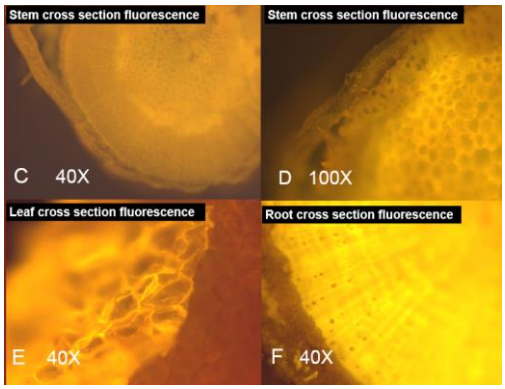

Figure 7. Stem section fluorescence shows three different anthraquinone concentrations: pith > woody stem > stem bark; (D) shows lowest anthraquinone concentration in stem surface; (E) leaf surface is dark while inside is bright yellow (anthraquinone derivatives); the leaf cell on leaf surface is estimated around 40 µm; and, (F) root section with high anthraquinone derivatives (bright yellow); surface cells show lower anthraquinone concentrations.

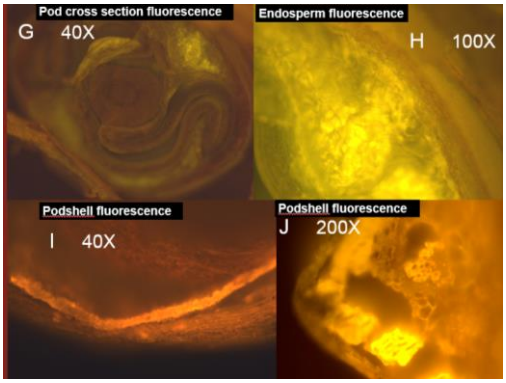

Figure 8. Pod cross-section: the radicle and plumule are relatively dark while the endosperm is bright yellow; (H) shows high anthraquinone concentration in endosperm; (I) shows podshell containing tissues with high anthraquinone derivative concentration (orange); and, (J) also shows some pod shell tissues contains high anthraquinone concentrations (bright yellow). The bright yellow cell section is around 28 µm.

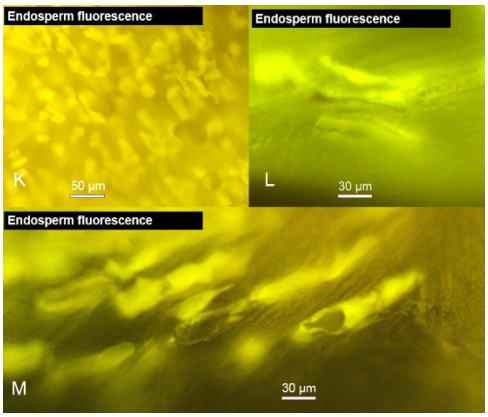

Figure 9. Pod cross-section: (K), (L), and (M) shows hollow membrane structures rich in anthraquinone derivatives in endosperm. They are irregularly shaped (K), and are isolated from each other or floating in the endosperm; 75% of which is galactomannans (Shang et al. 2012); they are not necessarily closed structure (L and M). Their section size is around 28 µm and length is around 55 µm.