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Ampelopsis brevipedunculata Berries are Simultaneously Attractive to Birds and Repulsive to Mammals

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Abstract: Berries are usually brightly pigmented with primary colors, for example *Ilex americana* (Holly) or *Solanum lycopersicum* (Tomato). In contrast, berries of *Ampelopsis brevipedunculata* (Maxim.) *Trautv* display bright patterns of pigments (red, green, blue and many intermediate shades) to attract animals and achieve seed dispersal. Our primary aim was to elucidate this unusual feature of *A. brevipedunculata*. In addition to this attractive feature, during our study we noticed two kinds of chemical structures that repel chewing animals and favor non-chewing animals; brittle (lignified) multicellular containers of poison (polyphenolic tannins) and idioblasts with raphides of calcium oxalate. Our secondary aim was to understand these contrasting features of *A. brevipedunculata* berries. Color changes may not be explained by simple changes in pH within pigment cells, titrating anthocyanins between various color forms. However, a RBVG (Red, Blue, Violet and Green) or Tetrachromatic Color model of animal vision can explain the formation of intermediate colors and pattern formation in these berries and suggests the target for berry attraction is birds not mammals because in addition to the three color receptors seen in humans, birds use a fourth near-ultraviolet receptors to achieve tetrachromatic vision. This is consistent with anatomical observations presented.

Key words: *Ampelopsis brevipedunculata*, invasive, oxalate, idioblast, anthocyanin

INTRODUCTION

Ampelopsis brevipedunculata (Maxim.) *Trautv.* (Porcelain Berry, vitaceae) is an understory vine whose seeds are distributed by birds that consume berries (Kominami *et al*., 2003). In New Jersey it is an invasive plant (Kaufman and Kaufman, 2007) which forms heavy tangled masses in the branches of competitor plants, dragging them down. This plant originated in East Asia and was not known in New Jersey during early plant surveys (Britton, 1881) i.e., this plant is a relatively recent arrival to New Jersey and is successfully invading this new habitat. It is a woody vine with a high lignin content in the form of xylem and bands of lignified fibers (Witty *et al*., 2010). The most conspicuous feature of *A. brevipedunculata* is the development of berries which show a complex series of colors with various shades of blue and a pattern of spots (Fig. 1a).

Ampelopsis brevipedunculata has several mechanisms besides gross anatomical features to account for its success. Crude extracts brown rapidly after maceration showing a general abundance of phenolic compounds such as tannins. In addition, this plant produces a range of novel oligostilbenes, ampelopsins (Oshima and Uenoa, 1993) whose natural function is assumed to be defensive. Some of these compounds have a potent effect on mammalian cellular processes as show by the effect of ethanol extracts (40% v/v in water) of *Ampelopsis brevipedunculata* berries that reduce proliferation in liver cell lines (Yabe and Matsui, 1997).

Ideoblasts are prominent in *A. brevipedunculata* and sharp calcium oxalate crystals (raphides) are a well known defense structure in many plant species (Franceschi and Nakata 2005; Korth *et al*., 2006) including other members of the vitaceae (DeBolt *et al*., 2004). Formation of calcium oxalate is associated with a matrix of macromolecules including protein which guides crystal growth (Bouropoulos *et al*., 2001; Li *et al*., 2003). Soluble oxalic acid is toxic to animals and insoluble calcium oxalate raphide crystals cause physical damage to vulnerable animal tissues (Naude and Naidoo, 2007) which means that a chemical titration of calcium oxalate to oxalic acid or separation of raphides from the matrix is required before it may become repulsive to animals.

This study describes some additional adaptations for defense in *A. brevipedunculata*; the formation of lignified envelopes of defense compounds. These were revealed using differential staining i.e., phloroglucinol to indicate

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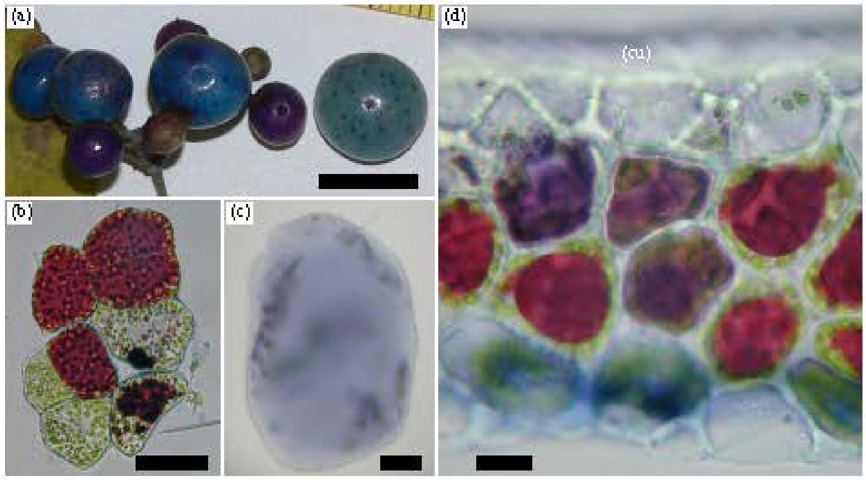

Fig. 1: Natural pigments of *Ampelopsis brevipedunculata*. (a) Mature berries (purple) and berries in transition to maturity showing patterns of pigmentation and non-primary colors such as cyan. Bar, 1 cm, (b) Macerated fruit showing a clump of closely juxtaposed red and green cells. Bar, 50 µm. (c) Macerated fruit showing a single purple cell. Bar, 10 µm. (d) section of exocarp showing closely juxtaposed red, blue and purple cells. Bar, 50 µm. cu, cuticle

lignified cells, vanillin to reveal tannins and other polyphenolic compounds (Zimmerman, 1893; Gardner, 1975; Peterson *et al*., 2008) and the polychromatic stain Toluidine Blue O (O'Brian *et al*., 1964).

We also describe a contrasting attractive feature of *A. brevipedunculata* i.e., a mechanism for color and pattern formation in berries. While three color receptors are seen in human vision (Rowe, 2002) most birds use a fourth near-ultraviolet receptor to achieve tetrachromatic vision (Chen *et al*., 1984; Honkavaara *et al*., 2002). We propose that these berries form intermediate colors and patterns which are more readily perceived by herbivores with tetrachromatic vision and suggest that the target for berry attraction is birds not mammals (Kelber *et al*., 2003; Rowe, 2002). Our explanation for *A. brevipedunculata* color uses an RBVG (Red, Blue, Violet and Green) hypothesis of superposition of four primary colors to attract birds, rather than three to attract mammals because all four of these pigments are present in *A. brevipedunculata* berries.

MATERIALS AND METHODS

Plant material was collected in October 2008 from the banks of Whale Pond Brook, West Long Branch, New Jersey and then sectioned either by hand or in a styrofoam clamp using the method of Carrington (2004). Maceration to remove individual pigment cells or groups of cells from mature berries was done under water, using a razor blade. Cells and tissues were stained as follows: Toluidine Blue O (TBO) using the methods described in O'Brian *et al*. (1964) and Peterson *et al*. (2008); Phloroglucinol and Vanillin-HCl staining using the methods of Peterson *et al*. (2008). Sudan IV and Sudan Black Stain were used at 0.5% $^{w}/_{v}$ in 70% ethanol. All other chemicals were reagent grade material purchased from the Sigma Chemical Company, St Louis.

For acid base titration of berries one berry was macerated as above in $\approx$500 µL dH_2O then decanted to a 20 mL beaker. The pulp was further extracted with 10 mL water and this was added to the pool of extract which was then filtered through Whatman paper. pH was measured using a meter with glass pH electrode (VWR Scientific Products models 8100 and 14002-772), then aliquots of 1N NaOH (10 µL) or 0.1 M HCl (100 µL) were added in a stepwise manner with pH measured at each step. pH was noted and a photograph taken with the addition of every aliquot.

RESULTS

Unlike most other berries, which have simple pigment tissues, *A. brevipedunculata* showed multiple layers of mesocarp cells with distinctly different pigment content that are closely juxtaposed (Fig. 1b-d). In addition, large structures in *A. brevipedunculata* fruit resembling the stone cell complexes of *Pyrus communis* (European Pear) were seen, but with a complex multilayer structure that includes lignified cells enveloping interior cells which accumulate tannins (Fig. 2a-c). These envelope structures displayed the typical pink color of phloroglucinol on

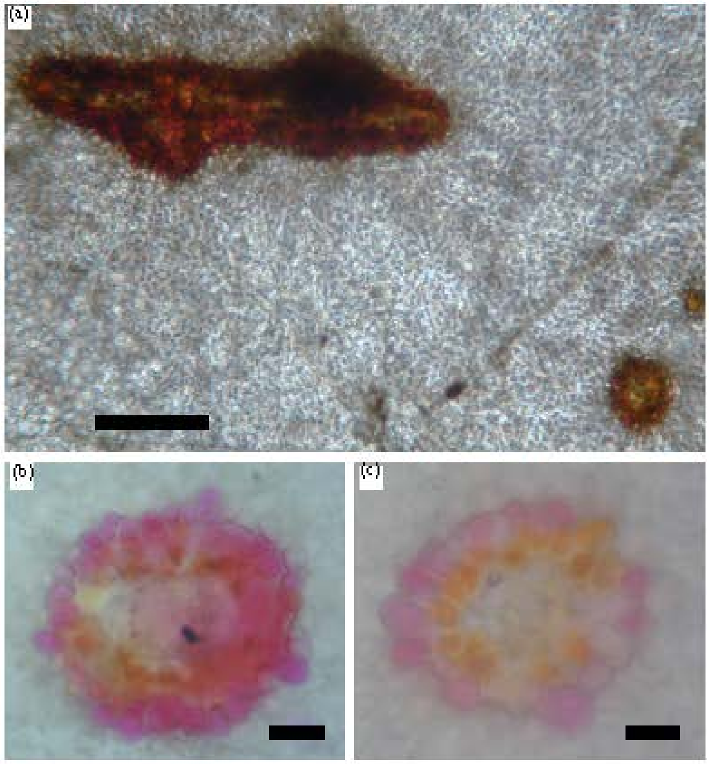

Fig. 2: Phloroglucinol stained sections of mesocarp. (a) Small and large groups of lignified cells are seen. Bar, 1 mm. (b) and (c) exterior cells stain pink because of reaction with phloroglucinol while interior cells develop a brown color because of endogenous phenolic browning. Central cells are not pigmented and show a hollow envelope of lignified cells. Bars, 100 µm

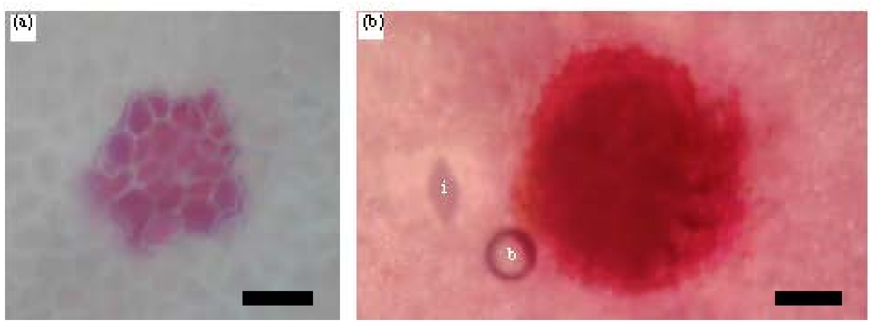

Fig. 3: Vanillin stained sections of mesocarp. (a) Small group of cells with transparent cell walls and containing polyphenolic tannins. These are assumed to correspond to the endogenously pigmented cells seen in Fig. 2. Bar, 120 µm. (b) large group of cells with no unstained central cells. Bar, 250 µm. i, idioblast. b, air bubble

exterior cells and also endogenous brown pigmentation from oxidation of phenolic compounds in interior cells. (Fig. 2c). Corresponding structures also reacted strongly with vanillin, confirming the phenolic nature of these bodies (Fig. 3a-b).

Idioblasts in the form of large cells containing large bundles of refractive raphide crystals were seen in unstained cells (Fig. 4c). Raphide bundles are visible in unstained material but also stain strongly with Sudan black (Fig. 4a) and TBO (Fig. 4d), suggesting these crystals are surrounded by a matrix of partially hydrophobic nature.

Titration of berry crude extracts showed an average pH of 4.5 for this organ and the formation of red at low pH

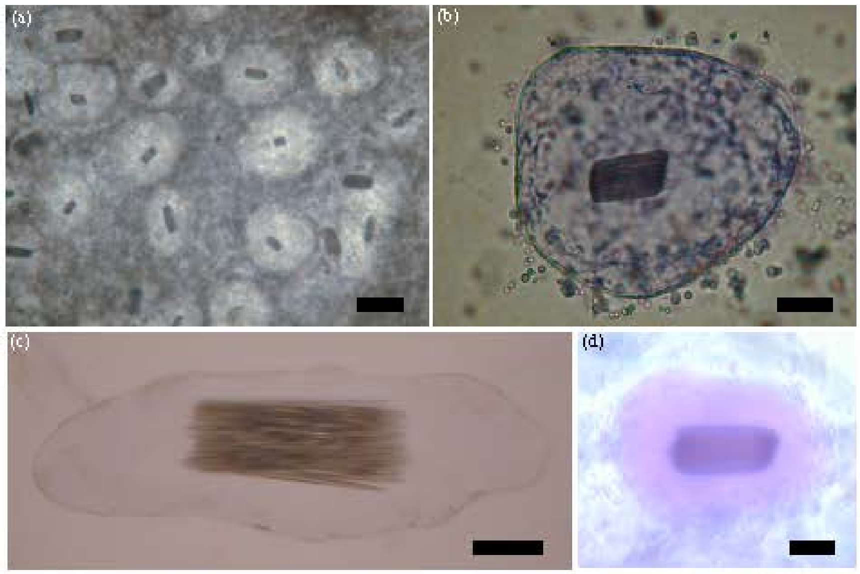

Fig. 4: Mesocarp idioblasts. (a) Mesocarp section showing large cells which contain central bundles of raphides in a hydrophobic matrix which stains with Sudan Black. Bar 50 µm. (b) Macerated fruit stained with TBO. Bar, 10 µm. (c) Macerated fruit, not stained. Bar, 5 µm. Small idioblast well stained with TBO. Bar, 10 µm

or purple at high pH i.e., no intermediate shades such as the cyan seen in some stages of berry development.

DISCUSSION

Most previous research has explained berry pigmentation in terms of one or a few anthocyanins or carotenoids conferring uniform pigmentation in primary colors (Giovannoni, 2001). *A. brevipedunculata* has unusual fruit with complex patterns of pigments, incorporating patterns of pastel shades more typical of some animal epidermi (Cloney and Florey, 1968). Titration experiments show that *A. brevipedunculata* fruit has an average pH of 4.5 and that anthocyanins can have only two colors; red or blue with a color transition at pH 7 (Fig. 5). However, the attractive shades and patterns of these berries includes intermediate shades between red and blue, such as cyan, which are not provided by simple absorption by anthocyanin. Microscopic examination of epidermal pigment cells was revealing. These cells have single pigments which are part of tissues where cells overlap in a way that, because of limited ocular resolution by animals, must be perceived as an average color, rather than an identifiable patch of macroscopic pigment; this provides intermediate color shades i.e., pastel shades (Hanlon, 2007; Honkavaara *et al*., 2002). The closely juxtaposed cells with four very different colors: red, blue, violet and green, suggest an RBVG coloration mechanism by this plant with

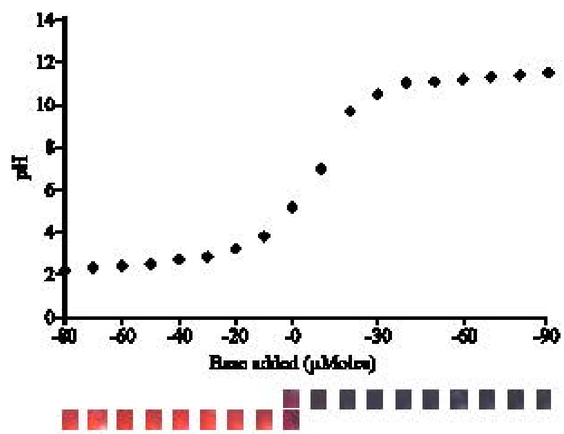

Fig. 5: Titration of *Ampelopsis brevipedunculata* anthocyanins. Only two primary colors and an intermediate shade were seen which is in contrast to the range of colors seen in Fig. 1

four pigments corresponding to the four color receptors of an avian eye better than the three color receptors of the mammalian eye.

The interior of *A. brevipedunculata* fruit is red and pH 4.5 (average, crude extract), near a pKa for oxalic acid ($pKa_1 = 1.27$ and $pKa_2 = 4.28$) and therefore, near the point at which calcium oxalate dissolution is possible. Because some compartments contain solid oxalate raphides (solid calcium oxalate, Fig. 4a-d) and some cells contain anthocyanin in the red form (Fig. 1d). I assume great variation in pH between cells or organelles and that a lower pH than the average of cytoplasm and vacuole prevails in the vacuole alone.

CONCLUSION

In general two kinds of herbivore are adapted to berries with an exocarp as thick as *A. brevipedunculata*: small mammals and birds. Because small mammals chew fruit before swallowing the repulsive features of *A. brevipedunculata* fruit are expressed: raphides are released causing physical damage to the mouth and oesophagus; mixing of solid oxalate salt and organic acids of plant origin forms soluble oxalic acid, a poison for mammals (Naude and Naidoo, 2007); chewing will also disrupt the large brittle (lignified) envelopes of tannin cell complexes. Birds, however, may be able to consume *A. brevipedunculata* berries unharmed because of their feeding habits. Birds swallow their food whole then macerate it in their gizzard. Raphides released in the acid gizzard would be rapidly dissolved, destroying the sharp raphides. Therefore birds, which are resistant to free oxalic acid poisoning (Verkoelen and Romijn, 1996; Nellis, 1998) and efficiently excrete it with their kidneys (Tremaine *et al*., 1985), also avoid *physical* damage to tissues by raphides.

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