PHYTOMELANIN: DEVELOPMENT AND ROLE IN HYBRID RESISTANCE TO *HOMOEOSOMA ELECTELLUM* LARVAE (LEPIDOPTERA:PYRALLIDAE).

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ABSTRACT

There was a highly significant negative correlation (r = − 0.93) between achene injury by larvae of *Homoeosoma electellum* (Hulst) and the presence of phytomelanin in pericarps during 4 years of field tests. Phytomelanin becomes evident microscopically as early as 3 days after achene fertilization in genotypes expressing the characteristic. The phytomelanin layer develops as a metabolic precipitate from the catabolism of hypodermal cells, and has a synergistic effect on early lignification and hardening of sclerenchyma cell walls. Young larvae of *H. electellum* showed a significantly greater feeding preference for RHA 266 pericarps lacking phytomelanin than for RHA 265 pericarps having phytomelanin in both no-choice and free-choice whole-achene feeding trials in the laboratory. Young larvae feeding on ground pericarps of RHS 265 and RHA 266 incorporated in a wheat-germ diet experienced a significantly higher mortality when feeding on the RHA 265 diet. Among released hybrid variety parental lines, the pericarp of 39% of the RHA's and 97% of the cmsHA's have phytomelanin.

INTRODUCTION

A phytomelanin (armored) layer develops between the hypodermis and sclerenchyma in the pericarp of some genotypes of cultivated sunflower, *Helianthus annuus* L. (E.D. Putt, 1944). Although the layer has been described as carboniferous, resinous, and amorphous (Sarkany, 1947; Kiewnick, 1964; and Putt, 1944), its chemical nature has not been defined nor have the physiological processes contributing to its development been clarified.

The phytomelanin layer is pliable and soft in the pericarp of immature achenes, but becomes rigid, dense, and hard in the pericarp of mature achenes (Kiewnick, 1964). The usefulness of the phytomelanin layer in protecting sunflower achenes from injury by larvae of *Homoeosoma nebulella* (Hubner) in Europe is well documented (Shapiro, 1975). However, the impact of phytomelanin in protecting achenes of hybrid varieties from injury by larvae of *Homoeosoma electellum* (Hulst) in North America is negligible, and its role as a useful resistance mechanism is largely discounted (Kinman, 1964). Recent investigations have indicated that we need to re-examine the potential role of phytomelanin as a useful tool in reducing economic losses caused by larvae of *H. electellum* in North America (Rogers, 1980).

MATERIALS AND METHODS

Field Trials — Several sunflower lines were planted in replicated, randomized blocks at Bushland, Texas, each year from 1976 to 1980. Plantings were made in late April to assure that flowering closely coincided with the seasonal peak of sunflower moth flight and ovi-positional activities. Fifteen plants from each of the 7.5 m rows were tagged and numbered to facilitate subsequent collection and summation of data. Detailed records were kept on flowering date, anthocyanin pigmentation of the pericarp and inflorescence, frassiness of the inflorescence, the presence of phytomelanin, and sclerenchyma and thickness. Data from each of the parameters were subjected to regression analyses to determine their correlation with achene injury by *H. electellum*. The correlation of larval injury with the parameters was accomplished by pairing achenes of plants within entries having coincident flowering dates across years.

Phytomelanin Development — The development of phytomelanin was studied by collecting achenes from adjacent heads within a line segregating for the characteristic. Three achenes were collected from the heads daily from day 0 though 18 post-fertilization, and at 3-day intervals thereafter through day 29 post-fertilization. As achenes were collected, they were cut into quarters and placed in either 90% ethanol or glutaraldehyde, where they remained until further processing. Fixed pericarps of each age grouping were subsequently processed, embedded in plastic, sectioned, and examined microscopically for phytomelanin development and other morphological differences.

The resistance of pericarps containing phytomelanin to penetration was determined in the greenhouse by a penetrometer. Immature achenes were collected 0, 5, 10, 15, and 20 days after fertilization, and the force required to penetrate the pericarp in the crown and equator areas was determined. Penetrometer readings were obtained for five achenes from five different plants at each age for about 100 lines segregating for the characteristic. Data were subjected to an analysis of variance and significantly different means were separated by Duncan's Multiple Range Test.

Laboratory Bioassays — The effect of phytomelanin on injury to immature achenes by larvae of *H. electellum* was studied by two bioassay techniques. In the first study, immature achenes were collected from RHA 265 (a phytomelanin line) and RHA 266 (a line lacking phytomelanin) at 2 to 3 day intervals through 9 days post-fertilization and stored in a freezer for subsequent feeding trials. Feeding trials were accomplished by placing whole achenes of desired age in 28 ml cups containing five newly hatched *H. electellum* larvae. In free-choice feeding trials, three achenes each of RHA 265 and RHA 266 were placed in each of five cups. In no-choice feeding trials, only achenes of either RHA 265 or RHA 266 were placed in a respective cup. On each of five consecutive days the achenes were removed from the cups and evaluated for pericarp injury and larval growth (or mortality). Three fresh achenes of each RHA line were put in the cups each day as the old ones were removed. Each combination of achene genotype versus age was replicated five times.

The second bioassay consisted of feeding newly hatched *H. electellum* on different-aged pericarps of RHA 265 and RHA 266 ground to a 60-mesh powder incorporated in a wheat germ diet. Pericarp mash of a desired age and concentration in the wheat germ diet was poured ca. 5 ml per each of 25 one-dram vials. Each vial was infested with two newly hatched larvae, the weaker of which was removed the following day. Detailed records were kept on larval development and mortality, and on pupal weight and mortality. Data from both bioassays were subjected to an analysis of variance test, and significantly different means were separated by the Duncan's Multiple Range Test.

Parental Lines — Achenes of male and female parental lines for currently used hybrid varieties were analyzed for phytomelanin. The occurrence of phytomelanin was determined by visual examination of immature achenes, by scraping away the epidermis of mature achenes, and by soaking mature achenes in a solution of potassium dichromate-sulfuric acid (Carlson *et al.*, 1972). Five achenes from each line were tested by the various methods, and recorded as having no, partial, or complete phytomelanization of the pericarp.

RESULTS AND DISCUSSION

Field Trials — Parameters possibly affecting injury by larvae

of *H. electellum* were assessed from 274 paired samples of achenes from heads with coincident flowering dates within entries. Achenes having low injury ratings were paired with achenes having a higher injury rating. There was a highly significant (1% level) negative correlation ($r = 0.93$) between percent injured achenes and percent pericarps with phytomelanin. The correlation of achene injury by *H. electellum* larvae with other parameters studied was not statistically significant at the 5% level. For example, percent frassiness of the inflorescence, anthocyanin pigmentation, and sclerenchyma thickness had r values of 0.55, −0.47, and 0.40, respectively. Hence, it appears that genotypes having phytomelanin in the pericarp suffered significantly less achene injury by *H. electellum* during the 4 years than genotypes lacking phytomelanin in the pericarp. The presence of phytomelanin in the pericarp had no significant effects on either percent oil in the achenes or on sclerenchyma thickness in the pericarp. In many cases, achenes having phytomelanin in the pericarp had a higher percent oil than achenes lacking phytomelanin in the pericarp. Also, sclerenchyma thickness averaged 239.43μ for pericarps having phytomelanin and 259.18μ in pericarps lacking phytomelanin. These data indicate that the incorporation of phytomelanin into the pericarp of hybrid varieties as a resistance mechanism would have no deleterious effects on oil production.

Phytomelanin Development — Microscopically, deposition of phytomelanin between the hypodermis and sclerenchyma of sunflower pericarps becomes evident by the 3rd day after achene fertilization (Rogers and Kreitner, 1981). Deposition of phytomelanin continues at a rapid rate from the 3rd through the 13th day after achene fertilization. Thereafter, phytomelanin deposition seems to wane, and hardening of the layer becomes more pronounced as the pericarp matures.

The expression of phytomelanin in the pericarp of sunflower is thought to be controlled by a single dominant gene (Pml) (Johnson and Beard, 1977). However, distinct morphological differences that occur in the pericarps with and without phytomelanin, and differences in quantity and earliness of phytomelanin deposition among pericarps with phytomelanin suggest that additional modifying genes may determine the phytomelanin characteristics of a pericarp. Hypodermal cells in the pericarp of achenes having phytomelanin undergo early, accelerated cell division and proliferation, resulting in cells that are impacted and disarranged. Hypodermal cells in pericarps lacking phytomelanin are relatively larger, thin-walled, and neatly arranged in defined columns. Also, the sclerenchymal cell walls in pericarps of achenes having phytomelanin lignify much earlier, and become thicker than cell walls in pericarps lacking phytomelanin.

Microscopic examinations suggest that inner cell layers of the hypodermis disintegrate and discharge cell contents which precipitate into the inter-cellular spaces between the hypodermis and sclerenchyma in the pericarps of genotypes forming the phytomelanin layer. Sarkany (1947) proposed that carbohydrates of hypodermal cell walls become hydrated by increased cellular metabolism of the "cell layer underneath the epidermis" to form the phytomelanin layer. Phytomelanin has no physical structure of its own, and assumes its layer morphology as it fills intercellular spaces between the hypodermis and sclerenchyma and hardens.

The juxtaposition of compacted hypodermal cells, the phytomelanin layer, and denser sclerenchyma cell walls result in significantly harder pericarps than occurs when phytomelanin is lacking. In fact, there is evidence that phytomelanin and the early lignification of associated sclerenchymal cell walls interact synergistically to increase pericarp hardness in immature achenes. Penetrometer studies showed that the crown and equatorial areas of RHA 266 achenes are much less resistant to penetration by the 5th day after fertilization than are achenes with phytomelanin (Table 1). In all achenes tested, the crown was much more resistant to penetration than the equatorial area on any given day during achene maturation. Although the crown of Peredovik achenes was equally resistant to penetration as the crown of other entries with phytomelanin, the equatorial area of Peredovik was no more resistant to penetration as the crown of penetration than was the equatorial areas of entries lacking phytomelanin. Perhaps the weak sides of Peredovik achenes explains why this variety appears to be injured as readily by larvae of *H. electellum* in the United States as are the achenes of hybrid varieties. Some experimental lines (e.g., entry 2522, Table 1) offer high potentials as parental lines for developing hybrid varieties that are resistant to injury by larvae of *H. electellum,* where both crowns and equatorial areas of achenes would be more resistant to penetration (Rogers, 1981).

Table 1. Force required to penetrate the pericarp of achenes at 5-day intervals after fertilization.

		Mean grams force required to penetrate pericarp on day[b/]					
	%	1		5		10	
Entry	achenes w/Pml[a/]	Crown	Side	Crown	Side	Crown	Side
Hybrid 894	20	49.8bcd	21.8abc	149.4bc	73.6ab	199.0bcd	132.1ab
RHA 266	0	57.5a-d	27.9ab	96.8d	18.8d	110.3d	51.3c
RHA 265	100	44.1cd	16.4bcd	141.4bc	91.5a	159.0bcd	124.3ab
Peredovik	100	70.6a	31.8a	122.3cd	52.5b	195.3bcd	51.8c
2522 #8	100	55.8a-d	7.2de	114.0cd	45.5bcd	260.1ab	160.0a

a/ Zygosity of Pml for entries were not known.
b/ Means followed by different letters are significantly different at the 5% level.

Laboratory Bioassays — European literature (Kiewnick, 1964 and Sarkany, 1947) states that the phytomelanin layer forms a physical barrier that prevents pericarp penetration by larvae of *H. nebulella.* Does the phytomelanin function solely as a physical barrier to larvae of *H. electellum?* Field observations indicate that earlier hardening of pericarps having phytomelanin plays a significant role in protecting maturing achenes from injury by *H. electellum* larvae. However, it appears that something other than hardness functions to reduce larvae injury to pericarps having phytomelanin during early stages of achene development (Table 2).

In whole-achene feeding trials in the laboratory, early-instar larvae of *H. electellum* showed a significantly (5% level) greater preference for RHA 266 achenes than for RHA 265 achenes following the 2nd day post-fertilization of achenes. The differential feeding preference for RHA 266 achenes was greater in free-choice trials, and appeared to be greater as achene age increased (Table 2). Pericarp hardness could have influenced results in the 5 to 9 day age category, but hardness should have had little influence on feeding preference in the soft, pliable pericarps of the 2 to 4 day through the 4 to 7 day age groupings. Physical evidence of feeding usually involved the total pericarp in RHA 266, but often only involved consumption of the epidermis and hypodermis of RHA 265 pericarps as larvae burrowed along the longitudinal axis of achenes. There was no significant difference in mortality of larvae feeding on whole-achenes of RHA 265 and RHA 266. Hence, acute toxicity of phytomelanin to early-instar larvae was not evident in the results of these trials. However, chronic toxicity or gustatory inhibition by phytomelanin could have influenced the apparent feeding preference of *H. electellum* larvae for RHA 266 achenes.

Responses of *H. electellum* larvae feeding on pericarp mash incorporated in a wheat germ diet at a 10% concentration are summarized in Table 3. The incorporation

of dry pericarp mash in the wheat germ diet at 10% concentration made the diet drier than the controlled diet, which may have biased the bioassay results. Nevertheless, significant differences (5%) in larval feeding responses on RHA 265 and RHA 266 pericarp mash suggested a chronic toxicity due to phytomelanin in immature pericarps. First-instar larvae had a significantly higher mortality when feeding on RHA 265 pericarp mash from 2 to 4 day-old and 3 to 5 day-old achenes than from other treatments. Also, total larval mortality was significantly higher for larvae feeding on RHA 265 pericarp mash of 2 to 4 day-old and 3 to 5 day-old achenes than for larvae feeding on some of the other entries. Pupal mortality was greatest from larvae feeding on the 3 to 5 day-old achenes.

Table 2. Injury to immature RHA 265 (w/phytomelanin) and RHA 266 (w/o phytomelanin) achenes by young larvae of *H. electellum* in whole-achene feeding trials in the laboratory.

Achene age (days)	RHA no.	x % pericarp destroyed/rep./day[a/] Free-Choice feeding	No-Choice feeding
5 — 9	265	1.88a	4.58a
	266	8.24b	41.42b
4 — 7	265	6.48a	16.14a
	266	11.02b	34.06b
3 — 5	265	6.22a	20.10a
	266	16.20b	27.90b
2 — 4	265	23.88a	20.00b
	266	34.12b	28.60b
1 — 2	265	15.80a	25.02a
	266	16.70a	19.80a

a/ Means followed by different letters within an "age-group" are significantly different at the 5% level (Chi square test).

Growth of surviving larvae was severely affected by the diet containing 2 to 4 day-old and 4 to 7 day-old achenes (Table 3). Larvae were usually smaller when feeding on the diet containing ground RHA 265 pericarps than when feeding on the diet containing ground RHA 266 pericarps. Larval size, developmental period, and pupal mass from surviving larvae were usually significantly greater for specimens reared on the standard diet than for specimens reared on the diet containing ground pericarps. Excessive drying of the diet or unpalatability of the test diets could have been responsible for biased results. Tests are just beginning using ground pericarps incorporated in the wheat germ diet at a 1% concentration. Early results indicate a better quality diet, and a higher mortality for first-instar larvae feeding on the diet containing ground RHA 265 pericarps.

Table 3. Results of laboratory bioassays incorporating ground pericarp in the larval diet at a 10% concentration.[a/]

Achene age and RHA line	% Mortality First instars	Total larva	Pupal	x Larval Length (mm) at: 4 days	7 days	x Larval duration (days)	x Pupal mass (g)
1 — 2 day							
265	4b	36d	18.7b	2.0abc	3.9b	23.5cd	21.8b
266	8b	24de	0.0c	2.1ab	3.7b	23.1d	24.0b
2 — 4 day							
265	48a	80a	0.0c	1.7c	2.4cd	28.0b	20.6b
266	12b	44cd	11.1bc	2.2a	3.5b	25.7bcd	19.8b
3 — 5 day							
265	64a	88a	33.3a	1.8bc	2.6cd	27.7bc	19.7b
266	20b	68abc	25.0b	1.7c	2.8bcd	26.8bcd	18.7b
4 — 7 day							
265	20b	64a-d	20.0b	1.7c	2.3d	28.9ab	20.5b
266	20b	56cd	0.0c	2.2a	3.3bc	32.3a	21.7b
Check[b/]	0b	8e	0.0c		6.6a	16.2e	31.1a

a/ Values followed by different letters are significantly different at the 5% level (Duncan's Multiple Range Test).
b/ Standard wheat germ diet with no ground pericarps.

Table 4. Phytomelanin rating in the pericarp of commonly used RHA and cmsHA parental sunflower lines.

Parental line		Assay method indicating phytomelanin[a/]	
		Scraping	Potassium dichromate — sulfuric acid
RHA	265	+	+
	266	—	—
	269	—	—
	270	+	+
	272	+	+
	274	+	+
cmsHA	60	+	+
	89	+	+
	99	+	+
	113	—	+
	224	—	+
	300	+	incomplete

a/ + indicates positive test for phytomelanin; — indicates negative test for phytomelanin; incomplete indicates part of pericarp "+" and part of pericarp "—" for phytomelanin.

Parental Lines — We have examined most of the public-released hybrid variety parental lines for phytomelanin. The pericarp of 39% of the RHA lines and 97% of the cmsHA lines have phytomelanin. American sunflower breeders have not actively selected for phytomelanin, and its zygosity among hybrid varieties is undetermined. Usually, the HA line has phytomelanin in its pericarp when the sister cmsHA line has phytomelanin, but there are exceptions. Also, the HA line may lack phytomelanin when the respective cmsHA line has it, and sometimes the HA line has phytomelanin while the cmsHA counterpart lacks it. The pericarp is most often positive or negative for phytomelanin, but sometimes only partial or incomplete development of phytomelanin is evident.

Phytomelanin expression in commonly used hybrid variety parental lines is summarized in Table 4. Scraping away the epidermis and hypodermis of mature achenes gave a positive response for phytomelanin in ca. 75% of the samples where soaking mature achenes in a potassium dichromate-sulfuric acid solution had indicated a positive response.

We feel that hybrid varieties developed from parental lines homozygous dominant for phytomelanin would result in considerable reduction in yield loss due to achene injury by feeding larvae of *H. electellum.*

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