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Proanthocyanidins: Key for Resistance to *Globisporangium* (Formerly *Pythium*) Seed Rot of Pea

Elmer E. Ewing
Horticulture Section, School of Integrative Plant Science, College of Agriculture and Life Sciences, Cornell University, Ithaca, NY 14853, USA

Norman F. Weeden
Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717, USA

Ivan Simko
US Department of Agriculture, Agricultural Research Service, Sam Farr United States Crop Improvement and Protection Research Center, 1636 East Alisal Street, Salinas, CA 93905, USA

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Abstract. Pea (*Pisum sativum*) dominant for the fundamental color gene *A* showed a high level of resistance to *Globisporangium ultimum* (formerly *Pythium ultimum*) seed rot. Reciprocal crosses demonstrated that, with our materials, such resistance was associated with the testa (seedcoat) phenotype but not the embryo phenotype. Dominance of *A* over *a* was complete for this trait. Neither wrinkled seed form (*r*) nor green cotyledons (*i*) diminished resistance when *A* was dominant, although both recessive alleles diminished resistance when seeds were borne on white-flowering (*a*) plants. The product of the *A* gene functions in the pathway leading to flavonoids, including proanthocyanidins (PAs) and anthocyanidins. We found that resistance to *G. ultimum* seed rot was closely associated with not only dominant *A* but also testa PAs and testa sclerenchyma. Even *A* testas that lacked anthocyanins but contained PAs and sclerenchyma showed a high level of seed rot resistance. Moreover, a mutation removing PAs and sclerenchyma in a narrow zone from the hilum to the radicle markedly increased susceptibility. The PAs in pea testas were predominantly prodelphinidins in seeds from purple-flowered plants (*A B*) and procyanidins from pink-flowered plants (*A b*). Compared with procyanidins, prodelphinidins have higher antioxidant activity but are more likely to sequester iron, a particular concern with dry pea. Although *A B* testas were more resistant than *A b* to seed rot, the difference seemed too slight to militate against growing pink-flowered pea. We stressed the need for more histological comparisons of *A B* and *A b* testas, and we indicated that genes and their phenotypic effects examined during the current study could be useful for modeling biosynthesis of PAs and related cell walls.

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I.S. is the corresponding author. E-mail: ivan.simko@usda.gov.

Seed rot (also known as pre-emergence damping off) incited by *Globisporangium ultimum* (formerly *Pythium ultimum*) and related species such as *G. debaryanum* is a soil-borne disease common to many crops. In pea (*Pisum sativum*), it is also associated with other soil-borne diseases, including *Aphanomyces euteiches* root rot. *Rhizoctonia solani* and especially various *Fusarium* species may also be involved in pea seed rot, but *G. ultimum* is most often the primary incitant (Flentje 1964; Jacks 1951; Leach and Smith 1945). Hyphae enter through openings in the testa (seedcoat) or penetrate it directly, attacking the cotyledons and then the embryonic axis (Hull 1937). High soil moisture content is typically the major environmental factor that increases disease severity (Hull 1937; Jones 1931; Kerr 1964); in wet weather or on poorly drained soils, the decay may result in crop failure. Consequently, most growers of white-flowered pea use seeds treated with fungicides.

The strong resistance to *G. ultimum* rot of pea seeds borne on plants with colored rather than white flowers was the focus of our study. Pea cultivars with white flowers have been available to growers for more than 700 years. The most common mutation (Mendel's clear testa gene, later given the symbol *a* by Tschermak 1912; White 1917) was shown to be produced by an intron splice site mutation in a bHLH gene homologous to the TRANSPARENT TESTA8 gene of *Arabidopsis thaliana* (Hellens et al. 2010). Numerous studies (Muehlbauer and Kraft 1978; Ohh et al. 1978; Stasz and Harman 1980) have noted an association between this

mutation and an increase in the susceptibility of pea to *G. ultimum* attack. Studies involving isogenic lines (Powell 1989) demonstrated the susceptibility was caused by the lack of a normal functioning bHLH protein rather than a product of change at a closely linked locus.

We examined the nature of seed rot resistance from pea plants with colored flowers (*A* phenotype) that was lost with the mutation to white flowers (*a*). The resistance is commonly accompanied by anthocyanins in the testa, which has led researchers to postulate that anthocyanins are at least partly responsible for the resistant phenotype (Muehlbauer and Kraft 1978). Therefore, it was of interest to test whether a line dominant for *A* but lacking testa anthocyanins would be as resistant to *G. ultimum* seed rot as lines that contained anthocyanins.

A second pea line also lacked testa anthocyanins, but for a different reason—it contained five pairs of recessive genes for partial coloration (Lamprecht 1947, 1957a, 1957b). Testas that were homozygous recessive for the genes *z* and *mp* lacked anthocyanins in a narrow zone from the hilum to the radicle. Lamprecht generously provided seeds of L578 to be tested for resistance. In addition to the requisite presence of both *mp* and *z*, L578 had three other partial coloration genes: *cal*, *dem*, and *pal*. Each of these three recessive genes increased the colorless zone produced by *mp z*, and the combined effect of the five homozygous recessive genes in L578 was a testa free from anthocyanin pigments. We investigated the ways in which these two *A* lines that lacked testa anthocyanins differed and the differences in seed rot resistance found in the offspring when they were crossed with one another.

We hypothesized that proanthocyanidins (PAs), also known as condensed tannins, played a major role, physically and chemically, in these differences, and that they were the dominant factors in the resistance of *A* testas. High concentrations of PAs have been observed in *A* pea testas (Mohamed et al. 2022; Quilichini et al. 2022; Troszyńská and Ciska 2002). The PAs most often found in pea testas have been prodelphinidins (Jin et al. 2012), but a few reports included procyanidins (Ferraro et al. 2014). Procyanidins consist of polymers and oligomers of catechin and epicatechin monomers. Prodelphinidins are similar, but they are polymers and oligomers of gallocatechin and epigallocatechin monomers. Hot acid treatment of procyanidins yields cyanidin (two hydroxy groups on the B carbon ring), whereas prodelphinidins yield delphinidin (with three hydroxy groups) (Bule et al. 2020; Gessner and Steiner 2005; Waterhouse et al. 2016). A recessive mutation at the *B* locus in pea inactivates flavonoid 3′, 5′ hydroxylase, producing pink, rather than purple, petals (Moreau et al. 2012). Based on this and the PA biosynthesis pathway proposed by Quilichini et al. (2022), one might surmise that in *A* plants, *B-b* will have a parallel effect on compounds other than anthocyanins in the pathway, including PAs. We investigated whether this mutation affects whether prodelphinidins or procyanidins are mainly present in the testas and examined whether such a change affects resistance to *G. ultimum* seed rot.

Field experiments were performed in Mexico with Rockefeller Foundation's Agricultural Program for Latin America. During the 1960s, when most of this work was ready for publication, there was little interest in resistance to seed rot conferred by *A*. In countries such as the United States, fungicides were thought to provide simple, economical control. Moreover, *A* in cooked green peas was linked with two problems: astringency (detected by some, but by no means all, tasters) and discoloration. Both traits relate to PAs. Presently, growers producing certified organic products cannot use certain chemical fungicides (e.g., metalaxyl, mefenoxam, and ethaboxam) to manage seed rot; therefore, there is a renewed interest in cultivar resistance. Moreover, there is enthusiasm for the health benefits of phytochemicals associated with colored produce (Bhagwat and Haytowitz 2015; Bule et al. 2020) and the antioxidant power of PAs (Dueñas et al. 2006). To obtain such benefits, consumers might accept the sensory and culinary effects of *A* seeds. Considering these developments, and by taking advantage of scientific advances that aid in chemical analysis and interpretation of our data, we were able to provide an updated report including the original work complemented with contemporary mass spectrometry techniques.

Materials and Methods

Timing of experiments. With the exception of the 2023 analysis using liquid chromatography with tandem mass spectrometry (LC-MS/MS) and the development of genetic materials assayed therewith, this research was performed before 1966. Field trials in Mexico had been performed beginning in Sep 1956, and most of the laboratory and greenhouse studies were included in a 1959 PhD thesis, with follow-up work performed during the next 6 years.

Genetic terminology. In this work, we use "genotype" to indicate alleles of genes relevant to our current topic and "phenotype" for what would be expressed by the genes. Thus, the genotype for wrinkled seeds borne by plants with colored flowers might be *AA rr* or *Aa rr*, but the phenotype for either would be *A r*. Table 1 summarizes the phenotypes to be discussed.

Pedigrees and sources of seeds. Seeds of white-flowering pea cultivars were donated by commercial seed companies. Two purple-flowering lines from the Cornell University Station at Geneva, NY, USA, were key to our research. Both were developed

Table 1. Summary of phenotypic traits and associated alleles included in our experiments with pea.

	Dominant		Recessive	
Trait	Allele	Phenotype	Allele	Phenotype
Flower color	*A*	Colored	*a*	White
Cotyledon color	*I*	Yellow	*i*	Green
Seed form	*R*	Round	*r*	Wrinkled
Internode length	*Le*	Tall	*le*	Dwarf
Flower color (if *A*)	*B*	Purple	*b*	Pink
Anthocyanin in testa (if *A*)	*Fs*	Flecks	*fs*	None
Brown marbling of testa	*M*	Pigmented	*m*	None
Testa partial color (if also *A mp*)	*Z*	Plain	*z*	Pattern[i]
Testa partial color (if also *A z*)	*Mp*	Plain	*mp*	Pattern[i]

[i] If *A* is dominant, then both *z* and *mp* must be recessive to develop a pattern in anthocyanins present in the testa. The pattern is a narrow, colorless band that forks up around the apex of the radicle and extends down past the hilum. When recessive, other partial color genes (*cal*, *dem*, *pal*) each enlarge a different part of the colorless area (Lamprecht 1947, 1957a, 1957b). We found that *z mp* also confers patterns in proanthocyanidins (PAs). Here, we designated the testa phenotype with a zone of any size free of PAs as "pattern," and the testa phenotype that contains PAs all over as "plain." Examples of plain and pattern in our materials are shown in Fig. 3.

by D.W. Barton for partial resistance to *Aphanomyces* root rot. He observed that they also were resistant to seed rot. Line G141 was a selection from PI 169604, which was collected in Turkey. Its phenotype was *A I R Le B Fs m.* Barton bred a second line from it, 55-567, as follows: G141 was crossed with 'Early Freezer', and the F_1 was crossed with 'New Era' (*a i r le).* Succeeding generations were selected to be a purple-flowered version of 'New Era', which it closely resembled. The phenotype was *A i r le B fs m.* During our experiments, the seeds of 55-567 were in the F_6 generation or later. Lines obtained from Lamprecht in Sweden included L578 and L19 (a parent of L578). According to Lamprecht (1957a), L19 was *A B M z Mp dem* and L578 was *A b m z mp dem cal pal*. We found both lines had round (*R*) seeds with yellow (*I*) cotyledons.

Location and environment of field experiments. All field experiments were performed from Sep 1956 to Aug 1957, at Rockefeller Foundation's Santa Elena Experiment Station in the Toluca Valley of Mexico (elevation 2700 m). The climate was cool, with an average noon temperature of 20 °C during the spring growing season. It rained almost every afternoon during the rainy season of mid-May to mid-October. On most days when there was no rain, plots were watered using a sprinkling can. The soil was alluvial and developed from volcanic ash parent material. Additionally, it was a deep, well-drained sandy loam (pH 6.5, 1.7% organic matter). It had been intensively cropped to various cool-season vegetables, including pea. There were many pea fields in the surrounding area consisting of commonly grown purple flower types.

Experimental design and implementation. Unless otherwise stated, experiments involving plant emergence had a randomized complete block design. Other details of field experiment procedures are presented in Supplemental Materials 1.

Collection and treatment of data. In addition to emergence counts, all seedlings were examined for disease symptoms, particularly on testas and cotyledons. Percentage values for plant emergence were transformed using the arcsine function before the analysis of variance (ANOVA) was performed, followed by Tukey's honestly significant difference post hoc tests. If transformed data did not meet the ANOVA requirements for normality of distribution and homogeneity of variances, then the nonparametric Kruskal-Wallis test was performed, followed by Steel-Dwass post hoc analyses.

We use the term "strong resistance" to describe seeds that, in the presence of conditions that favored seed rot, provided at least 90% emergence and produced surviving plantlets in which testas and cotyledons remained mostly intact.

Causal organism. During field experiments, *G. ultimum* was isolated as the chief causal organism. The identity was verified by an authority on the organism (Middleton JT, personal communication). When pea seeds were planted in autoclaved greenhouse soil that had been infested with the isolate, they developed disease symptoms typical of those found in the field, and the organism was re-isolated from the decaying cotyledons. Although there were occasional isolates of what appeared to be *Fusarium* species, efforts to incite seed rot with these were not successful. Other soil-borne pathogens may have been present; however, as widely reported by studies of pea seed rot under conditions of high soil moisture, *G. ultimum* or closely related species appeared to be the chief causal organisms.

Procedures for the greenhouse test of resistance of F_2 plants from 55-567 × L578. A different culture of *G. ultimum* than the one isolated in Mexico was obtained from the Cornell University Department of Plant Pathology. Mycelia were grown in 10 flasks. Each flask contained 100 mL of autoclaved pea broth. After 2 d, mycelial colonies had grown to approximately 8 cm in diameter. Contents of each flask were macerated in a blender for 1 min on low speed and returned to the flask. Then, each suspension was poured evenly over the growth medium in one of 10 metal flats (53 × 31 cm). The growth medium was composed of vermiculite and peatmoss plus mineral nutrients (Sheldrake and Boodley 1966), which had been autoclaved for 2 h before applying inoculum. The experiment was planted 10 d after inoculation, during which interval flats were watered to saturation every other day in a mist chamber. With the aid of a spotting strip, seeds were planted 4 cm deep, with five of a kind in each row. Seeds were spaced 2.5 cm apart in rows separated by 6 cm. Each of seven flats received 11 rows from 107 plants of the F_2 of 55-567 × L578; three flats received only 10 rows plus a "filler" row of *A* seeds for balance. All 10 flats received one row each of L19 and L578. The order of the rows in each flat was randomized. Flats continued to be misted to saturation every other day starting at planting. Plants were counted, dug, and examined 18 d after planting.

Comparison of resistance to *G. ultimum* and *R. solani*. During an experiment conducted at the same time as the preceding one, resistance to *G. ultimum* and *R. solani* was compared. Both cultures were obtained from the Cornell Department of Plant Pathology, and the procedures for growing the mycelia and flat inoculation for each pathogen were as described previously for *G. ultimum*. A control (pea broth only) was added as a third treatment in a randomized split plot design. Main plots consisted of pathogen treatments, with each flat representing a plot, arranged as two randomized complete blocks. Flats were each divided into quarters. Two quarters per flat were assigned at random to seeds with *A* testas and two were assigned to seeds with *a* testas. Sixteen seeds were planted per quarter. Seeds were from F_2 plants of G141 (*A I R*) × 'Yukon' (*a i R*). No selection was made for cotyledon color. Yellow and green cotyledons segregated at random in all treatments. Cotyledon color was difficult to assess accurately with *A* testas.

Chemical studies. Hide powder (American Standard No.1872, Drawer 239, Lot 28; F. F. Marshall, Ridgway, PA, USA) was used to test for tannins and remove them from extracts. Water was purified by passage through ion exchange resins. The vanillin and hot acid tests were performed as outlined by Bate-Smith and Lerner (1954). A bright red reaction from the vanillin reagent is produced by a phloroglucinol residue with no carbonyl group next to the ring and has long been used to test for PAs (Gardner 1975).

Qualitative determinations for prodelphinidins and procyanidins were made for testas from *A B* and *A b* siblings on two occasions using two different methods. The first time, they were made for siblings from the F_2 of 55-567 × L578. Because all genes that confer anthocyanins in the testa were recessive in this population, none was present to interfere with the analysis of PAs via hot acidic treatment—any anthocyanidins produced would have come from PAs. Paper chromatography of anthocyanidins produced by hot acid treatment of testas was performed using the method described by Bate-Smith (1954) for what were then believed to be polymers of leucoanthocyanins but are now known to be PAs (Supplemental Materials 2).

An F_2 population unrelated to the earlier one was used for our 2023 test of the difference between testa PAs in purple-flowered (*A B*) and pink-flowered (*A b*) siblings. It was derived from a cross

between two breeding lines obtained from G.A. Marx at Cornell University. The line used as the female parent had the phenotype *A I R Le b Fs m z mp*, whereas the pollen donor had the phenotype *A I R Le B Fs m Z* (the phenotype of *mp* could not be determined because of masking by dominant *Z*; however, based on segregation data of the F_2, it could be assumed to be recessive). Treatment with $K_2Cr_2O_7$ indicated PAs covered the entire testas of both siblings sampled; therefore, both were dominant *Z*, but one was *A B* phenotype, and the other was *A b*.

Assays, including sample preparation, were performed by the Proteomics and Metabolomics Facility at Cornell University. Testa fragments from three seeds of each phenotype were powdered using a mortar and pestle, weighed, and washed three times. The first two washes used water, and the third used 50% methanol (volume:volume) acidified with 0.1% HCl (volume:volume). Gentle vortexing was performed after each wash, followed by centrifuging. The washings were saved for later analyses, and the precipitates containing PA polymers were treated with 2 N HCl, heated 20 min in a 95 °C water bath, and extracted twice with iso-amyl alcohol. The organic fractions were allowed to dry and resolubilized in 5% methanol (volume:volume). An LC-MS/MS method was used for identification and relative quantitation of cyanidin and delphinidin in the two samples using cyanidin and delphinidin standards. A C18 column with an MS/MS high-field asymmetric waveform ion mobility spectrometer was operated under positive ion data-dependent acquisition and parallel reaction monitoring mode for targeted identification and relative quantitation. Later, the saved washings from each of the two samples, which contained anthocyanins along with soluble PAs and their monomers, were allowed to dry, resolubilized in 2 N HCl, and heated 20 min; then, iso-amyl alcohol extractions were performed, and they were similarly assayed for anthocyanidins (Supplemental Materials 2).

Inhibitory effects of extracts on *G. ultimum*. We examined whether *A* testas contained substances inhibitory to *G. ultimum*. *A* and *a* testas were ground in a Wiley mill and soaked in water. The methodology is presented in Supplemental Materials 3.

Histology. A small cut was made through testas of 'Creole' (*a*) seeds and seeds grown on F_2 plants of 55-567 × L578. Seeds were placed between moist pieces of filter paper. When the testas were thoroughly moistened, they were slipped off the cotyledons. Testa sections were immediately cut with a freezing microtome. Some sections were treated with vanillin reagent before examination. Other sections were cut (7 μm) with a conventional microtome, stained with thionin and Orange G, and embedded with paraffin.

Results

Outline of experiments. We performed field studies of differences among white-flowering plants in terms of susceptibility to *G. ultimum* seed rot, compared field resistance of seeds borne on purple-flowering and white-flowering plants, and used reciprocal crosses to show that strong resistance resided in the testa, not in the embryo. We examined physical and chemical properties of the testa that contributed to strong resistance and found that PAs were involved in both. Confirmation of the role of PAs was determined based on the segregation of alleles that control the distribution of testa PAs and another allele that confers a different type of PA. Finally, we found that strong resistance to *G. ultimum* did not carry over to *R. solani*.

Background summary for white-flowering plants. Seventeen crosses using 14 white-flowering cultivars from five commercial seed companies were made to examine the effects of round (R) and wrinkled (*r*) and of yellow (*I*) and green (*i*) cotyledons on field resistance to seed rot. During every comparison, wrinkled seeds were significantly more susceptible than round seeds of F_2 siblings. The advantage of yellow over green was somewhat less consistent; however, when the difference was significant, green was always more susceptible than yellow. Table 2 summarizes the results of one of these trials.

Purple-flowered and white-flowered pea were compared to determine resistance. Strong field resistance to pea seed rot occurred with seeds grown on plants with purple (*A*) rather than white (*a*) flowers. Table 3 shows emergence during one experiment. Line 55-567 was equal to its G141 ancestor in terms of resistance. Unlike G141, it lacked anthocyanin pigment in the testa, had green cotyledons and wrinkled seeds, and had a dwarf (*le*) stature. The testas and cotyledons on 55-567 and G141 were generally intact when dug-up. In contrast, testas on the three cultivars were almost all disintegrated, and cotyledons, especially on the two wrinkled seeded ones, were rotted or missing.

Role of testa in resistance. The next experiment used reciprocal crosses and selfed crosses to determine whether the strong resistance conferred by *A* was a function of the testa (maternal tissue) or the embryo, and whether dominance was complete. The results (Table 4) could be divided into two distinct groups based on emergence as well as on the condition of testas after exposure in the soil. Testas from the six genotypes with maternal parents of *A* phenotype (derived from 55-567) were almost all intact and provided good emergence; testas of *a* phenotype (derived from 'New Era') were, mostly, disintegrated, and plant emergence was poor. Thus, whether *A* was present in the embryo had no detectable effect on susceptibility to seed decay, and the dominance of *A* testas for the strong resistance trait was complete.

Physical differences. Testas constituted 10.0% of the weight of seeds from 62 F_2 plants segregating as *A*; however, they constituted 8.2% of the weight of seeds from 60 plants from the same F_2 segregating as *a*. This difference was significant ($P \leq 0.01$). The seeds selected for the study were matched; for every seed chosen with an *A* testa, an *a* seed of similar weight was taken. Similar to other studies (Ohh et al. 1978; Stasz and Harman 1980; Taylor and Dickson 1987), we found that a small cut in an *A* testa made the seed much more susceptible to rot (data not shown).

The status of *A* (compared to *a*) had a marked effect on the anatomy of the testa. When sections of *A* and *a* testas were cut by the freezing microtome and stained with vanillin reagent, only *A* cell walls turned bright red—an indication of the presence of PAs (data not shown). We also examined sections of G141 (*A*) and 'Perfection' (*a*) testas (kindly provided by R. Provvidenti, who had stained them with safranin). The macrosclereids in G141were darker than the ones in 'Perfection', and a brown material filled the lumens of many G141 cells (not shown). During a third study, we stained sections with thionin and orange G. Below the cuticle and its supporting layers of macrosclereids and osteosclereids, testas from white-flowering plants were mainly filled with parenchyma cells (Fig. 1A and C), whereas testas from purple-flowering plants contained only sclerenchyma (Fig. 1B and D).

Inhibition of *G. ultimum* by aqueous extracts from testas. Vials were examined 2 d after inoculation (Table 5). All three

Table 2. Two-way table of a factorial field study of the effects of round (*R*) and wrinkled (*r*) seeds combined with the effects of yellow (*I*) and green (*i*) cotyledons on emergence of pea seedlings. Seeds were segregating for these two traits in F_2 generations. The soil was a well-drained sandy loam. The cool, wet climate was well-suited for pea, except for the problem with seed rot. The causal organism for the rot most often isolated was *Globisporangium ultimum*. Seeds were planted by hand, and soil was kept moist through frequent showers and hand sprinkling. There were six replications of 18 seeds per treatment arranged in a randomized complete blocks. Among the six replications, three pedigrees and five parents were represented; however, within a replication, the ancestry was the same. All plants were white-flowered (*a*). The experiment was planted Aug 1957, at the Santa Elena Experiment Station in Mexico.

	Round seeds (*R*)	Wrinkled seeds (*r*)	Mean (arcsine degrees)	Equivalent percent emergence
Yellow cotyledons (*I*)	60.02[i]	42.02[i]	51.02[ii]	60.4
Green cotyledons (*i*)	51.28[i]	26.98[i]	39.13[ii]	39.8
Mean (arcsine degrees)	55.65[ii]	34.50[ii]		
Equivalent percent emergence[i]	68.2	32.1		

[i] To improve homogeneity of variance, individual values for percent emergence were arcsine-transformed to degrees before statistical analyses (see Materials and Methods). The percentages shown have been transformed back from arcsine degree values.
[ii] Mean differences between *R* and *r* and of *I* and *I* were significant at $P \leq 0.01$, as determined by single degree of freedom. The interaction between the two factors was not significant.

concentrations (undiluted, diluted 1:1 with sterile water, or diluted 1:2) of *A B Z* testa extracts inhibited the growth of *G. ultimum* mycelia in vitro, with no discernable effect of dilution. Treatment of *A B Z* leachates with hide powder before sterilization eliminated the inhibition. Dense mycelial mats filled the bottoms of all vials that had *A B z*, *A b Z*, *A B Z* plus hide powder, *a*, and *a* plus hide powder.

Chemical nature of extracts. Aqueous extracts from *A* testas provided positive results with reagents known to develop strong colors with tannins. Of these, a 2% (weight/volume) solution of potassium dichromate ($K_2Cr_2O_7$)—which has a long history as a simple test for tannin (Shull 1913)—was chosen as a quick test. Soaking *A* seeds in it overnight produced an intense brown color in testas, but only traces of color in *a* seeds. Tests more specific for tannins also resulted in positive results, such as the precipitation of gelatin from saline solution and the ability to combine with hide powder, for *A* testa extracts. After treatment with hide powder and filtration, the reactions of extracts from *A* testas to potassium dichromate and to the vanillin reagent were negative. Equivalent extracts from *a* testas were negative for dichromate before and after hide powder treatment and for the other reagents mentioned.

Regarding the type of tannins, treatment with hot acid yielded anthocyanidins, indicating the presence of PAs. Typically, PAs vary widely in degree of polymerization, and aqueous extracts of the compounds in *A* testas appeared to consist of a mixture of molecular sizes. A small fraction of the material was soluble and dialyzable; however, most was colloidal or of larger molecules, and many were seemingly inseparable from tissues.

Genes for the partial color of testas. The results that Lamprecht (1947, 1957a, 1957b) found regarding the absence of anthocyanins in L578 also applied to the distribution of PAs. Despite the presence of *A*, L578 testas did not develop a dark brown color after treatment with dichromate. In this regard, they also resembled the *a* phenotype (Fig. 2).

Population derived from L578. L578 was crossed with 55-567 and F_2 plants were examined for phenotypes. Both parents were recessive for genes that produce anthocyanins or similar pigments in the testa; as expected, all their offspring also lacked these testa pigments. Testas from 81 (76%) of the 107 plants resembled 55-567 testas. They were a drab, pale green that matched the "grape green" illustrated by Ridgway (1912) (Plate XLI) and were less translucent than testas from *a* plants. When immersed overnight in a 2% (weight/volume) solution of potassium dichromate, the testas turned dark brown, whether *B-b* was dominant (Fig. 3, row 1) or recessive (Fig. 3, row 4).

Testas from the 26 remaining F_2 plants were somewhat translucent, especially in the zone between the hilum and the radicle. It was difficult to detect patterns on these testas unless they were soaked in dichromate or other PA reagents; however, after such treatment, patterns were distinct. One pattern (Fig. 3, second row, right) had a shape similar to that in previous pictures of a forked zone (*furca*) free from anthocyanin pigments (Lamprecht 1947; his Fig. 1). The anthocyanin pattern is conferred by *A mp z*, but it is visible only if genes such as *M* or *Fs* are present to produce the anthocyanin or related pigments. Correspondingly, during our experiment, the pattern for PAs was evident only if treated with dichromate, vanillin, or other reagents that elicit a color when reacting to PAs.

Other patterns in our F_2 population had zones larger than *furca* that were free from PAs, and seeds from two plants had a pattern like that of the L578 parent, with very little darkening in any part of the testa (Fig. 3, row 3, right). Presumably, such

Table 3. Field emergence of two pea breeding lines, both with purple flowers (*A*), compared with that of three white-flowering (*a*) cultivars. The experiment was planted in Sep 1956, at the Santa Elena Experiment Station in Mexico. Seeds were planted by hand and watered by sprinkling can immediately after planting. There were four replications of 25 seeds per treatment arranged in randomized complete blocks. The soil was a well-drained sandy loam. The cool, wet climate was well-suited for pea, except for the problem with seed rot. The causal organism for the rot most often isolated was *Globisporangium ultimum*.

Cultivar/line	55-567 *A i r le fs*	G141 *A I R Le Fs*	'Alaska' *a i R Le*	'Perfection' *a i r le*	'New Era' *a i r le*
Percent emergence[i]	99.8 x	98.5 x	85.3 y	15.9 z	6.6 z

[i] To improve homogeneity of variance, individual values of the emergence rate were arcsine-transformed before statistical analyses (see Materials and Methods). Values followed by the same letter were not significantly different ($P \leq 0.05$).

Table 4. Emergence of pea progenies derived from reciprocal and selfed crosses among 55-567 (*AA*), 'New Era' (*aa*), and the F_1 (*Aa*) of 55-567 × 'New Era'. Both parents had the *i r le* phenotype. The maternal parent in each cross is shown on the left. The experiment was planted by hand Aug 1957, at the Santa Elena Experiment Station in Mexico. The soil was a well-drained sandy loam. The cool, wet climate was well-suited for pea, except for the problem with seed rot. The causal organism for the rot most often isolated was *Globisporangium ultimum*. There were six replications of 20 seeds per treatment in randomized complete blocks. Rows were 10 cm apart with 2.5 cm between seeds. Soil was kept moist through frequent showers and hand sprinkling.

Cross	Testa	Embryo (expected segregation ratio)	Percent emergence[i]	Condition of testas
AA × *AA*	*AA*	*AA* (all)	99.2 x	Mostly intact
Aa × *aa*	*Aa*	1 *Aa:* 1 *aa*	98.9 x	Mostly intact
AA × *Aa*	*AA*	1 *AA*: 1 *Aa*	97.6 x	Mostly intact
AA × *aa*	*AA*	*Aa* (all)	95.8 x	Mostly intact
Aa × *Aa*	*Aa*	1 *AA*: 2 *Aa:* 1 *aa*	93.3 x	Mostly intact
aa × *AA*	*aa*	*Aa* (all)	46.5 y	Mostly disintegrated
aa × *aa*	*aa*	*aa* (all)	36.7 y	Mostly disintegrated
aa × *Aa*	*aa*	1 *Aa:* 1 *aa*	29.9 y	Mostly disintegrated

[i] To improve homogeneity of variance, individual values for the rate of emergence were arcsine-transformed before statistical analyses (see Materials and Methods). Values followed by the same letter were not significantly different ($P \leq 0.05$).

patterns can be attributed to segregation of one or more of the other three genes for partial coloration that were present in L578. All these other patterns were enlargements of the PA-free *furca* zone, where the radicle breaks through the testa during germination.

Because of the similarity between the *furca* patterns we observed for PAs and the *furca* patterns Lamprecht (1947, 1957a, 1957b) depicted for partial coloration of anthocyanins, it was reasonable that, as in Lamprecht's case, both *mp* and *z* would

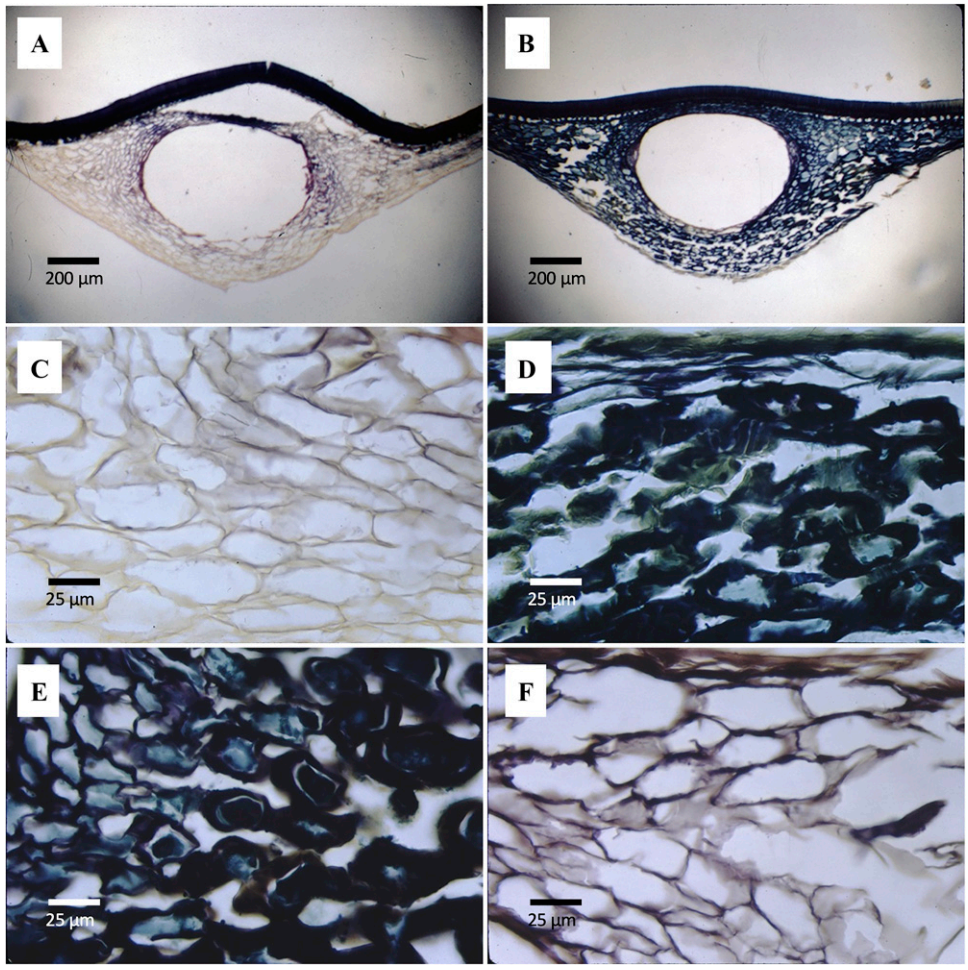

Fig. 1. Cross-sections of the radicles of pea testas displaying differences in cell structures among phenotypes. Testas were cut and pea seeds were allowed to imbibe water overnight before sectioning. Cross-sections through the radicle were obtained. "Plain" denotes testas that would have turned completely dark brown if treated by the dichromate test for tannins and related compounds. "Pattern" denotes testas that would have developed a zone free from the dark brown color. Thionin and orange G were used to stain the sections shown here. (**A**) Overview of a section cut from 'Creole', an *a* (white flowering) phenotype, free from testa tannins. (**B**) Similar view of a testa from a plant with the plain phenotype conferred in *A B* (purple-flowering) testas. (**C**) Higher magnification of the *a* phenotype filled with parenchyma cells. (**D**) Higher magnification of the sclerenchyma cells found in *A B* with the plain phenotype. (**E**) Sclerenchyma in *A b* (pink-flowering) with the plain phenotype. (**F**) Parenchyma in *A b* with the pattern phenotype free from tannins in a forked (*furca*) zone surrounding the radicle. *A B* pattern tissues (not shown) resemble *A b* pattern tissues. The principal tannins were proanthocyanidins.

Table 5. Effects of phenotype and hide powder (HP) treatment on in vitro growth of *Globisporangium ultimum* in aqueous extracts of pea testas. Testa powders were eluted with water (30 mL·g^{-1}) and stored for 2 d at 1 °C. Eluates were filtered, filtrates from *A B Z* and 'Creole' (*a*) were divided in half, and half of each was treated with HP (20 mg·mL^{-1}). After 3 d at 1 °C, HP was filtered-off, and all extracts were passed through sintered glass bacterial filters. Aliquots (0.9 mL) from each of the six treatments were aseptically transferred to small vials at three different concentrations: undiluted, diluted 1:1, and diluted 1:2 with water. Seven days (9 d for one replication) after extraction, vials were inoculated with *G. ultimum* cut from Petri plates of water agar. Mycelial growth at room temperature was rated 2 d later. There were five replications of each treatment.

		No. of replications with the given rating of mycelial growth after 2 d			
Phenotype	Dilution	Profuse	Medium	Trace	None
A B Z	None		1	2	2
	1:1	1			4
	1:2		1	1	3
A B Z + HP	None	5			
	1:1	5			
	1:2	5			
A b Z	None	5			
	1:1	5			
	1:2	5			
A B z	None	5			
	1:1	5			
	1:2	5			
'Creole' (*a*)	None	5			
	1:1	5			
	1:2	5			
'Creole' (*a*) + HP	None	5			
	1:1	5			
	1:2	5			

have been required for PA patterns. However, based on the experience of the second author, and his search of the literature, dominant *Mp* is rare. Almost all pea breeding lines, including materials derived from lines used by Barton (the donor of 55-567), have been *mp* for many years. Therefore, it could be expected that, of the two genes, only *Z-z* would segregate in the F_2 testas, leading to the 3:1 segregation we found for plain:pattern traits. A brief history of the discoveries of *Mp* and *Z* is presented in Supplemental Materials 4.

Other F_2 populations. In addition to 55-567 × L578, a second F_2 population provided evidence that *z* and *mp*, which together confer patterns of anthocyanin pigments in testas, conferred in a similar manner the distribution of PAs (Table 6), whether *Z* or *Mp* was segregating. Data (not shown) regarding a small F_2 population of G141 × L19 were consistent with these pleiotropic effects of *z* and *mp* as well (Supplemental Materials 5).

Anatomical differences from partial coloration genes. For *A b z* (pink-flowered, pattern; Fig. 1F) and *A B z* (purple-flowered, pattern; not shown), cells in the zone surrounding the radicle resembled those for the *a* phenotype (Fig. 1C), with thin-walled parenchyma rather than the thick-walled sclerenchyma otherwise present in *A* testas.

Effects of genes for flower color and partial testa color on resistance. Seeds borne on the 107 F_2 plants of 55-567 × L578 were planted in 10 flats that previously had been

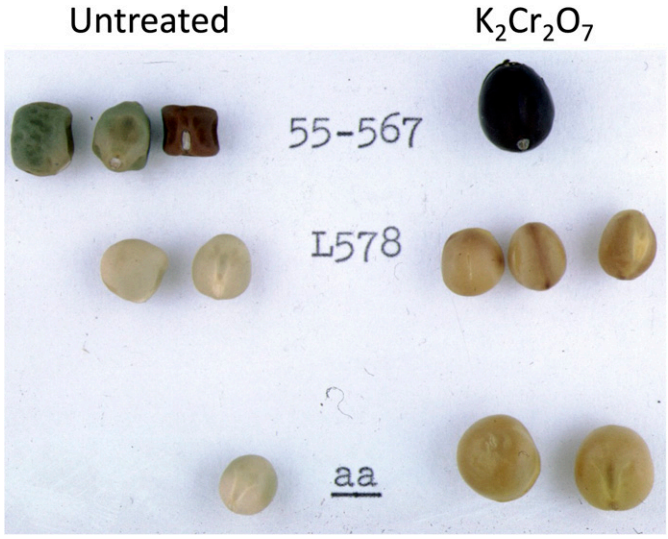

Fig. 2. Effects of partial coloration genes on proanthocyanidin (PA) distribution in pea. Shown are seeds of 55-567 (typical *A*), L578 (*A*, but resembling *a* because of five pairs of recessive alleles for partial coloration), and a typical *a* phenotype. Note the variation in color of untreated 55-567. This may occur in seeds from the same pod. Such darkening is associated with the oxidation of PAs (Quilichini et al. 2022), and dichromate treatment (right side) intensifies the color in all seeds.

autoclaved and inoculated with *G. ultimum*. In one flat, inoculation with *G. ultimum* failed—all seedlings emerged in all treatments. These data are not included in the results.

When seedlings were dug-up, some rows of seeds had good emergence, with testas mainly intact; nevertheless, testas were

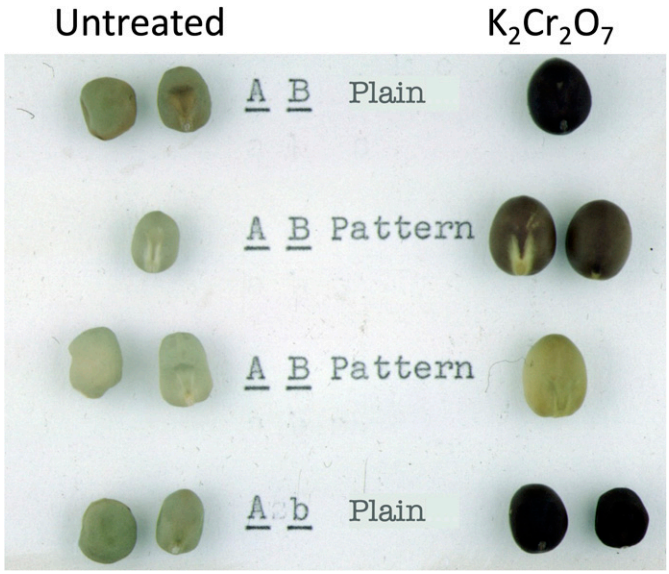

Fig. 3. Pea seeds grown on F2 plants of 55-567 (*A B r i*) × L578 (*A b R I z mp cal dem pal*) illustrating segregation after dichromate treatment. Testas from 81 of the plants had no pattern; they turned "solid" dark brown all over ("plain" phenotype), regardless of *A B* (top row, purple flowering) or *A b* (bottom row, pink flowering). Most of the 26 with the "pattern" phenotype were like those in the second row (*furca*), but some had larger zones free from tannin. Two plants bore seeds (row 3) that, like the L578 parent (see Fig. 2, center row, right), developed only traces of brown after dichromate treatment. *A b* pattern seeds are not shown but resembled *A B* pattern seeds. The principal tannins were proanthocyanidins, and their plain and pattern appearances were pleiotropic traits conferred by the genes *Mp-mp* and *Z-z*, which also confer similar distributions of anthocyanins.

Table 6. Comparisons of two F_2 populations of pea involving L578 and L19, a parent of L578. Phenotypes for these two lines were those published by Lamprecht (1947, 1957a, 1957b). The phenotype for 55-567 was assigned by the authors based on evidence from the crosses and published information. All lines were *A*.

Cross	Phenotypes	Segregating allele(s)	Plain:pattern ratio expected and (observed)[i]
55-567 × L578	*Z mp* × *z mp*	*Z*	3:1 (81:26)
L19 × L578	*z Mp* × *z mp*	*Mp*	3:1 (74:25)

[i] Observed segregating ratios had $P = 1$ when compared with the expected segregating ratios using the chi square test.

soft and easily rubbed away. After consulting the field notes and dichromate test results, such testas turned out to be of the *b Z* phenotype (pink flowers, plain). This prompted us to separate the F_2 emergence data into *B* and *b* phenotypes (Table 7). All *B* seeds testing as plain (*Z*) had nearly complete emergence. Of 278 seeds, the only three failures had girdled plumules, and their testas were still completely sound. Seeds with *b Z* testas had good emergence; however, in this instance, it was not quite as good as that of seeds with *B Z* testas. In contrast, both *B z* and *b z* had poor emergence, and surviving seedlings had testas that were completely disintegrated, with cotyledons that were often infected or destroyed. L19 testas showed softening very similar to that of the *b* Z testas, both in control flats and in infested flats. L19 resembled *b Z*, not only in testa softening but also in emergence. L578 resulted in poor emergence that was much lower than that shown in the field, indicating that conditions favored pathogenicity.

To further test the apparent small difference in emergence between *A B* Z and *A b* Z testas, another experiment was performed with seeds borne on F_2 plants of the same F_2 population. Based on 41% emergence of a cultivar with wrinkled seeds and green cotyledons, which were included to indicate the extent to which conditions favored pathogenicity, the disease was less severe than that in the preceding experiment but serious enough to have had an economic impact if the cultivar had been grown on a farm without seed treatment. Although *A b* testas at the end of the trial were again softer than *A B* testas, emergence of the two phenotypes was essentially identical (*A B*, 99%; *A b*, 97%) under this lower disease stress. Notably, the softening of *A b* testas occurred in control flats as well as in the infested ones.

Chemical differences between testas on purple-flowered and pink-flowered plants. In the F_2 of 55-567 × L578, testas borne on purple-flowered and pink-flowered plants were treated with hot acid; after extraction, the resulting anthocyanidins were examined by paper chromatography. The R_f values of anthocyanidins for testas from three different *A B* plants were 0.34, 0.36, and 0.36. For three *A b* plants, they were 0.56, 0.57, and 0.56. Published values (Bate-Smith 1954) of delphinidin and cyanidin were 0.30 and 0.50, respectively. Although authentic standards were not available, the difference between *A B* and *A b* was clear. Considering that the only PAs reported to exist in pea testas are prodelphinidins and procyanidins, it seemed likely that *A B* conferred the former and that *A b* conferred the latter.

Confirmatory results were obtained using LC-MS/MS to analyze testas of F_2 siblings from a completely different F_2 population (Table 8). Unlike the first population, all plants in the second population were dominant for *Fs*, conferring small purple (*B Fs*) or reddish (*b Fs*) spots on testas. Thus, washings of the testas contained soluble PAs and anthocyanins, both of which yielded anthocyanidins from the hot acid treatment. The washings also included PA monomers, which would not have been converted to anthocyanidins (Thompson et al. 1972). In both fractions, the estimated amounts of anthocyanidins produced by *A B* testas were predominantly delphinidins; in contrast, for *A b* testas, almost all were cyanidins. In the soluble fractions, the estimated concentration of anthocyanidins from *A B* testas was 50-fold more than that from *A b* testas. In contrast, the insoluble fractions of the two phenotypes appeared to be essentially equal, except that *A B* was mainly delphinidin and *A b* was mainly cyanidin.

Lack of resistance to *Rhizoctonia*. In control flats, all 64 plants emerged for each type of testa. The same was true for *A* testas in the *G. ultimum* flats; additionally, as might be expected, only 26 of 64 emerged for *a* testas. However, in the *R. solani* flats, five of 64 seedlings with *A* testas emerged, and none with *a* testas emerged.

Discussion

Susceptibility differences among seeds borne on white-flowered pea. Initial experiments (not described here in detail) established that in white-flowering (*a*) lines, wrinkled (*r*) seeds were more susceptible to *G. ultimum* rot than were their round (*R*) F_2 siblings. A wide range of pedigrees was involved, and the outlook for finding a solution through conventional breeding techniques was not promising. The association between cultivars with wrinkled seeds and susceptibility to this disorder have been reported widely (Flentje and Saksena 1964), but cultivars vary in many other traits at the same time; therefore, a comparison of

Table 7. Emergence in *Globisporangium ultimum*-infested flats of pea seeds borne on F_2 plants of 55-567 × L578. Seeds that developed a solid, dark brown color when samples were treated with dichromate (*Z*) are compared with those that developed a pattern (*z*), indicating the absence of proanthocyanidins in a zone that reached from the hilum up to and around the tip of the radicle. Seeds were also sorted for the flower color, purple (*B*) or pink (*b*), of the parent plant. Data regarding emergence are from seeds untreated with dichromate. Also shown for comparison are emergence data for L578 and its parent, L19.

Phenotype	Fraction emerged	Percent emergence[i]	Condition of testas 18 d after planting
A B Z *mp* (plain)	275/278	98.9 x	Almost all completely intact and firm
A b Z *mp* (plain)	73/79	92.4 y	Mostly intact, but easily rubbed away
A B z *mp* (pattern)	25/75	33.3 z	Disintegrated
A b z *mp* (pattern)	9/45	20.0 z	Disintegrated
L19 (*A B R z Mp*)	40/45	88.8 y	Mostly intact, but easily rubbed away
L578 (*A b R z mp cal dem pal*)	4/45	8.9 z	Disintegrated

[i] To improve homogeneity of variance, individual values for the rate of emergence were arcsine-transformed before statistical analyses (see Materials and Methods). Values followed by the same letter were not significantly different ($P \leq 0.05$).

Table 8. Estimated amounts of delphinidin and cyanidin produced by hot acid treatment of *A B Fs* and *A b Fs* pea testas. Testa fragments from three seeds per phenotype were washed with water and dilute methanol, and the washings were treated with hot acid to convert proanthocyanidins (PAs) and anthocyanins to anthocyanidins. The insoluble fraction was also treated with hot acid. Amounts of delphinidin and cyanidin from each phenotype are estimates obtained by liquid chromatography with tandem mass spectrometry analysis.

	Delphinidin from hot acid treatment		Cyanidin from hot acid treatment	
Testa phenotype	*A B* (purple flowers) ng/mg testa	*A b* (pink flowers) ng/mg testa	*A B* (purple flowers) ng/mg testa	*A b* (pink flowers) ng/mg testa
Soluble fraction[i]	1327.2	0.9	83.4	86.8
Insoluble fraction[ii]	24.5	1.6	0.2	20.9

[i] Includes anthocyanidins derived from anthocyanins as well as from PA oligomers.
[ii] Anthocyanidins derived from PA polymers.

siblings is a better test. We also showed that in *a* phenotypes, green (*i*) cotyledons are more susceptible than yellow (*I*) ones. We have found no other reports of this effect.

Role of the testa in plants with colored flowers. Importantly, unlike the situation for seeds from *a* plants, seeds borne on *A* plants were highly resistant to *G. ultimum* rot regardless of the status of *R-r* and *I-i.* Reciprocal crosses involving nine combinations of *AA*, *Aa*, and *aa* genotypes indicated this resistance was conferred by *A* of the maternal parent only, and that dominance was complete. Because the nucellar tissue of the pea ovule is digested 2 to 3 d after fertilization, and because the endosperm—which is never definitely cellular at any stage of development—is completely digested 11 to 12 d after fertilization (Hayward 1938; Quilichini et al. 2022; Reeve 1946), the only two parts of the dry pea seed are the testa (maternal tissue) and embryo. We concluded that the genotype of the testa determined the strong resistance conferred by *A* in our experiments, and we found no evidence indicating that the status of *A-a* in the embryo played a role.

Stasz and Harman (1980) came to a different conclusion about *A-a* in the embryo. During experiments with seeds from which testas had been removed, they were able to detect a statistically significant degree of protection by the presence of *A* in the embryos of some lines. They did not detect an advantage of *A* embryos during their field experiments, however. We do not know whether the explanation for their different conclusion are dependent on the fact that our reciprocal cross experiments were performed in the field or on other factors. In any case, because there may have been circumstances in which the presence of *A* in the embryo contributed to resistance—perhaps especially after the radical has broken through the testa—during our study, the presence of *A* in the testa was of overriding importance.

Presence of PAs in *A* testas. Similar to other studies, *A* testas were positive according to the traditional tests for tannins and were able to bind with hide powder (Zabel 1948). Additionally, a positive acidic vanillin test and the production of anthocyanidins after hot acid treatment provided evidence indicating that PAs were present in both soluble and insoluble forms, typical of the oligomers and polymers usually found with PAs. We found that pea with pink flowers (*A b*) had testas containing mainly procyanidins, whereas purple-flowered (*A B*) ones had mainly prodelphinidins. The latter are much more common; however, in the literature, pea flowers often have been referred to as white or "colored," with no further identification.

Physical factors in resistance. In mature seeds, the testa comprises dead cells, and the strong resistance in question depends on the physical and chemical properties of such cells, as opposed to interactions with the pathogen that involve living host cells. There is reason to conclude that both the physical and chemical properties of the testa play a role (Stasz and Harman 1980). Subjectively, it is more difficult to cut *A* testas with a blade than it is to cut *a* testas with a blade, and our own evidence of a physical role includes a higher ratio of testas to total seed weight of *A* seeds than of *a* siblings. During anatomical comparisons of the two types, *A* testas displayed darker safranin staining of macrosclereids, positive test results for PAs throughout the testa tissue, and the presence of sclerenchyma where *a* testas had parenchyma (Fig. 1). These observations are consistent with those reported by Stoll (1950), who found that *A* testas were more difficult than *a* testas to macerate, whether buried in soil, exposed to macerating bacteria, or treated with hot calcium hypochlorite solution. Ferraro et al. (2014) suggested that PAs are bound to pea cell walls in *A* testas. Quilichini et al. (2022) came to a similar conclusion. In *A. thaliana,* Demonsais et al. (2020) established that PAs are constituents of testa cell walls, providing extra strength and protection. If the same holds true for *A* pea testas, then that would help explain the barriers they present to hyphal penetration.

Obstruction to hyphal penetration is not the only way that a strong barrier might reduce infection. The barrier is also likely to reduce leaching from the embryo of nutrients that favor hyphal growth (Flentje and Saksena 1964; Perry 1973; Short and Lacy 1976). Perhaps it would be equally important or more important to block the escape of vapors. Gorecki et al. (1985) detected ethanol and acetaldehyde gases fugitive from *a* pea seeds as soon as imbibition of water was initiated. Nelson (1987) found that the escaping ethanol vapor stimulated *G. ultimum* sporangia to germinate, leading to rapid penetration of testas by the hyphae. A third possibility is that the barrier provided by *A* testas slows the imbibition of water by the cotyledons, which, in turn, reduces cracking and solute leakage that, for *a* testas, would weaken the host and promote pathogenicity (Kraft 1977; Matthews and Whitbread 1968; Powell and Matthews 1978, 1980; Reid and Ross 2011).

Chemical factors. Evidence (as noted, likely from *A B* testas) of a chemical role in the resistance of *A* testas is provided by the fact that their extracts are toxic to pathogens. This was demonstrated by Sörgel (1952) for *Ascochyta* foot rot of pea and confirmed by Clauss (1963) for *Ascochyta* and *Mycosphaerella*. Experiments by other researchers have reported similar results for *G. ultimum* (Kraft 1977; Stasz and Harman 1980). *A B* pea testas are rich in phenolics that, in vitro, could be toxic to *G. ultimum*, and various phenolics have been suggested (Kraft 1974, 1977; Kraft and Roberts 1970; Troszyńská and Ciska 2002).

Consistent with these reports, *G. ultimum* growth in vitro was inhibited in aqueous extracts of testas from purple-flowered

plants (*A B Z*). Seven days elapsed from the time when the powdered testas were placed in water until the first four replications were inoculated, and 9 d elapsed before inoculation of the fifth replication. These delays may have promoted oxidation of PAs and other phenolics in the extracts. However, this did not eliminate the inhibitory effects of the extracts, and the fifth replication, which was delayed 2 d more than the others were delayed, had zero growth in all three concentrations. Growth inhibition was eliminated by prior removal of tannins with hide powder, which is a technique that was used by Zabel (1948) to demonstrate the role of tannins in resistance to a fungal disease in oak (*Quercus alba*) wood. In contrast, extracts from *a* testas supported abundant mycelial growth with or without hide powder treatment. This points to tannins as a principal factor, and tannins in *A* testas are mainly PAs.

If any inhibitors escaped the hide powder, then, even collectively, they were not sufficiently concentrated to affect *G. ultimum* during our tests. However, along with PAs and their monomers, hide powder may have removed other soluble phenolics that would have contributed to the inhibition. Jha et al. (2019) measured the concentrations of polyphenols in testas of one white-flowered and two purple-flowered pea lines. A number of polyphenols were present in higher concentrations in the *A B* line than in the *a* line. Some of these may have tanning attributes, but none was present in concentrations that began to approach those of the PA monomers (gallocatechins). More importantly, PA oligomers were not included in their assays, and estimates of *A B* extracts (Table 8) indicated very high levels of soluble PAs in our materials. Jha et al. (2019) found only trace amounts of anthocyanins in testas of a phenotype that seemed to be *A B Fs* (purple flowers with purple-spotted testas), like the one in our second experiment; therefore, it is unlikely that its anthocyanins made a significant contribution to the delphinidins that resulted from hot acid treatment. Based on our studies and those in the literature, although other compounds may have contributed, there is reason to believe that PAs and their monomers were the principal compounds responsible for the inhibition of *A B* testa extracts to *G. ultimum*.

To our knowledge, the toxicity of *A b* extracts has not been tested by others; however, during our experiment, no inhibition was detected. The much lower concentrations of soluble PAs in *A b* than in *A B* testas (Table 8) may explain the lack of inhibition by *A b* extracts; however, it would be desirable to examine more concentrated extracts from *A b* testas to determine the effects on the growth of *G. ultimum*.

The effects of soluble prodelphinidins suggest that the polymers would similarly inhibit hyphal attack. In this regard, Stasz and Harman (1980) speculated that high concentrations of fungistatic compounds in the testa tissues, rather than in the spermosphere, might explain why hyphae failed to penetrate *A* testas, even though they had colonized the testa surface. Our microscopic examination of testas stained with acidic vanillin showed PAs throughout the testa, indicating that along with soluble fractions, PA polymers were present. If so, then the polymers might react with invading hyphae by precipitating enzymes and other proteins, just as PAs of all sizes precipitate the proteins of animal hides when tanning leather (Scalbert 1991).

There are also other ways in which PAs may be toxic to pathogens (Demonsais et al. 2020); however, in terms of both toxic effects and physical barrier effects, more information about the anatomical differences in testas is necessary (Fig. 1). In *a* phenotype and in *A z* phenotype, along with the absence of PAs, we found parenchyma instead of sclerenchyma—thin cell walls rather than thick ones. As mentioned, it has been indicated that PAs are components of the cell wall. This suggests "tannic cells" may be present in pea testas, as found by Demonsais et al. (2020) in *A. thaliana*. The use of their histological techniques for pea testas could be very informative, especially if both *A B* and *A b* testas were examined.

Failure of resistance against *Rhizoctonia*. During our only test of resistance to *Rhizoctonia*, the severity of the *Rhizoctonia* attack was surprising, especially because all seeds in the experiment were round rather than wrinkled. An examination of the seedlings that did not survive indicated that those with *a* testas had been killed at an earlier stage than those with *A* testas. Whether more substantial evidence of *A* resistance would be found under conditions less favorable for *Rhizoctonia* is an interesting question. The pathologist who supplied the *Rhizoctonia* culture explained in advance that it was an exceptionally virulent one.

Partial coloration genes. Our study of genes provided further evidence of the importance of PAs and cell structure for resistance to pea seed rot incited by *G. ultimum*. A cross was made between the two *A* lines, 55-567 and L578. Testas of 55-567 were rich in PAs and highly resistant to seed rot; in contrast, testas of L578 had only traces of PAs and were not resistant. Lamprecht (1957a) stated that L578 was *A b z mp cal dem pal*; therefore, it was lacking in testa anthocyanins. In the F_2 testas from 55-567 × L578, there was a 3:1 segregation for the partial coloration gene *Z* that was identifiable not by testa anthocyanins (absent in all) but was identifiable by PA distribution, as detected by dichromate treatment. We concluded that the *A z mp* phenotype confers the pattern of not only anthocyanins but also PAs. More evidence of this conclusion is presented in Supplemental Materials 5.

Segregation in F_2 permitted us to examine the association between the presence of PAs and resistance (Table 7). Seeds with patterned testas lacked PAs in the *furca* (forked) zone, where the emerging radicle breaks the testa open. In the same zone, sclerenchyma was replaced by parenchyma (Fig. 1F). Thus, the pocket of PA-filled sclerenchyma cells in *A* testas that normally protect the radicle until it breaks through the testa was missing. These changes in a relatively small part of the testa eliminated the strong resistance normally conferred by *A*. The timing may be critical—for some of the genotypes studied by Stasz and Harman (1980), embryos from which testas had been removed were much less susceptible to *G. ultimum* attack if the testas remained in place until the radicle had broken through.

L578 contributed three other partial coloration genes: *dem, cal*, and *pal*. Lamprecht (1947, 1957a, 1957b) demonstrated that in the presence of *mp z*, each of these other recessive alleles enlarged the zone lacking anthocyanins. As might be expected, some of our F_2 testas responded to dichromate treatment by exhibiting PA-free patterns larger than the forked area. Presumably, this occurred when one or more of these three genes were homozygous recessive, along with *z mp*. The number of seeds with such patterns in the F_2 population was too small to determine whether expanding the PA-free zone beyond the forked pattern led to a corresponding increase in disease, and this would be interesting to investigate. However, even the small *furca* zone conferred by *z mp* resulted in the loss of strong resistance. Like the previously mentioned effects of a small cut in the testa, this

suggests that the presence of PAs within the entire intact testa may be more essential to strong resistance than the toxicity of the leachates. Kantar et al. (1996) reached a similar conclusion for fava bean (*Vicia faba*), for which purple flowers were associated with resistance to seed rot, high PAs, and strong testas.

Effects of pink flower color. Flower color, controlled by *B-b*, also segregated in this cross. Flowers are purple on *A B* plants (three hydroxy groups on the B carbon ring of the delphinidin anthocyanin) but pink on *A b* plants (only two hydroxy groups on the B ring for cyanidin). Our results were consistent with the hypothesis that PAs would be affected in a manner parallel to anthocyanins by segregation at the *B* locus. In two different F_2 populations, testas borne on purple-flowered plants contained predominantly prodelphinidins, yielding delphinidin after hot acid treatment; siblings with pink-flowered plants had testas with procyanidins, yielding cyanidin. The biosynthesis pathway allows for "leakage" of cyanidins into the *A B* phenotype, evident in the soluble fraction. Even though the effect of *B-b* on resistance to *G. ultimum* seed rot was small, the results provide one more piece of evidence that PAs have a role.

We have not found in the literature that the *b* allele confers procyanidins in pea testas. Therefore, our evidence could be important for dietary reasons, especially for those whose diets tend to be low in iron—such as in parts of the world where dry pea that are dominant for *A* are a major source of protein. Elessawy et al. (2021) pointed out that, compared with procyanidins, the extra hydroxyl group on the B ring of prodelphinidins makes them higher in antioxidants; unfortunately, this also makes them stronger in chelating iron. Differences in concentrations of PAs and other phenolics also affect chelation, but the type of PA is a major factor, and pink flowers would be a simple, although not perfect, indicator of better iron bioavailability in testas.

Because of this connection, it could be useful that seeds borne on pink-flowered plants were very resistant to *G. ultimum* seed rot, although not to the same degree as seeds borne on purple-flowered plants. Dominant *B* provided testas that were completely intact 18 d after planting, even when exposed to conditions most conducive for the disease (i.e., continuous high moisture content of autoclaved soil mixtures that had been heavily infested with *G. ultimum* before planting). In *G. ultimum* flats, *A b* testas were present 18 d after planting, although they could be wiped away by soft rubbing. During a follow-up experiment, the testa softening also occurred in control flats.

Plant emergence in *G. ultimum* flats was only slightly lower than that of *A B* testas. For practical purposes, the level of resistance by *A b* testas should be adequate—infestation of steamed soils with *G. ultimum* leads to more severe disease than ordinarily encountered in the field (Stasz and Harman 1980). If the resistance pattern described in the current study is maintained across genetic backgrounds and environments, then green pea breeders could develop dwarf *A* lines, whether *B* or *b*, and increase their table quality by incorporating wrinkled seeds with green cotyledons while still maintaining strong resistance to *G. ultimum* seed rot. Similarly, breeders of dry pea could select for pink flower color to increase dietary iron with minimal concern regarding the loss of resistance to seed rot.

Early plant vigor. During our experiments, most testas had disappeared from *a* seedlings that survived seed rot. This was accompanied by severe rotting or total loss of cotyledons. In contrast, *A* testas were intact, and the protected cotyledons not only were free from lesions but also were shriveled because of translocation of nutrients into the developing plants. The consequent boost in early plant vigor, like the boost reported for fungicide treatment (Brett et al. 1937), might help explain why *A* lines often contribute resistance to related diseases such as root rot (Muehlbauer and Kraft 1978).

Relevance for modeling PA and lignin biosynthesis. Flavonoid biosynthesis has long been intensively research (Winkel-Shirley 2001), but Dixon and Sarnala (2020) called for better plant models to study the pathways of PA and lignin biosynthesis and suggested poplars (*Populus* spp.) as test plants. Quilichini et al. (2022) stressed the need for more modeling of PA biosynthesis in pea testas. Ferraro et al. (2014) pointed out that the large seeds and rich genetic diversity of pea would be advantageous for such modeling. Ellis et al. (2018) noted that *A-a* has a role in regulating compounds beyond anthocyanins, and our data provided examples of this: *A* not only confers the presence of anthocyanins in testas, flowers, and nodes but also confers both PAs and sclerenchyma in testas. Moreover, the *A b* phenotype that confers pink instead of purple flowers is pleiotropic; it changes the testa PAs from prodelphinidins to procyanidins, slightly reduces resistance to *G. ultimum* seed rot, and confers softening of testas during exposure to the soil, even in the absence of *G. ultimum*. In contrast, *A z mp* phenotypes confer a zone around the radicle that is free not only from anthocyanins but also from PAs and sclerenchyma. Other recessive alleles enlarge this zone, each in a different manner. Thus, aside from their role in disease resistance, the apparent association of PAs with lignification in pea testas controlled by these classical genes may be of interest for modeling biosynthesis pathways.

Conclusions

To return to the question of why *A* confers resistance to *G. ultimum* pea seed rot, it confers a testa that serves as a strong physical barrier to external attack while also blocking exudates and volatiles beneficial to the pathogen. This is combined with lumens containing PAs and thick cell walls fortified by PAs that are inhibitory to the pathogen (as small molecules eluted into the spermosphere and likely as insoluble ones remaining in place). The combination would seem to afford potent protection for the embryo. Focus has long been on the role of anthocyanins in seed rot resistance (Muehlbauer and Kraft 1978). We do not dismiss the possibility that, under other circumstances, anthocyanins may contribute to this resistance (Kraft 1977); however, in the absence of genes for testa pigmentation, under conditions extremely favorable to the pathogen, 98.9% emergence was obtained from *A B* seeds, and resistance was associated with testa PAs. Our results suggest that more attention should be focused on PAs and accompanying cell structure, attributes less noticeable than coloration.

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