Anthocyanins in Leaves and Other Vegetative Organs: An Introduction

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ABSTRACT

Although anthocyanins are most recognized as pigments contributing to coloration in fruits and flowers, they are also present in leaves and other vegetative organs. Although their presence has long been recognized, particularly because of their contribution to autumn coloration, the phenomenon has been poorly studied and is not well understood. In this chapter we review the history of research on anthocyanins in leaves, emphasizing the flurry of research at the end of the 19th century as well as the growing body of contemporary research on the topic. We emphasize the various hypotheses of anthocyanin function that were mainly developed more than a century ago, and emphasize recent research that takes advantage of our dramatically increased understanding of whole plant physiology.

Advances in Botanical Research Vol. 37
incorporating Advances in Plant Pathology

I. INTRODUCTION

Anthocyanins (Fig. 1) are red, purple or blue pigments derived from the flavonoid pathway. They are synthesized in bryophytes and vascular plants, most notably among the angiosperms, but are absent from the algae. In the flowering plants, anthocyanins are most conspicuous in the coloration of flowers and fruits, although they are also to be found in the leaves, stems and roots of many species. The economic importance of anthocyanins to the fruit and cut-flower industries has driven intensive research into the molecular control of cyanic coloration (Mol *et al.*, 1996). However, despite the widespread distribution of anthocyanins among vegetative organs, and the long history of interest in anthocyanins, relatively little research has been directed towards their function in leaves. There are several reasons for this lack of research; these are best understood by reviewing the history of study of red pigmentation in plants.

II. HISTORY

A. EARLY

Interest in the red coloration of leaves and other plant parts can be traced back to the beginnings of western science. Red coloration in leaves was observed by Aristotle (Barnes, 1984) in his writings on the theory of colour, and by Theophrastus (1918) in his descriptions of different plants. During the renaissance, Nehemiah Grew (1682) observed the red pigmentation in vegetative organs and conducted simple experiments on them, described in his "The Anatomy of Plants". Various scientists observed the distribution of 'coloured cell sap' in plant organs with the light microscope, for which Marquart (1835) coined the term anthocyanin, derived from the Greek *anthos* (flower) and *kyanos* (blue). Many long-standing misconceptions on anthocyanin function were derived

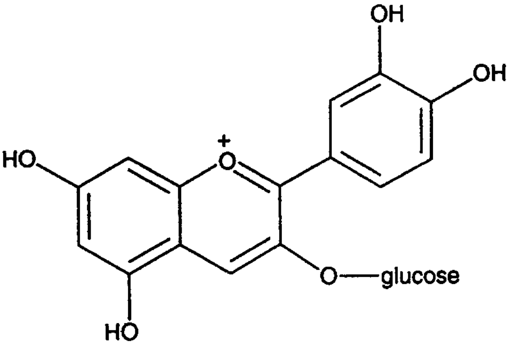

Fig. 1. The structure of cyanidin-3-glucoside, the most commonly encountered anthocyanin in leaves.

from these early observations. For instance von Mohl (1837) held that anthocyanins were derived from the breakdown of chlorophyll, based on his observations of autumn leaf coloration. Clearly, this is incorrect, yet the adjunct hypothesis, that anthocyanins are revealed during leaf senescence as chlorophylls degrade (which is also incorrect) persists in the contemporary literature. The early history of anthocyanin research was reviewed in detail by Wheldale (1916), many of whose findings are highlighted in this chapter.

B. LATE 19TH AND EARLY 20TH CENTURY

The dramatic expansion of research on anthocyanins during this period can be ascribed to three factors. First, there was the rise of organic chemistry, particularly in Germany, that made possible the determination of the chemical nature of anthocyanins and other pigments. Second was the development of a 'school' of plant research in Europe known as physiological plant anatomy. Third was the re-discovery of Mendel's laws of inheritance.

The attachment of anthocyanins to sugar molecules was first described by Lidforss in 1891 (Wheldale, 1916). Improvements in the techniques for the elucidation of chemical structures culminated in the classical studies of Willstatter and colleagues in which the formula and structure of an anthocyanin molecule was determined, and a biochemical pathway of its synthesis was proposed (Willstätter and Everest, 1912; Willstätter and Mallison, 1914).

During this time, work on anatomy, morphology and physiology in plants became fused as a new discipline: physiological plant anatomy (Haberlandt, 1914; Cittadino, 1990). Much of the inspiration for this research was the experience of European botanists in the tropics, particularly in Southeast Asia. There, they observed the presence of red pigmentation in tropical plants, particularly the rapidly expanding foliage of trees and the undersurfaces of shade plants. The striking coloration in leaves of these plants, rarely observed in temperate regions where speculation was primarily derived from observations of autumn leaf colour, stimulated much research on anthocyanin function in leaves.

Three hypotheses on the effects of anthocyanins on leaf function were proposed at this time: photoprotection of photosynthesis; photoprotection of starch hydrolysis and translocation; and temperature regulation.

The German physiologist Pringsheim (1879–1882) had shown that chlorophyll in leaves was damaged by exposures to white light of high intensity, but not to red light. This led him to suggest that anthocyanin might protect the photosynthetic machinery by screening out the most damaging wavelengths of light, thus establishing the photoprotection hypothesis. He was influenced by many earlier observations, summarized by Kerner von Marilaum (1897), that sun exposure promoted the synthesis of red pigmentation. Others noted the relationship between sugar accumulation and anthocyanin formation, and hypothesized that anthocyanins might protect against damaging effects of intense light on starch hydrolysis and sugar translocation (Pick, 1883).

Since anthocyanins absorb radiation at wavelengths different than chlorophyll, some scientists argued that these pigments might moderate leaf temperature. The most prominent among the physiological plant anatomists was Ernst Stahl. Based on his research in Java, Stahl (1896) concluded that anthocyanins could contribute to increased absorption of light by leaves, thereby elevating leaf temperatures and increasing rates of transpiration and metabolism. Stahl's hypothesis directly opposed that of Keeble (1895), who had previously suggested that in the flushing and hanging red leaves of tropical trees, high temperatures would be moderated by the red pigments. The publication of Stahl's hypothesis stirred controversy among the scientific community. In Ewart's (1897) critique, Stahl's hypothesis was denounced, and evidence presented instead in favour of the photoprotection hypothesis. Direct evidence supporting Stahl's hypothesis was provided by Smith (1909) who, using thermocouple sensors to measure leaf temperatures, reported that red leaves were up to several degrees warmer than green leaves under comparable environmental conditions.

Shortly after the turn of the century, the laws of Mendel were re-discovered. Contemporaneous discoveries in the chemistry of anthocyanins and other flavonoid pigments led to the use of these pigments as biochemical characters in genetics. The control of flower and fruit colour presented an economic incentive for research in the biochemical genetics of anthocyanins. This was well documented by Wheldale (1916, later as Onslow, 1926), who was an active participant in the research. Advances in the inheritance of anthocyanins led to greater understanding of the pathways of synthesis of these pigments, and this research continued during the first half of the 20th century.

C. THE LAST FIFTY YEARS

Dramatic advances in the understanding of flavonoids and anthocyanins followed the Second World War, particularly due to the incorporation of carbon 14 into tracer molecules to resolve pathways of synthesis. This research was particularly centred in the laboratory of Hans Grisebach at the University of Freiburg (Grisebach, 1957; Grisebach and Patschke, 1961), although others also contributed (Geismann and Swain, 1957; Watkin *et al.*, 1957). Much of this research was completed in the 1950s, and the pathway was further refined with the advent of high performance liquid chromatography in the 1980s (Stafford, 1990). During this period of chemical research, there were few studies on the functions of anthocyanins in leaves – only their roles in pollinator attraction to flowers and disperser attraction to fruits were considered.

D. WHY NOW?

The bulk of research on anthocyanins has concentrated on those plant organs of greatest economic significance: flowers and fruits. There has been, and continues to be, considerable commercial incentive to control flower colour for floriculture, and fruit colour for the horticultural industry. However, the past decade has also seen a significant increase in research on the functions of anthocyanins in leaves and other vegetative organs. There are several reasons for the long stagnant period and the recent resurgence in activity. These relate to the history of research, economic pressures, trends towards reductionist approaches in biology, and recent advances in whole-plant physiology and molecular genetics.

Early observations that anthocyanins in leaves were inducible by light led to an expansion in research into plant photobiology. The pathway of anthocyanin biosynthesis, with the induction of enzymes at different points, has became a model system in photobiology (Mancinelli, 1985; Beggs and Wellman, 1994) and plant molecular biology (Westhoff, 1998), and is a key component of research on phytochrome (Sage, 1992). Thus, it is not surprising that we have a detailed knowledge of the biosynthesis of these pigments, including the molecular genetics of their control at different points in their biosynthetic pathway. In contrast, we have relatively little knowledge of anthocyanin function(s) in plant organs, other than their potential to attract animals for pollination and seed dispersal. Moreover, the focus on important crop plants has meant neglect in research on the functions of these molecules in vegetative organs of non-crop plants. There is a parallel in the history of research on anthocyanins in that for phytochrome. The discovery of the red:far-red effects led to the elucidation of the pigment molecule, and eventually to its molecular biology. Yet, research on the function of phytochrome in nature (Smith, 1994) lagged far behind.

In the past decade, interest in the questions of anthocyanin function in vegetative organs has definitely increased. There are several reasons for this. First, there have been steady advances in whole plant physiology and physiological ecology, partly made possible by the development of sophisticated field-portable instrumentation. Foremost among these are fluorometers used in dissecting photosynthetic function by the analysis of the kinetics of chlorophyll fluorescence decay. We now have a more detailed understanding of the impact of high irradiance on photosynthetic processes, and the potential mitigating effects of anthocyanins as light filters. Second, with the realisation that global levels of UV-B radiation are rising, researchers have actively searched for UV-screening agents. The UV-absorbing properties of most flavonoids, including those of the anthocyanins, are obvious targets for research (Shirley, 1996). A third development is the growing awareness of the dietary importance of flavonoids as antioxidants (Rice-Evans and Packer, 1998). Anthocyanins in particular are potent scavengers of most reactive oxygen species. Given their documented effects in human diet, the scientific community has begun also to realise the importance of anthocyanins in the amelioration of plant defence responses to oxidative damage.

III. HYPOTHESES FOR ANTHOCYANIN FUNCTION IN LEAVES

Given the long, if somewhat fragmented, history of research into anthocyanins, it is not surprising that many different hypotheses have been proposed for the functions of these pigments in leaves. Here we summarize the chief hypotheses that have been debated over the past decade or so. Since most of these are explored in far greater depth in the following chapters, we succinctly summarize them and expand on those not covered in other chapters.

A. UV-B PROTECTION

Anthocyanins, particularly acylated forms, absorb UV-B radiation (Harborne, 1988), are induced and accumulate in plants subjected to UV-B (Kakegawa *et al.*, 1991; Klaper *et al.*, 1996) and reduce photoinhibition and DNA damage in UV-irradiated material (Takahashi *et al.*, 1991; Stapleton and Walbot, 1994; Burger and Edwards, 1996; Klaper *et al.*, 1996). Flavonoid-deficient mutants are hypersensitive to UV-B (Li *et al.*, 1993; Lois and Buchanan, 1994). However, anthocyanins are less effective UV-protectants than other flavonoids and hydroxycinnamic acid cunjugates (Woodall and Stewart, 1998) and in many leaves anthocyanins are not optimally located for UV-B screening (Lee *et al.*, 1987; Gould and Quinn, 1999; Lee and Collins, 2001) compared to other flavonoids (Burchard *et al.*, 2000).

B. LEAF WARMING

Quanta absorbed by anthocyanins may be converted into heat, serving to elevate leaf temperatures. Cyanic leaves are common in cold locations, particularly at high altitudes (McClure, 1975; Ganders *et al.*, 1980; Hoch *et al.*, 2001). Modest temperature increases were noted for cyanic versus green conifer needles (Sturgeon and Mitten, 1980), though no differences were found among tropical species (Lee *et al.*, 1979, 1987). Lee *et al.* (2002) found no differences in leaf temperatures between senescing green and red leaves of *Quercus rubra* and *Vaccinium corymbosum* in a New England forest. Anthocyanins are not required for freezing tolerance in *Arabidopsis* (Leyva *et al.*, 1995; McKown *et al.*, 1996). This hypothesis will be discussed in this volume in chapters by Chalker-Scott as well as by Starr and Oberbauer.

C. DEFENCE AGAINST HERBIVORES AND PATHOGENS

As an end-product of the flavonoid pathway, anthocyanins have often been assumed to be biologically active along with other flavonoid compounds. Insect attacks may induce the production of anthocyanins around the lesions on leaves (Costa-Arbulú *et al.*, 2001), as they are produced in response to mechanical damage and fungal attack. However, the direct evidence for such activity is not very strong. Nonetheless, anthocyanins have been hypothesized to function defensively for such putative activity, and also in their alteration of the appearances of leaves.

A wide variety of flavonoid compounds has been implicated in defensive activity against herbivores, particularly as toxins and anti-feedants against insects, and against fungal pathogens, often as phytoalexins (Rosenthal and Janzen, 1979; Harborne, 1997; Lambers *et al.*, 1998). However, such evidence is not strong. In fact anthocyanins are known to be well-tolerated in the diets of higher animals, including humans.

Anthocyanins are frequently produced around lesions from fungal pathogens (Harborne, 1976; Chalker-Scott, 1999 and this volume). This is particularly well-documented for diseases of maize, for instance by Hammaerschmidt and Nicholson (1977). However, evidence for actual inhibition of fungal growth is weak. Interestingly, strong evidence for the antifungal activity of a 3-deoxy anthocyanin has been shown for soybeans against several pathogens (Nicholson *et al.*, 1987), but not for anthocyanins.

Coley and Aide (1989) reported leaf cutting insects (that remove leaf parts and carry them to nests to cultivate fungal gardens) in feeding tests to discriminate against both young red leaves and food laced with anthocyanic leaf extracts. They speculated that such a food collection strategy would logically protect their fungal gardens against antifungal activity. However, they did not directly demonstrate such activity or compare the total flavonoid chemistry of the leaf pieces. Numerous attempts to show such antifungal activity for anthocyanins in a variety of contexts have produced negative results (Harborne and Grayer, 1988; Hoagland and Boyette, 1994).

Similar attempts to show biological effects on herbivorous insects have also largely been unsuccessful. However, Hedin and colleagues (1983) showed that cyanidin-3 glucoside, commonly produced in leaves, including cotton, inhibited the growth of larvae of the tobacco budworm, *Heliothis viridis*, an important pest of cotton and other crops. This is an exception to the general lack of results of inhibitory or toxic activity by anthocyanins.

Anthocyanins may have a more important role in reducing herbivory by altering leaf appearance. Anthocyanins and chlorophylls combine to produce brown colours in leaves, almost black if in sufficient concentrations. Of course, in the absence of chlorophylls and in the right placement in the leaf, anthocyanins produce a very bright colour at rather low concentrations, given the high extinction coefficients of anthocyanins in the wavelengths around 510–550 nm and absorbances of other ubiqutous pigments at shorter wavelengths. Stiles (1982) has argued that red leaves attract dispersal agents when associated with less conspicuous ripe fruits.

Stone (1979) hypothesized that the accumulation of anthocyanins in young fronds of certain species of palms could disguise the leaves and thus reduce rates of herbivory during development. Some rainforest understory herbs produce mature leaves with combinations of chlorophylls and anthocyanins, and the leaves are indistinguishable from the adjacent dead leaves, such as *Psychotria ulviformis* in French Guyana (unpublished research). Juniper (1993) also argued that such coloration could disguise young leaves of temperate plants during the growing season, to make them less susceptible to herbivores. Another possible camouflage of leaves is variegation, not exclusively but often through the production of anthocyanic spots. Givnish (1987, 1990) argued that such spots may make leaves a difficult search image for potential herbivores. Smith (1986) had shown in *Byttneria aculeata* (Sterculiaceae, now Malvaceae), a small understory herb of the neotropics, that variegation could reduce herbivory by leaf mining insects as well as improve the energy properties of the leaves, depending on the location of the plants. This is an important study because it is unique in backing up this hypothesis with experimental evidence. However, the source of variegation was not due to anthocyanins. Ganders *et al.* (1980) worked out the controls of inheritance of variegation due to anthocyanin spots in three coniferous forest herbs. They observed gradients in the frequency of variegation, but concluded that such spots increased leaf temperatures and did not affect herbivory. Variegation, often due to anthocyanin spots, is common among understory plants in both temperate and, particularly, tropical forests. We need more experiments and less speculation.

In the absence of chlorophylls, anthocyanins could also make leaves more apparent to potential herbivores, and warn them away. Such an appearance could warn of some negative consequence, as poor nutrition or toxicity. Coley has argued that the young leaves of many tropical trees delay greening (and the production of nutritious tissue) as a strategy to reduce herbivory during leaf expansion. Such leaves frequently produce anthocyanins and are brilliantly colored during expansion. This is a commonly observed phenomenon in tropical trees. She has speculated that such coloration may be a signal of this low palatability and deter herbivores (Kursar and Coley, 1992; Coley and Barone, 1996). In a large survey of tropical woody taxa, the majority of species with flushing red leaves produced appreciable levels of chlorophyll during development (Lee and Collins, 2001) and two well-known red-flushing species, mango and cacao, produce increasing amounts of chlorophylls during leaf expansion (Lee *et al.*, 1987). Before his untimely death, William Hamilton developed the hypothesis that red autumn coloration protected against egg-laying and future herbivory by aphids through warning. Brown and Hamilton (2001) recently reported correlation between incidence of aphid attacks and anthocyanic coloration in a survey of 262 tree species. However, their conclusions are based on surveys of the literature and not on direct observation and experimentation. Archetti (2000) has constructed a model of coevolution that demonstrates how such a phenomenon could occur.

Thus, the hypotheses of anthocyanin involvement in defence against attack by pathogens and herbivores are interesting, but not very well supported by experimental data.

D. DROUGHT RESISTANCE

Anthocyanins may confer resistance to water stresss by osmotic adjustment of the vacuolar sap (Chalker-Scott, 1999 and this volume). Cell cultures and whole-plant systems subjected to osmotic stressors often accumulate anthocyanins (Do and Cormier, 1991; Murray *et al.*, 1994; Tholakalabavi *et al.*, 1997; Mita *et al.*, 1997). Plants that are drought-tolerant commonly have cyanic leaves (Bahler *et al.*, 1991; Sherwin and Farrant, 1998).

E. LIGHT BACKSCATTERING

Abaxially located anthocyanins might enhance light capture in understory plants by reflecting photons that would otherwise penetrate the leaf back to the chlorenchyma (Lee *et al.*, 1979). The hypothesis is mechanistically difficult to understand, and has been refuted (Gould *et al.*, 1995; Neill and Gould, 1999).

F. PHOTOPROTECTION

By absorbing quanta that would otherwise be intercepted by chloroplasts, anthocyanins may reduce both the requirements for non-photochemical quenching, and the structural damage associated with chronic photoinhibition under high irradiances and low temperatures. Enhanced photosynthetic performance under strong light has been reported for red versus green leaves in several species (Gould *et al.*, 1995; Krol *et al.*, 1995; Smillie and Hetherington, 1999; Hoch *et al.*, 2001; Feild *et al.*, 2001), although no differences were found among red- and green-leafed *Coleus* varieties (Burger and Edwards, 1996). This hypothesis will be discussed in some detail in the chapter by Timmins *et al.*, in this volume.

G. ANTIOXIDANT PROTECTION

Anthocyanins may mitigate oxidative damage in leaves subjected to biotic or abiotic stress (Yamasaki, 1997; Neill *et al.*, 2002). Anthocyanins scavenge most species of reactive oxygen (Bors *et al.*, 1994). They could also reduce photooxidative stress by reducing the light flux incident on chloroplasts (Neill, 2002), and by chelating transition metals in the cell vacuole (van Acker *et al.*, 1996). This hypothesis will be discussed in more detail in the chapter in this volume by Gould *et al.*

IV. AN EVOLUTIONARY PERSPECTIVE

All of the above hypotheses predict advantages for plants as protection against the loss of photosynthetic tissue, thereby enhancing the ability of plants to assimilate carbon over the long run. However, to show that such a mechanism is evolutionarily significant it is necessary to demonstrate that such a mechanism increases the ***fitness*** of the organism, seen as the greater survival and/or reproduction of offspring with such a defensive feature, over a period of time. We have virtually no such evidence of function of anthocyanins in such an evolutionary context.

Given the appearance of anthocyanins, as part of flavonoid metabolism, with the arrival of terrestrial plants, well over 400 million years ago, it seems that there should be one or more such functions. Their current importance in attracting pollinators and dispersers would not have been possible 250 million years before the arrival of the angiosperms. It seems probably that one or more of these explanations (or perhaps others not mentioned) could have functioned in these early plants, and these pigments could have assumed other functions much later. Research providing evidence on a molecular, cellular, or integrated physiological level is an important first step. However, it is useful to speculate on the kind of research that will be necessary to put hypotheses of anthocyanin function in a stronger evolutionary perspective.

Research on the evolutionary significance of anthocyanin function will require the performance of traits of anthocyanin production in plant organs in natural field settings. Mark Rausher and collaborators (Fineblum and Rausher, 1997) have demonstrated the kinds of experiments that can yield evidence of evolutionary significance. Such research requires the understanding of the genetic control of anthocyanin production in plant organs, and then the evaluation of the field performance of such genes. This research has also revealed the importance of considering the interactive or conflicting effects of anthocyanins produced in different organs. Thus, a gene producing an anthocyanic flower may also produce purple leaves. The anthocyanic flowers may be better pollinated (and leave more progeny) while the anthocyanic leaves could also influence rates of herbivory and affect the numbers of progeny. Simms and Bucher (1996) did detect such pleiotropic effects of flower colour intensity on herbivore performance in *Ipomoea purpurea*.

Mutants for anthocyanin production in plant organs can be used in research on function (and fitness) in two ways. Careful observations within natural populations can reveal considerable variation in the production of anthocyanins in vegetative organs. Breeding experiments can determine the patterns of inheritance, and pure lines can be used for the kinds of physiological research described in this volume. Then these plants can be grown in mixed populations in semi-natural settings to determine the production of progeny and their survivorship. Secondly, mutants (particularly those ‘knocking out’ anthocyanin production at specific points in the biosynthetic pathway) can be developed using the techniques of molecular genetics to produce plants for physiological and population experiments.

In certain cases natural selection may have already 'performed' such experiments and the comparative biological approaches may provide useful evidence. For instance, the hypothesis of protective anthocyanin function during leaf senescence (see 'Anthocyanins in Autumn Leaf Senescence' in this volume) stipulates that such protected leaves will have less nitrogen in tissues at leaf fall because relatively more has been resorbed by the parent plant. A comparative study could focus on a taxon of closely related species, with annual and perennial growth habits. We would expect that the annuals would not produce anthocyanins during leaf senescence and their leaf nitrogen levels might be higher, and the perennials should produce these pigments and have lower leaf nitrogen levels at senescence. Similar scenarios can be envisaged for other functions, as herbivore defence or drought tolerance.

Finally, it is interesting to focus on the evolution of the genes responsible for the synthesis of anthocyanins in specific tissues. Patterns and rates of evolution may reveal much about the importance of these different gene products (Rausher *et al.*, 1999). In this long history of research on anthocyanin function in vegetative organs, we are now poised to solve some of the physiological and biochemical challenges and will then need to put this research in an evolutionary context.

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