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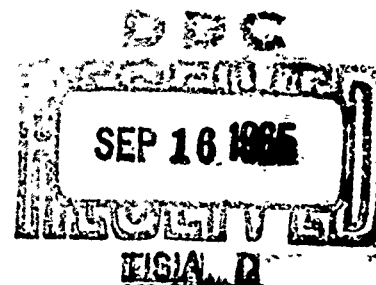
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IMMUNITY OF PIGMENTED PLANTS AND ANTIBIOTICS

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IMMUNITY OF PIGMENTED PLANTS AND ANTIBIOTICS

[Following is the translation of an article by A. F. Kapustinskiy (Leningrad), published in the Russian-language periodical Uspekhi Sovremennoy Biologii (Successes of Modern Biology), Vol XXIX, No 3, 1950, pages 370-378. Translation performed by Sp/7 Charles T. Ostertag Jr.]

The present report is an attempt at a systematization and critical interpretation of the separate disconnected facts which have accumulated in literature concerning the immunity of plants, connected with their anthocyanin pigmentation, supplemented by the author's account of personal observations and investigations, with the simultaneous revelation of the existence of a common biological foundation for the antibiotic effect of antocyanins from higher plants and antibiotics of fungus origin.

Indications of cases of the resistance of plants to diseases which are connected with their pigmentation we find already in the works of Charles Darwin. "On the island of North Mauritius," he writes, "the red sugar cane suffers much less from one disease than the white cane. In recent years the white sugar cane suffered so drastically from disease that many planters were compelled to give up the cultivation of this variety and cultivate only the red cane. The white onion and verbena are subject most of all to mildew. In Spain, grapes with green fruits were harmed more than varieties of other colors. (Even when red and blue grapes had sick plants intermixed among them, they did not suffer at all.)"

Many investigators have pointed out the existence of a correlation between the presence of pigments in plants and their resistance to various diseases. Some time ago Walker (1925) established the fact of the high resistance of pigmented sorts of onion (yellow and in particular red) to Colletotrichum circicans and the high susceptibility to the stated disease of the white (nonpigmented) sorts. Protocatechuic acid is indicated as the substance exerting a toxic effect on the fungi. It is produced in the pigmented, outer dry scales of red and yellow onions which are immune to the fungus.

The author established that the pigment of the scales, which proved to be quercetin (flavanol), as well as the protocatechuic acid obtained from its hydrolysis, are toxic to an unusual degree for the spores of a number of fungi, including those mentioned above.

Rieman (1931) also reports about the same case of immunity of pigmented onions to Colletotrichum. He presents his personal considerations that, in regard to toxicity, the active substance, which it was possible to isolate --

protocatechuic acid -- is colorless, in spite of the fact that its formation is caused by red and yellow pigments. On this basis he suggests that toxins may be produced independent of the development of red and white pigments, that is, white sorts of onions may be obtained which are immune to fungus disease.

The mistakenness of this proposal becomes completely clear in the light of existing opinions on the biochemical nature of the stated pigments.

Based on chemical structure, protocatechuic acid is 3,4-dioxybenzoic acid and is very close to flavone. The formula of flavone has been established by synthesis from benzoic acid and orthocetophenone. Anthocyanin is made up of oxonium bases, derivatives of benzo-pyrylium. Based on their chemical nature, pigments from the group of anthocyanins are usually considered as being close to flavone and flavanol, which often accompany them in plants, giving a light yellow pigmentation, and also soluble in the cellular sap. Under the influence of light the flavanols, which are rich in oxygen, are reduced to anthocyanins. Kostychev considers anthocyanins as combinations of the sugars of glucose and rhamnose with anthocyanidins, which are the carriers of the coloring properties of anthocyanin and are close to flavone derivatives. In living plant tissues the constant transformation of flavone derivatives into anthocyanidins and vice versa is being accomplished.

Thus the opinion concerning the possibility of the formation of toxins without regard to the presence of pigments is little convincing. As regards the supposition concerning the possibility of obtaining white colorless sorts of onion which are immune to disease, then from the point of view of modern views on the nature of pigmentation of organic compounds, a substance is pigmented or colored, in the wide sense of these words, if it absorbs light selectively, that is, it possesses absorption not necessarily in the visible portion of the spectrum, but also in the ultraviolet or infra-red portion (invisible) and reflects all other rays, due to which for our eyes it seems unpigmented or of a white color. In the given case of flavones, standing close to anthocyanins as substances possessing the capability of absorbing ultraviolet rays, in the light of this theory it may not always appear pigmented for our eyes, thus Rieman's conclusions in part, regarding the assumption of the possible existence of white nonpigmented sorts of onion, immune to Colletotrichum, find a certain degree of justification. We find indications of the capability of flavone to absorb ultraviolet rays in the works of Breslavets (1933), which fully agrees with the established capability of plants of an alpine (high mountain) flora for the intensified formation of anthocyanin, which here is given the role of the protective function of absorbing part of the ultraviolet rays and warding off their injurious effects, if it is kept in mind that based on their clinical nature, anthocyanins are derivatives of flavones (see Znamenskiy, 1935).

A number of investigators share the inclination to look for the presence of colorless substances in the nature of pigments; thus, Timiryayev

considers that the source of all pigments are colorless bodies -- chromogens and the enzymes which cause their transformation.

Vilshtetter and his students are also inclined to an analogous opinion. They point out that the capability to endure modifications connected with the change of coloration is inherent for each anthocyanin. In one and the same plant there may exist four modifications of one and the same anthocyanin: The violet, blue and red modifications and a colorless isomer. This colorless modification is an isomeric transformation of pigmented forms of anthocyanins, easily originating in water. Thus the lack of coloration of the contents of cells may be caused by the presence of the colorless isomer of anthocyanin pigments.

The reaction (isomerization) proceeds with the addition of a molecule of water and consists of the destruction of the quinoidine bond (Lyubimenko, 1924), with which the yellow pigmentation of flavones is usually connected.

As will be seen from further accounts, the processes of oxidation of phenols are connected with the phenomena of resistance, based on the chemotropic properties of quinones; in this case the colorless isomeric forms of pigments can hardly be looked at as possessing toxic properties.

Komes also presents data confirming the importance of anthocyanin pigments as factors of resistance. Thus, species of Ribes with pigmented berries are affected less by Cronartium than species with white berries. The sort of carrot with roots with red-violet pulp are less subject to disease than the sort with yellow pulp. A sort of apples with red fruit are more resistant to Fusicladium than sorts with yellow or green fruits. Many authors note the greater resistance to oidium, perenospora and anthracosis of sorts of grapes with black berries in comparison with white berry sorts. Together with these cases of parallelism between the immunity of plants and their anthocyanin pigmentation, there are cases, for the most part among the grasses, when this correlation is not observed. As an illustration it is possible to cite a race of spring barley Hordeum vulgare (var. pallidum) f. jarenskianum, the leaves, stems and spikes of which stand out sharply with anthocyanin pigment, but are strongly affected by all the rusts. Several races of wheat, belonging to the varieties of Tr. vulgare var. cutescens, which by the time of ripening have leaves and stalks with a clearly expressed anthocyanin pigmentation, are strongly affected by brown and linear rusts. However, here it must be kept in mind that the infection might have taken place as the result of a non-coincidence of the moment of the most intensive accumulation of anthocyanin in the plant (that is, the most resistance) with the moment of the most favorable development of the rust, that is, when the plant still has not accumulated the necessary amount of anthocyanin in order to withstand the harm of the rust spores, which is also the cause of the sensitivity of these sorts to rust. Here it will be appropriate to cite the point of view of Naumov (1940), who considers that in the searches for an explanation of the phenomenon of fungus specialization, the decisive significance will not be the composition of substances in the plant, but the peculiarities of their formation and trans-

formation at the moment of infection; for example, rust fungi, in his opinion, react to the course of the process of assimilation at the moment of infection and do not infect a plant if it is not carrying out photosynthesis. Sukhorukov (1936) also points out that the exclusion of photosynthesis through the removal of CO₂ (under experimental conditions) created the temporary immunity of plants to rust.

Such an interpretation of the problem fully agrees with the fact that it is known in the respect of increased formation of anthocyanin, which is accompanied by a reduction of chlorophyll and the related disruption and suppression of the process of assimilation.

1. Resistance, Connected with the Differentiation of Sorts Based on Anthocyanin Pigmentation

Fedotov (1936), in his work on the genetic investigation of anthocyanin pigmentation of peas, points out that the factor of the anthocyanin pigmentation of the pod on a background of other factors displays the capability to increase the resistance of the peas to *Ascochyta* infection, and comes to a conclusion concerning the existence of a parallelism in the differentiation within the various species of plants.

Anthocyanin pigmentation of peas has a systematic importance. On the basis of a differentiation of forms of peas, a histological localization of anthocyanin takes place in the cells found in the leaves of pea buds. Thus, cells filled up with anthocyanin in the leaves of pea buds from Abyssinia belong to the mesophyll of the leaf. In peas from Afghanistan the anthocyanin is localized in cells belong to the epidermis, and in peas from India, the cells containing anthocyanin are found both in the mesophyll and in the epidermis. Naumov (1940) cites the data of Veselovskiy who clears up the dependency between the immunity of various sorts of potatoes to cancer and the histological location of anthocyanin pigmentation within the tubers. These authors point out that out of the sorts of potatoes with pigmented tubers, only one sort -- the Berlikhingen -- is cancer resistant. In it the pigmenting substance -- anthocyanin, is deposited not only in the upper cell layers of the tuber but is also found in its thin peel, while other sorts -- Rannyaya Roza, Voltman and Geroy, in which anthocyanin is not contained in the peel, are not resistant to cancer.

Thus we have direct indication of immunity in a potato, conditioned by the differentiation of sorts based on anthocyanin pigmentation.

One of the examples, supporting the fact of the resistance of pigmented sorts of plants to fungous diseases, is the anthocyanin form of sunflower, which is usually encountered among wild growing groups of sunflowers. Out of the selected sorts of this group the so-called Fuksinka 10.3 and 62 are known, in which anthocyanin appears in the form of a violet coloring on the sprouts, on the stems (at the internodes) and peduncles, veins and along the edges of leaves, or only on the hypocotyl (sort VNIMK, 1646).

In comparison with the others, this sort of sunflower is mildly affected by rust and not affected at all by sclerotinose. Thus, the

above presented facts testify to the resistance of plants, connected with the differentiation of sorts based on anthocyanin pigmentation. Darwin in his works points to the existence of immunity, connected with intra-species differentiation of varieties based on their coloring. In presenting his collected material on the relationship between the coloring of fruits and other organs of plants and their resistance to diseases, he writes, "When all the specimens of any one variety possess immunity, we do not know that it stands in any kind of relationship with their coloring, but when they are distinguished by several similarly pigmented varieties of one and the same species, the varieties of another color are deprived of this advantage and we should believe in the existence of such a relationship."

It was established by the works of Pospelov (1946) that populations of the Turetskiy subspecies of poppy are distinguished by their exceptional resistance in comparison with other sorts (based on the data of two years observations, in them there was no formation of peronospora within the tissues of the oospore); the available data concerning the presence within the limits of these populations of plants with violet flowers made a foundation for proposing that these populations are the producers of anthocyanin. Stemming from these reasons the above indicated populations of the Turetskiy subspecies were taken by us as test objects for experimentation with the aim of studying the nature of their resistance.

For the identification of the pigment which is carried by these populations with the typical pigment belonging to the group of anthocyanins, we conducted a series of qualitative reactions of water extraction from the flowers of the poppy which are representative for the typical anthocyanin. Namely, these tests were: 1) the change of coloration of the aqueous extract in organic acids (dyeing), 2) discoloration of an acid solution of pigment by zinc dust, and also by hydrogen peroxide, 3) obtaining an olive color by the addition of 25% alkali to an aqueous solution of the pigment, 4) precipitation of crystals during hydrolysis with hydrochloric acid, which by their structure correspond to those described for typical anthocyanin. All these qualitative reactions give a foundation to say that in the stated case we are dealing with a pigment which belongs to the anthocyanin group.

For the testing of the antibiotic activity of the pigment yielded by the poppy flowers and identified by us with typical anthocyanin, we took the spores of peronospora (Peronospora arborescens) and conducted a test for germination in an aqueous extract of pigment from poppy flowers on slides (by the method of exposed drops in Petri dishes). No germinated spores were detected, while at the same time both in water as well as in an aqueous extract from the flowers of other sorts (Tyan-Shan) of poppy, the spores germinated normally. Such an antibiotic action of the extract was also observed on spores of brown wheat rust and also spores of Fusarium sp.

2. Biological Factors of Anthocyanin Formation

Taliyev (1930) considers the development of anthocyanin as a diagnostic sign, accompanying various diseased conditions of plants which are

included in the broad group of blotches (limited blotches). These have a central part which is more subject to change under the influence of the pathogenic factor, and a peripheral, less changed, colored part, in which it is most often possible to establish the accumulation of starch and the formation of anthocyanin; as an example of such a type of blotch he cited the blotch causing the crown rust of oats.

Mer (1877), Mirande (1907) and Lippmaa (1927) also point to the formation of anthocyanins in the leaves of plants due to the parasitic activity of fungi.

In exactly the same manner, the harm caused by insects is one of the factors causing the formation of anthocyanins in plants. Aleksandrov and Aleksandrova (1935) detected that pea seeds (*Pisum elatus*) damaged by moth larvae were pigmented. During the investigation of the pigmentation of these seeds, the presence was established of an anthocyanin pigment in the cells of the palisade epidermis.

Trebushchenko (1939), when studying the phenomena of the inheritance of anthocyanin coloring in apple trees, detected that in affected places (on the fruit of the apple tree) pigmented fruit intensified the anthocyanin coloring around the site of injection of San Jose scale. Popova (1938) also points out that San Jose scale forms red spots on the fruits (when the fruit ripens). During this, the contents of the large parenchyma cells of the flesh of the fruit change; acids are substituted by sugar. Tannic substances disappear and pigments from the anthocyanin group appear.

During the phenomena of oat pupation, which is a viral disease transmitted with the help of the dark cicada (*Delphax striatella*), together with leaf mosaic, anthocyanin develops intensely in the leaves (Sukhov, 1940). The above stated facts lead to the conclusion concerning the existence of a parallelism in the reactions of pigment formation, by which the plants answer any attempts of injection into their tissues both on the part of a fungous organism as well as on the part of a pest from the world of insects. In either case, this reaction is directed at the localization of the influence of the pathogenic factor and not permitting the spreading of this influence beyond the sector of tissue which was changed by the disease.

3. Characteristics of the Parallelism in the Biochemical Properties of Anthocyanins (Flavones) from Higher Plants and Antibiotics of a Fungous Origin

Prints made an attempt to connect the resistance of the grape vine to phylloxera with the content of flavones (quercetin) in the leaves of this plant, attributing the formation of a red ring around the site of puncture of phylloxera to the precipitation here of the product of flavone oxidation.

Blagoveshchenskiy (1945), while studying the biochemical nature of the interrelation between the enzymes of the cellular sap of grape leaves and the enzymes given off by phylloxera, found that the formation of red spots around the site of injection occurred as the result of the penetration of atmospheric oxygen into the cells which were harmed by the injection and the oxidation of flavones into quinones. It is interesting to note that resistance to parasitic fungi in sorts of grapes is often connected with resistance to phylloxera. Thus, the American grape vines which are most resistant to the majority of fungal parasites are at the same time resistant to phylloxera. The oxidation of flavones into quinones is a very conclusive fact, speaking in favor of the structural nearness of flavones and phenols. It is known that quinones, just as the other phenols, are derivatives of benzenes and are obtained as a result of the oxidation of hydroquinones (para-dihydroxybenzenes). The yellow pigmentation of flavones is usually connected with their quinoid structure.

The greatest toxicity is possessed by phenols with para and ortho positions of the hydroxyls (hydroquinone and pyrocatechol). One of the reasons for the increase in toxicity of phenol derivatives during an increase of pH is perceived to be the fact that under these conditions the process of phenol oxidation is speeded up, as a result of which many more toxic products of the quinone type are formed (Rubin and Artsikhovskaya, 1948).

As is known, an important group of newly discovered antibiotics is characterized by quinone structure and contains a benzene nucleus (for example, fumigatin, citrinin); these pharmacological properties approach them with flavones.

Anderson (1932), while investigating the yellow pigment of the Khapli sort of wheat (Tr. dicoccum) as a form which is distinguished from other sorts by a greater content of phenols and a greater resistance to rusts, found the flavone -- tritsin in the leaves of this wheat. Kargopolova's investigations (1935) established also a correlative bond between the presence of phenol compounds and resistance to rusts in sorts of Tr. Timopheevi, which as is known occupies an intermediate position between Tr. dicoccum and Tr. spontaneum. The sulfur-ether fraction gave a very sharply expressed reaction for protocatechuic acid in these sorts of wheat.

Analogous indications are cited in the work by Ewans (1943), who established the dependency between the resistance of the sugar cane to red mold and the amount of aminophenols of the tyrosine series, formed in the infected tissue of the plant-host. Recently in the laboratory of Prof. Gauze (Malaria Institute of the USSR Academy of Medical Sciences) a strain of actinomyces -- blue proactinomycete -- was obtained from a sample of soil. It yields a special pigment, the so-called litmocidin, which produces a red coloration in an acid medium and blue in alkaline. It turned out that this pigment possessed bactericidal properties. A study of it showed that it is very similar to the vegetable pigment due to which the rose has a red

color and the cornflower - blue. In accordance with the viewpoint of modern biochemistry, in spite of the great diversity in the coloration of flowers and the fruits of plants, the color of which is determined by anthocyanidins included in the composition of anthocyanins, the number of anthocyanidins is very insignificant. The coloring of the rose red and cornflower blue is caused by the same cyanide. Depending on the reaction of the medium, anthocyanidin reacts both as a substance of a base nature (oxonium salt) and as a weakly acidic substance (metal derivatives); of course all varieties of shades are not caused by only one reaction of the medium. A great role is also played by the composition and method of binding carbohydrates in the anthocyanins. For the group of anthocyanidins, there is a series of qualitative chemical reactions. It turned out that lithomycin produces all these reactions (that is, it behaves similar to anthocyanidins), and on the basis of this it can be suggested that the pigment group of lithomycin is constructed according to the type of anthocyanidins; however, it is not connected with carbohydrate.

Kriss (1936) established the similarity of the blue pigment given off by Actinomyces violaceus with typical anthocyanins.

According to the data of Krasilnikov (1939), Actinomyces violaceus possesses especially expressed antagonistic properties in respect to many microorganisms, including pathogenic staphylococci. The active substance isolated from Act. violaceus was named mycetin.

The introduction of the methyl group OCH_3 into the nucleus of quinone, as a study of antibiotics showed (Vaksman, 1948), leads to a significant increase in antibacterial activity. From this aspect, it is necessary to note that among the pigments of the anthocyanin group there is a series of methyl derivatives containing the OCH_3 group: Peonidin (cyanidin), petunidin, malvidin, chirazutidin (delphinidin).

Oxydases, which are formed by many fungi and actively operate in the presence of glucose or other carbohydrates, also belong to the number of antibiotics (for example, notatin); on the other hand it is known that anthocyanins are compounds, having a pigment group combined with some hydrocarbon, most often with glucose, to which they easily detach. The formation of antibiotic substances is increased with the presence of vitamins in the nutrient medium (Vaksman, 1948). Such an action of vitamins is also observed in the formation of anthocyanin; thus, as an example, in young pea sprouts there is noted a stimulating action of nicotinic acid (vitamin PP) on the formation of anthocyanin in them.

In the base of a molecule of patulin (identical to clavacin, claviformin, expansin) lies tetra- β -pyrone; flavones are also considered as phenol derivatives of 1--4 pyrone.

Tokin (1928), who discovered antibiotic substances in the onion (phytoncide), notes that red sorts of onion possess stronger bactericidal

properties than sorts of a yellow color. This speaks in favor of the greater biological activity of pigmented sorts of onion.

The above presented facts led us to the conclusion concerning the existence of a parallelism in the biochemical properties of anthocyanins (flavones) from higher plants and antibiotics of a fungous origin, and to the notion of the necessity of directing the search for antibiotics among sorts of poppy, as crops having anthocyanin varieties which possess the properties of immunity.

The antibiotic activity of the pigment from flowers of resistant sorts of poppy which we detected, compelled us to assume the presence in them of antibiotically active agents in respect to microorganism which are pathogenic for man. With this aim, the sap from the flowers of these same sorts of poppy was tested in respect to certain species of pathogenic microbes. As the test cultures we took Micrococcus luteus and Mycobact. citreum. Positive results were obtained for the bacteriostatic action of the sap for Mycobact. citreum with a width of the action zone of the preparation (inhibiting the growth of bacteria) in 10 mm and a complete bactericidal effect in regard to Microc. luteus with a width of the action zone (stopping the growth of the microbe) in 10 mm with a simultaneous bacteriostatic action in 15 mm.

The resulting facts of the antibiotic action of the sap from flowers of the poppy we are inclined to evaluate as evidence of a general biological basis for the action of sap from plants -- carriers of anthocyanin, both in respect to fungous organisms as well as to microbes which are pathogenic for man, or, in other words, supporting the bond between the immunity of plant-anthocyanin carriers and their antibiotic activity in respect to pathogenic microbes.

The above presented data from our search for antibiotics from higher plants permits the conclusions to be made concerning the necessity of introducing a rational foundation in the empirical searches for antibiotics among the higher plants, directing our search for antibiotics among plants which are anthocyanin carriers, isolating them not from accidentally selected objects, but in connection with their immunity to fungous diseases (stemming from the theoretical basis concerning the presence of a general biological foundation for the antibiotic action of the sap from plants -- carriers of anthocyanin -- to fungous parasites and to microorganisms which are pathogenic for man), and looking at this bond between the immunity of pigmented plants and antibiotics as a particular question of the overall problem of the connection of plant immunity with their antibiotic activity. Recently the discovery was made of the antibiotic tomatine which is an example of such a bond. The source of obtaining tomatine, as is known, are the leaves of the most resistant forms of tomatoes, which possess the capability to produce tomatine. Susceptible forms of tomatoes do not produce a sufficient amount of this antibiotic, which is capable of stopping the development of the causative agent Fusarium oxysporum, and also other pathogenic fungi and bacteria.

The practical results achieved with the working hypothesis assumed by us, making it possible to systematize the phenomenon of the resistance of pigmented plants, connected with their antibiotic activity, may serve as the basis for the trend of investigations put forward by us. It would be little promising, for example, to take as the object of testing for antibiotic activity the berries of the red raspberry -- carrier of the least active chemical modification of anthocyanin -- pelargonidin, which do not have methyl derivatives which contain the OCH_3 group, with which the antibacterial activity of an antibiotic is usually connected.

Encouraging results should not have been expected from this object also based on the reason of a lack of data concerning the resistance of the red raspberry, connected with the presence in it of anthocyanin.

On the other hand, among the medicinal plants, possessing pharmacological properties, there are a number of plants -- carriers of the most toxic isomers of anthocyanin, such as, for example, valerian, containing cyanidin (in the flowers), foxglove (luteolin); among the various sorts of these cultures there are also sorts which are immune to fungous diseases and consequently promising in respect to antibiotic activity. Disease resistant forms of perilla, among which there are also anthocyanophores, are of such promise.

Conclusions

1. The trends put forth by us in the investigations of antibiotics do not pretend to be a universal approach to the detection of the latter and concern only those of them whose actively working substance is caused by the presence of compounds of a quinoid structure, having in the capacity of the initial product a pigment group of the anthocyanin (flavone) type.

2. Directed searches for antibiotic substances make it possible to avoid blind experiments in the affair of studying the given problem, having speeded up the survey for antibiotic activity of the most promising representatives of different systematic groups of plants -- carriers of the most active modifications of anthocyanin pigments, in connection with immunity to fungous diseases.

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Note: The original journal was tightly bound and on the last page of the xerox copy some of the reference works could not be made out completely. These are items q. to dd. above.