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THE EFFECT OF VIRUS INFECTION ON PHENOLIC COMPOUNDS IN FLOWERS OF *MATTHIOLA INCANA* R. BR.

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Abstract—Quantitative analysis of *p*-coumaric, caffeic, ferulic and sinapic acids, kaempferol and anthocyanin pigment has been made in the flowers of healthy and virus infected plants of *Matthiola incana* R. Br. Virus infection results in the formation of white flower parts in plants whose flowers are normally colored pink or red by acylated perlargonidin glycosides; in pink types there may also be an intensification of the color. The virus-infected white parts bear a great resemblance to normal white flowers with respect to their phenolic constituents: both contain kaempferol, sinapic acid and a small amount of ferulic acid. It is assumed therefore that the action of the virus consists mainly in a blocking of the anthocyanin synthesis.

In healthy red flowers larger amounts of cinnamic acids are present, bound to the acylated anthocyanins and other compounds, and possibly also as free acids. When anthocyanin synthesis is blocked, the formation of cinnamic acids is also inhibited, except for small amounts of sinapic and ferulic acids. The amounts of kaempferol present in the genotypes examined are roughly the same in all healthy types but almost doubled in the white flowers of virus-infected cyanic plants. It appears, however, that there is no direct relationship between the effects of the virus on flavonol and on anthocyanin synthesis.

INTRODUCTION

NORMAL flowers of the cruciferous plant *Matthiola incana* R. Br. have uniformly coloured petals, and among the garden varieties of this species there is a large genetic colour variation from white to various shades of red and purple.[1] Purple flowers contain cyanidin glycosides, red flowers pelargonidin glycosides.[2] It has been shown for purple flowers that the anthocyanins may be acylated with *p*-coumaric, caffeic, ferulic, or sinapic acids[3]; the anthocyanins from red types are likewise complex.[2]

As the result of a virus infection, petals of red- and of purple-flowered types may show white spots of various sizes, from small dots or streaks to whole sectors; in extreme cases the whole petal can be white. In strongly infected plants, whole flowers spikes may be almost entirely white.[4]

In pink and lilac types, however, virus infection can cause either reduction or intensification of pigmentation. It is not known whether both effects are due to the same virus, since these observations were on naturally infected material, and in experimental studies in these laboratories a crude plant extract was used for making inoculations. However, the same decolourization and intensification in pink types has been noticed by one of the authors (W. J. F.) in lines of *Matthiola incana* of completely different origin grown in the Netherlands.

It seemed probable that, besides affecting anthocyanin pigmentation, the virus might also affect other phenolic compounds in the petals. A comparison, by means of paper chromatography, of genotypically white, and healthy and infected red and pink types,

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[1] H. KAPPERT, *Zuchter* **19**, 289–297 (1949).
[2] E. JUNGFER, *Zuchter* **27**, 140–145 (1957).
[3] W. SEYFFERT, *Z. Pflanzenzucht.* **44**, 4–29 (1960).
[4] B. L. JOHNSON and D. BARNHART, *Proc. Am. Soc. Hort. Sci.*, **67**, 522–533 (1956).

indicated quantitative differences with regard to brown, yellow, and blue ultra-violet fluorescing substances. Hydrolysates of excised brown and yellow spots from two dimensional chromatograms on Whatman 3 MM paper contained a flavonol, which, on chromatographic and spectrophotometric data, proved to be kaempferol. In the hydrolysates of blue-fluorescing spots the cinnamic acids, *p*-coumaric, ferulic, and sinapic acid were found.

The anthocyanins in the red and pink types yielded pelargonidin upon acid hydrolysis, confirming Jungfer's observations.[2] Spectra of anthocyanin fractions from red flowers, according to Harborne's criteria,[5] and the decrease in Rf-values as a result of short alkaline hydrolysis of the pigments, showed the anthocyanins in this flower type to be acylated; also in accordance with Jungfer's results.[2]

Cinnamic acids or closely related C_9 compounds play a role in the biosynthesis of flavonoids,[6-8] and thus one might expect them to increase in concentration when anthocyanin formation is blocked. Moreover, it seemed of interest to know whether flower types devoid of anthocyanin pigmentation contain the same cinnamic acids, either free or bound as glycosides or esters,[9] which in pigmented types are bound in the anthocyanins. It was decided therefore to carry out quantitative analyses of *p*-coumaric, caffeic, ferulic, and sinapic acids in the flower types shown in Table 1.

TABLE 1. DESCRIPTION OF THE FLOWER TYPES STUDIED

Number	Description
1	White; resistant
2	White; susceptible
3	Pink; resistant
4h	Pink; susceptible; healthy
4^s/w	Pink; susceptible; infected, white flower parts
4^s/r	Pink; susceptible; infected, red flower parts
5	Red; resistant
6h	Red; susceptible; healthy
6s	Red; susceptible; infected, white flower parts
7h	Red; susceptible; healthy
7s	Red; susceptible; infected, white flower parts

The cinnamic acids bound to the anthocyanins were separated from those occurring free or bound in other forms by extraction of the aqueous solution of the petal pigments with ethyl acetate. This treatment removes most of the cinnamic acids not bound in the anthocyanins from the aqueous solution.[3] The amount of kaempferol in the various flower types was also determined.

A parallel study of normal and virus-infected purple and lilac varieties led to conclusions that did not differ materially from those that are presented here, and are not reported in detail.

RESULTS AND DISCUSSION

As is shown in Table 2, the total amounts of cinnamic acids in the white petals of virus-infected genotypically red types (6s and 7s), are considerably lower than those in the comparable healthy types (6h and 7h). Both in the healthy susceptible and in the resistant

[5] J. B. HARBORNE, *Biochem. J.* **70**, 22–28 (1958).
[6] T. A. GEISSMAN and T. SWAIN, *Chem. & Ind. London* 984 (1957).
[7] H. GRISEBACH, *Z. Naturforsch.* **13b**, 335–336 (1958).
[8] E. W. UNDERHILL, J. E. WATKIN and A. C. NEISH, *Can. J. Biochem. Physiol.* **35**, 229–237 (1957).
[9] J. BIRKOFER, C. KAISER, W. NOUVERTRÉ and U. THOMAS, *Z. Naturforsch.* **16b**, 249 (1961).
J. B. HARBORNE (unpublished results).

red types (5) the major part of the cinnamic acids is bound in the anthocyanin fraction. Apparently these acids are not present as "free" compounds in virus infected types where anthocyanins are absent. This suggests that the synthesis of the anthocyanins proper and the synthesis of the cinnamic acids are positively correlated. Whether the acids are synthesized to acylate the anthocyanins, or that vigorous anthocyanin synthesis is only possible when there is a sufficient amount of cinnamic acid available to acylate them, cannot be deduced from the available data.

TABLE 2. THE CONTENT* OF ANTHOCYANIN, KAEMPFEROL AND CINNAMIC ACIDS IN THE PETALS OF HEALTHY AND VIRUS-INFECTED FLOWERS OF *Matthiola incana*

Plant number	Flower type	Antho-cyanin‡	Flavonol	Cinnamic acids†								Total
				Bound to anthocyanin				Extractable by ethyl acetate				
				Co	Ca	Fe	Si	Co	Ca	Fe	Si	
1	White-resistant	—	0·9	—	—	—	—	—	—	—	0·5	0·5
2	White-susceptible	—	1·2	—	—	—	0·2	—	—	0·2	1·2	1·6
3	Pink-resistant	0·19	1·3	0·2	—	0·5	1·0	—	—	0·2	1·0	2·9
4h	Pink-susceptible-*healthy*	0·09	1·3	—	—	0·2	0·4	—	—	0·2	0·2	1·0
4^s/w	Pink-susceptible-inf. white	0·02	2·3	—	—	—	0·2	—	—	0·4	1·6	2·2
4^s/r	Pink-susceptible-inf. red	0·51	2·0	0·5	0·5	1·3	1·5	0·5	—	1·0	0·9	6·2
5	Red-resistant	1·3	1·4	1·1	0·7	2·5	2·7	0·9	—	1·1	0·7	9·7
6h	Red-susceptible-*healthy*	3·6	1·3	6·7	4·3	4·8	3·8	3·3	—	1·3	0·8	25·0
6s	Red-susceptible-inf. white	0·12	3·0	—	—	0·2	0·5	—	—	0·6	1·3	2·6
7h	Red-susceptible-*healthy*	1·4	1·2	1·4	0·9	2·9	2·6	1·0	—	1·0	0·5	10·3
7s	Red-susceptible-inf. white	0·03	2·2	—	—	—	0·4	—	—	0·3	1·9	2·6

* Each figure is the average of replicate analyses on separate samples.
† Co = *p*-coumaric; Ca = caffeic; Fe = ferulic; Si = sinapic.
‡ Anthocyanins are expressed as optical densities at 515 mμ of 1 g petals/100 ml in 0·2% HCl; flavonol and cinnamic acids are 10^{-3} mM/1 g petals.

Sinapic acid is the only cinnamic acid occurring in the "free" form in the genotypically red types in higher amounts in the virus-infected white flowers than in the healthy coloured ones. This compound and ferulic acid are the only acids occurring in genotypically white flowers (1 and 2); the amount of sinapic acid here is of the same order as the amount in the flowers which are white due to virus infection.* The amounts of ferulic acid in normal white and virus-infected white flowers are of the same order. Furthermore, the total amounts of cinnamic acids in all the virus-infected white flowers are approximately equal, whereas the amounts of anthocyanin and cinnamic acids in healthy plants vary considerably. These results suggest that virus infection simulates the substitution of a recessive gene, which does not permit anthocyanin formation,[1] for its dominant allele which gives synthesis of the pigment; in other words, the presence of the recessive allele and virus infection both lead to blocking of the anthocyanin synthesis.

Blocking of the anthocyanin synthesis in all cases leads to a small increase of the amount of "free" methylated cinnamic acid, mainly sinapic acid. Due to the fact that the

* Small amounts of ferulic and sinapic acids were found in the fraction not extractable in ethyl acetate of genotypically white (2) and virus-infected white flowers (4^s/w, 6s and 7s). In the case of the infected flowers the major part of these acids will have originated from the small amount of anthocyanin still present in the coloured areas of the petals of these types. Incomplete extraction of the bound forms[9] from the aqueous extract by ethyl acetate may be another reason; the latter effect is probably responsible for the occurrence of acid in the "anthocyanin" fraction of the genotypically white-susceptible types (2).

healthy pink flowers contain only a small amount of cinnamic acid, blocking of the pigment synthesis here leads to an increase of the total amount of cinnamic acid present.

Besides decolourization of pink flowers as a result of infection, an intensification of pigmentation has been observed (Table 1, No. $4^s/r$). The analysis shows (Table 2) that parallel with the increased anthocyanin is an increase in cinnamic acids, both attached to the anthocyanins and occurring in the "free" form. The pink infected-red type is thus very similar to the healthy red types. As has already been mentioned, it cannot be deduced at present, whether both blocking and intensification of the anthocyanin synthesis are caused by the same virus, and there is, therefore, little use in trying to explain these phenomena.

All virus-infected types show an increased flavonol content, whereas the healthy types, regardless of pigmentation, show little variation in the amount of flavonol (Table 2). The increase of flavonol content is greatest in the genotype showing the largest amount of anthocyanin in the healthy flowers (6s and 6h, Table 2). This increase, however, cannot be explained as the result of a shift in the equilibrium between anthocyanin and flavonol formation for two reasons. First, there is no indication of any constant ratio of increase of flavonol to decrease of anthocyanin among the various genotypes (Table 3); second, in the type pink infected-red, both anthocyanin and flavonol show a rise, when compared to pink healthy (Nos. $4^s/r$ and 4h, Table 2).

TABLE 3. RATIOS OF INCREASE OF FLAVONOL TO DECREASE OF ANTHOCYANIN CAUSED BY VIRUS INFECTION

Types compared	Decrease of anthocyanin (units optical density)	Increase of flavonol (10^{-3} mM)	Increase of flavonol per unit decrease of anthocyanin
Pink 4h–$4^s/w$	0·07	1·0	14·30
Red 6h–6s	3·48	1·7	0·48
Red 7h–7s	1·37	1·0	0·73

It thus seems likely that the effects of the virus on the synthesis of flavonol on the one hand, and on the formation of anthocyanin and cinnamic acids on the other, are more or less independent. For further studies of these phenomena in *Matthiola incana* flowers, a purification of the virus(es) is necessary, in order to establish whether one, or more type, is involved.

EXPERIMENTAL

Material and methods

Pure lines of garden varieties of *Matthiola incana* were grown in the experimental garden of the Department of Floriculture of the University of California at Los Angeles. A number of plants in each susceptible line were inoculated with the colour-breaking virus in order to obtain material clearly showing the symptoms of infection in the flowers. The types investigated are shown in Table 1.

Extraction

Fresh petals, separated from the green portion at the base, were collected from a number of plants in a pure line. Slightly over 4 g of petals were mixed and two samples of about

1 g taken for analysis. The weighed petals were crushed in a mortar, quantitatively transferred to a 20-mm sintered—glass funnel and repeatedly extracted with small amounts of 0·2% ethanolic HCl. The total volume of extraction solvent used was 35 ml; the last portions remained colourless after the extraction. The petal debris of red types was still slightly coloured after this treatment.

The ethanol was partly removed in a rotary vacuum evaporator at 35–40°, 10 ml water was added and the distillation continued until the solution was completely aqueous. The final solution was made up to 100 ml with 0·2% aqueous HCl.

Determination of the relative anthocyanin concentration

The optical density of the extract was measured at 515 mμ in a Beckman DU spectrophotometer, using 1 cm. cuvettes. Extracts of red types were diluted when necessary in order to obtain convenient readings on the spectrophotometer.

Determination of the relative flavonol concentration

Two millilitres of the extract were pipetted into a 10×125 mm test tube; concentrated HCl was added so as to make the final acid concentration about 2%. The tubes were heated in a boiling water bath for 30 min, cooled, and the contents evaporated to dryness in a vacuum desiccator over KOH. The residue was taken up in a few drops of water and spotted on a paper chromatogram, along with spots containing 10 and 40 μl of an ethanolic solution of kaempferol (2×10^{-3} M). The chromatogram was run in 2% aqueous acetic acid. The above hydrolysis was at least sufficient to remove any sugar present at the 3-position of the flavonol; flavonols and flavonol glycosides with a free OH at C-3 have Rf ~0 in 2% acetic acid,[10] whereas other compounds which could interfere in the photometric determination of the flavonols are mobile. Only a trace of anthocyanin stayed at the starting line.

Rectangular pieces of filter paper of equal size containing the flavonol spots were cut from the chromatogram, placed in a test tube and immersed in 70% aqueous ethanol (3·5 ml). A blank piece of filter paper of the same size was cut from the chromatogram from a position alongside the flavonol spots and treated in the same way for use as a blank. After 48 hr the optical density of the extracts was determined at 370 and 515 mμ in a 1 cm quartz cuvette. The flavonol had λ_{max} 370 mμ, but as anthocyanins also absorb at this wavelength the optical density due to any such compounds (if present) had to be subtracted. The magnitude of this correction was calculated from the optical density of the extract at 515 mμ, the relation between the optical densities of the anthocyanins at 370 and 515 mμ being determined in a solution of the anthocyanin containing no flavonol. The amounts of kaempferol present were calculated from the optical densities of known standards. A possible error in the calculation is that not all the yellow pigment was present as the aglycone, but glycosidation other than in the 3-position does not appreciably affect the intensity of the absorption.[11]

Determination of the amounts of p-*coumaric, caffeic, ferulic and sinapic acids*

Ten milliliters of the aqueous petal extract were continuously extracted with ethyl acetate for 5 hr. To prevent heating of the aqueous solution during extraction, the apparatus

[10] E. A. H. Roberts, R. A. Cartwright and D. J. Wood, *J. Sci. Food Agr.* **7**, 637–646 (1956).
[11] B. Skarzynski, *Biochem Z.* **301**, 150–169 (1939).

was equipped with a cooling jacket. After removal of the ethyl acetate (B), nitrogen was passed through the residual solution (A) for 2 min, after which 5 ml of 6 N NaOH was added and nitrogen was bubbled through for another minute. The closed apparatus was left for 3 hr at room temperature; then 7 ml ice-cold 5N aqueous HCl was added in four portions, with cooling, and mixed with the pigment solution by bubbling nitrogen through the solution. Care was taken to prevent heating of the solution as the cinnamic acids, especially *p*-coumaric acid, tend to decompose when heated under acid conditions.[12]

The resulting acid solution was continuously extracted with ether for 5 hr. The ethereal layer was removed and the solvent was evaporated under vacuum at room temperature. The residue was taken up in a few drops of 95% ethanol and quantitatively transferred to a 1 ml volumetric flask by means of a small pipette and the volume adjusted to 1 ml. Two samples of 200 μl of this ethanolic solution were applied by means of a micro pipette as spots to paper chromatograms, along with spots containing 10 and 40 μl of ethanolic solutions, containing *p*-coumaric, caffeic, ferulic and sinapic acids, each in the concentration 2×10^{-3} M.* The chromatograms were equilibrated overnight with the aqueous phase of the solvent toluene–acetic acid–water (5 : 3 : 5 by vol.) and then developed (descending) with the organic phase for about 4 hr. The use of a toluene–acetic acid–water mixture (4 : 1 : 5 by vol.) for the separation of cinnamic acids was originally suggested by Bate-Smith,[13] but the above composition gave a somewhat better spreading of the R_f values.

Ferulic, sinapic and *p*-coumaric acids moved in this solvent with R_f values of 0·41, 0·28 and 0·12 respectively; caffeic acid remained at the starting line.[13] The spots of the former three acids were marked under U.V. light, cut out, and extracted from the paper as described previously for the flavonols. Aqueous ethanol (70%), acidified with hydrochloric acid (1 ml concentrated acid per 200 ml solvent) was used in the extraction. The optical densities of extracts containing *p*-coumaric acid were read at 312 mμ, of those containing ferulic or sinapic acids at 325 mμ, the λ_{max} of the respective pure acids. The amounts of acid present were calculated by comparing their optical densities with those given by the standards).†

Caffeic acid could not be measured in the same way as the other acids due to the presence of other compounds, especially flavonol, which also remained at the starting line and interfered with the determination of the optical density of caffeic acid. The latter therefore was eluted from the paper with 1% aqueous acetic acid; the flavonol stayed in the paper as it did not move in this solvent. The eluate from this chromatogram was directly applied to a second chromatogram by serrating the end and placing the pointed ends of the strip on the new starting line. To promote evaporation of the solvent the second chromatogram was placed with its starting line over a slit which had been cut in a piece of wide rubber tubing through which air with a temperature of about 35° was blown. The second chromatogram was run in a mixture of butanolacetic acid–water (6 : 1 : 2 by vol). The caffeic acid spots were marked and cut out in the usual way, and the amount of acid determined as

* Caffeic acid of sufficient purity was available; *p*-coumaric, ferulic and sinapic acids were synthesized by usual methods from the corresponding substituted benzaldehydes and malonic acid.

† This method proved to be simpler and more accurate than one in which strip chromatograms were made, the resulting bands cut out and the compounds eluted from the paper and subsequently determined spectrophotometrically.

An attempt was also made to carry out the analyses by measuring spot areas, but the results of experiments with known amounts of acids indicated that this method gave completely unreliable results.

[12] T. A. GEISSMAN, *Arch. Biochem. Biophys.* **60**, 1 (1956).
[13] E. C. BATE-SMITH, *Chem. & Ind. London* 1457–1458 (1954).

described previously for the other cinnamic acids. The optical density was read at 328 mμ.

The ethyl acetate solution (B) was taken to dryness in a rotary vacuum evaporator at room temperature, and the residue was treated under nitrogen with 12 ml 0·5 N NaOH. After hydrolysis at room temperature for 3 hr the solution was cooled, acidified with ice-cold 5 N HCl, and continuously extracted with ether for 5 hr. The cinnamic acids in the etheral extract were quantitatively determined as described above.

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