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FLAVONOID COMPLEXES IN *PISUM SATIVUM*—III.

THE EFFECT OF LIGHT ON THE SYNTHESIS OF KAEMPFEROL AND QUERCETIN COMPLEXES

W. BOTTOMLEY,* HARRY SMITH† and A. W. GALSTON

Department of Biology, Yale University, New Haven, Conn.

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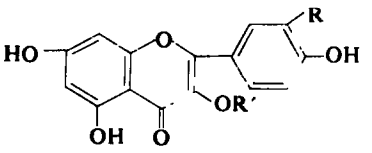
Abstract—A method has been developed for the separation and estimation of kaempferol-3-*p*-coumaroyl-triglucoside (KGC) and quercetin-3-*p*-coumaroyltriglucoside (QGC) from *Pisum sativum*. A study of these two flavonoids in dark grown and in light treated pea plants has shown that the KGC concentration is unaffected by low intensity red light or by high intensity white light. On the other hand the synthesis of QGC is triggered by red light and thus appears to be under the control of the phytochrome system which might therefore control 3′-hydroxylation. In young green plants the highest concentration of the flavonoids is in the fully expanded leaves.

INTRODUCTION

THE major flavonoid components of the terminal buds of dark grown peas (*Pisum sativum* cv. Alaska) have been shown to be kaempferol-3-*p*-coumaroyltriglucoside (KGC. I: R = H, R′ = *p*-coumaroyl-glucosyl-glucosyl-glucose) and kaempferol-3- triglucoside (KG. I: R = H;



(I)

R′ = glucosyl-glucosyl-glucose).[1] In pea plants grown in high intensity light, in addition to KGC and KG, two quercetin derivatives have been found[1] and tentatively identified as quercetin-3-*p*-coumaroyltriglucoside (QGC. I: R = OH; R′ = *p*-coumaroyl-glucosyl-glucosyl-glucose) and quercetin-3-triglucoside (QG. I: R = OH; R′ = glucosyl-glucosyl-glucose).

Furuya and Thomas[2] have reported that KGC synthesis in dark grown peas is enhanced following a brief exposure to weak red light and that this stimulation is reversed by far-red. We set out to investigate the synthesis of QGC under the influence of high intensity light. The discovery that KGC concentration was virtually unaffected by high intensity light led us to re-examine the variation in KGC and QGC under the influence of low-intensity red light. We then found that KGC synthesis is insensitive to red light whereas the synthesis of QGC is under red-far red control.

Present addresses: * Division of Plant Industry, C.S.I.R.O., Canberra, A.C.T., Australia; † Dept. of Botany, Queen Mary College, London University, London, E.1.

[1] M. FURUYA, Ph.D. Thesis, Yale University (1962).
[2] M. FURUYA and R. G. THOMAS, *Plant Physiol.* **39**, 634 (1964).

RESULTS

Estimation of KGC and QGC

The separation of kaempferol and quercetin derivatives was accomplished by extraction from the plant with hot *n*-butanol and chromatography on a silica gel column. The isolated KGC and QGC were then separately hydrolysed with alkali and the liberated *p*-coumaric acid estimated spectrophotometrically. When known amounts of KGC and QGC were added either separately or together, to the plant material before extraction, between 90 and 98 per cent of the added flavonoids were recovered.

The identity of the KGC and QGC was checked during each assay by examining the shape of the u.v. absorption spectrum curve of each fraction before hydrolysis. The QGC spectrum has a pronounced shoulder at 256 mμ which is absent from KGC thus enabling the two to be readily distinguished. The *p*-coumaric acid obtained by hydrolysis of KGC and QGC was identified by comparison with the u.v. spectra of authentic *p*-coumaric acid in both neutral and alkaline solutions.

QGC in Dark Grown Peas

It was found that the terminal buds of pea plants grown in absolute darkness contained a small amount of QGC as well as larger amounts of KGC. For example, in 7-day old etiolated plants, there was usually about 0·1 μM QGC/g fresh tissue and about 0·5–0·8 μM KGC/g. Although the plumular hook and internodes contained traces of KGC, no QGC could be detected in any part of the etiolated plant other than the terminal buds.

Response of the Terminal Bud of Dark Grown Plants to Low-intensity Red Light and High-intensity White Light

Six-day-old, dark grown peas were exposed to red light of 546 ergs/cm^2/sec for 10 min (= 327 kergs/cm^2) and then returned to the dark. Samples of terminal buds were harvested and assayed at various times during the subsequent 24 hr. It was found (Fig. 1) that the KGC

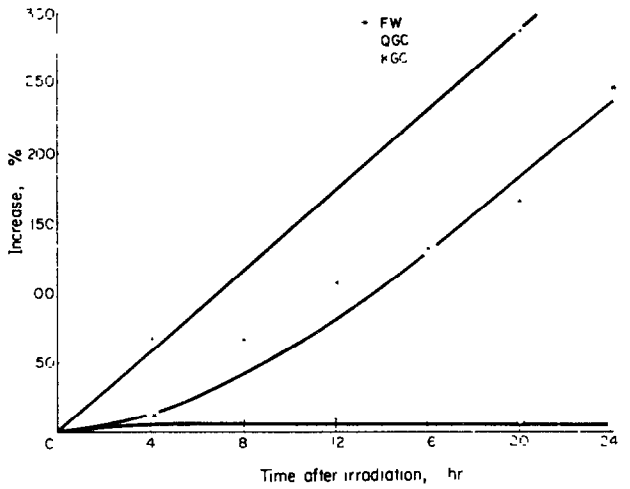


FIG. 1. VARIATIONS IN KGC AND QGC CONTENT AND GROWTH OF DARK GROWN PEA SEEDLINGS FOLLOWING IRRADIATION WITH RED LIGHT OF 327 kergs/cm^2.

Flavonoid contents and growth are expressed as a percentage of dark control. Fresh weight (FW) was measured as the average weight of the terminal buds. KGC and QGC contents were measured as μmoles/g fresh weight.

level was virtually unaffected by this treatment whereas the QGC content increased markedly, and in fact, paralleled the increase in growth as measured by fresh weight of the excised buds.

When, in place of a short irradiation with weak red light, the plants were placed in continuous relatively high intensity (50 lux) white light for 24 hours, then essentially the same results were obtained (Fig. 2) despite the fact that visible greening had taken place after 8 hours.

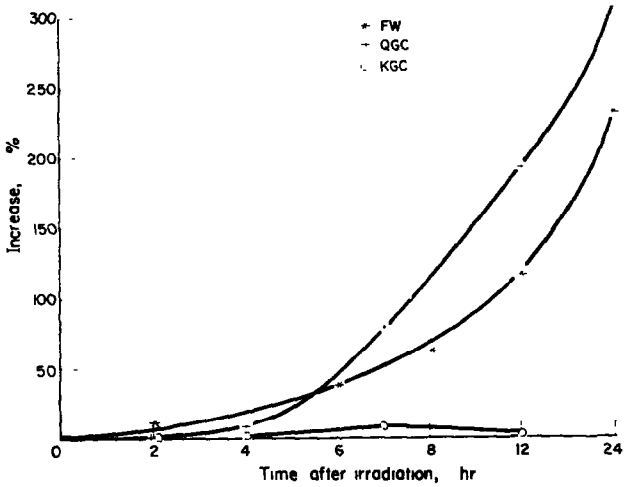


FIG. 2. VARIATIONS IN KGC AND QGC CONTENT AND GROWTH OF DARK GROWN PEAS AFTER TRANSFER TO CONTINUOUS WHITE LIGHT OF 50 lux.

Flavonoid content and growth are expressed as a percentage of dark control. Fresh weight (FW) was measured as the average weight of the terminal buds. KGC and QGC contents were measured as μmoles/g fresh weight.

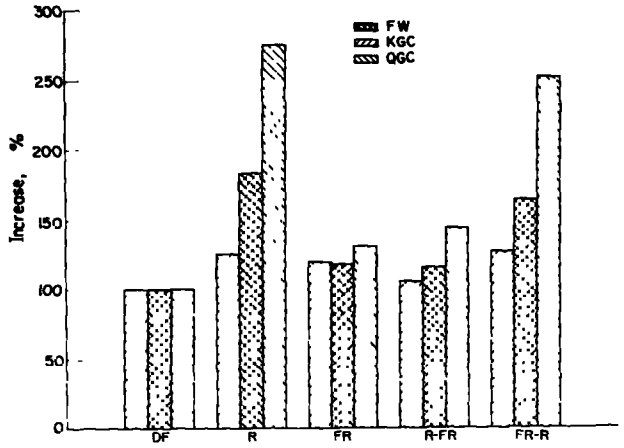


FIG. 3. VARIATIONS IN KGC AND QGC CONTENT AND OF GROWTH OF DARK GROWN PEAS AFTER IRRADIATION WITH COMBINATIONS OF RED (R) AND FAR RED (FR) LIGHT.

Flavonoid content and growth are expressed as a percentage of dark control (DF). Red light exposure; 546 ergs/cm²/sec for 3 min (98 kergs/cm²). Far-red light exposure: 1173 ergs/cm² sec for 12 min (845 kergs/cm²). Fresh weight (FW) was measured as the average weight of the terminal buds. KGC and QGC contents were measured as μmoles/g fresh weight.

Red–Far Red Reversibility of KGC and QGC Synthesis

When a short exposure to red light (98 kergs/cm^2) was immediately followed by far red light (845 kergs/cm^2) or vice versa, then the response in terms of both growth and QGC synthesis was determined by the nature of the final irradiation. Red light increased both growth and QGC synthesis and far red reversed these effects. KGC synthesis was unaffected by either red or far-red irradiation (Fig. 3).

The Effect of Red-light Dose on QGC Synthesis

To determine the effect of the dose of red light on flavonoid synthesis, 6-day-old dark grown peas were subjected to red light from a source of 600 ergs/cm^2/sec for varying times so that doses between 0 and 1000 kergs/cm^2 were administered. The terminal buds were harvested 21 hr later. Once again KGC concentration was unaffected, while the QGC concentration increased rapidly up to about 30 kergs/cm^2 after which the rate of increase rapidly diminished (Fig. 4).

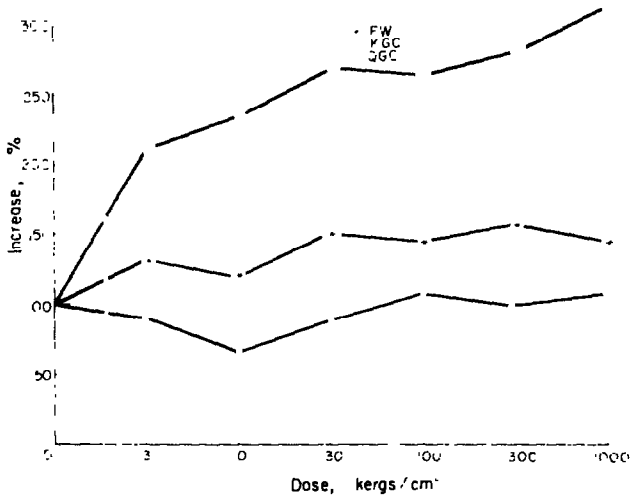


FIG. 4. VARIATION IN KGC AND QGC CONTENT AND GROWTH WITH INCREASING DOSES OF RED LIGHT. Flavonoid content and growth are expressed as percentage of the dark control. Fresh weight (FW) was measured as the average weight of the terminal buds. KGC and QGC contents were measured as μmoles/g fresh weight.

TABLE 1. DISTRIBUTION OF KGC AND QGC IN VARIOUS TISSUES OF THE GREEN PEA PLANT

Plant part	μM/g fresh wt. KGC	QGC
Terminal bud	0·23	0·18
Petiole and tendril of youngest leaf	0·19	1·07
Youngest leaf and stipule	1·06	0·80
Youngest internode	0·16	0·39
Second youngest petiole and tendril	0·11	0·74
Second youngest leaf and stipule	1·21	1·61
Second youngest internode	0·09	0·17

KGC and QGC Distribution in the Green Pea Plant

Because KGC and QGC were confined to the terminal buds of dark grown peas and the response of buds to light in terms of KGC and QGC were different, it was of interest to see if there were variations in the concentrations of these two compounds in different parts of the green plants. The results are shown in Table 1.

DISCUSSION

With essentially the same plant system as used here, Furuya and Thomas[2] claimed to show that KGC concentration increases with red light treatments. The present work indicates however that KGC concentration is relatively stable during a variety of light treatments while QGC varies significantly. The two sets of data may be reconciled by assuming that the paper chromatographic procedure of Furuya and Thomas failed to distinguish between KGC and QGC. This has since been confirmed by Dr. M. Furuya by re-examination of his original chromatograms. The total amount of flavonoids detected and the magnitude of the variations with different light regimes obtained by Furuya and Thomas were somewhat greater than we have described. However, in our hands, their method gave similar results to ours, suggesting that their plant material was in some way different from ours.

The role of flavonoids in plant metabolism is of interest for several reasons. Their widespread occurrence in higher plants suggests a possible significant function in the metabolism of the plant. Also, recent suggestions[3–6] that flavonoids may affect plant growth by controlling the activity of the indole-acetic acid oxidase system make it desirable to understand how flavonoids change under varying conditions of growth. In this context it may be significant that, whereas at low concentrations KGC acts as a cofactor for IAA oxidase *in vitro*, QGC inhibits the activity of the enzyme at all concentrations.[1] Thus, an increase in QGC concentration could lead to an inhibition of IAA-oxidase activity and to a consequent sparing of auxin. So far it has not been possible to demonstrate any direct effect of flavonoid compounds in a plant on the *in vivo* IAA oxidase activity or on growth. Because terminal buds that have been stimulated to grow have a constant KGC content while the QGC content appears to parallel the growth, it might be postulated that QGC is mediating growth through its effect on IAA oxidase.

In 14-day-old green plants the concentrations of flavonoids varied between tissues being very low in the internodes and rather high in the leaves. With the exception of the petioles and tendrils the concentrations of KGC and QGC in the tissues were about equal. In the petioles and tendrils the QGC content was approximately fivefold that of KGC. An examination of the petioles and tendrils separately showed that the tendrils contained relatively large amounts of QGC (about 1·3 μM/g of fresh tissue) whereas there was only a very low concentration of KGC (0·1–0·2 μM/g). Tronchet[7] has suggested that the twining habit of some creepers may be related to variations in their flavonoid content and our evidence is, at least, consistent with this view.

As the leaves contribute the greatest part of the fresh weight of the plant, it is apparent from the results that the older leaves contain the bulk of the total flavonoids present in the

[3] F. E. Mumford, D. H. Smith and P. G. Heytler, *Biochem. J.* **91**, 517 (1964).
[4] A. W. Galston, In *Photoperiodism and Related Phenomena in Plants and Animals* (Edited by R. B. Withrow). Am. Assoc. Advancement Sci., Washington, D.C. (1959).
[5] M. Furuya, A. W. Galston and B. B. Stowe, *Nature* **193**, 456 (1962).
[6] J. P. Nitsch and C. Nitsch, *Bull. Soc. Botan., France* **108**, 349 (1961).
[7] J. Tronchet, *Conges. des Soc. Savantes* **86**, 493 (1961).

plant. Because these leaves are no longer growing the bulk of the flavonoids are obviously not functioning as regulators of growth.

The present work provides no support for a causal relationship between flavonoid synthesis and growth or for a connection between these two processes as affected by light. Our observations may, however, provide some information on the role of phytochrome in the biosynthesis of flavonoids. The reversibility of red-light induced QGC synthesis by far red light demonstrates clearly that the synthesis of QGC in Alaska peas is controlled by the phytochrome system. Thus, the presence of phytochrome in its presumed enzymatically-active, far-red absorbing form (P_{FR}), is apparently necessary for appreciable synthesis of the quercetin derivative, but is not required for the synthesis of the kaempferol derivative. The quercetin derivative (QGC) differs from the kaempferol derivative (KGC) only in the presence of a 3′-hydroxyl group (I, R = H or OH). This indicates that one of the metabolic functions of P_{FR} is to control the rate of 3′-hydroxylation of flavonoids. The suggestion of Stafford[8] of a possible involvement of phytochrome in the 3′-hydroxylation of *Sorghum* flavonoids lends support to this suggestion. The question of where in the biosynthetic pathway of flavonoids 3′-hydroxylation occurs is still unresolved, but it is generally accepted that the hydroxylation pattern is fixed early.[9] Thus P_{FR} might exert its control at the level of *p*-coumaric acid → caffeic acid, or of 4-hydroxylated chalcone → 3,4-dihydroxylated chalcone. Since the sugar residues of KGC and QGC are thought to be identical, the same enzyme systems are probably capable of building up these onto either molecule. The alternative possibility, that KGC is hydroxylated directly to QGC through a P_{FR} controlled reaction, is contrary to the current view of the establishment of the hydroxylation pattern early in the biosynthetic pathway. The fact that the synthesis of quercetin derivatives has been shown to be under the control of the phytochrome system in other plants,[10] also suggests that this control is exercised at some point during the synthesis of the quercetin ring system rather than after the elaboration of any of the glycosidic patterns.

Our data of course, provide no evidence on whether the function of P_{FR} in controlling the hydroxylation pattern of flavonoids is near to its primary function, or merely a remote manifestation of the primary function. However, it is possible that P_{FR} may control the rate of 3′-hydroxylation through the activation or synthesis of the enzymes responsible for the reaction. Since this biochemical effect of phytochrome is rather more precisely defined than those previously described, it may well be a valuable tool in further investigations into the biochemical and morphogenetic consequences of the photoactivation of phytochrome.

EXPERIMENTAL

Plant Materials and Light Treatments

The growing conditions for the plants (*Pisum sativum* c.v. Alaska) and the lights used in the various treatments were the same as those described by Furuya and Thomas.[2]

Extraction of Plant Tissue

Approximately 500 mg of plant tissue was homogenized with 5 ml of *n*-butanol in a Potter-Elvehjem tissue macerator. The suspension was heated in a boiling water bath for 10 min and separated by centrifugation. After washing the residue with a little butanol the combined supernatants were extracted (3 × 3 ml) with 0·1 M NH_4OH. The ammonia

[8] H. A. STAFFORD, *Plant Physiol.* **40**, 130 (1965).
[9] J. B. HARBORNE, In *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN) p. 593. Pergamon Press, Oxford (1962).
[10] H. MOHR, *Biol. Rev.* **39**, 87 (1964).

solution was carefully acidified with 6 N HCl, extracted with *n*-butanol (3 × 3 ml) and the butanol evaporated to dryness. After solution in Solvent B (see below), the residue was applied to the silica gel column.

Silica Gel Column

The solvents used were: A; 0·5 M H_3BO_3 adjusted to pH 7·0 with 2 N KOH and then saturated by shaking with *n*-butanol: B; The supernatant layer from a mixture of *n*-butanol (840 ml) and Solvent A (160 ml): C; Acetic acid (200 ml) and methanol (800 ml).

Ten grammes of silica gel (Merck- 0·05–0·2 mesh), which had been washed with 6 N HCl to remove a yellow impurity, was slurried with Solvent A and packed into a 2 cm diameter glass column. The column was washed with Solvent B until a translucent zone, which formed at the top, had travelled to the bottom of the column. The extract was dissolved in Solvent B (1 ml), applied to the top of the column and the first fraction eluted with Solvent B (150 ml). This fraction contained KGC. QGC was then eluted with Solvent C (50 ml).

Each fraction was evaporated to dryness, taken up in *n*-butanol (10 ml) and its u.v. spectrum determined to check its identity.

Hydrolysis of KGC and QGC

Each butanol solution was extracted with N KOH (2 × 3 ml) and the alkaline solution stored at room temperature for 30 min. Spectral evidence showed that hydrolysis was complete in 15 to 20 min. The solution was carefully acidified and extracted with ether (3 × 3 ml). The ether solution was made up to 10 ml and the *p*-coumaric acid content determined spectrophotometrically on a Perkin Elmer 350 U.V. spectrophotometer. The extinction coefficient for *p*-coumaric acid in ether was found to be $2{\cdot}05 \times 10^4$ at 309 mμ.

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