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EFFECTS OF INDOLEACETIC ACID AND KINETIN ON SCOPOLETIN-SCOPOLIN LEVELS IN RELATION TO GROWTH OF TOBACCO TISSUES IN VITRO[1,2]

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The levels of both scopoletin and its glycoside, scopolin, in tobacco tissues cultured in vitro have been shown to be markedly influenced by the supply of 3-indoleacetic acid (IAA) and kinetin in the nutrient medium, and these substances likewise influence the release of scopoletin to the medium (15). A more detailed quantitative study has, therefore, been carried out to determine possible causal relationships between the actions of IAA and kinetin in these phenomena and the effects of these chemicals on growth and differentiation of the tissues.

MATERIALS AND METHODS

In the earlier work scopoletin was extracted from the agar medium. In order to measure the concentration of scopoletin in the medium directly by fluorimetry a liquid culture technique similar to one described by Heller (10) has been adopted. A 19 × 50 mm Pyrex test tube containing 10 ml of modified White's medium (14) less agar, was used for the culture of a single explant. The tissue was supported on a filter pad. A 5.5 cm disc of Whatman No. 2 filter paper suitably folded to form a cylinder with one end open, was inserted, open end first, into the tube and the pad so formed pushed down until its surface was just level with that of the medium.

Nicotiana tabacum L. (var. Wisconsin #38) tissues were used throughout these studies. Pith sections of approximately 65 mg fresh weight were prepared according to (12) and callus explants of approximately 20 mg fresh weight were cut from stock tissue grown in Erlenmeyer flasks on agar medium containing 2 mg/l IAA and 0.2 mg/l kinetin. The tissues were cultured from 2.5 to 24 days. Except as stated the tubes were kept in batches on shelves exposed to high (ca. 80 %) relative humidity and continuous weak light from fluorescent ceiling fixtures. The room temperature was 28° C. In all experiments ten replicate cultures were pooled for fresh and dry weight determinations. The media were combined, made up to 100 ml with distilled water and assayed fluorometrically.

For each determination of scopoletin and glyco-

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side in the tissue approximately 250 mg samples of fresh tissue were frozen at −15° C and homogenized with 10 ml of 80 % ethanol in a Potter-Elvehjem homogenizer. The homogenate was centrifuged, the supernatant decanted, and the procedure repeated twice with 5 ml volumes of 80 % ethanol. The last supernatant showed little fluorescence under ultra violet light. The three supernatants were pooled and evaporated to dryness in a 30 ml beaker in a stream of air. The residue was then dissolved in about one milliliter of 80 % ethanol and applied to an 11 cm starting line of a Whatman 3 MM paper chromatogram. The chromatogram was developed in *n*-butanol : acetic acid : water, 4 : 1 : 2 v/v, mixed immediately before use. Development was allowed to proceed overnight and stopped when the front had moved about 40 cm from the origin. Under these conditions scopoletin and the glycoside separated as two brightly fluorescing bands which, after drying, were cut out and eluted with 0.14 M boric acid buffer adjusted to pH 10.0 with sodium hydroxide. Each eluate was made up to 5.0 ml and its fluorescence measured. The photofluorometer was a Coleman 12C fitted with Corning 5874 primary and 3389–4308 secondary filters and adapted to carry 10 × 77 mm Pyrex test tubes as cuvettes. With each series of measurements a sample of scopoletin (2 μg) was chromatographed, eluted, and used to calibrate the instrument. In measurements of scopoletin directly in the medium the standard sample was dissolved in fresh nutrient solution. The standard scopoletin had been prepared in a crystalline form from hydrolyzed tobacco root extract by a method similar to that of Andreae (1). The fluorescence of the glycoside as such was used for its determination. In pH 10.0 borate buffer the intensity of fluorescence of the glycoside was found to be only 0.048 that of the scopoletin released from it on hydrolysis and determined in the same medium. All steps in the extraction, separation, and determination of these photolabile compounds were carried out in darkness or in weak diffuse light.

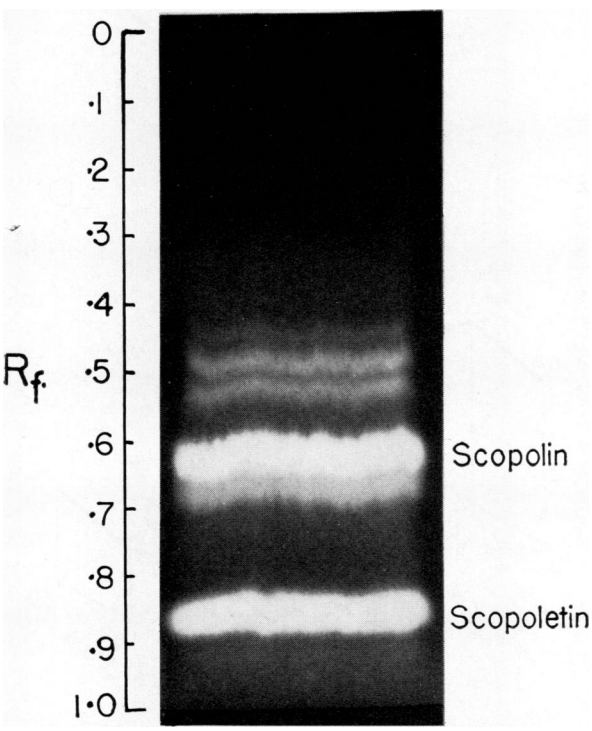


FIG. 1. Paper chromatogram of ethanolic extract of tobacco callus photographed under ultraviolet light. Paper: Whatman No. 3 MM. Solvent: *n*-butanol : acetic : acid : water = 4 : 1 : 2 v/v. Direction: descending.

RESULTS

A typical separation of fluorescent components obtained by chromatography of an ethanolic extract is shown in figure 1. The chromatogram was photographed under ultra violet light. (u.v.) Analysis of the glycoside has shown it to be scopolin. In the butanol : acetic : water solvent it separated as a distinct band at R_f 0.61, while scopoletin ran farther ahead at R_f 0.85. When conditions of culture favored the formation of chlorogenic acid in the tissue (21) this compound was found to run close to scopolin, at R_f 0.67. A number of less intense bands running slower than scopolin have been analysed and shown to be glycosides of scopoletin. Details of analysis of these and of scopolin will be reported in a later paper.

The fresh weight and scopoletin status of a series of pith sections cultured for 24 days in IAA and kinetin concentration ranges of 1 to 100 mg/l and 0.02 to 2.0 mg/l, respectively, are shown in figure 2. Dry weight increases closely paralleled those of fresh weight, and only the latter are shown in figure 2A. From the data it is clear that vigorous growth is limited to a rather narrow range of IAA concentrations (around 1 mg/l) and that kinetin exerts a marked growth promoting effect in the presence of this concentration of IAA. Kinetin alone does not promote growth of the pith tissue. The relatively quiescent cells of the pith as removed from the tobacco stem are low in both scopolin (equivalent to 14.9 μg/G.F.W. scopoletin) and scopoletin (0.27 μg/G.F.W.) and begin to produce these substances in quantity only when cultured under conditions which lead to tissue growth. The striking feature of the data shown in figure 2B is the rapid rise in scopolin content of the tissue as the exogenous supply of kinetin is increased. This rise is paralleled by one in the endogenous scopoletin content of the tissue suggesting a state of equilibrium between the aglycone and its glycoside, but a comparison of the ordinate scales of figures 2B and 2C (ratio 13 : 1) shows this equilibrium to be strongly towards the latter. Under conditions of maximal growth (1 mg/l IAA and 0.2 mg/l kinetin in this case) a depression in the curve relating scopolin concentration with kinetin supply was consistently observed. This low level of scopolin could not be correlated with a rise in the endogenous

scopoletin level (a depression was likewise observed), neither was there an increase in the amount of scopoletin released to the medium under these conditions. It should be emphasized that here, as in earlier studies (15), scopolin was never detected in the external medium.

The free scopoletin released to the medium (figure 2D) is expressed in terms of fresh weight of the tissue rather than as concentration in the medium. In general very low external scopoletin levels are associated with vigorously growing tissue, and it will become clear that scopoletin is lost only by cells in an abnormal or dead state.

Two other points are of interest. First, high, yet not toxic IAA concentrations shift the scopolin/scopoletin equilibrium somewhat toward scopoletin; and second, in the absence of kinetin and with scopolin formation at a minimum, relatively high levels of scopoletin are found both within the tissue and in the external solution.

The above experiment was repeated under identical conditions except that callus tissue was substi-

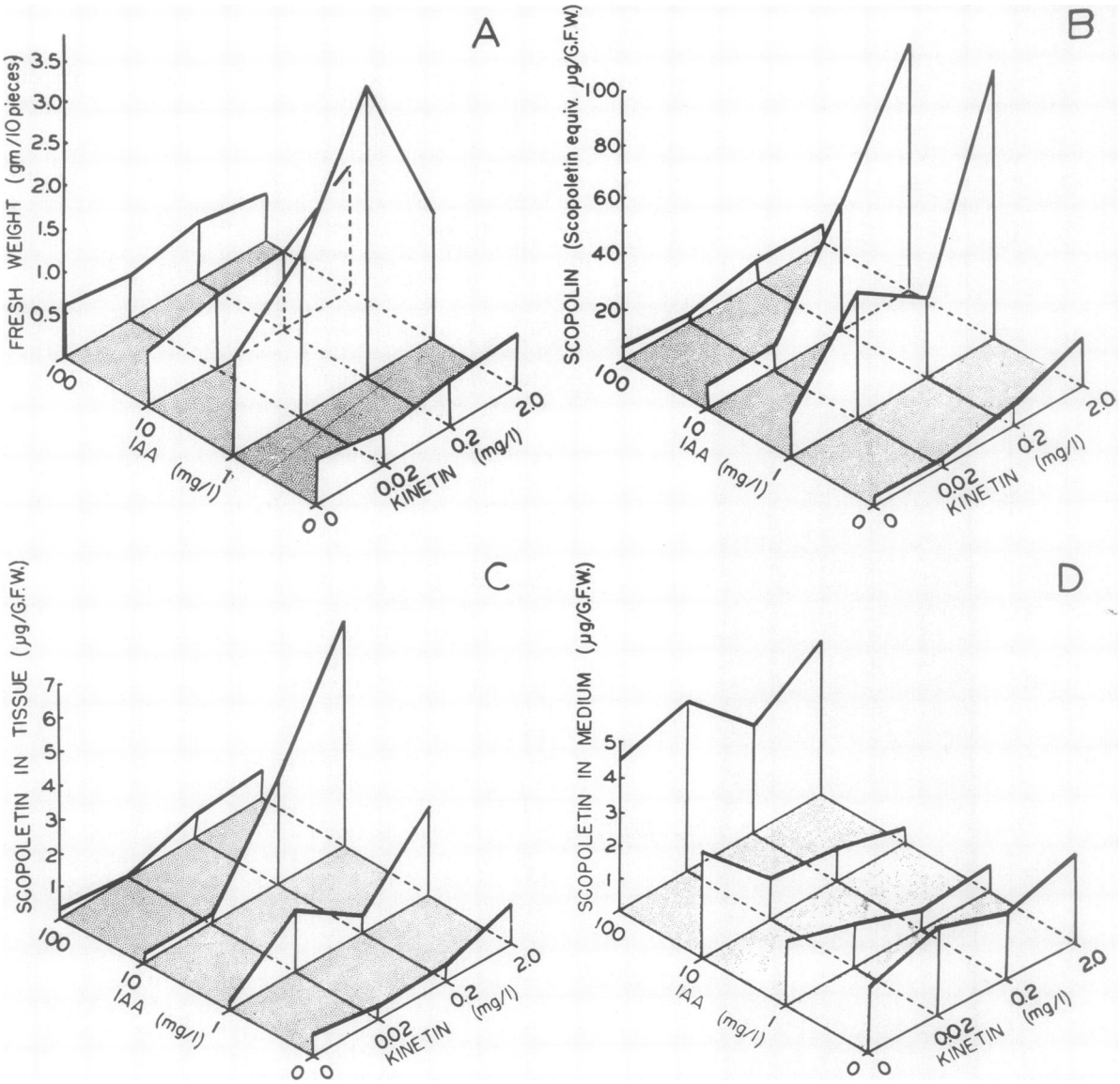


FIG. 2. Effect of IAA and kinetin on the fresh weight and scopolin and scopoletin levels in pith tissue after 24 days. A: Fresh weight of tissue (Dry weight = 4–7 % of fresh weight). B: Scopolin level in the tissue. C: Scopoletin level in the tissue. D: Scopoletin released to the medium. Experiment started 9 January 1959. Original fresh weight ca. 0.65 g/10 pieces.

tuted for pith and sampling was done at 3, 6, 12, and 24 days after the cultures were started. This experiment was performed three times, and, although there was some variation between experiments, the influence of IAA and kinetin on the scopoletin status of this tissue was marked, consistent, and parallel to that observed in pith. Representative results obtained after 3 and 24 days in one experiment are shown in figures 3 and 4, respectively.

In comparing figure 4 with figure 2 it is immediately obvious that scopolin which is produced in relatively small amounts in pith in the absence of exogenous IAA, occurs in high concentrations in callus also when the auxin is not supplied. This no doubt reflects the relatively greater ability of the callus tissue to produce its own auxin. It is also clear that in callus, kinetin becomes inhibitory to growth at a lower concentration than in pith. When these characteristic differences between the two tissues are considered it is apparent that the changes in scopoletin status associated with the supply of IAA and kinetin in pith occur also in callus.

The excision of explants from stock callus pieces damages many cells at the cut surfaces. Associated with such wounding is a rapid rise in the scopoletin level of the tissue, no doubt through enzymatic hy-

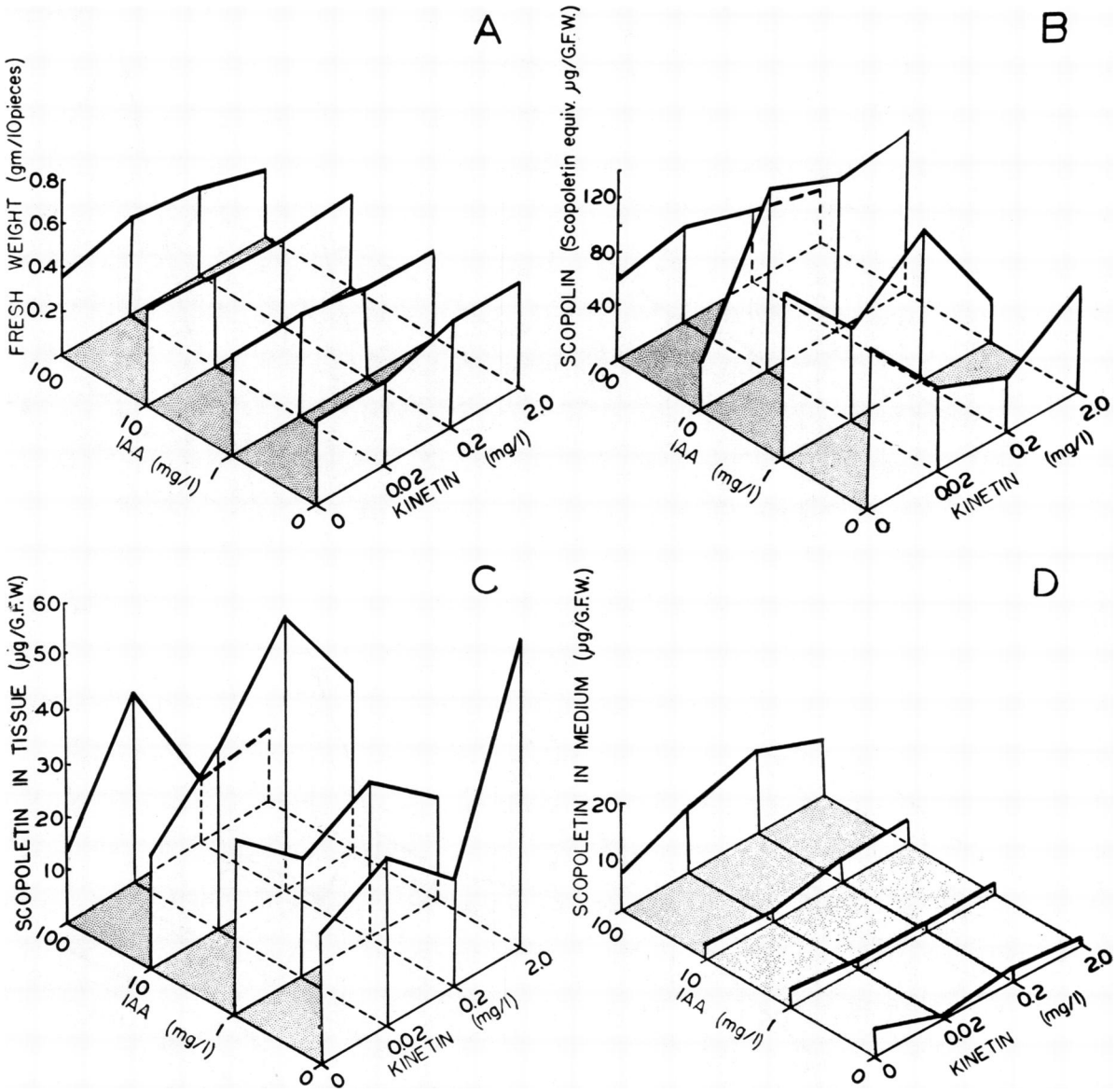


FIG. 3. Effect of IAA and kinetin on the fresh weight and scopolin and scopoletin levels in callus tissue after 3 days. A, B, C, and D, as in figure 2. Experiment started 7 February 1958. Original fresh weight ca. 0.30 g/10 pieces.

drolysis of scopolin. This generally high level of scopoletin still exists after 3 days (figure 3C) but already at this time when little growth differential is apparent, changes are beginning to occur in the levels of scopolin in response to the different treatments (figure 3B). Moreover, the scopoletin which is maintained in equilibrium with the scopolin in the tissue contributes to the pattern of total endogenous scopoletin, shown in fig 3C. A loss of scopoletin to the medium following the supply of toxic concentrations of IAA to the tissue is clear from the effect of 100 mg/l IAA, in figure 3D.

Analyses of the 6 and 12 day samples showed a general reduction in the level of endogenous scopoletin along with a development of the differential growth response and scopolin and scopoletin levels observed after 24 days and illustrated in figure 4. The high levels of scopoletin in the medium in the absence of kinetin after 24 days was preceded by a high level of scopoletin in the tissue after 12 days.

Further evidence for the interpretation that scopolin is degraded to scopoletin and that this is then released to the medium as a result of injury to the tissue or physiologically unfavorable conditions is

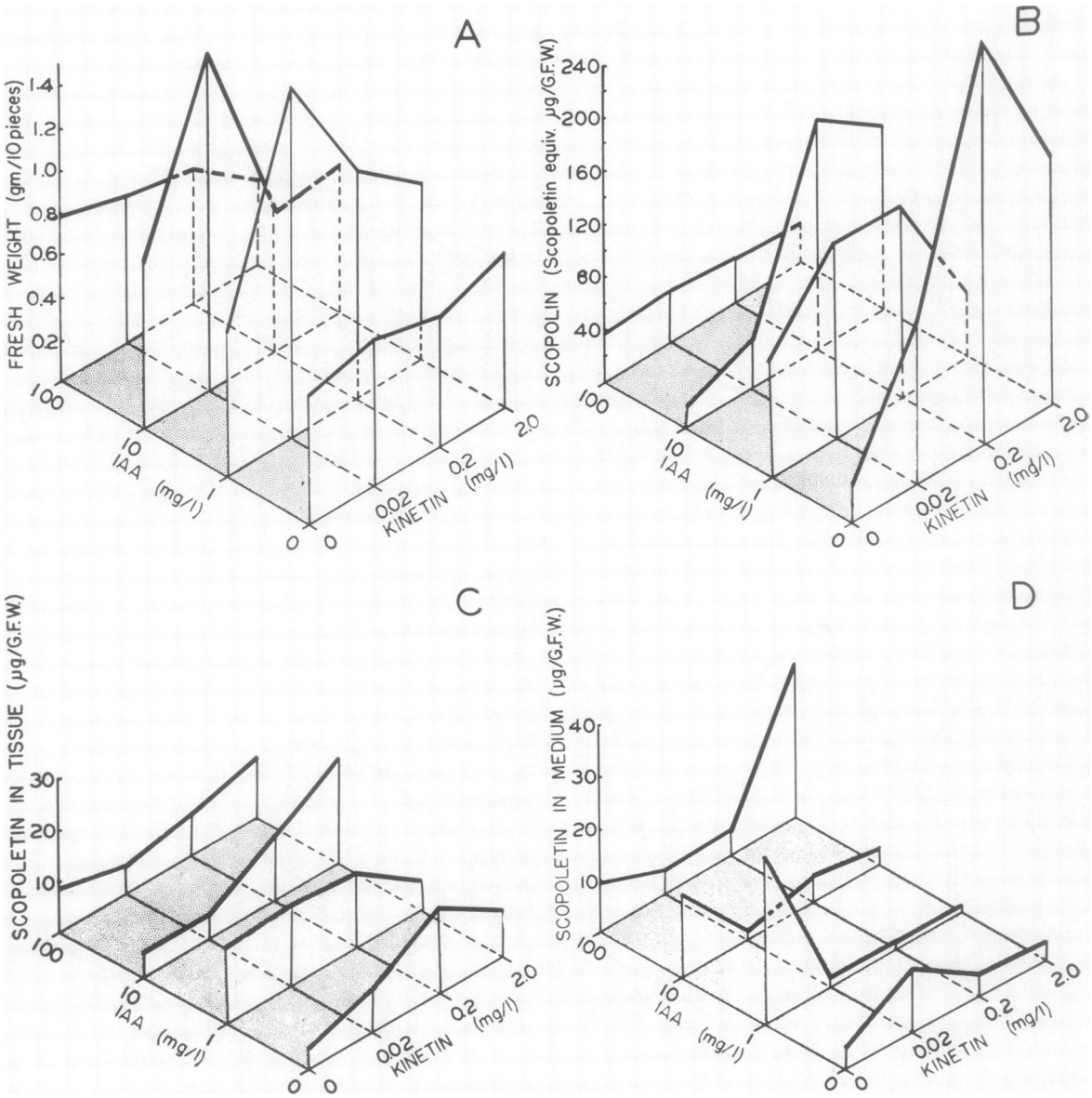


FIG. 4. Effect of IAA and kinetin on the fresh weight and scopolin and scopoletin levels in callus tissue after 24 days. A, B, C, and D, as in figure 2. Experiment started 7 February 1958. Original fresh weight ca. 0.30 g/10 pieces.

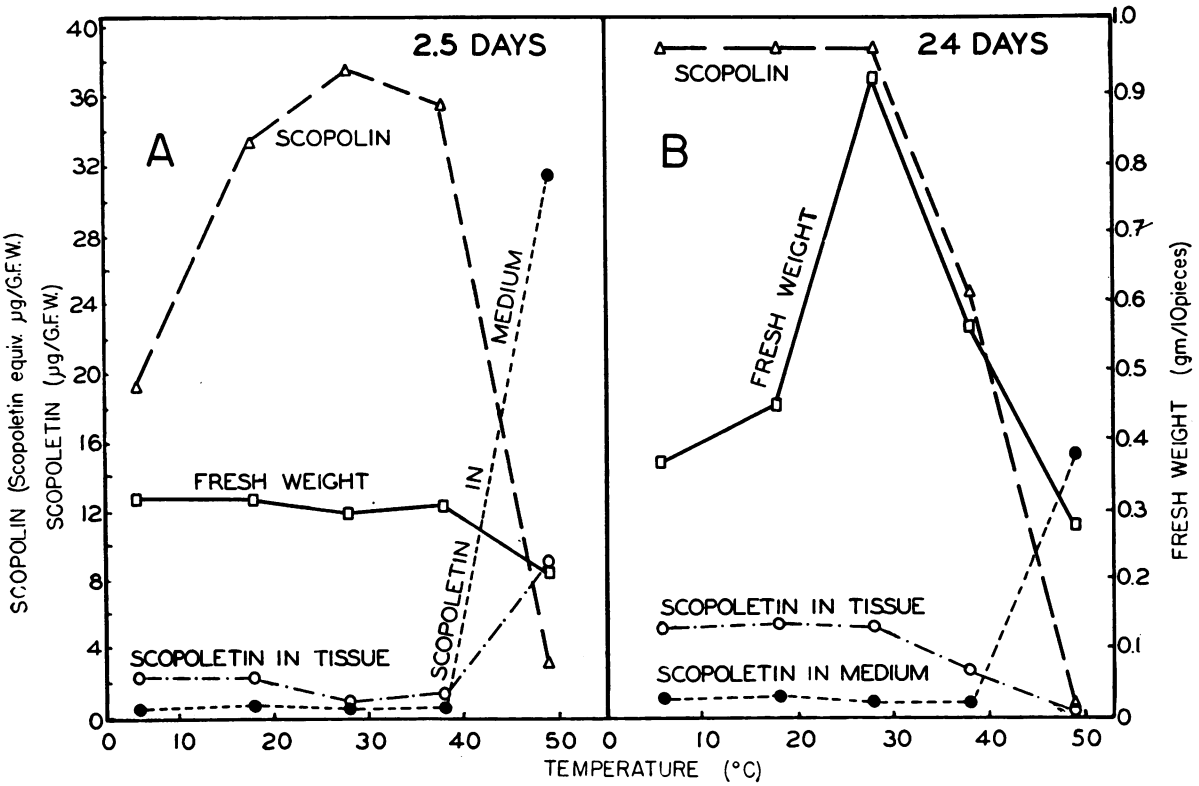


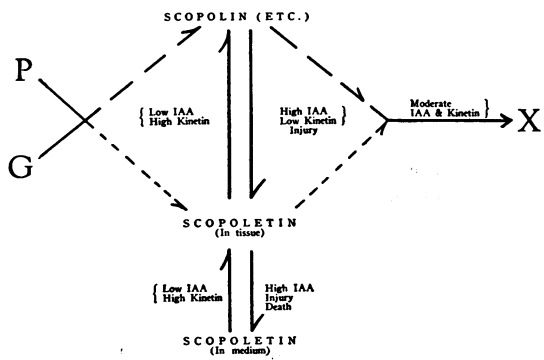
FIG. 5. Effect of temperature on the fresh weight and scopolin and scopoletin levels in callus tissue. A: After 2.5 days. B: After 25 days. Experiment started 7 January 1959. Original fresh weight ca. 0.30 g/10 pieces.

afforded by two experiments in which callus was grown at different temperatures on a medium favorable for growth. The temperatures ranged from 6 to 49° C as shown in figure 5. In one experiment (fig 5A) the tissues were harvested after 2.5 days, in the other (fig 5B) after 24 days. It is clear from both figures that as the temperature increased from 6 to 38° C the level of scopoletin in the medium remained unchanged. However, at 49° C a very high external level had been attained, strongly indicating that the release of scopoletin from the tissue is due to the death of component cells. After both time intervals the level of scopolin in the tissue was observed to fall as the temperature rose from 28 to 49° C. In the 24 day old cultures, this fall was paralleled by a drop in scopoletin level in the tissue. In the young cultures (2.5 days) there was actually a rise in the level of free scopoletin which was almost certainly the result of degradation of scopolin under conditions unfavorable for growth.

Also when toxic concentrations of cyanide, coumarin, maleic hydrazide, ascorbic acid, or triiodobenzoic acid were supplied to the callus a sharp increase was observed in the release of scopoletin to the medium.

DISCUSSION

A diagram is presented to summarize possible influences of auxin and kinetin levels and proportions on quantitative relationships between scopoletin and scopolin and between these substances and their close metabolic relatives.



The formation of scopolin from a precursor, P, and glucose or a glucose derivative, G it is suggested, is promoted as a function of the concentration of added kinetin. The question is left open whether

the synthesis proceeds via scopoletin or some other pathway. It is clear, however, from unpublished work (Sargent and Skoog) that other carbohydrates may substitute for glucose to form a variety of scopoletin glycosides (several are evident as fluorescent bands in figure 1). The level of scopolin attained in the tissue is also dependent on the auxin content. A minimal level of auxin of either endogenous or external origin would seem to be required for the accumulation of very high levels of scopolin, but as the concentration of added IAA is increased the scopolin level falls drastically. With moderate, growth-promoting concentrations of IAA, scopolin is converted in large part to some non-fluorescent product, X, which presumably is utilized in cellular synthesis; likely in lignin formation. Again the question is left open whether or not this transformation occurs via scopoletin as an intermediate. Another possibility would be that scopoletin merely is set free in the process. In any case some free scopoletin is present in the tissue, and as the IAA level is raised, higher amounts appear. With toxic levels of IAA or under otherwise unfavorable conditions for growth much scopoletin is released to the external medium. In such cases, it is proposed, the free scopoletin in the tissues and medium definitely is derived at least for the most part from degradation of scopolin and/or other scopoletin glycosides which would normally have been utilized in growth.

The reverse process, i.e., the incorporation of exogenous scopoletin, has not been studied in detail here, but a marked growth effect of scopoletin added together with kinetin has been observed in tobacco callus cultures (15). This is evidence that scopoletin is in fact assimilated, and suggests that the process requires a kinin; at least it is promoted by kinetin.

In the above diagram kinetin-IAA levels are indicated to affect the direction of equilibrium reactions between scopoletin and its different combined forms or derivatives. In view of the time relations in the present experiments, it is of course likely that steady states of synthesis and degradation rather than true, reversible equilibrium reactions are being observed. It should be pointed out that not only do the levels of scopoletin and scopolin vary, but also the fluorescent bands of other scopoletin glycosides, shown in figure 1, change in relative intensities with variations in the kinetin and IAA supplies in the medium.

It is clear, therefore, that complex quantitative relationships exist between free scopoletin and its several bound forms which are intimately dependent on the availability, levels, and proportions, of these growth substances.

A large number of plants are known to accumulate scopoletin especially under conditions unfavorable for growth (8). Best (3, 4, 5) who observed an increase in scopoletin content of tobacco plants following injury or viral infection put forward a number of hypotheses to explain this, but he favored one which postulated the diversion of normal metabolism resulting in scopoletin accumulation. The path, P → scopolin → X, in the scheme presented here as the major one in healthy rapidly growing tobacco tissue would be in accord with this and with Best's observations that the level of scopoletin in healthy tobacco plants is minimal when the growth rate is maximal. An accumulation of a blue-fluorescing material (presumably scopoletin) around mechanical cracks or around lesions induced by boron deficiency in celery has been recorded by Spurr (18). That viral infection per se did not result in scopoletin formation in potato tubers (1) but that physical damage to the cells of the tuber was necessary has been shown by Sanford and Grimble (17). Andreae working with a variety of potatoes and Best with Nasturtium could not detect scopoletin (or fluorescence) in these plants prior to injury. It may be assumed, therefore, that these plants also exhibit a rapid normal turnover of glycoside to X. Of interest in this connection was the finding of Andreae and Andreae (2) that the fluorescence observed in the deformed portions of potato leaves infected with leaf-roll virus was associated with a local accumulation of starch. From physical properties it appears certain that the scopoletin glycoside reported in Avena roots is identical with the scopolin obtained from tobacco (9, 7). Also, in potato tubers the presence of scopolin seems likely from chromatographic properties of fluorescent substances extracted by Burton (6); and Housley and Taylor (11) have recently claimed that scopoletin is a component of inhibitor β extracted from the tubers. The role of scopoletin as a natural growth regulator in roots has been studied by Goodwin and his students (16) and in tissue cultures as a substitute for IAA by Montaldi and Skoog (15). No determination of the effect of scopolin on growth has been reported, but it is of interest that the root-inhibiting properties of esculetin are not shared by its glucoside esculin. Pertinent to this is the recent finding by Towers et al (19) that maleic hydrazide supplied to wheat leaf segments becomes detoxified by the formation of a β-glycoside. Finally, Johnson and Fults (13) have demonstrated the formation of high concentrations of free scopoletin in a number of plants which they treated with high concentrations of 2,4-dichlorophenoxyacetic acid and have suggested this as a possible cause of the herbicidal effect of the chemicals. (20). Evidence derived from diverse sources, therefore, points to the participation of a scopoletin system associated with normal plant anabolism, to possible disruption of this system under conditions of disease or injury, and to its apparent control by growth substances. Although the exact composition, interconversion and other relationships of many of its components, including unidentified fluorescent intermediates and the postulated P and X still remain to be determined, at least it is clear at this stage that the scopoletin status within tobacco tissue is markedly influenced by the supply of IAA and kinetin and, furthermore, that the functioning of scopoletin in the growth process must include the participation of its glycosides and their derivatives.

Summary

The levels of scopoletin and its glucoside, scopolin, have been investigated in both tobacco pith and tobacco callus grown in vitro and supplied with various concentrations of IAA and kinetin over a period of 24 days. The amount of scopoletin released to the medium under these conditions has also been determined. The effect of temperature on these levels has been observed. It is concluded that the formation of scopolin by the tissue is dependent upon a supply of kinetin and that a steady state equilibrium exists between this glycoside and its aglycone. In healthy tissue the scopolin: scolopetin ratio is extremely high, a value of ca. 13:1 was observed. Under conditions which favor growth, scopolin is converted to a non-fluorescent substance (X) presumed to be utilized in the growth process. Degradation to scopoletin is accelerated by a high supply of IAA or injury to the tissues. Death or abnormally functioning cells result in release of scopoletin to the medium. The data are discussed in relation to earlier findings.

Acknowledgment

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