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# PLANT PHENOLS AND POLYPHENOLOXIDASE IN *NICOTIANA TABACUM* DURING GREENHOUSE GROWTH, FIELD GROWTH AND AIR-CURING*

R. A. ANDERSEN, R. LOWE and T. A. VAUGHN

Crops Research Division, Agricultural Research Service, U.S. Dept of Agriculture and Dept. of Agronomy, Univ. of Kentucky, Lexington, Kentucky 40506, U.S.A.

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**Abstract**—A difference was noted between greenhouse *Nicotiniana tabacum* plants and field plants in the amounts of total soluble phenols, chlorogenic acids and rutin distributed among the leaves from upper, middle and lower stalk positions after flowering. Field plants contained more soluble phenols in upper leaves; greenhouse plants contained more in lower leaves. Total soluble phenols did not decrease during air-curing, but chlorogenic acids and rutin decreased with time during curing-chamber (primed leaves) and conventional (leaves on stalks) air-curing. Polyphenoloxidase (PPO) activity increased as plants matured. PPO activity showed an additional transient rise during the first 7–9 days of air-curing. Thereafter, the enzyme activity decreased for 3–4 weeks, reaching very low levels. Leaves from greenhouse plants contained lesser amounts of total soluble phenols, chlorogenic acids, rutin and PPO activity than field plants of the same variety during growth and air-curing. These differences between greenhouse and field plants may be related to the light transmission characteristics of glass in the wavelength region 290–330 nm.

## INTRODUCTION

SOLUBLE plant phenols in mature greenhouse *Nicotiana tabacum* were estimated to be lower in quantity than corresponding leaf tissue of field-grown plants.[1–3] Although the precise cause of this anomaly is unknown, photocontrol, temperature, stalk position and other factors may be involved.[2,4] There are considerable data on the qualitative and quantitative composition of individual soluble plant phenols in tobacco at given stages of maturity, but there has been less emphasis on the quantitative analysis of soluble plant phenols throughout a growth and curing cycle. Sheen and Calvert[5] have recently determined levels of plant phenols at a few stages of maturity for several varieties of *N. tabacum*. They also reported on activities of polyphenoloxidase and peroxidase in primed leaves of field tobacco at intervals throughout simulated air-curing in a curing-chamber; conditions were employed which approximated those used in conventional air-curing of the Burley variety.[6]

The purpose of our studies was to compare leaf tissue quantitatively at various stalk positions of greenhouse and field-grown *N. tabacum* throughout growth, and during both a conventional and a simulated air-curing cycle. The following variables were studied with respect to their possible interdependences: total plant phenols, the principal individual plant phenols, i.e. chlorogenic acids and rutin, and the associated enzyme, polyphenoloxidase.

* The investigation reported in this paper (No. 69-3-49) is in connection with a project of the Kentucky Agricultural Experiment Station and is published with approval of the Director.

[1] P. T. PENN and J. A. WEYBREW, *Tobacco Sci.* **2**, 68 (1958).
[2] H. V. LOTT, *Planta* **55**, 480 (1960).
[3] T. P. FURGUSON and A. S. WEAVING, *Nature* **183**, 64 (1959).
[4] E. K. WALKFR and T. T. LEE, *Can. J. Plant Sci.* **48**, 381 (1968).
[5] S. J. SHEEN and J. CALVERT, *Tobacco Sci.* **13**, 10 (1969).
[6] S. J. SHEEN and J. CALVERT, *Plant Physiol.* **44**, 199 (1969).

## RESULTS

### *Total Phenols*

Greenhouse "F.C. 402" leaves at the same stalk position and sampling date contained only 66 ± 29 per cent (mean ± standard deviation) of the amount of total soluble phenols in field "F.C. 402" leaves (Table 1). Another difference between greenhouse plants and field plants was in the relative amounts of total phenols distributed among the leaves from upper, middle and lower stalk positions. In greenhouse plants the lower leaves contained more total phenols than leaves in other positions from 5–11 weeks after transplanting (Table 1). In field leaves, however, there was no consistent relationship during this period. At harvest, i.e. 9 weeks after transplanting, upper leaves of "F.C. 402" and "Ky. 16" contained relatively more total phenols than leaves from lower stalk positions (Table 1).

TABLE 1. % TOTAL PLANT PHENOLS* IN GREENHOUSE AND FIELD-GROWN TOBACCO LEAVES

| Weeks after transplant | Leaf position | % Plant Phenols | | |
|---|---|---|---|---|
| | | "F.C. 402" greenhouse grown | "F.C. 402" field grown | "Ky. 16" field grown |
| 3 | Upper | 2·25 | 3·20 | |
| 3 | Middle | 1·98 | 2·58 | |
| 3 | Lower | — | 2·77 | |
| 5 | Upper | 1·49 | 3·07 | 1·53 |
| 5 | Middle | 1·25 | 3·07 | 1·88 |
| 5 | Lower | 2·05 | 2·98 | 1·98 |
| 7 | Upper | 1·16 | 2·48 | |
| 7 | Middle | 1·53 | 1·92 | |
| 7 | Lower | 2·09 | 2·90 | |
| 9 | Upper | 1·33 | 3·63 | 2·43 |
| 9 | Middle | 1·50 | 3·19 | 1·41 |
| 9 | Lower | 2·03 | 2·85 | 1·61 |
| 11 | Upper | 1·15 | | |
| 11 | Middle | 1·34 | | |
| 11 | Lower | 1·95 | | |

* The results are expressed in terms of chlorogenic acid equivalent in the PVP-Folin–Ciocalteau spectrophotometric method. Each value represents the average result of a duplicate determination.

"Field "Ky. 16" leaves at the same stalk position and sampling date contained 58 ± 9 per cent (mean ± standard deviation) of the amount of total soluble phenols in field "F.C. 402" leaves (Table 1).

During the 40-day curing cycle, there was no apparent change in levels of total phenols in middle leaves of either field-grown variety. Leaves of greenhouse plants cured under simulated conditions for 27 days did not differ in this respect from the conventionally air-cured field leaves. Total phenolic content was approximately two times higher in field leaves than in greenhouse leaves throughout the curing cycles. This was consistent with the higher level of total phenols at harvest in the middle leaves of field "F.C. 402" as contrasted with greenhouse leaves (Table 1).

### *Individual Phenols*

Chlorogenic acids and rutin were lower in greenhouse leaves than in "F.C. 402" field plants at each corresponding date during plant growth (Table 2). Rutin levels were generally 25 per cent or less of chlorogenic acid levels at a given sampling date in both field and greenhouse samples.

TABLE 2. % PLANT PHENOLS* IN GREENHOUSE AND FIELD "F.C. 402" LEAVES

| Compound | Leaf position | Weeks after transplant: 3 Greenhouse | 3 Field | 5 Greenhouse | 5 Field | 9 Greenhouse | 9 Field |
|---|---|---|---|---|---|---|---|
| Chlorogenic acids | Upper | 0·28 | 1·38 | 0·39 | 1·43 | 1·06 | 1·40 |
| Chlorogenic acids | Middle | 0·85 | 1·37 | 0·59 | 0·92 | 0·76 | 1·24 |
| Chlorogenic acids | Lower | — | 1·05 | 0·73 | 0·86 | 0·90 | 0·96 |
| Rutin | Upper | 0·07 | 0·32 | 0·07 | 0·32 | 0·05 | 0·22 |
| Rutin | Middle | 0·08 | 0·32 | 0·03 | 0·27 | 0·04 | 0·13 |
| Rutin | Lower | — | 0·32 | 0·07 | 0·25 | 0·07 | 0·12 |

* Individual phenols were determined by spectrophotometric analysis after paper chromatographic separation. Chlorogenic acid isomers were pooled prior to spectrophotometric measurement.

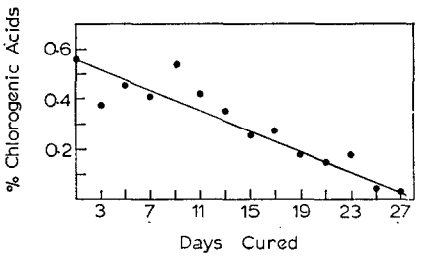


FIG. 1. % CHLOROGENIC ACIDS IN CURING-CHAMBER AIR-CURED, PRIMED GREENHOUSE "F.C. 402" MIDDLE LEAVES.

Chlorogenic acid isomers were isolated by paper chromatography, extracted from the paper, pooled, and determined by spectrophotometry.

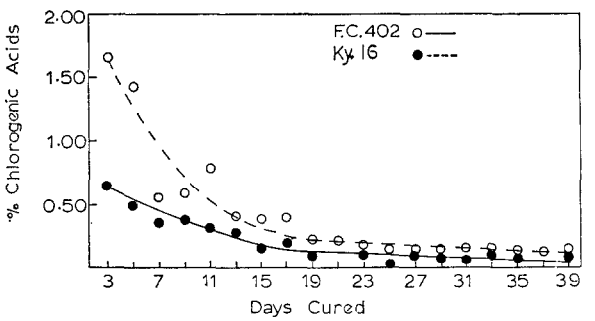


FIG. 2. % CHLOROGENIC ACIDS IN CONVENTIONALLY AIR-CURED FIELD "F.C. 402" AND "Ky. 16" MIDDLE LEAVES.

Chlorogenic acid isomers were isolated by paper chromatography, extracted from the paper, pooled, and determined by spectrophotometry.

In field "F.C. 402" plants, the amounts of chlorogenic acids and rutin in the leaves at 5 and 9 weeks after transplanting varied according to stalk position (Table 2). The order of decreasing amounts of these compounds was upper leaves, middle leaves, lower leaves.

During curing, chlorogenic acids in greenhouse "F.C. 402" middle leaves declined from an initial value of *ca.* 0·55 per cent to nearly zero after 27 days (Fig. 1). A similar pattern of decline for this compound occurred in field middle leaves of the same tobacco variety and those of "Ky. 16" (Fig. 2). Rutin, in middle leaves of conventionally air-cured "F.C. 402" and "Ky. 16" field tobacco, decreased in a similar manner. Rutin levels declined to about 50% of their initial values after 21 days and then remained at these lower levels.

*Polyphenoloxidase*

At corresponding dates of maturity, PPO activity was higher in "F.C. 402" field leaves than in greenhouse leaves of the same variety (cf. Figs. 3 and 4). The activity increased as

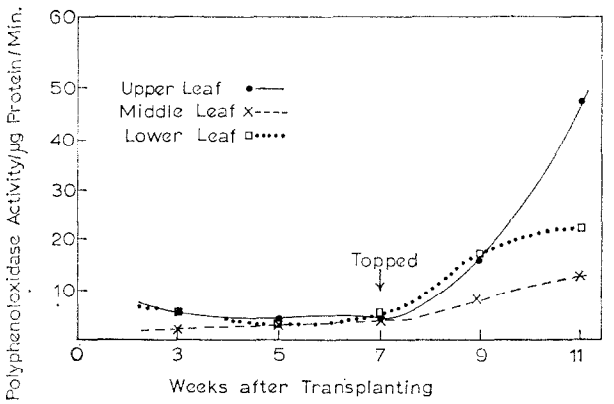


FIG. 3. POLYPHENOLOXIDASE ACTIVITY IN GREENHOUSE "F.C. 402" LEAVES.

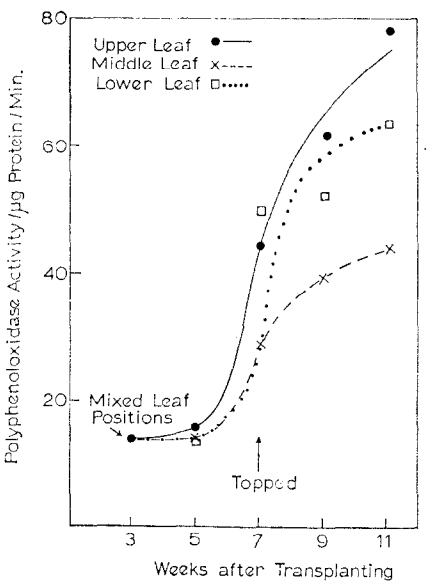


FIG. 4. POLYPHENOLOXIDASE ACTIVITY IN FIELD "F.C. 402" LEAVES.

plants matured in greenhouse and field "F.C. 402" leaves and in field "Ky. 16" leaves. PPO activity varied with stalk position in both greenhouse and field "F.C. 402" leaves 9–11 weeks after transplanting. Top leaves contained more activity than bottom leaves, and bottom leaves showed more activity than middle leaves. This relationship was not evident in field "Ky. 16" leaves sampled 11 weeks after transplantation (Table 3).

TABLE 3. POLYPHENOLOXIDASE ACTIVITY* IN FIELD "Ky. 16" LEAVES

| Weeks after transplant | Leaf position | Polyphenoloxidase activity/μg protein/min |
|---|---|---|
| 7 | Upper | 44·8 |
| 7 | Middle | 49·0 |
| 7 | Lower | 45·8 |
| 11 | Upper | 92·5 |
| 11 | Middle | 73·4 |
| 11 | Lower | 110·9 |

* A partially purified form of the enzyme was assayed by a spectrophotometric procedure employing 3,4-dihydroxyphenylalanine as substrate.

During curing, an initial rise of PPO activity in middle leaves lasted for 7–9 days; thereafter, PPO activity declined (Figs. 5 and 6). PPO activities in greenhouse "F.C. 402" plants were similar to those in field "F.C. 402" plants at corresponding dates of cure. However, PPO activity in field "Ky. 16" leaves was higher than that in "F.C. 402" at corresponding dates (Fig. 6). In both varieties, very low PPO activities were present after 33 days of conventional air-curing. To determine whether the initial rise in the specific activity of PPO

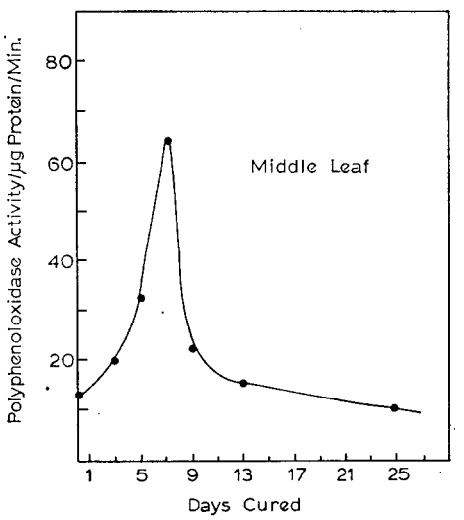


FIG. 5. POLYPHENOLOXIDASE ACTIVITY IN PRIMED, CURING-CHAMBER AIR-CURED GREENHOUSE "F.C.402" MIDDLE LEAVES.

during air-curing (Figs. 5 and 6) was caused by a differential rate of protein breakdown, comparable analyses were performed for PPO activity in terms of the weight of leaf tissue. There was a comparable transient increase for 7–9 days followed by a subsequent decline of activity in greenhouse and field "F.C. 402" and in field "Ky. 16" leaves.

In general, there was a direct correlation among PPO activities and amounts of individual phenols during the curing cycles (Figs. 1, 2, 5 and 6). However, the initial rise in PPO activity during curing was not correlated to the chlorogenic acids and rutin contents.

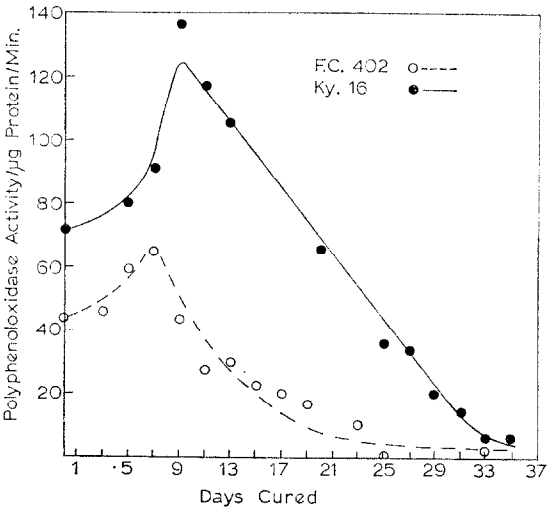


FIG. 6. POLYPHENOLOXIDASE ACTIVITY IN CONVENTIONALLY AIR-CURED FIELD "F.C. 402" AND "Ky. 16" MIDDLE LEAVES.

## DISCUSSION

An interesting difference between greenhouse and field plants in our experiments concerned the relative amounts of total and individual phenols in mature leaves from upper, middle and lower stalk positions. At harvest, greenhouse lower leaves contained more total phenols than did leaves from other stalk positions, while in field plants the upper leaves contained more total phenols. Further, greenhouse plants at harvest showed no significant correlation between stalk position and amounts of chlorogenic acids and rutin. However, field plants contained more chlorogenic acids and rutin in upper leaves than in lower leaves. This agreed with the results of Walker and Lee.[4] These findings may relate to the observation that greenhouse lower leaves do not appear senescent after flowering, while field lower leaves lose chlorophyll, appear to shrivel, and brown considerably at this time. Plant phenols in senescent leaves may undergo structural changes in browning reactions or enzymatic degradation prior to harvest.[7–9]

Amounts of total phenols did not vary with the length of air-curing, but chlorogenic acids and rutin declined with curing time. Total phenols were assayed by their ability to hydrogen bond to insoluble polyvinylpyrrolidone at pH 3·5.[10, 11] Thus, structural changes in

[7] H. E. WRIGHT, JR., W. W. BURTON and R. C. BERRY, JR., *Phytochem.* **3**, 525 (1964).
[8] H. E. WRIGHT, JR., W. W. BURTON and R. C. BERRY, JR., *Archs. Biochem. Biophys.* **86**, 94 (1960).
[9] T. J. WESTON, *Phytochem.* **7**, 921 (1968).
[10] R. A. ANDERSEN and J. R. TODD, *Tobacco Sci.* **12**, 107 (1968).
[11] R. A. ANDERSEN and J. A. SOWERS, *Phytochem.* **7**, 293 (1968).

soluble phenols during curing were probably not accompanied by loss of aromatic hydroxyl functional groups which hydrogen bond to PVP.[10,11] One explanation for the decline of individual phenols as curing progressed is their role in the formation of brown pigment.[7,8] It is difficult to speculate why total soluble phenols do not decrease concurrently with decrease of chlorogenic acids and rutin. It is thought that soluble plant phenols are covalently bound to protein moieties in brown pigment. Hence, they would not be expected to react with PVP at pH 3·5 unless aromatic hydroxyl groups are free to hydrogen bond and the free protanated amino groups of the protein moieties do not overcome this bonding.[10,11] The net amounts of total phenols may reflect amounts of lower molecular weight phenols and aglycones formed by the action of enzymes on soluble phenols, lignins and tannins during curing; these compounds may not enter into pigment formation. Further, they may continue to be synthesized during air-curing. Perhaps by these means the levels of phenols as determined by the non-specific assay for aromatic hydroxyl compounds remain unchanged. Further quantitative studies in progress which will compare levels of individual phenols and amounts of isolated brown pigment may resolve the relationships among brown pigment formation, chlorogenic acids and rutin during air-curing.[12]

Polyphenoloxidase activity was found to increase with plant age in our experiments. This increase did not appear to relate to the amounts of chlorogenic acids and rutin which are substrates for PPO, or to levels of total phenols which may be substrates. However, during the curing cycles PPO activities and amounts of chlorogenic acids and rutin generally declined (Figs. 1, 2, 5 and 6). The initial transient rise in PPO activity for 7–9 days was not, however, accompanied by similar increases of these phenols. It is difficult to offer an explanation for this transient rise which was also noted by other investigators who measured PPO activity in *Nicotiana tabacum* during air-curing[6] and flue-curing.[9] An explanation offered by Weston[9] for this rise in PPO was that it is a response to infection and pigment formation processes.

Our experiments suggest that the chemical composition and maturation of *N. tabacum* are changed by differences in the growth environment that exist between greenhouse and field experiments. Because it is difficult to duplicate conditions exactly between glasshouse and field growth, further experimentation is necessary to verify the following speculations about the lesser amounts of total phenols, chlorogenic acids, rutin and PPO in greenhouse plants as noted in our experiments. Rutin levels were particularly affected by growth under glasshouse conditions. Photocontrol of plant phenol synthesis was most probably a factor involved in these quantitative differences. Other investigators noted similar lower levels of chlorogenic acid and rutin in greenhouse plants,[1–3] and Lott[2] showed that u.v. light may be a critical factor. Greenhouse glass absorbs light less than 330 nm but transmits nearly all light of wavelengths 330 nm to beyond 800 nm.[13,14] Studies of the relative amounts of sun radiations reaching the earth indicate that a small proportion lies in the range 290–330 nm.[13] Light between 290 nm and 390 nm has been shown to influence plant phenol synthesis in other plant tissues.[15,16] While there is another action spectrum in the longer wavelength region,

[12] R. A. ANDERSEN, R. LOWE and T. VAUGHN, unpublished data.
[13] M. J. KASPERBAUER and W. E. LOOMIS, *Crop. Sci.* **5**, 193 (1965).
[14] Beckman Instruments, Inc. Bulletin 796A.
[15] J. M. ARTHUR, in *Biological Effects of Radiation* (edited by B. M. DUGGAR), Vol. II, p. 1109, McGraw-H ill, New York (1936).
[16] E. G. GROENWALD, P. LEE and J. A. D. ZEEV AART, Michigan State University, Atomic Energy Commission Plant Research Laboratory Report (1967).

namely, 600–750 nm,[17] radiations in this range are transmitted through glass.[14] Further experiments which would relate photocontrol to enzymes involved specifically in plant phenol (phenylpropane) biosynthesis such as phenylalanine ammonia lyase[18] might elucidate photocontrol mechanisms. Other differences between greenhouse and field growth which may have influenced growth and levels of plant phenols in our experiments include differences in overall light intensity, temperature, moisture, soil, soil leaching, and time of year. These factors need to be precisely controlled before any definitive conclusions can be drawn concerning the photocontrol.

## EXPERIMENTAL

*Plant Growth, Curing and Sampling Conditions*

A high phenol variety of *Nicotiana tabacum*, Ky. Iso 6 F.C. 402 ("F.C. 402") and a low content variety, Ky. Iso 1 Ky. 16 ("Ky. 16") were used.[6,19] Seeds were germinated and the seedlings grown to transplant size on vermiculite contained in 50-ml polypropylene tubes with a small opening at the bottom. The plants were watered by sub-irrigation with Hoagland's Nutrient Solution No. 1.[20] After 5 weeks the plants, about 10 cm in height, were transplanted.

In the greenhouse experiment, "F.C. 402" seedlings were transplanted to a soil floor on 1 February 1968. The soil had a high content of K and P; N was added at the level of 70 lb N/acre as $KNO_3$.

In the field experiment, "F.C. 402" and "Ky. 16" seedlings were transplanted to a field plot on 28 June 1968. The soil had a fertilization application of 140 lb N, 70 lb P and 200 lb K/acre.

The greenhouse and field plots consisted of ten rows of each variety of tobacco with trim plants around the periphery. Samples were taken at random on a given day from upper, middle and lower stalk positions of one plant in each of the rows. Border plants were not sampled. Samples were taken every 2 weeks from the time of setting to the final sampling date 11 weeks after transplanting. The tobacco was topped 7 weeks after transplanting, and 4 weeks later the tobacco was harvested for curing.

Middle leaves from greenhouse plots were primed and cured in a chamber maintained at 21° and 70% relative humidity. During the curing, samples of three to four leaves were collected at 2-day intervals during a 4-week period.

The field tobacco was air-cured on the stalks in a conventional Burley tobacco air-curing barn. Samples of two to three leaves were taken from the middle leaf positions of three plants of each variety at 2-day intervals during a 6-week period.

After sampling, midribs were removed and the leaf tissue was immediately freeze-dried and stored *in vacuo*.[21] Field samples were stored in dry ice during transfer to the laboratory.

*Total Phenols*

Total soluble plant phenols were determined by the method of Andersen and Todd.[10]

*Chlorogenic Acid, Rutin, Scopolin and Scopoletin*

Individual soluble plant phenols were determined by a paper chromatographic–spectrophotometric method as described by Sheen and Calvert.[5]

*Polyphenoloxidase* (PPO)

A partially purified PPO was prepared by a modification of the procedure described by Sheen and Calvert.[6] Plant material was homogenized in 0·15 M phosphate buffer at pH 6·8 containing 0·01 M ascorbic acid (15 ml/g) in a glass tissue grinder at 4°. The homogenate was immediately centrifuged at 20,000 *g* for 20 min at 0° in a Sorvall RC2-B Superspeed Centrifuge.* The residue was discarded. $(NH_4)_2SO_4$ was added to the supernatant fluid to make a final salt concentration of 10% (w/v). After centrifugation at 10,000 *g* at 0° the precipitate was discarded and the supernatant adjusted to a final concentration of 30% (w/v) $(NH_4)_2SO_4$.

* Trade names are given as part of the exact experimental conditions and not as an endorsement of products.

[17] H. W. SIEGELMAN and S. B. HENDRICKS, *Plant Physiol.* **33**, 185 (1958).
[18] H. SCHERF and T. N. ZENK, *Z. Pflanzenphysiol.* **57**, 401 (1967).
[19] G. W. STOKES, *Science* **141**, 1185 (1963).
[20] D. R. HOAGLAND and D. I. ARNON, *California Agr. Exp. Sta. Cir.* **347** (1950).
[21] C. J. KELLER and M. J. KASPERBAUER, *J. Agric. Food Chem.* **17**, 327 (1969).

The resulting precipitate was separated, dissolved in the same phosphate buffer at 4° used for the extraction and dialyzed overnight at 4° against water.

An aliquot of the dialysate was used to determine PPO by spectrophotometric measurement of the absorbancy change employing a final concentration of 0·022 M 3,4-dihydroxyphenylalanine as the substrate in 0·1 M phosphate buffer, pH 6, according to the method of Constantinides and Bedford.[22] Activity was determined in terms of the amount of protein present, or in terms of the amount of tissue used in the aliquot of dialysate used for the PPO assay. In the former case, protein was determined by the method Lowry *et al.*[23]

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[22] S. M. Constantinides and C. I. Bedford, *J. Food Sci.* **32**, 466 (1967).
[23] O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).