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CHANGES IN THE AMOUNTS OF CHLOROGENIC AND CAFFEIC ACIDS DURING TOBACCO SEED MATURATION

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; seed; maturation; chlorogenic acid; caffeic acid.

Abstract—During maturation of tobacco seed, the amount of chlorogenic acid rapidly increased between days 7 and 11 after anthesis and then decreased until day 15. Chlorogenic acid was not detected in fully mature seed. On the other hand, caffeic acid appeared from day 15, reached a maximum at day 21, and rapidly disappeared by day 25 although the amount was always low in comparison with that of chlorogenic acid. Changes in dry wt, water content, testa color and germination of tobacco seeds during maturation were also investigated. The results suggested that there were rapid biochemical changes in seed between days 11 and 15 after anthesis.

INTRODUCTION

Chlorogenic acid is the major phenolic of tobacco leaf [1, 2]. Previously, Sheen studied the distribution of phenolics in the various parts of the tobacco plant and detected large amounts in the flowers, especially in the anthers and pistils [3]. He also reported that the chlorogenic acid content successively increased during growth of flower and capsule until two weeks after flowering although no phenols were detected in the mature seed [4]. This raises the question of whether immature seeds, which occur inside the capsule, contain these compounds. In our studies on seed maturation, we detected two major compounds in the immature seeds which were absent from ripe seed. These compounds were identified as chlorogenic and caffeic acids after isolation in crystalline form. This finding led us to study how these compounds change during tobacco seed maturation.

This report deals with changes in chlorogenic and caffeic acids during tobacco seed maturation in relation to changes in dry wt, water content, testa color and germination of the seeds.

RESULTS

Some properties of seeds during maturation

In comparison with the extensive studies on seed formation in legumes and cereals, little is known about tobacco seed formation. Therefore the first of our experiments was conducted to obtain basic knowledge on tobacco seed maturation. Thus, in the case of *Nicotiana tabacum* cv BY-4, seed maturation occurred *ca* one month after anthesis. As shown in Fig. 1, the dry wt of a seed increased until 25 days after anthesis and then remained constant at *ca* 80 μg/seed. Rapid accumulation of dry matter in the seed occurred between 7 and 18 days after anthesis; by contrast, water content declined constantly through the maturation period and finally reached *ca* 7% in the mature seed. Thus the period from day 25 to 33 appears to be the drying stage in the ripening of tobacco seeds.

In terms of testa color (Fig. 1), 7 and 11 day-old seeds were white but gradually turned to yellow from day 11 to 18. By day 21, the testas of several seeds started to turn brown but most seeds turned brown on day 25 and became gradually darker until full maturation. A microscopic observation showed that seed size increased until day 15 but did not change

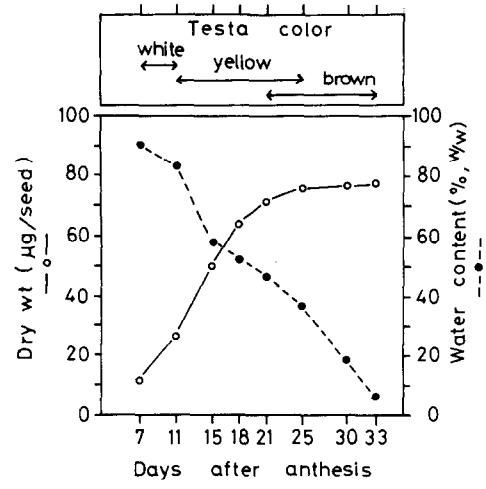

Fig. 1. Changes in dry wt, water content and testa color during tobacco seed maturation. Changes in testa color are located in the upper part of this figure.

after that (length × width; 0.39 × 0.30 mm at day 7 and 0.72 × 0.51 mm on day 15).

Using seeds at different stages, a germination test was carried out and the results are shown in Table 1. Very young seeds obtained from days 7 and 11 did not germinate, whereas *ca* 50% of the seeds from day 15 germinated. This result suggests that rapid biochemical changes occur in seeds between days 11 and 15 when expansion occurs. The ability to germinate successively increased until more than 90% of seeds germinated after day 28.

Changes in chlorogenic and caffeic acids during tobacco seed maturation

Quantitative determination of chlorogenic and caffeic acids in seeds was carried out by HPLC as described in the Experimental and the results are shown in Fig. 2. Chlorogenic acid content rapidly increased from day 7 to 11 after anthesis and then decreased from day 11 to 15. The content of the day 15 seeds was *ca* 25% of that of the day 11 seeds and continued to decrease from day 15 to 21 and chlorogenic acid was not detected in seeds after day 25. These results may indicate some correlation between ability to germinate and chlorogenic acid content. On the other hand, young seeds until day 15 lack caffeic acid. Caffeic acid appears on day 15, increases and reaches a maximum on day 21 and disappears on day 25. Thus, caffeic acid was only present between days 15 and 25 of seed maturation when the testa color is yellow. The production of caffeic and chlorogenic acids occurs in immature seeds of cultivars with white and yellow testa color (e.g. cv Shiroenshu, cv MC, cv Wisconsin 38) and also in immature seeds of *N. rustica*.

Table 1. Percentage germination of tobacco seeds at different stages of maturation

Days after anthesis	% germination
7	0
11	0
15	50.0
18	64.9
21	77.4
25	77.7
28	90.8
30	95.3
33	96.9

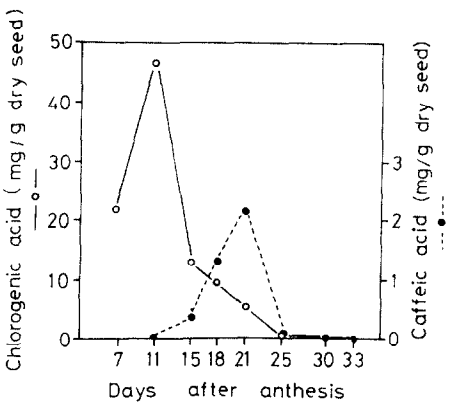

Fig. 2. Changes in chlorogenic and caffeic acid amounts in tobacco seed maturation.

DISCUSSION

Sheen reported that the quantity of chlorogenic acid increased rapidly with the onset of capsule growth and reached a maximum in the large green capsules two weeks after anthesis. The present experiments showed that the chlorogenic acid content in seeds reached a maximum 11 days after anthesis and this corresponds to Sheen's results on capsules. As seed growth accompanies capsule growth, the chlorogenic acid accumulation in the capsule is largely due to seed chlorogenic acid. Since brown colored capsules, containing mature seeds, contained very little chlorogenic acid in comparison with green capsules, Sheen suspected that the disappearance of chlorogenic acid is due to oxidative polymerization to produce precursors of the hard brown protective coat formed at the end of capsule development. However, the results presented here show that the rapid decay of chlorogenic acid in seeds occurs when the seeds are still white. This suggests that chlorogenic acid is not directly concerned with brown seed-coat formation. An interesting result is that caffeic acid appears after the rapid decrease in chlorogenic acid although it is present in a very small amount compared with the initial amount of chlorogenic acid.

EXPERIMENTAL

Sample preparation. Tobacco plants (*N. tabacum* cv BY-4) were grown in a greenhouse during early spring. Tobacco seeds were collected after anthesis according to the time schedule described in Figs. 1 and 2 and Table 1. Dry wt and H_2O content of the seeds were calculated from the wt before and after lyophilization. Each sample was homogenized with MeOH in a blender. After filtration, the residue was again extracted with 70% aq. MeOH. The filtrate and washings were combined and evaporated to dryness *in vacuo* below 40°. The residue was dissolved in H_2O and the insoluble materials filtered off. The aq. soln was stored at −20° until used.

Germination test. Ca 200 seeds collected from several capsules at different stages, were immediately placed on wet paper in Petri dishes and their germination measured after standing for 10 days at 28° under 12 hr illumination (2000 lx) a day.

Isolation procedure for chlorogenic and caffeic acids. Green capsules were collected from field-grown tobaccos and *ca* 500 g fr. wt immature seeds with yellow testas were extracted with MeOH as described above. After evaporation, the residue was dissolved in 300 ml H_2O. The aq. soln was adjusted to pH 3 with 1 N HCl and the phenolic acids extracted with EtOAc (300 ml × 3). The EtOAc extracts were combined, evaporated to dryness (3.6 g) and the residue dissolved in 30 ml hot H_2O. The soln was applied to a 3 × 30 cm column of Dowex 50 × 4(Na^+) and eluted with H_2O. Every 15 ml of eluent was collected. Each fraction was examined by Avicel TLC (*n*-BuOH–HOAc–H_2O, 4:1:2) and

Si gel TLC (MeOH–EtOAc, 1:1). Fractions 16–55 showed only one fluorescent spot by either Avicel (R_f 0.65) and Si gel TLC (R_f 0.35). Fractions 56–130 showed two fluorescent spots by either Avicel (R_f 0.76 and 0.65) or Si gel TLC (R_f 0.77 and 0.35). The former fractions were combined, acidified and extracted with EtOAc. Concn of the soln gave 500 mg solid. Three recrystallizations from EtOAc–hexane gave 100 mg needles of chlorogenic acid (**1**). The latter fractions were combined and extracted as above. The concd EtOAc soln was applied to a 1.5 × 20 cm Si gel column and **1** was separated from the second compound. Two recrystallizations from hot H_2O gave *ca* 22 mg yellow crystals of caffeic acid.

Quantitative determination of chlorogenic and caffeic acids. HPLC was performed on a Waters' 244 liquid chromatograph with a μBondapak C_{18} (300 × 4 mm i.d.) and a UV detector at 254 nm (0.5 AUFS). 15% (v/v) MeOH–0.1 N KH_2PO_4 was used as a solvent at a flow-rate of 0.5 ml/min. RR_ts of chlorogenic and caffeic acids were 21 and 33 min, respectively. Authentic standards of the two compounds gave a linear relationship in height in the range of 0–15 μg.

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