Studies on Sporopollenin Biosynthesis: The Effect of Inhibitors of Carotenoid Biosynthesis on Sporopollenin Accumulation

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1. The unequivocal chemical composition of sporopollenin is unknown. It was often hypothesized that sporopollenin represents a derivative of carotenoids and/or carotenoid esters. Proceeding from this working hypothesis the influence of inhibitors of carotenoid biosynthesis (DPA, nicotine, Sandoz) on the sporopollenin accumulation was studied in order to test whether an intact carotenoid metabolism is involved.

2. Drastic changes in carotenoid spectra as well as in carotenoid patterns in pollen extracts were used as a "marker" for the uptake and transport of the inhibitors into the anther loculus, the site of sporopollenin biosynthesis. Of the three inhibitors of carotenoid metabolism, DPA, nicotine and Sandoz, only Sandoz appeared to influence carotenoid metabolism in the anthers.

3. After application of Sandoz the sporopollenin accumulation in anthers and pollen was only slightly affected. The amount of sporopollenin after treatment is similar to, or possibly even marginally higher than the control, if dry weight is chosen as reference. If the amount is expressed with respect to one pollen grain, the content is very slightly decreased in the case of pollen from treated plants.

Therefore, it is concluded that severe interference in carotenoid biosynthesis does not result in a subsequent drastic inhibition of sporopollenin biosynthesis.

4. A significant increase of PAL activity under the influence of Sandoz indicates that effects upon phenylpropanoid metabolism are possible.

Introduction

The outer pollen wall, the exine, is one of the most complex wall systems in higher plants. It consists of sporopollenin, a substance of extraordinary resistance against chemical agents. It has been postulated that sporopollenin is a biopolymer composed of carotenoids and/or carotenoid esters. This hypothesis is supported by the similarity between naturally occurring sporopollenin and a polymer synthesized from carotenoids which was observed following elemental and spectroscopical analyses [1, 2]. Both substances also show similar resistance to acetolysis. In tracer experiments using [14]C- and/or ^{3}H-labelled acetate, mevalonate or β-carotene, substantial incorporation of label into a so-called sporopollenin-fraction was observed in several microbiological systems (ascospores of *Neurospora crassa*; zygospores of *Mucor mucedo*; [3−5]). The microbial evidence is supported by observations on *Chlorella fusca* which exhibits a correlation between the capacities to synthesize keto-carotenoids and sporopollenin. Mutants which lack the ability to produce keto-carotenoids are not able to accumulate sporopollenin [6, 7].

In spite of these results with microbial and unicellular algal systems, the chemistry of sporopollenin in higher plants is still an unresolved problem. Tracer experiments which were carried out upon higher plants and which were instrumental in the establishment of the carotenoid hypothesis give little unequivocal information [8, 9]. Therefore it is not surprising that there still exists a great uncertainty about the metabolic pathways which produce sporopollenin [10, 11].

Assuming, as a working hypothesis, that carotenoids and/or carotenoid esters are involved in biosynthesis of sporopollenin we applied known inhibitors of carotenoid biosynthesis in order to investigate whether an intact carotenoid biosynthesis system is a prerequisite for undisturbed sporopollenin accumulation.

Materials and Methods

Plant material

Seeds of *Cucurbita pepo* "Gelber Zentner" were purchased from Bruno Nebelung (Münster). The plants were cultivated in the greenhouse of the Botanical Garden of Münster at 19−25 °C under a light/dark period of 16 h/8 h; the light flux was 2500 lux. The plants were fertilized once a week (polyfertisal; Nebelung, Münster, 5 g/l).

Chemicals

Diphenylamine was obtained from Boehringer (Mannheim, FRG), and nicotine from Roth (Karlsruhe, FRG). Norflurazon (Sandoz 9789) was a kind gift from Sandoz AG (Switzerland). NADP$^+$ and glucose-6-phosphate were purchased from Boehringer (Mannheim, FRG).

Methods

The application of inhibitors of carotenoid biosynthesis

Inhibitors, partly previously dissolved in acetone (diphenylamine, Sandoz 9789), were applied in the appropriate concentration in a nutrient solution (composition see [12]). For the control the nutrient solution alone was applied. The application by a wick (Fig. 1) was the most efficient method. The following concentrations were tested:

10^{-4}M, 10^{-5}M, 10^{-6}M Sandoz
10^{-4}M, 2×10^{-4}M, 10^{-5}M diphenylamine
10^{-2}M, 10^{-3}M, 10^{-4}M nicotine.

All flower buds with anthers in postmeiotic developmental stages were removed at the beginning of the experiments. The first new flower buds developed one week after application of the experimental treatment.
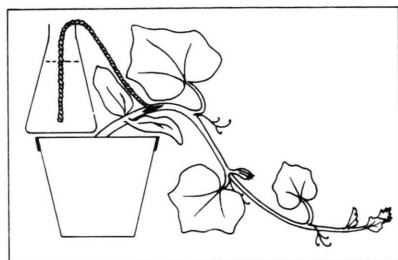

Fig. 1. Scheme demonstrating the method used to apply inhibitors.

Determination of the sporopollenin content

The sporopollenin content was determined gravimetrically. The sporopollenin was obtained by acetolysis [13]; in this case the entire acetolysis resistant material was considered to be sporopollenin. The plant material was harvested either one day before the flowers opened (anthers) or immediately after anthesis (pollen) and was then lyophilised. Following numerous preliminary experiments the purification procedure presented in Table I was established. If the experiments were performed strictly under the conditions as shown in this table the results were highly reproducible.

Table I. Procedure of the sporopollenin isolation.

40−100 mg pollen or ca. *200 mg anthers* (definite number); ground in 30 ml 96% acetic acid.
▼
Centrifugation (16000 × g; 10 min).
▼
All but 0.5 ml supernatant was discarded.
▼
Sediment was resuspended in the residual supernatant prior to the addition of 20 ml acetolysation medium*.
▼
The suspension was slowly (10 min) heated up to 100 °C.
▼
Treatment at 100 °C for 10 min; regular resuspension.
▼
The suspension was cooled down (10 min) to 4 °C, and then diluted with 10 ml 45% acetic acid (Banerjee, 1967) [29]; regular resuspension.
▼
Centrifugation (20000 × g; 15 min).
▼
All but 5 ml supernatant was discarded.
▼
Sediment was resuspended and diluted with 30 ml aqua dest.
▼
Centrifugation (16000 × g; 10 min).
▼
Sediment washed in sequences: 5 times with 30 ml aqua dest. (95 °C) 5 times with 30 ml methanol (56 °C) 3 times with 30 ml acetone 2 times with 30 ml diethylether.
▼
Dried at room temperature over phosphorpentoxide to a constant weight.

* Composition of the acetolysation medium: acetic anhydride/sulphuric acid: 8/2.

Carotenoid extraction

The extraction of carotenoids was performed according to [14]. The extracts were analysed by standard spectrophotometric procedures (Uvikon 810; Tegimenta, Rotkreuz (Switzerland)). Carotenoids were separated and purified according to [19].

Enzyme assay

Glucose-6-phosphate dehydrogenase activity was determined according to a method optimised by [15]. PAL activity was assayed by a technique described by Zucker [16].

Protein determination

Protein was determined either by the biuret method modified by [17] or by the method of [18]. Bovine serum albumin or ovalbumin "Turkey egg" were used as standards.

Results

Sporopollenin acccumulation during microsporogenesis and pollen ripening

In order to apply inhibitors most effectively it is of importance to know the developmental stages during which sporopollenin accumulation is most marked. The course of sporopollenin accumulation in anthers of *Cucurbita pepo* is shown in Fig. 2. Individual developmental stages have been characterised by cytological criteria. Extensive sporopollenin accumulation takes place between a phase immediately after the degradation of the tetrades when the pollen is only weakly sculptured (Fig. 2, stage 4) and the stage when binucleate microspores have developed (Fig. 2, stage 7).

Effects of inhibitors of carotenoid biosynthesis

It was necessary to determine whether the various inhibitors penetrate to the site of sporopollenin synthesis, *i.e.* the loculus of the anthers and especially the pollen. We have used inhibitor-stimulated changes in carotenoid biosynthesis as "markers" for the uptake and transport of the inhibitors into the anther loculus and to the pollen.

Therefore UV-spectra of carotenoid extracts of anthers and/or pollen were measured. In the presence of diphenylamine or nicotine neither bleaching of leaves and petals nor significant differences in carotenoid and sporopollenin contents could be ob-
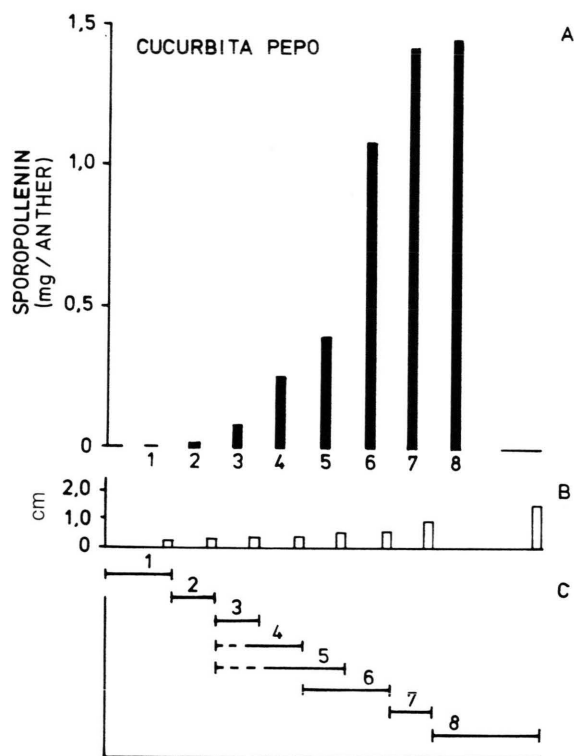

Fig. 2. Correlation between length of the anthers, stages of development and the amount of sporopollenin. A: sporopollenin accumulation; B: the length of the anthers; C: stages of development: 1: pollen mother cells, 2: meiosis, 3: tetrades in callose, 4: decay of the tetrades, 5: young pollen grains with a weakly sculptured exine, 6: mononuclear pollen grains, 7: two-celled pollen grains, 8: mature pollen grains.

served. Therefore, in order to study a possible correlation between carotenoid synthesis and sporopollenin accumulation, we restricted ourselves to the use of Sandoz, which, in contrast to the above-mentioned substances, caused clear effects when applied. The effects manifested themselves as extensive changes in the carotenoid spectra of the pollen extracts (Fig. 3). These changes are due to variations in the proportions of various carotenoids. As shown in Table II saturated carotenoids such as phytoene and phytofluene are found instead of desaturated pigments; only very small traces of yellow carotenoids are present.

Effects of Sandoz on sporopollenin accumulation

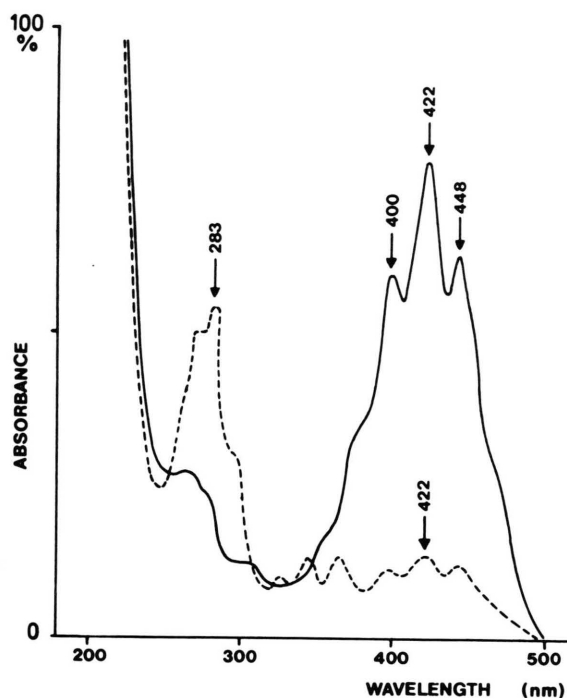Following the application of Sandoz extensive changes in the carotenoid spectra indicated a severe

Fig. 3. Absorption spectra of carotenoid extracts of pollen from treated (– – – –) and nontreated plants (———).

Table II. Carotenoid patterns of pollen extracts from treated and non-treated plants (*Cucurbita pepo* "gelber Zentner") (+: detected; –: non-detectable).

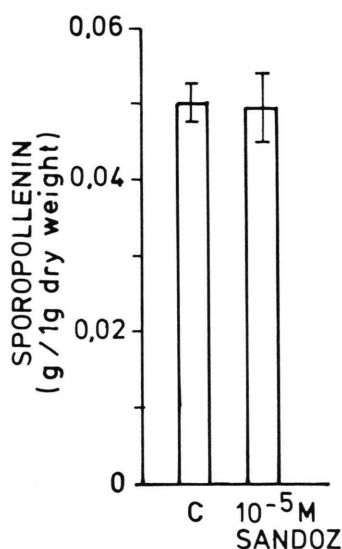| Carotenoid	Control	10^{-4} M Sandoz
phytoene	–	+
phytofluene	–	+
β-carotene	+	
cryptoxanthin	+	
β-carotene-5,6,5,6-diepoxide	+	traces
zeaxanthin	+	
antheraxanthin	+	
violaxanthin	+	
neoxanthin	+	

Fig. 4. Sporopollenin content of anthers of *Cucurbita pepo* after application of Sandoz expressed as a function of dry weight; C: control.
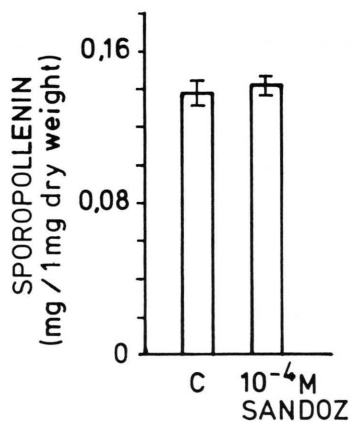

Fig. 5. Sporopollenin content of pollen from *Cucurbita pepo* after application of Sandoz expressed as a function of dry weight, C: control.

disturbance of the carotenoid metabolism in the anther loculus. This was considered as proof that the inhibitor had reached the site of sporopollenin synthesis, the loculus and pollen. By this, an important prerequisite for the study of the effect of Sandoz on sporopollenin accumulation was successfully fulfilled. No significant difference in the sporopollenin content was detected between anthers from treated and non-treated plants (Fig. 4). These investigations

were extended to an analysis of the pollen itself. Fig. 5 shows that the amount of sporopollenin after treatment is similar to, or possibly even marginally higher than the control, if the dry weight is chosen as a reference. If one expresses the amount of sporopollenin as μg/pollen grain the plants treated with 10^{-4} M Sandoz appear to contain slightly less sporopollenin (Fig. 6).
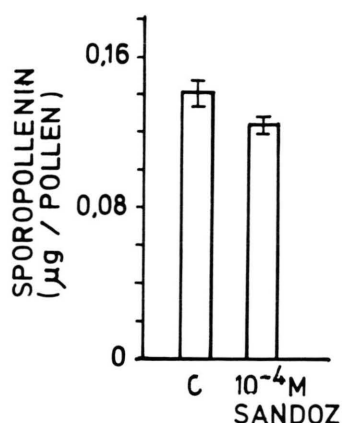
Fig. 6. Sporopollenin content of pollen from *Cucurbita pepo* after application of Sandoz with respect to one pollen grain; C: control.
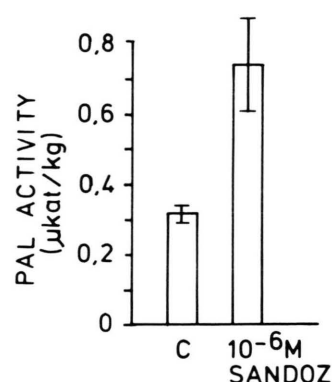
Influence of Sandoz on glucose-6-phosphate dehydrogenase (G6P-DH) and phenylalanine ammonia-lyase (PAL) activity

In order to study if the inhibitor affects other metabolic pathways, *e.g.* primary metabolism and phenylpropanoid metabolism, the activities of G6P-DH and PAL were measured. As shown in Fig. 7 no significant differences were observed in the G6P-DH activity between anthers from treated plants and control plants. In contrast, PAL activity is clearly influenced. Application of Sandoz results in a drastic increase of PAL activity in anthers (Fig. 8). This indicates that Sandoz may influence phenyl-propanoid metabolism, too.
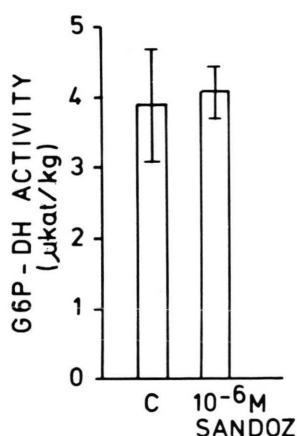

Fig. 7. Glucose-6-phosphate dehydrogenase activity of anthers of *Cucurbita pepo* after application of Sandoz; C: control.

Fig. 8. Phenylalanine ammonia-lyase activity of anthers of *Cucurbita pepo* after application of Sandoz; C: control.

Discussion

As stated in the introduction there are several observations, particularly data from labelling experiments, which do not unequivocally support the hypothesis that sporopollenin is derived from carotenoids and/or carotenoid esters [1–3, 8, 9].

Inhibitor studies have often been useful for the elucidation of metabolic pathways in plants. For example α-aminooxy-β-phenylpropionic acid (AOPP), a very potent inhibitor of phenylalanine ammonia-lyase (PAL) has been successfully used in the investigation of flavonoid metabolism [20–22]. Therefore as an obvious approach, it was studied by using inhibitors of carotenoid biosynthesis whether an intact carotenoid biosynthesis system is a prerequisite for undisturbed sporopollenin accumulation.

Of the three inhibitors of carotenoid metabolism supplied in the nutrient medium, DPA, nicotine and Sandoz, only Sandoz appeared to influence carotenoid metabolism in the anthers. The application of Sandoz resulted in extensive changes in the UV-spectra of the carotenoid extracts of pollen. In accordance with these findings we concluded that the inhibitor had been transported into the anther loculus, the site of sporopollenin biosynthesis.

The application of the inhibitor Sandoz hinders the desaturation of carotenoids. As a consequence saturated precursors such as phytoene and phytofluene accumulate [23–25]. The data obtained with pollen extracts support these results.

Since it was demonstrated by these results that the inhibitor penetrates to the site of sporopollenin

biosynthesis, the anther loculus, the question as to its influence on sporopollenin biosynthesis arose. As the results demonstrate, the effects caused by this inhibitor are very slight. The amount of sporopollenin after treatment is similar to, or possibly marginally higher, than the control, if dry weight is chosen as reference. Differences were minimal when the sporopollenin content is expressed as µg per pollen grain; in this case, the amount of sporopollenin was slightly lower. In order to ascertain the possible sporopollenin content of other anther wall systems, such as the tapetal and peritapetal walls [26], we also measured the sporopollenin content in whole anthers.

The results allow the conclusion that a complete carotenoid biosynthesis is not a prerequisite for an undisturbed sporopollenin accumulation. Our observations are in good agreement with those of Heslop-Harrison and Dickenson [27]. These authors were unable to detect carotenoids during the stages of pollen ripening when sporopollenin undergoes an intensive accumulation.

As Sandoz only affects final sequences in the pathway of carotenoid biosynthesis one cannot exclude the possibility that sporopollenin synthesis is supplied by a pool of saturated carotenoids or other precursors. Finally, it may be that unsaturated carotenoid synthesis is not completely abolished by Sandoz and that the traces of unsaturated carotenoids produced allow a normal, complete sporopollenin biosynthesis.

These uncertainties do not detract from our principal observation, that severe interference in carotenoid biosynthesis does not result in a subsequent drastic inhibition of sporopollenin accumulation.

A significant increase of PAL activity under the influence of Sandoz indicates that effects upon phenylpropanoid metabolism are possible. Similar results were reported by Reiss *et al.* [28].

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