

The Effect of Phytohormones and Sucrose Supply on the Induction of Tryptophan Decarboxylase in Developing Embryos of *Juglans regia* L.

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Z. Naturforsch. **45c**, 785–792 (1990); received February 2, 1990

Dedicated to Professor Wilhelm Menke on the occasion of his 80th birthday

Juglans regia L., Tryptophan Decarboxylase, Phytohormones, Enzyme Induction, Regulation

The *de novo* synthesis of tryptophan decarboxylase (TDC) (EC 4.1.1.27) in cotyledons of *Juglans regia* L. was accelerated at an earlier stage of development by harvesting immature fruits. After a lag phase of 6 h TDC activity became detectable in isolated cotyledons and reached values of about 5 nkat · g FW⁻¹ following a 24 h incubation in double-distilled water. The TDC synthesis was suppressed to about 20% by supplying the incubation medium with 2 to 6% sucrose. Phytohormones were also involved in the regulation of TDC synthesis. A highly sensitive reaction was observed with a 50% suppression resulting from an external supply of 1×10^{-8} M gibberellic acid (GA₃). Absciscic acid (ABA) was much less effective, reaching a similar level of suppression only at an unphysiologically high concentration of about 6×10^{-5} M. ABA, however, became one order of magnitude more effective when supplied together with sucrose. In contrast to GA₃ and ABA, an exogenous supply of indole-3-acetic acid (IAA) stimulated TDC synthesis significantly at a concentration of 1×10^{-7} M. From these *in vitro* studies and earlier measurements of internal ABA concentrations as well as incorporation of [³H]tryptophan into IAA, we hypothesize a regulatory effect of assimilate supply and phytohormones on TDC biosynthesis in developing embryos of walnut (*Juglans regia* L.).

Introduction

The enzyme tryptophan decarboxylase (TDC) is an adaptive enzyme which is synthesized *de novo* in the cotyledons of developing walnut seeds [1]. It is the first and the key regulating enzyme of serotonin (5-HT) biosynthesis, which leads in a 2-step-pathway from tryptophan (TRP) *via* tryptamine (TNH₂) to the indole moiety containing proto-alkaloid serotonin [2]. During seed development about 18 weeks after anthesis, the metabolical exchange between the tree and seed are cut off by the lignification of the endocarp, the TDC becomes detectable in the cotyledons, followed by 5-HT accumulation. In the kernels, 5-HT is seen as an ammonia detoxification compound [3–6] which may provide protection against predation. There-

fore, the regulation of 5-HT accumulation by TDC expression is of great interest. In addition, such studies will prove useful in elucidating the regulation mechanism of the synthesis of all indole alkaloids. From earlier results [2], we suppose that both the assimilate supply of the seeds or the plant hormone exchange between the tree and the seeds may suppress the metabolism of serotonin. The effects of sucrose as well as ABA, GA₃ and IAA upon the expression of the TDC activity during feeding experiments will be reported now.

Materials and Methods

Plant material

The seeds of *Juglans regia* L., used in experiments of varying incubation time, were supplied by the Botanical Garden of Cologne City (1984). For the remaining studies, seeds of *Juglans regia* L. were taken from trees thriving in the garden of the Botanical Institute of the University of Cologne (1988/1989). The fruits were harvested during a period of two weeks about 14 weeks after bloom, when the tissue inside the shell had formed the kernel. The husks were removed immediately from the nuts, the seeds were freed aseptically

Abbreviations: ABA, (+-)*cis-trans* absciscic acid; BSA, bovine serum albumin; DTT, 1,4-dithiothreitol; FW, fresh weight; GA₃, gibberellic acid; 5-HT, 5-hydroxytryptamine = serotonin; IAA, indole-3-acetic acid; TDC, tryptophan decarboxylase (EC 4.1.1.27); TNH₂, tryptamine; TRP, L-tryptophan; nkat (nanokatal), 10⁻⁹ mol substrate used per sec.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341-0382/90/0700-0785 \$ 01.30/0



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from the shells and integuments, and the cotyledons cut into pieces of about 0.05 to 0.1 g FW.

Incubation experiments

Aliquots of 2 to 2.5 g FW of cut cotyledons, mixed from 4 seeds, were placed in sterilized Erlenmeyer flasks (50 ml Duran; Schott, Mainz, F.R.G.), containing 10 ml of autoclaved, double-distilled water or 2% sucrose in water (sterilized by membrane filtration using Minisart 0.2 µm pore size; Sartorius, Göttingen, F.R.G.). For the feeding experiments the content of IAA, GA₃, ABA or sucrose was varied as described below. After 24 h of incubation at 25 °C in the dark on a rotary shaker (RO 20, Gerhardt, Bonn, F.R.G.) the cotyledon pieces were rinsed with double-distilled water, shock-frozen with liquid N₂ (−196 °C) and stored below −18 °C until their TDC activities were determined.

Extraction and determination of TDC activity

The cotyledons (2 to 2.5 g FW) were homogenized in the presence of 0.4 g polyvinylpyrrolidone (Polyclar AT), 2 g arenaceous quartz and 8 ml buffer solution (50 mM sodium phosphate, 1 mM pyridoxal 5-phosphate, 0.25 mM 1,4-dithiothreitol (DTT), pH 7.5) for at least 7 min at 0 °C. After spinning down insoluble material (15 min 48,000 × g, Sorvall RC-5B refrigerated superspeed centrifuge, Du Pont Instruments, Bad Homburg, F.R.G.), the lipids were extracted from the supernatant by a 1,1,2-trichloro-trifluoroethane (Freon 113) treatment. The proteins were then precipitated by 50% ammonium sulfate (0 °C, saturated solution), sedimented and resuspended in 3 ml buffer solution. The protein content of each enzyme extract was determined according to Bradford [7].

The activity of the TDC was assayed by measuring the rate of TNH₂ formation in the presence of pargyline hydrochloride, an inhibitor of mono-amino oxydases. The assays were performed with a buffer substrate mixture containing 28 mM Tris/acetate (pH 7.5), 29 mM L-tryptophan, 29 mM pargyline hydrochloride, 0.18 mM DTT, 29 µM pyridoxal 5-phosphate, 4.6 mg BSA · ml^{−1}, and enzyme extract of up to 0.6 mg protein · ml^{−1}. After 2 h at 37 °C the incubation was stopped by adding 1.5 N hydrochloric acid to a final concentration of 4%,

and centrifuging 5 min at 8000 × g (centrifuge type 5413, Eppendorf, Hamburg, F.R.G.).

Serotonin (5-HT) content and TDC activity calculated from the TNH₂ content in the supernatant were determined by HPLC according to Grosse *et al.* [2] but using a flow rate of 1.5 ml · min^{−1} and methanol instead of acetonitrile for TNH₂ measurements.

Results

From earlier studies we know that 5-HT accumulation starts with a lag phase of more than 6 h in cotyledons of seeds which are harvested just after finishing their milky stage [2]. During an incubation period of 24 h an increase of the TDC activity occurred (Fig. 1), similar to the 5-HT accumulation observed earlier [2]. A maximal value of 430 ± 80 nmol 5-HT · g · FW^{−1} and a specific TDC activity of 2.9 ± 0.2 nkat · g FW^{−1} were detected within seeds from a tree thriving in the Botanical Garden of Cologne City (Fig. 1). Within seeds of a different cultivar, thriving in the garden of the Botanical Institute, the specific activity was nearly twice as high (Fig. 2A).

Due to the decrease of the soluble proteins during incubation (Table I), the FW of the cotyledonary tissue at the start of the experiments was used to calculate specific activities. The change in the

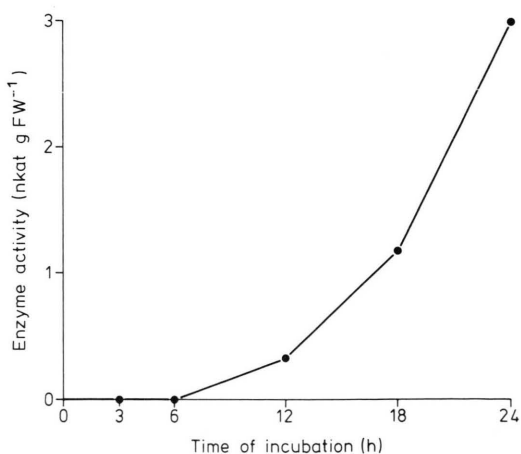


Fig. 1. Synthesis of tryptophan decarboxylase in cotyledons of immature *Juglans regia* L., following the time course of incubation in double-distilled water at 25 °C in the dark. Specific activity (nkat · g FW^{−1}) was determined at the end of several time intervals of incubation.

content of extractable proteins was similar, regardless of the incubation media or additives used.

The biosynthesis of TDC was partially suppressed when cut cotyledons were incubated in solutions of 2 to 6% sucrose supplied to double-distilled water. After 24 h of incubation the TDC activity reached only 20 to 35% of that obtained from incubation without sucrose (Fig. 3), but no significant differences in suppression resulted from varying the sucrose concentration.

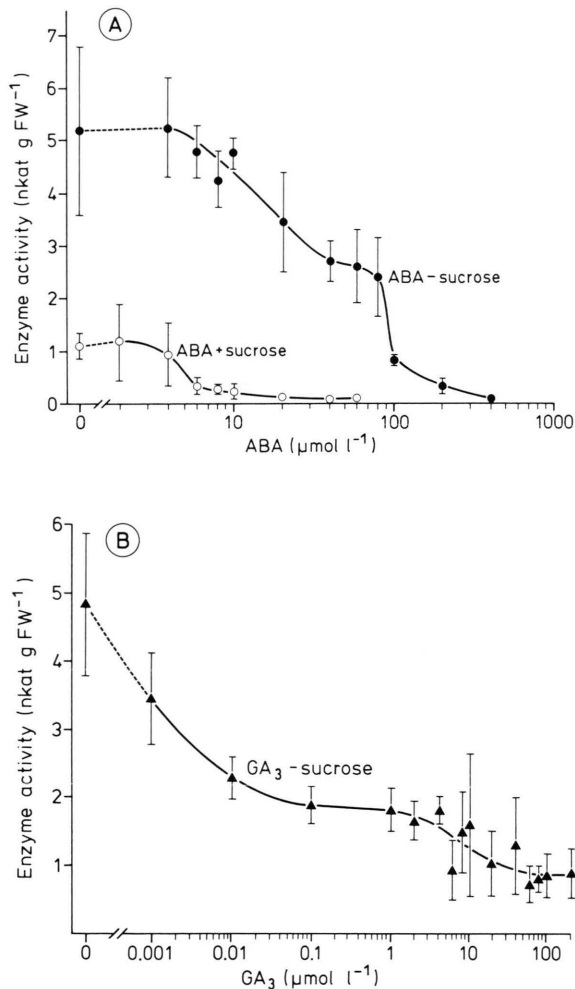


Fig. 2. Effect of exogenous ABA and GA₃ upon the synthesis of TDC in cotyledons of immature seeds of *Juglans regia* L. The specific activity of TDC in the cotyledons as determined after a 24 h incubation at 25 °C in the dark: (A) ABA in double-distilled water (●---●) or aqueous sucrose (2%) (○---○), and (B) GA₃ in double-distilled water (▲---▲). Specific activities are given as a mean value ± the S.D. of 5 replicates.

The amount of TDC activity detectable in the cotyledons after a 24 h period of incubation was affected significantly when different phytohormones were supplied to the basic incubation media. As shown in Fig. 2, the synthesis of TDC activity was reduced by ABA as well as GA₃, while the expression of TDC activity was negatively correlated with the concentrations of exogenous ABA and GA₃. After the 24 h incubation of the cotyledons in aqueous solutions of ABA, a moderate decrease of specific activity, corresponding to the increase of exogenous ABA, was obtained over a wide range of ABA concentrations. At concentrations higher than 8×10^{-5} M a rapid decrease of activity occurred (Fig. 2A). The effective concentration of ABA for 50% suppression of the TDC synthesis was about 6×10^{-5} M, while maximal inhibition was observed at 4×10^{-4} M of exogenous ABA (Table II). With a 2% sucrose basic medium, although there was a lower initial TDC activity, a similar decrease in enzyme activity occurred even when lower concentrations of ABA were supplied to the medium (Fig. 2A). Under these conditions, a 50% suppression of TDC activity was observed at about 6×10^{-6} M ABA and minimal values of specific activity (0.11 ± 0.01 nkat · g FW⁻¹) at an exogenous ABA concentration of 4×10^{-5} M (Ta-

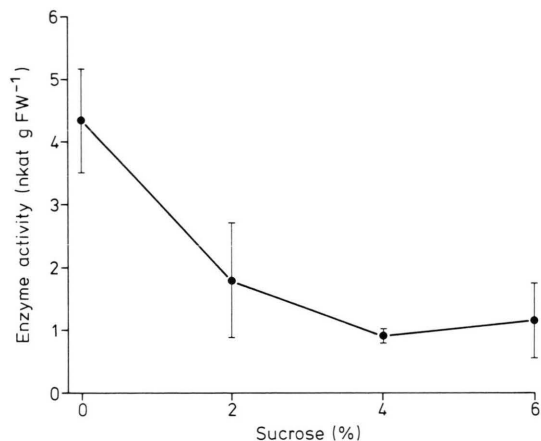


Fig. 3. Effect of exogenous sucrose upon the induction of TDC activity in cotyledons of immature seeds of *Juglans regia* L. harvested about 14 weeks after anthesis. Specific activity of TDC was determined after a 24 h incubation in double-distilled water containing several concentrations of sucrose (% w/v). The values are given as a mean value ± the S.D. of 5 replicates.

Table I. The effect of incubation on the content of extractable proteins in cotyledons of maturing seeds of *Juglans regia* L. harvested during a period of two weeks starting about 14 weeks after anthesis. Cotyledons were isolated aseptically and incubated for 24 h at 25 °C in the dark in double-distilled water (A) or 2% sucrose in water (B) with varying contents of phytohormones. Control = content of protein without incubation; evaluations of protein content are given as mean \pm S.D.

Additions	Concentration [M]	Soluble proteins [mg · g FW ⁻¹]	n	Amount relative to control [%]
Control		3.0 \pm 0.4	31	100
A none	—	2.2 \pm 0.2	21	73
GA ₃	4 \times 10 ⁻⁶	2.3 \pm 0.2	5	77
ABA	4 \times 10 ⁻⁴	2.0 \pm 0.1	5	67
IAA	1 \times 10 ⁻⁵	2.4 \pm 0.2	5	80
B none	—	2.0 \pm 0.2	12	67
ABA	4 \times 10 ⁻⁵	2.5 \pm 0.1	4	83
IAA	1 \times 10 ⁻⁵	2.2 \pm 0.2	4	73

ble II). Since the minimal values of TDC activity after ABA incubation with or without sucrose were in the same range, the reported data show that the effective concentrations of ABA was shifted to 10-fold lower values when the metabolite sucrose was present (Fig. 2A).

In contrast to the general growth stimulating effect of the gibberellins, our results underscore the well known suppressing effect of GA₃ upon the metabolism of secondary plant products. The amount of TDC activity, accumulated during 24 h

of incubation, was reduced to 50% by less than 1 \times 10⁻⁸ M exogenous GA₃ (Fig. 2B). Thus, the effective concentration for 50% suppression seems to be 4000-fold lower for GA₃ compared to ABA under the same conditions. Nevertheless, the suppression of TDC activity at higher concentrations of GA₃ was not as drastic as for ABA and led to a final value of 0.9 \pm 0.3 nkat · g FW⁻¹ at 6 \times 10⁻⁵ M exogenous GA₃ (Table II). No further decrease in TDC activity could be detected at GA₃ concentrations up to 2 \times 10⁻⁴ M.

Table II. The 50% suppression and maximal effect of ABA, GA₃, IAA and sucrose upon the synthesis of TDC activity in cotyledons of maturing *Juglans regia* L. seeds. The fruits were harvested during a period of two weeks starting about 14 weeks after anthesis. Cotyledons were isolated aseptically and incubated for 24 h in double-distilled water (A) or 2% of sucrose in water (B) with different additions. Values of specific activity are given as mean values \pm S.D. n = number of replicates. Changes in relative amounts (\pm [%]) are related to the specific activities developed during incubations in water (A) or 2% sucrose (B) without additives, respectively.

Additions	Concentration for		n	Specific activity (maximal effected)	
	50% suppression	maximal effect		[nkat · g FW ⁻¹]	relative amount \pm [%]
A sucrose	2%	4%	5	0.80 \pm 0.20	-80
ABA	6 \times 10 ⁻⁵ M	4 \times 10 ⁻⁴ M	5	0.11 \pm 0.02	-98
GA ₃	1 \times 10 ⁻⁸ M	6 \times 10 ⁻⁵ M	5	0.90 \pm 0.30	-85
IAA	—	1 \times 10 ⁻⁷ M	5	10.00 \pm 1.00	+35
B ABA	6 \times 10 ⁻⁶ M	4 \times 10 ⁻⁵ M	4	0.01 \pm 0.01	-91
IAA	—	1 \times 10 ⁻⁷ M	4	3.40 \pm 0.60	+115

Experiments carried out with different amounts of exogenous ABA and GA₃ used simultaneously show that the inhibitory effect of ABA and GA₃ upon the synthesis of TDC was very similar when 50 to 100 × 10⁻⁶ M ABA or GA₃ in aqueous solution was added to the cotyledons (Fig. 4). During the 24 h incubation no differences in the relative amounts of TDC activity could be found when ABA concentrations of 10 to 50 × 10⁻⁶ M were supplied to the GA₃ containing media. Only the addition of 100 × 10⁻⁶ M ABA to 100 × 10⁻⁶ M GA₃ clearly decreased the activity of TDC relative to either phytohormone alone. In this case the specific activity reached 0.3 ± 0.1 nkat · g FW⁻¹, 6% of that of the incubations in the aqueous basic medium without phytohormone supply (Fig. 4).

As shown above, a distinct inhibition of the synthesis of the TDC was obtained in 24 h incubations with ABA, GA₃ and sucrose, respectively. On the other hand, a significant enhancement of the expression of TDC activity was detected when indole-3-acetic acid (IAA) was supplied to the incubation media. The incubation of cotyledonary tissue in water containing several amounts of IAA increased the specific activity of TDC up to 138%

relative to the specific activity detected in water without IAA (Fig. 5). The maximum value of TDC activity was 10 ± 1 nkat · g FW⁻¹ at a concentration of 1 × 10⁻⁷ M of exogenous IAA (Table II). When supplied to the 2% sucrose medium, IAA caused an increase in TDC activity of up to 215% (Fig. 5), relative to the values reached after 24 h incubation in the sucrose medium alone. The maximum value of specific activity detected was 3.4 ± 0.6 nkat · g FW⁻¹ (Table II). As opposed to the results of the investigations done with GA₃ and ABA, no concentration-dependent effect of IAA upon the synthesis of TDC could be observed.

Discussion

Growth and expression of secondary plant products are often countercurrent processes in the development of plants. In cotyledons of developing walnut seeds, the differentiation of the tissues after the milky stage is followed by an accumulation of storage compounds. The vacuoles are replaced by oleosomes and protein bodies, while starch is synthesized in the amyloplasts of the coty-

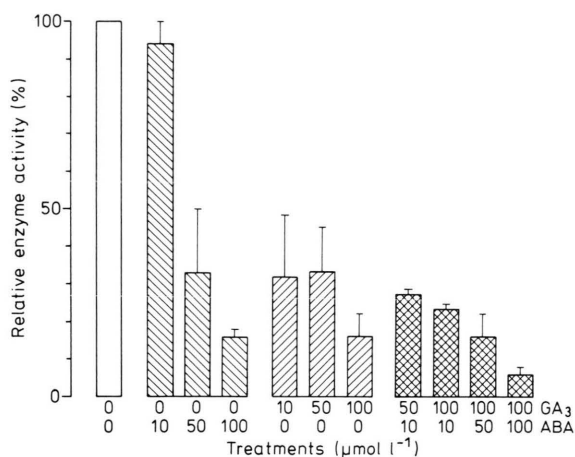


Fig. 4. Interacting effects of exogenous GA₃ and ABA in aqueous solution upon the TDC activity developing in cotyledons of immature walnut seeds during incubation. The enzyme activities were determined after 24 h of incubation (25 °C, dark) in media supplied with different concentrations of GA₃ or/and ABA. TDC activity is reported relative to the specific activity developed during incubation in double-distilled water (= 100% activity). Relative enzyme activities are given as a mean value ± the S.D. of 5 replicates.

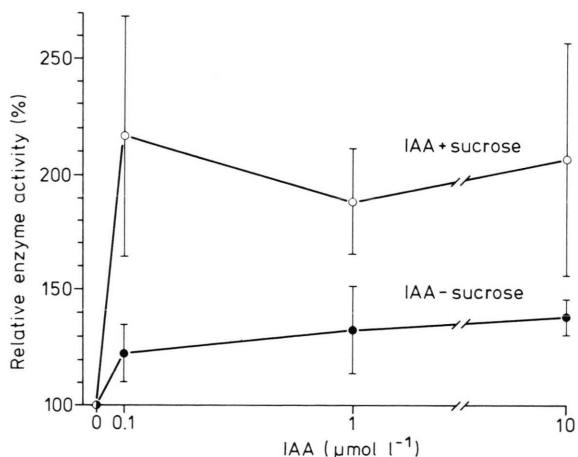


Fig. 5. Effect of exogenous IAA and its dependence on the presence of sucrose (2%, w/v) upon the synthesis of TDC activity in cotyledons of immature seeds of *Juglans regia* L. The specific activity was determined after 24 h of incubation (25 °C, dark) in double-distilled water or aqueous sucrose (2%, w/v), containing several amounts of IAA. TDC activity is reported relative to the specific activity developed during incubation in double-distilled water or aqueous sucrose (2%, w/v), respectively (= 100%). Relative enzyme activities are given as a mean value ± the S.D. of 5 replicates.

ledonary tissue [6]. At this stage of development the TDC activity and accumulation of 5-HT [8] into the protein bodies [6] were not yet detectable in the kernels. Later, while the physiological abscission of the seeds was occurring by lignification of the endocarp, TDC was synthesized *de novo* [1] and 5-HT accumulation started [2]. Their high ammonia as well as TRP content did not accelerate the expression of the 5-HT metabolism.

When the fruits were harvested just after solidification of the cotyledons, TDC activity and 5-HT accumulation [2] were observed in the embryos with a lag phase of about 6 h after isolation. We conclude, therefore, that the assimilate supply of the seeds is important for suppression of 5-HT metabolism. This is in accordance with both the high TDC activity detected in isolated cotyledons after incubation for 24 h in water, and the low TDC activity developed in cotyledons incubated in aqueous sucrose solution. On the other hand mineral nutrients seem to effect the expression of TDC synthesis less. The TDC activity in the cotyledons was in the same range, when water is replaced in the incubation experiments by a mineral culture medium as described by Murashige and Skoog [9], but without any organic additives.

Cereals, rapeseeds and soybeans are reported to germinate when the immature embryos are isolated and cultivated with a 1 to 3% sucrose supply. When the osmolarity of the culture medium is increased by adding more sucrose, mannitol or sorbitol up to a final concentration of about 10% w/v, this precocious germination is prevented and the accumulation of storage proteins continues [10–13]. In the incubation experiments with immature cotyledons of walnut seeds, we observed a suppression of TDC synthesis at 2% sucrose, while raising the sucrose concentration to 6% did not change the level of suppression. We suggest, therefore, that the suppressing effect upon the synthesis of TDC is the result of sucrose supply itself, simulating natural conditions at the tree, regardless of the changes of the osmolarity of the incubation medium. As long as an assimilate transport from the tree to the seed is established or the isolated cotyledons are supplied by exogenous sucrose, the biosynthesis of storage material may go on and the metabolism of secondary plant products will be suppressed. When the sucrose import is ceased naturally by lignification of the endocarp or arti-

cially by harvest, TDC biosynthesis is expressed and TRP, which will be needed no longer for storage protein formation, can be converted to 5-HT.

The stimulation of TDC biosynthesis [14–16] and indole alkaloid production [14, 17] by sucrose in nutrient-deficient cell suspension cultures of *Catharanthus roseus* is not contradictory to our findings. In these cell cultures TDC is constitutive at a low level, and its activity is increased by sucrose during mineral nutrient deficiency. This higher alkaloid production may result from an improved carbon skeleton supply by exogenous sucrose. These findings may delineate the differences in the expression of the secondary metabolism in storage organs and the stimulation of enzyme biosynthesis in cell suspension cultures.

It is well established that phytohormones are involved at many different levels of plant embryogenesis, and they may take part in the regulation of secondary metabolism. ABA promotes the synthesis of seed-specific storage proteins and suppresses both premature germination of the embryo and enzymes specific for germination [11, 18, 19]. In embryos of *Juglans regia* the ABA content is low in immature cotyledons about 14 weeks after anthesis. It leveled off at about 4 nmol of ABA per g FW embryo during the following two weeks, remained nearly constant for the next four weeks, and declined in the final four weeks of fruit ripening to about 1 nmol per g FW in the fully developed embryo [20]. In concert with the concept that the ABA content and water status of the embryo act together to prevent precocious germination [10], it was assumed that ABA in developing walnut seeds may perform the same function by reducing the water uptake of the embryo [20].

When the fruits remained attached at the tree, the TDC activity became detectable during the phase of highest ABA concentration without any remarkable changes in the ABA content at that time. We believe, therefore, that under natural conditions ABA is primarily responsible for storage protein biosynthesis, and not for expression of 5-HT metabolism. Our experiments with supplementary exogenous ABA underscore these suggestions. When external ABA concentrations are low, not significant differences in TDC expression were obtained. This may result from varying internal ABA concentrations. Only when external ABA concentrations were unnaturally high a decrease in

TDC activity was observed with increasing ABA concentrations.

Gibberellins are generally reported at much higher concentrations in immature seeds than in other plant tissues, but nothing is known about their specific function in the seeds (*cf.* [21]). In peas, a function for GAs was suggested for early embryo development, but the main increase in GA content was obtained when the embryo was fully developed (*cf.* [22]). Wang and Sponsel [23] have correlated this increase of GAs with the mobilization of assimilates during the stage of rapid seed growth. In contrast to this effect upon the primary metabolism, we hypothesize that the effect of GA₃ in immature walnut seeds, represented by a reduced TDC synthesis, indicates a sensitive mechanism in suppressing a pathway of secondary metabolism. A 50% suppression of TDC synthesis occurred even at exogenous GA₃ concentrations lower than 1×10^{-8} M, while a similar effect for ABA could be observed only at a 4000-fold higher concentrations (Table II), unusually high for phytohormone-induced effects. Regardless of the high sensitivity of the cotyledonary tissues to GA₃ at low concentrations, even high concentrations of GA₃ produces only incomplete suppression. As described for peas, biologically active GA₂₀ accumulates during the period of rapid growth leading to maximal values near to the attainment of maximum FW. On the other hand, bioactive GAs normally begin to decline from their maximum, when the ABA content increases (*cf.* [21]). Considering the increasing ABA content within the developing walnut seed [20], we suggest that the only partial suppression of TDC may result from an inactivation of some of the exogenous GA₃ following uptake, in addition to the potentially limiting rates of GA₃ uptake and transport to the center of the incubated cotyledon pieces. A higher degree of suppression was observed when in addition to GA₃ an unphysiologically high amount of ABA was supplied to the incubation medium (Fig. 4). This points out that GA₃ and ABA are cumulative, inhibiting TDC expression at different levels of metabolism.

In contrast to GA₃ and ABA the phytohormone IAA stimulates TDC expression. The degree of stimulation is relative low with the water incubation experiments, but much greater when expression is limited by the sucrose supply (Fig. 5). We

think that the expression was near its top level in the water incubation and could not be stimulated substantially. The inhibiting effect of sucrose, however, was neutralized by auxins at external concentrations of 1×10^{-7} M. Thus, these results give evidence for an inductive effect of IAA upon the synthesis of TDC *in vitro*.

Studies on tryptophan transaminase, and the incorporation of [³H] into IAA after the supplementation of immature seeds of *Juglans regia* L. [2] with exogenous [³H]Trp suggest that the biosynthetic pathway of IAA from TRP *via* indole-3-pyruvic acid and indole-3-acetaldehyde was present even in the immature cotyledons. Thus, we assume that TRP, due to its decreased incorporation into proteins when the synthesis of storage proteins declined, accumulates to high enough values to overcome the unfavorable substrate specificity of the tryptophan transaminase (unpublished results). Hence, the synthesis of IAA will be enhanced while the biochemical pathway to 5-HT is blocked by the absence of TDC. IAA, therefore, may be involved in TDC induction *in vivo* as well.

In summation, the present results point out that the supply of nutrients like sucrose can suppress the expression of the secondary metabolism, represented by TDC synthesis and 5-HT metabolism. While ABA may be involved in the regulation of embryo growth and in preventing precocious germination, the role of GA₃ may consist mainly in supporting early embryo growth, but also in suppressing TDC synthesis in the early maturation stage of the seed, thereby keeping the TRP available for storage protein formation. At the start of the desiccation period, the lack of an assimilate supply along with an increased IAA synthesis, promoted by TRP accumulation following the cessation of storage protein synthesis, may induce the 5-HT biosynthetic pathway for ammonia detoxification.

Acknowledgement

We are indebted to E. Moll, Botanical Garden of the City of Cologne for the seed supply, and to J. Frye, Department of Environmental Sciences, University of Virginia, U.S.A., for reviewing the manuscript. The financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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