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SHIKIMATE ACCUMULATION IN GLYPHOSATE-TREATED BUCKWHEAT COTYLEDONS: EFFECT OF THE PREILLUMINATION OF THE SEEDLINGS

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Abstract. The rate of shikimic acid accumulation in excised cotyledons of etiolated buckwheat seedlings during their incubation with glyphosate was markedly higher in the light than in the dark. In seedlings preilluminated before glyphosate treatment, the extent of light/dark differences depended on the duration of the preillumination. The accumulation rate of shikimic acid during incubation with glyphosate in the light increased substantially when the preillumination was prolonged, reaching its maximum at a 16-h preillumination; further prolongation of preillumination weakened this stimulatory effect. In case of dark incubation no such increase was observed. The coefficient of light effect, calculated as a ratio of the accumulation rates of shikimate in the light and in the dark, increased with the duration of preillumination. The results give evidence of two different mechanisms of light action that control the operation of the shikimate pathway in greening buckwheat cotyledons.

Key words: *Fagopyrum esculentum* Moench, glyphosate, light, shikimic acid pathway.

It has long been known that in etiolated seedlings a transient enhancement of the synthesis of phenolic compounds derived from L-phenylalanine, such as hydroxycinnamic acids and flavonoids, takes place after the onset of illumination (Scherf & Zenk, 1967; Beggs et al., 1987; Brödenfeldt & Mohr, 1988). In search of the regulatory mechanisms of light-induced phenolic biosynthesis, earlier investigations have been focused mainly on the concomitant induction and subsequent activity changes of the enzymes of phenylpropanoid and flavonoid pathway in illuminated seedlings (Scherf & Zenk, 1967; Amrhein & Zenk, 1970; Hahlbrock et al., 1976; Ulbrich et al., 1976; Brödenfeldt & Mohr, 1988). However, a number of reports (for a review see Margna, 1977) suggest that phenylalanine supply might be the limiting factor controlling the level of phenolic biosynthesis in several plant objects. Phenylalanine is one of the major products of the shikimic acid pathway. Recent findings that the activity of the first enzyme of the pathway, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase (EC 4.1.2.15), can be induced by wounding, elicitor treatment, and light (references in Schmid & Amrhein, 1995) suggest that the formation of phenylalanine might also be

regulated by environmental stimuli. Indeed, light stimulation of the activity of the pathway has been found in etiolated buckwheat hypocotyls (Holländer et al., 1979; Amrhein & Holländer, 1981) and cotyledons (Тохвер & Пальм, 1986; Margna et al., 1989). Moreover, a closer examination of illumination effects on shikimate accumulation in buckwheat seedlings after blocking the pathway with its specific inhibitor, glyphosate, has led us to the conclusion that in buckwheat cotyledons, besides a photoactivation of the shikimic acid pathway (as judged by light-dependent differences in shikimate accumulation in glyphosate-treated tissues), light affects the operation of the pathway by an additional mechanism that is susceptible to glyphosate treatment (Laanest & Vainjärv, 1992). The nature of this phenomenon has remained unclear.

In the present contribution an attempt is made to get an insight into the changes in the operation of the shikimic acid pathway in etiolated buckwheat cotyledons after the beginning of illumination when the most pronounced changes in the accumulation rate of phenolic substances take place. The aim of the work was to differentiate between the possible mechanisms of light action on the shikimate pathway.

MATERIALS AND METHODS

The experiments were carried out with etiolated buckwheat (*Fagopyrum esculentum* Moench cv. Victoria) seedlings grown in darkness at 25 °C in tap water. For determining the time course of shikimic acid accumulation in glyphosate-treated cotyledons, the cotyledons were excised from 80-h-old etiolated seedlings, placed into Petri dishes on filter paper moistened with 2 mM solution of glyphosate, and incubated either in a light chamber (light from fluorescent tubes of white light, 30 Wm^{-2}) or in darkness at 25 °C. The content of shikimic acid was estimated at certain time intervals.

In preillumination studies, seed coats were removed from 80-h-old etiolated seedlings and the seedlings were illuminated in the phytotron (30 Wm^{-2}). After 6, 12, 16, 24, 48, and 72 h the cotyledons were detached from seedlings, transferred into Petri dishes to 2 mM solution of glyphosate, and incubated either in the light or in the dark. The content of shikimic acid was estimated after 2 and 8 h of incubation and its accumulation rate was calculated from the difference. So, accumulation curves principally similar to those presented in Fig. 1 were used to derive the data presented in Fig. 2. The main difference was that (except for the first data points corresponding to etiolated seedlings) now the zero point of the curves was preceded by a preillumination period of different duration.

Shikimic acid was assayed in the fresh material which was ground with a small amount of ethanol and then extracted first with dilute ethanol (1 : 1 v/v) and thereafter with hot water. The combined extract was clarified by adding some more ethanol, centrifuged, evaporated to dryness in air stream at room temperature, and the dry residue was dissolved in a small volume of distilled water. The resulting mixture was applied to a column of polycaprolactam and the alicyclic acids were eluted with water. The content of shikimic acid in the effluent was determined according to Gaitonde & Gordon (1958). This method gives a colour reaction with both shikimic and quinic acid. However, it was established previously that the content of quinic acid in buckwheat seedlings was close to the lowest limit of the assay (Тохвер & Пальм, 1986) and did not interfere with the shikimic acid determination.

The experiments were run in four replicate series, three sets of 20—25 pairs of cotyledons in each.

RESULTS

The accumulation of shikimic acid in the cotyledons of buckwheat seedlings incubated with glyphosate started without an apparent lag phase both in the light and in the dark and proceeded at a constant rate for 12 to 15 h (Fig. 1). Such a prolonged linearity indicates that the accumulation rate of shikimic acid in glyphosate-treated tissues, characterizing the process during at least the first 12 h of treatment, can indeed serve as a criterion of the potency of the shikimic acid pathway at the onset of the treatment. However, as shikimate accumulation showed different rates during the dark and light incubation, two different estimates of the potency could be obtained.

In seedlings preilluminated prior to glyphosate treatment the general pattern of shikimate accumulation during the light and dark incubation was similar to that observed when the experiments were carried out with etiolated seedlings: a long-time linearity and light stimulation of the process were characteristic also in that material. However, the difference in the accumulation rates, i.e. in the light activation of shikimic acid accumulation, depended substantially on the duration of the preillumination (Fig. 2). In the light the accumulation rate of shikimic acid during incubation with glyphosate as a function of the duration of preillumination had a pronounced maximum at a 16-h preillumination (curve *1*). Surprisingly enough, in case of dark incubation no similar increase in the accumulation rate was observed (curve *2*). Prolongation of the preillumination resulted even in its gradual decrease. Although the maximum of curve *1* was followed by a decline, and in case of prolonged illumination the differences in light/dark accumulation rates of shikimate in absolute terms did not differ much, the relative light effect became more pronounced. The ratio of the accumulation rates of shikimic acid in the light and in the dark (curve *3*), characterizing the light stimulation of this process, increased markedly when the preillumination time was prolonged.

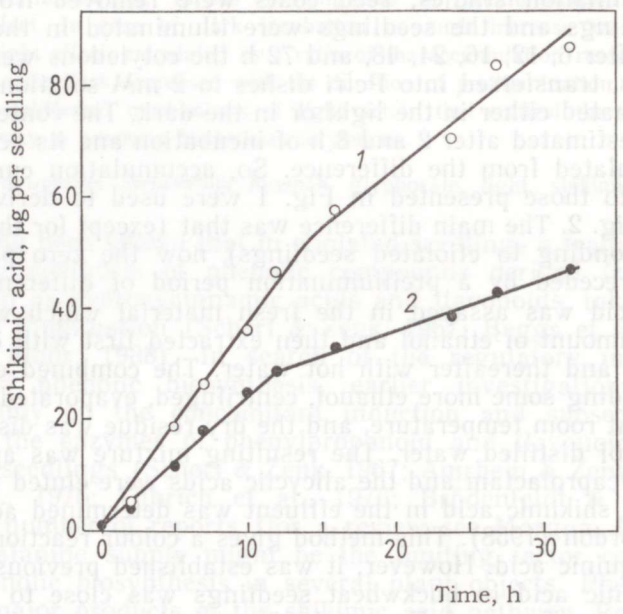

Fig. 1. Time course of shikimic acid accumulation in excised buckwheat cotyledons placed at zero time into a 2 mM glyphosate solution and incubated subsequently in the light (*1*) or in the dark (*2*).

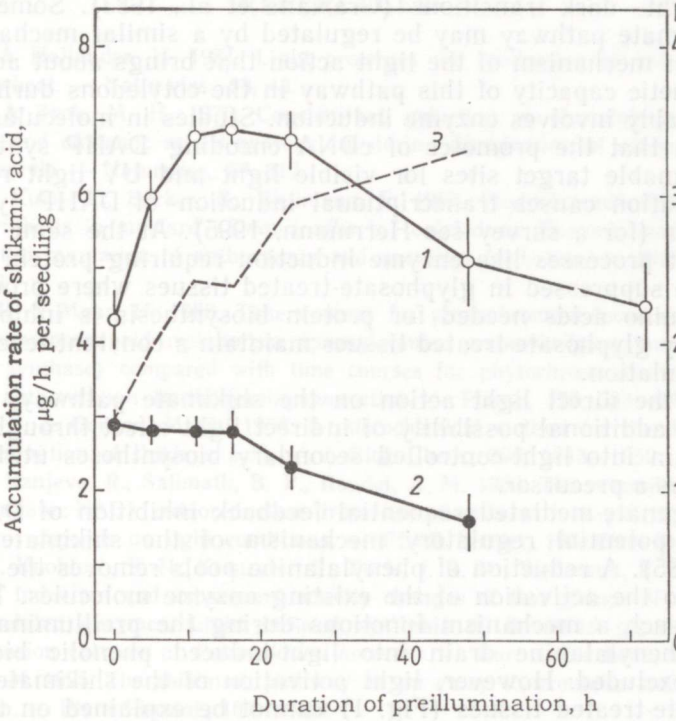

Fig. 2. The rate of shikimic acid accumulation in glyphosate-treated buckwheat cotyledons as a function of preillumination without glyphosate. Dark-grown seedlings were transferred into a light chamber at zero time. After time intervals indicated on the axis of abscissas the cotyledons were detached and placed into a 2 mM glyphosate solution to determine shikimate accumulation during the subsequent incubation in the light (*1*) and in the dark (*2*). Curve (*3*) shows the ratio (*R*) of light/dark accumulation rates of shikimic acid. Bars represent the standard deviation.

It is remarkable that this light effect can be observed only when the operation of the shikimic acid pathway is tested in the light (Fig. 2) as both curves *1* and *2* correspond to the same preilluminated material differing only in the testing conditions (light/dark incubation with glyphosate). This means that the process responsible for the stimulation of shikimate formation is not only light-induced but it can proceed only in the light.

DISCUSSION

The results presented here give evidence of the existence of two mechanisms of light action on the shikimate pathway in buckwheat cotyledons.

One of them is the activation of the pathway revealed by the difference between accumulation rates of shikimic acid during incubation of the cotyledons with glyphosate in the light and in the dark. This light stimulation of shikimate accumulation was studied in our earlier work where its dependence on fluence rate in different spectral regions was recorded (Тохвер, 1990). No direct photoactivation of the enzymes of the shikimate pathway has been established so far. However, reversible changes in the regulatory and structural properties of quinate : NAD^+ oxidoreductase (EC 1.1.1.24), which reversibly converts dehydroquinate into quinate, a by-product of the shikimate pathway, have been found to

occur at light—dark transitions (Graziana et al., 1983). Some enzymes of the shikimate pathway may be regulated by a similar mechanism.

The other mechanism of the light action that brings about an increase in the synthetic capacity of this pathway in the cotyledons during illumination probably involves enzyme induction. Studies in molecular genetics have shown that the promoter of cDNA encoding DAHP synthase contains presumable target sites for visible light and UV light regulators, and illumination causes transcriptional induction of DAHP synthase in parsley cells (for a survey see Herrmann, 1995). At the same time it is obvious that processes like enzyme induction requiring protein synthesis *de novo* are suppressed in glyphosate-treated tissues where production of aromatic amino acids needed for protein biosynthesis is inhibited. This explains why glyphosate-treated tissues maintain a constant level of shikimate accumulation.

Besides the direct light action on the shikimate pathway, one must consider an additional possibility of indirect light effect through the end-product drain into light-controlled secondary biosyntheses utilizing phenylalanine as a precursor.

The arogenate-mediated sequential feedback inhibition of DAHP synthase is a potential regulatory mechanism of the shikimate pathway (Jensen, 1985). A reduction of phenylalanine pools removes the inhibition and leads to the activation of the existing enzyme molecules. The possibility that such a mechanism functions during the preillumination as a result of phenylalanine drain into light-induced phenolic biosynthesis cannot be excluded. However, light activation of the shikimate pathway in glyphosate-treated tissues (Fig. 1) cannot be explained on this basis: the level of arogenate/phenylalanine is low both in darkness and under illumination. The differences in the rate of shikimate accumulation in the light and in the dark may only be explained by a direct action of light on the activity of the respective enzyme(s) of the pathway.

There is also evidence of the increase in the DAHP synthase activity as a result of induction (transcription and *de novo* synthesis) by the light-induced drain of phenylalanine pool (McCue & Conn, 1990). This process may be responsible for the development of the established preillumination effect. However, during the glyphosate test this mechanism cannot function, either, because glyphosate blocks phenylalanine formation and therefore stops its flow into the phenylpropanoid pathway, and hinders enzyme synthesis *de novo* as well.

Presently it is not possible to decide which of the two alternative mechanisms of light induction of the shikimate pathway — a direct transcriptional induction of its light-sensitive enzyme(s) or a secondary effect caused by a change in the pool size of phenylalanine resulting from light-induced secondary biosyntheses — is responsible for the established preillumination effect.

The results of the present work indicate that the onset of illumination brings about a transient increase in the capacity of cotyledons for the synthesis of shikimic acid. Further investigations are needed to elucidate the molecular basis of this phenomenon. At the same time, our results demonstrate that in the glyphosate-treated tissues, light may probably exert its action by a direct photoactivation of the respective enzyme(s).

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TATRA IDULEHTEDES GLÜFOSAADI MÕJUL TOIMUVA ŠIKIMAADI AKUMULATSIOONI SÕLTUVUS IDANDITE EELNEVAST VALGUSTAMISEST

Ants TOHVER, Tiiu VAINJÄRV, Lembe LAANEST

On leitud, et šikimihappe akumulatsiooni kiirus tatra isoleeritud idulehtedes nende inkubeerimisel glüfosaadiga on valguses tunduvalt suurem kui pimeduses. See erinevus sõltus intaktsete idandite inkubatsioonieelsest valgustamisest. Valguses toimuva inkubatsiooni puhul suurenes šikimihappe kogunemiskiirus eelvalgustamise pikendamisel tugevasti, saavutades maksimumi 16-tunnise eelvalgustuse korral; eelvalgustusaja edasisel pikenemisel see stimulatsioon nõrgenes. Pimeduses inkubeerimisel šikimihappe sünteesikiiruse tõusu ei täheldatud. Saadud tulemused lubavad järeldada, et šikimaatse tee funktsioneerimist etioleerunud faasist väljuvates tatra idulehtedes kontrollib valgus kahe mehhanismi kaudu.

ЗАВИСИМОСТЬ НАКОПЛЕНИЯ ШИКИМАТА В ОБРАБОТАННЫХ ГЛИФОЗАТОМ СЕМЯДОЛЬНЫХ ЛИСТЬЯХ ГРЕЧИХИ ОТ ПРЕДВАРИТЕЛЬНОГО ОСВЕЩЕНИЯ ПРОРОСТКОВ

Антс ТОХВЕР, Тийу ВАЙНЯРВ, Лембе ЛААНЕСТ

Обнаружили, что скорость накопления шикимовой кислоты в отделенных семядольных листьях при их инкубировании с глифозатом на свету гораздо выше, чем в темноте. Эта разница зависела от продолжительности предварительного освещения интактных проростков. Скорость накопления шикимовой кислоты в семядольных листьях при их инкубировании на свету значительно увеличивалась с длительностью прединкубационного освещения проростков и достигала максимума при 16-часовом освещении, дальнейшее продолжение прединкубационного освещения приводило к ослаблению стимулирующего эффекта света. В случае темновой инкубации такой стимуляции не наблюдалось. Полученные результаты показывают, что существуют два разных механизма световой регуляции работы шикиматного пути в зеленеющих семядольных листьях гречихи.