PHOTOSYNTHETICA 39 (4): 597-601, 2001

Induction of porphyrin biosynthesis by 5-aminolevulinic acid, glutamic acid, and 1,10-phenanthroline and their possible photodynamic action in wheat and mustard plants

V. TONEVA, T. GECHEV, and I. MINKOV

Plant Physiology and Molecular Biology Department, University of Plovdiv,
24 Tsar Assen Str., 4000 Plovdiv, Bulgaria

Abstract

The photodynamic damage of the sensitive plants wheat and mustard, treated with chlorophyll (Chl) precursors 5-aminolevulinic acid (ALA) and glutamic acid (Glu) and with 1,10-phenanthroline (Phen), was caused by tetrapyrroles, which accumulated after 17 h in the dark period, followed by 12 h of irradiation with "white light". The effect of accumulated Chl in mustard plants was accompanied by changes in the amounts of the Chls and carotenoids and by dehydration of the tissues, partial chlorosis, and necrosis. The molecular nature of the specific photodynamic sensitivity of the mustard and wheat plants under the influence of Phen and Chl precursors was important: accumulation of tetrapyrroles was a necessary, but not only reason for photodynamic damage of the plants. The degree of leaf damage was related to the amount and chemical nature of accumulated tetrapyrroles and to the greening group to which the investigated plant belongs.

Additional key words: protochlorophyllide; photoherbicide effect; protoporphyrin IX; *Triticum aestivum*; *Sinapis alba.*

Introduction

The tetrapyrrole-dependent photodynamic herbicides are substances, which selectively stimulate green plants to synthesise large amounts of metabolite precursors of the chlorophyll (Chl) biosynthetic pathway. When irradiated, the tetrapyrroles cause a formation of singlet oxygen, which damages the sensitive plants mainly by oxidation of cell membranes (Rebeiz *et al.* 1984, Becerril and Duke 1989, Scalla *et al.* 1990, Härtel *et al.* 1993). This group of substances includes 5-aminolevulinic acid (ALA), which is the main tetrapyrrole precursor (Beale *et al.* 1975, Castelfranco and Beale 1983). ALA does not have any toxic effect because it is the natural precursor of all tetrapyrroles in plant and animal cells (Rebeiz *et al.* 1984, 1988, Nandihalli *et al.* 1993). The second compound of the photodynamic herbicides is usually a modulator, which changes the type of tetrapyrrole accumulation (Rebeiz *et al.* 1987).

In plants, fungi, and cyanobacteria ALA is formed from the intact carbon backbone of the glutamic acid (Glu) (Beale *et al.* 1975, Castelfranco and Beale 1983, Beale 1990). Using this early Chl precursor, a new way for producing photodynamic herbicides can be developed. Glu is more available and cheaper than ALA. The introduction of exogenous Glu may remove the substrate deficit of ALA and stimulate the dark accumulation of protochlorophyll (Proto) and Mg-protochlorophyllide (Averina *et al.* 1989). Plants show different sensitivity to photodynamic herbicides, due to which greening group they belong to (Rebeiz *et al.* 1984, 1988, 1999). For example, wheat belongs to the DMV/LDV (dark-monovinyl/light-divinyl) greening group and herbicide response type III. Plants of the photodynamic herbicide response type III synthesise and accumulate certain amounts of tetrapyroles without noticeable photodynamic damage. Mustard plants, which belong to the DDV/LDV group, herbicide response type I, can store large amounts of tetrapyrroles but suffer a substantial photodynamic damage (Rebeiz *et al.* 1984).

Therefore it is important to investigate the action of combinations of precursor-modulator on Chl synthesis and determine their potential for photoherbicidal damage in different plants. In this paper we compared the influence of ALA and Glu together with a metal chelator 1,10-phenanthroline (Phen) on the accumulation of porphyrins

---

Received 1 June 2001, *accepted* 10 December 2001.

Fax: 00359-32-629-595; e-mail: minkov@pu.acad.bg

Acknowledgements: This work was supported by the Bulgarian National Science Fund and the University of Plovdiv.

and the content of photosynthetic pigments in leaves and cotyledons, and studied the visual photoherbicide effects on wheat and mustard plants.

Materials and methods

Germinated seeds of wheat (*Triticum aestivum* L.) and mustard (*Sinapis alba* L.) were sown in soil in vegetation pots and grown in controlled conditions at 25-26 °C and an irradiance of 48 W m^{-2} (12-h photoperiod). Soil humidity was kept at about 60-70 %. Wheat was treated at the third leaf stage and mustard on the third to fourth real leaf stage as follows: (*1*) ALA (5 mM); (*2*) ALA (5 mM) + Phen (15 mM); (*3*) Glu (20 mM); (*4*) Glu (20 mM) + Phen (15 mM); (*5*) Phen (15 mM).

Phen, Glu, and ALA were dissolved in a mixture of acetone : ethanol : *Tween 80* : water (0.45 : 0.45 : 0.1 : 9, v/v) as described by Rebeiz *et al.* (1988). Leaves were sprayed until total wetness of the leaves was achieved. The control plants were treated in the same way with distilled water. The plants were kept in darkness for 17 h at 25-26 °C. To study the photodynamic effect, the plants were irradiated for 12 h with 48 W m^{-2}.

Protochlorophyllide (Pchlide) and Proto were extracted with 85 % acetone containing 0.1 M NH_4OH. For separation of the hexane-soluble phytolised pigments from Mg-porphyrins, Mg-phorbins, and Pchlide we used the method described by Rebeiz *et al.* (1984). The water-acetone phase was washed twice with an equal volume of hexane; then it was once more covered with a hexane layer (water : acetone 1 : 3, v/v). The Proto and Pchlide were measured fluorometrically as described by Shlyk *et al.* (1982) on an *LS-3B Perkin-Elmer* fluorescent spectrophotometer. The total amount of Chls and carotenoids (Car) were measured in 80 % acetone as described by Lichtenthaler and Wellburn (1983).

The protein content in the samples was measured after pigment extraction. The pellet after centrifugation was resuspended in water and the determination was performed in 0.2 cm^3 of homogenate according to Lowry *et al.* (1951).

The photodynamic effect caused by the tetrapyrole precursors in light was assessed according to Rebeiz *et al.* (1984) as a percentage of the necrotised treated leaves, in response to irradiation. For example, if 10 out of 10 sprayed leaves died as a consequence of irradiation, the photodynamic damage was considered to be 100 %. If only 5 out of the 10 sprayed leaves died, the photodynamic damage was considered to be 50 %.

The mean deviation (SE) was calculated by the Student's *t*-test.

Results

In wheat leaves we observed a considerable (3-4 times) increase of Proto content in almost all experiments (Fig. 1*A*). The treatment of plants with Glu only caused an increase by about two times. Phen induced a larger accumulation (391 % of the control plants) compared with Glu (191 %) and the combination Glu+Phen (330 %) but less than the combination ALA+Phen (430 %).

In mustard leaves we found a large increase in the content of precursor (5-9 times) for ALA and ALA+Phen (Fig. 1*A*). Under the influence of Phen the dark accumulation of Proto (205 %) was slightly increased compared to exogenous Glu (191 %), but it was still lower than the result of the combination Glu+Phen.

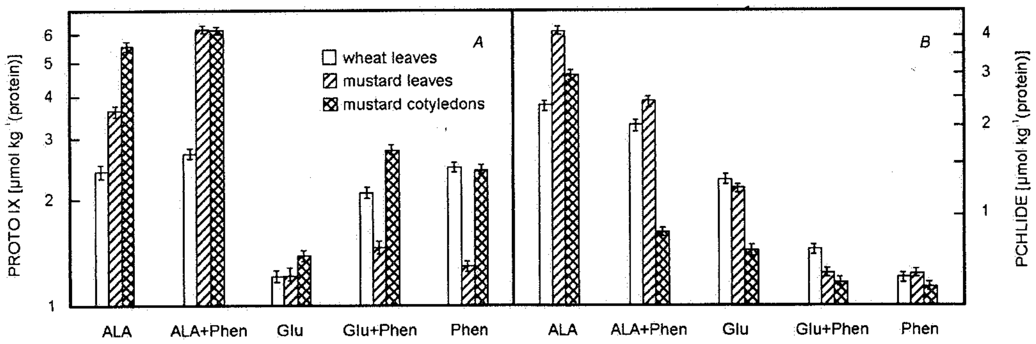

Fig. 1. Accumulation of protoporphyrin IX (Proto) (*A*) and protochlorophyllide (Pchlide) (*B*) in wheat leaves and in leaves and cotyledons of mustard under the influence of different combination of 5-aminolevulinic acid (ALA), glutamic acid (Glu), and 1,10-phenanthroline (Phen), darkened after the treatment for 17 h.

In mustard cotyledons we found a very large increase of the content of Proto (9-10 times) for ALA and ALA+Phen (Fig. 1*A*). Considerable accumulation of the precursor was observed also by the sole action of Phen (385 %) compared with the sole action of Glu (219 %), which was still lower than the combination Glu+Phen (439 %).

Pchlide is one of the photosensibilisers, which induce a destruction of cell membranes and plant death (Rebeiz *et al.* 1990). We investigated its accumulation in leaves of wheat and in leaves and cotyledons of mustard (Fig. 1*B*). The changes in content of this precursor were in one direction in all variants of the experiment. The highest dark content of Pchlide was found after treatment with ALA and was 481, 853, 691 % for leaves of wheat and leaves and cotyledons of mustard, respectively. Glu induced a considerable Pchlide accumulation (272 % for wheat leaves, 255 % for mustard leaves, and 158 % for mustard cotyledons), compared with the controls. The amount of the precursor was larger in this variant than in the one affected by the modulator. The separate functions of ALA and Glu were stronger than their combinations with a modulator.

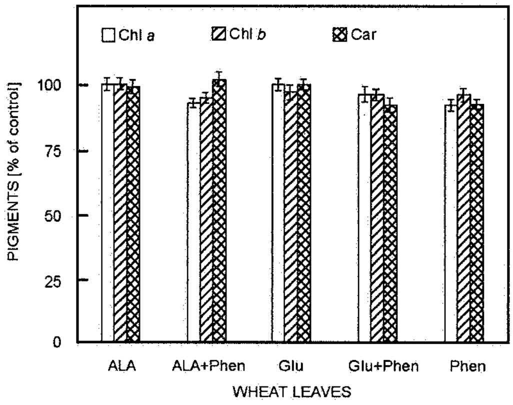

Fig. 2. Changes of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid (Car) contents in wheat leaves under the influence of different combinations of 5-aminolevulinic acid (ALA), glutamic acid (Glu), and 1,10-phenanthroline (Phen). They were darkened after the treatment for 17 h and irradiated for 12 h with 48 W m^{-2}.

Next we studied the effect of the tested herbicide combinations on the quantity of the photosynthetic pigments. Fig. 2 shows the changes of the amounts of Chl *a*, Chl *b*, and Car in leaves of wheat, influenced by the applied compounds and irradiated for 12 h by 48 W m^{-2}. The largest decrease from the control probe in Chl *a* amount was observed under Phen treatment (82 %). Used separately, ALA and Glu did not cause changes while their combinations with Phen showed a small decrease (92 and 95 %). In different variants of the experiment, Chl *b* content fluctuated within the limits of experimental error. The quantity of Car decreased under the influence of Glu+Phen and Phen (92 and 91 %, respectively). Generally, the tested combinations did not affect the quantity of Chl *a* in wheat leaves. In mustard leaves (Fig. 3*A*) the content of both Chls decreased when treated with Phen, the larger change being found in Chl *a* (up to 84 %). In Chl *b* such tendency appeared under Glu treatment (92 %). The content of Cars increased slightly under ALA and ALA+Phen treatments (108 %) and decreased under treatment with Phen (92 %). The largest changes were observed in Chl *a* in mustard cotyledons (Fig. 3*B*). Phen separately and especially in combination with ALA and Glu decreased much the Chl *a* amount (92, 69, 74 %, respectively). Glu slightly increased the amount of Chl *a* (108 %). Changes in the amount of Chl *b* were insignificant: there was some decrease in the Glu+Phen and Phen treatments. The content of Cars increased slightly when treated with Glu (108 %), but decreased when treated with Glu+Phen (92 %).

Visually we established no photodynamic effect in wheat by the used photodynamic combinations. The plants did not differ from the controls neither by habitat nor by leaf colour. The mustard leaves were especially sensitive to the treatment with tetrapyrrole-dependent photodynamic herbicides. In all treated plants we observed loss of turgidity of leaves and cotyledons compared to the controls. In the leaves we observed partial chlorosis and necrosis. An acute form of the symptoms of photodynamic damage appeared when plants were treated with ALA and ALA+Phen. There was a dehydration of the stems in these cases. Slightly expressed photodynamic effect was observed in plants treated with Phen only.

We examined the amount of total protein in order to investigate possible toxic influence of different photoherbicide combinations (values not shown). The protein amount did not change in the different variants of the experiment. The results proved that the used combinations of chemicals have a specific effect upon the synthesis of the precursors rather than any inhibition of the protein synthesis. This was also proved by the fact that the conversion of Chl precursors was not considerably repressed.

Discussion

In green plants the pool of Proto is only a few percent of the amount of the precursors of Chl biosynthesis (Rebeiz *et al.* 1988). The separate usage of Phen as well as in combination with Glu caused a large increase in the content of Proto in the dark. The straight correlation between the increased content of Proto and the photodynamic damage to leaves showed that it is the main inducer of the photodynamic effect (Averina *et al.* 1988). Though the content of Proto accumulated in the dark was quite large in the mustard cotyledons too (Fig. 1*A*), its photodynamic effect was not evident in light. This could be due to the different morphology of the two organs, their different sensitivity, and the rates of tetrapyrrole turnover found previously by Rebeiz *et al.* (1984). More efficient changes of precursors were found in mustard than in wheat plants. In organ comparison there were greater changes in leaves than in cotyledons.

Chelators have a porphyrinogenic mechanism of action that involves the induction of porphyrin synthesis *via* 5-ALA accumulation in bean (Duggan and Gassman 1974). Glu, the substrate of ALA biosynthesis, may be the limiting factor in the activated ALA-synthesising enzyme system (Averina *et al.* 1989). Its introduction in the mentioned conditions may remove the deficiency of the substrate and induce the accumulation of protoporphyrin IX (Proto IX) in darkness (Averina *et al.* 1989).

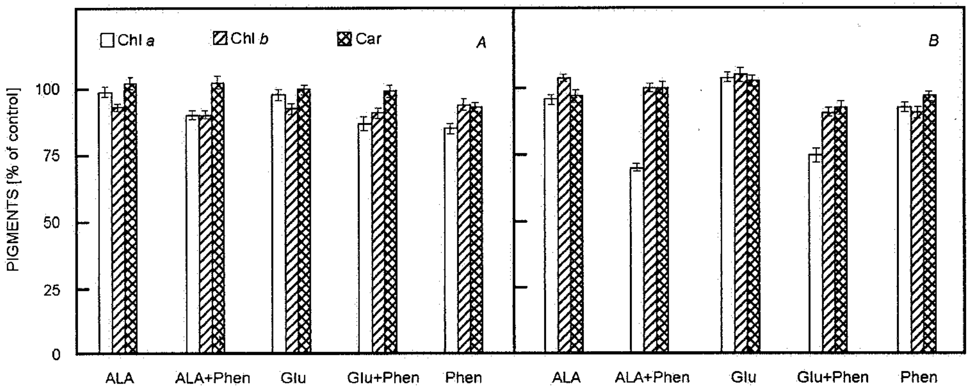

Fig. 3. Changes of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid (Car) contents in mustard leaves (*A*) or cotyledons (*B*) under the influence of different combinations of 5-aminolevulinic acid (ALA), glutamic acid (Glu), and 1,10-phenanthroline (Phen). They were darkened after the treatment for 17 h and irradiated for 12 h with W m^{-2}.

There is a correlation between the changes in the amount of Proto IX and Pchlide under the influence of the tested substances. Comparing the quantities of Proto IX and Pchlide in wheat and mustard leaves we found (Fig. 1) that ALA and Glu induced higher accumulation of Pchlide while Phen led to a higher accumulation of Proto IX (Nandihalli and Rebeiz 1991).

Chl *a* is synthesised as the main metabolite in the biosynthetic path, while Chl *b* is a product of some additional transformation (Kotzabasis and Senger 1989). In the investigated plant species and specific organs, Chl *a* was more sensitive than Chl *b*. Similar results, *i.e.* strong degradation of Chl *a* while Chl *b* content changes only slightly, were observed after the use of other modulators such as 2,2'-dipyridyl (Toneva *et al.* 1997) and in ALA-treated leaves of wheat and lettuce (Härtel *et al.* 1993).

The high sensitivity of Chl *a* to Phen (with ALA or Glu) could be explained with the fact that Phen induces the process of pheophytinisation and causes the dissociation of the pigment. Chl *a* changes into pheophytin considerably faster than Chl *b* because the last one is steadier as it possesses a –CHO group in the second pyrrole ring (Averina *et al.* 1991).

There was a clear visible photodynamic herbicide effect in mustard leaves. Dehydration of the tissues, partial chlorosis, and necroses were observed. No such effect was noticed in wheat. The biochemical basis of this organ- and species-dependent photodynamic herbicidal selectivity depends on the rates of tetrapyrrole turnover and on a differential enhancement of monovinyl and divinyl tetrapyrrole biosynthetic pathways in different plants (Rebeiz *et al.* 1984).

References

Averina, N.G., Shalygo, N.V., Linnik, N.N.: [Chlorophyll pheophytinisation by metal pyridine chelators.] – Fiziol. Rast. **38**: 1059-1065, 1991. [In Russ.]

Averina, N.G., Shalygo, N.V., Yaronskaya, E.B.: Effect of glutamic acid and 1,10-phenanthroline on the accumulation of chlorophyll precursors in green *Phaseolus* leaves. – Photosynthetica **23**: 383-385, 1989.

Averina, N.G., Shalygo, N.V., Yaronskaya, E.B., Rassadina, B.B.: [Photodynamic effect and porphyrin accumulation in plants treated with δ-aminolevulinic acid and α,α'-dipyridyl.] – Fiziol. Rast. **35**: 679-686, 1988. [In Russ.]

Beale, S.I.: The biosynthesis of the tetrapyrrole pigment precursor, δ-aminolevulinic acid, from glutamate. – Plant Physiol. **93**: 1273-1279, 1990.

Beale, S.I., Gough, S.P., Granick, S.: Biosynthesis of δ-aminolevulinic acid from intact skeleton of glutamic acid in greening barley. – Proc. nat. Acad. Sci. USA **72**: 2719-2723, 1975.

Becerril, J.M., Duke, S.O.: Protoporphyrin IX content correlates with activity of photobleaching herbicides. – Plant Physiol. **90**: 1175-1181, 1989.

Castelfranco, P.A., Beale, S.I.: Chlorophyll biosynthesis: recent advances and areas of current interest. – Annu. Rev. Plant

Physiol. **34**: 241-247, 1983.

Duggan, J., Gassman, M.: Induction of porphyrin synthesis in etiolated bean leaves by chelators of iron. – Plant Physiol. **53**: 206-215, 1974.

Härtel, H., Walter, G., Hanke, T.: Photoinduced damage in leaf segments of wheat (*Triticum aestivum* L.) and lettuce (*Lactuca sativa* L.) treated with 5-aminolevulinic acid. I. Effect on structural components of the photosynthetic apparatus. – J. Plant Physiol. **142**: 230-236, 1993.

Kotzabasis, K., Senger, H.: Biosynthesis of chlorophyll *b* in pigment mutant C-2A' of *Scenedesmus obliquus*. – Physiol. Plant. **76**: 474-478, 1989.

Lichtenthaler, H.K., Wellburn, A.R.: Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. – Biochem. Soc. Trans. **11**: 591-592, 1983.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. – J. biol. Chem. **193**: 265-275, 1951.

Nandihalli, U.B., Duke, S.O.: The porphyrin pathway as a herbicide target site. – In: Duke, S.O., Menn, J.J., Plummer, J.R. (ed.): Pest Control with Enhanced Environmental Safety. Pp. 62-78. American Chemical Society, Washington 1993.

Nandihalli, U.B., Rebeiz, C.A.: Photodynamic herbicides. 9. Structure activity study of substituted 1,10-phenanthrolines as potent photodynamic herbicide modulators. – Pestic. Biochem. Physiol. **40**: 27-46, 1991.

Rebeiz, C.A., Ioannides, I.M., Kolossov, V., Kopetz, K.J.: Chloroplast biogenesis 80. Proposal of a multibranched chlorophyll *a/b* biosynthetic pathway. – Photosynthetica **36**: 117-1128, 1999.

Rebeiz, C.A., Montazer-Zouhoor, A., Hopen, H.J., Wu, S.M.: Photodynamic herbicides: 1. Concept and phenomenology. – Enzyme Microb. Technol. **6**: 390-401, 1984.

Rebeiz, C.A., Montazer-Zouhoor, A., Mayasich, J.M., Tripathy, B.C., Wu, S.M., Rebeiz, C.C.: Photodynamic herbicides and chlorophyll biosynthesis modulators. – In: Heitz, J.R., Downum, K.R. (ed.): Light Activated Pesticides. Pp. 295-328. American Chemical Society, Washington 1987.

Rebeiz, C.A., Montazer-Zouhoor, A., Mayasich, J.M., Tripathy, B.C., Wu, S.M., Rebeiz, C.C.: Photodynamic herbicides: Recent developments and molecular basis of selectivity. – CRC crit. Rev. Plant Sci. **6**: 385-436, 1988.

Reddy, K.N., Nandihalli, U.B., Velu, J.: Tetrapyrrole-dependent photodynamic herbicides. – Photochem. Photobiol. **6**: 1099-1117, 1990.

Scalla, R., Martinge, M., Camadro, J.M., Labbe, P.: Recent advances in the mode of action of diphenyl ethers and related herbicides. – Z. Naturforsch. **45c**: 503-511, 1990.

Shlyk, A.A., Averina, N.G., Shalygo, N.V.: Metabolism and intermembrane location of magnesium-protoporphyrin in monomethyl ester in centers of chlorophyll biosinthesis. – Photobiochem. Photobiophys. **3**: 197-223, 1982.

Toneva, V., Koleva, A., Minkov, I.: Influence of glutamic acid and 2,2'-dipyridyl on the protochlorophyllide and protoporphyrin IX accumulation and their photodynamic action in green monocotyledons and dicotyledons. – J. Plant Physiol. **150**: 57-62, 1997.