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INFLUENCE OF NITROPYRINE® ON THE EARLY STAGES OF CHLOROPHYLL SYNTHESIS IN WHEAT

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Summary. The effect of NITROPYRINE® on the chlorophyll biosynthesis in wheat plants was studied. A stimulating effect on the early stages of chlorophyll synthesis was found, concerning the synthesis of d-aminolevulinic acid (ALA) and the activity of ALA-dehydratase, which catalyzes condensation of two molecules of ALA giving rise to porphobilinogen. The protochlorophyllide (Pchlide) formation, as well as chlorophyll *a* and chlorophyll *b* contents were also increased. Further implementation of NITROPYRINE® as a tool in investigating plant metabolism was discussed.

Key words: Nitrate, protochlorophylide, chlorophylls, wheat (*Triticum aestivum L.*), δ-aminolevulinic acid, δ-aminolevulinic acid dehydratase

Abbreviations: ALA – δ-aminolevulinic acid, ALAD – ALA-dehydratase, Phclide – protochlorophyllide, Chl – chlorophyll.

Introduction

How to balance the need of increased crop production with the control of pollution caused by underutilisation of applied nutrients is an active area of research (Shaffer et al., 1991, Matson et al., 1997, Pang et al., 1998). Nevertheless, the nitrates and other nutrient pollution has become a major ecological problem world-wide (Smil V., 1997, Vitonsek et al., 1997). The problem of nitrates regulation in plants is an important issue in agriculture and food industry as well. The accumulation of free nitrates in plant tissues is a chain in the entire mechanism of adaptation of the plants to the

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environment. From one side, the equilibrium of the nitrates in the soil and in the plants is maintained by the enzyme nitrate reductase, which plays an important role in the regulation of nitrates uptake. Being an NADPH co-factor enzyme, it could be regulated by exogenous precursors of the NADPH, like nicotine amide, and other co-factors. On the basis of this assumption we prepared a combination of required enzyme co-factors and $(NH_4)_2MoO_4$ (Zhelyazkov, Minkov, 1994, Zhelyazkov, Minkov, Patent No 60 673/1996) with the trade name of NITROPYRINE®, which can regulate the nitrates in the higher plants, maintaining their content in a species specific level and its action is strongly dependent on the amount of nitrogen (N) and potassium (K) in the soil (Zhelyazkov, Minkov, Patent No 60 673/1996). In conditions of high nitrates amounts in the soil, it maintains their uptake at a physiological level. By this the plants can overcome the intensive nitrates accumulation in the cells. The assumption is that the NITROPYRINE® can also regulate other NADPH-dependent enzymes in the cell, especially those playing a crucial role in chlorophyll synthesis, such the δ-aminolevulinc acid dehydratase (ALAD) and the NADPH-protochlorophyllide oxidoreductase (Pchlide-reductase).

The main purpose of this work is to investigate in more detailed way the process of pigments accumulation in some plants, under the influence of NITROPYRINE®, and specially on the early stages of chlorophyll synthesis and chlorophyll precursors accumulation.

Materials and Methods

The seeds of wheat (*Triticum aestivum* L.) were sown in vegetative pots containing 3 kg of soil (N – 6,8 mg/100 g soil, K – 11,3 mg/100 g soil) and plants were grown in growth chamber under controlled conditions with 12 h light/12 h dark cycles, at a light intensity of 45 $W.m^{-2}$ at 22–25 ºC. The etiolated plants were grown in a similar chamber in darkness.

The plants were treated in the stage of third leaf by spraying with NITROPYRINE® solution with concentration of 1 g/l, until the complete wetness was achieved. Part of the plants were supplied with 50 mM KNO_3 for a pot and another – without it. The analyses were performed 24 h after the treatment.

The amount of δ-aminolevulinic acid (ALA) was measured by Miller et al. (1979). For calculations the molar extinction coefficient of $6{,}8.10^4\ M.cm^{-1}$ was used (Mauzerall and Granick, 1956).

The activity of the enzyme δ-aminolevulinic acid-dehydratase (ALAD) was measured by the amount of porphobilinogen synthesized (nM) for one hour, from 1 g of fresh leaves. The enzyme preparation was purified and analyzed according to Mauzerall, Granick, (1956) and Shemin (1962). The total amount of porphobilinogen, accumulated during the incubation time is presented as a total of its amount and the amount of

the porphobilinogen converted to uroporphyrinogen III. It has to be pointed out, that for the synthesis of one molecule of uroporphyrinogen four molecules of porphobilinogen are required.

The amount of protochlorophyllide (Pchlide) was measured after its extraction from the leaves with 85% acetone, containing 0.1 N NH_4OH, as described by Rebeiz et al., (1984). Pchlide was measured spectrophotometrically (Shlyk et al 1982). The chlorophylls a and b content, as well as the content of carotenoids were calculated, using the molar extinction coefficients by Lichtentaler, Wellburn (1983).

Results

Fig. 1 shows the changes of the amount of ALA in wheat leaves, treated with NITROPYRINE® and grown in soil fertilized with KNO_3. The treatment of the leaves resulted in an increase of ALA amount, which was 25,5 nM/g fresh weight, while the non-treated leaves, grown in not fertilized soil, accumulated considerably low amount of ALA – 18,5 nM/g fresh weight.

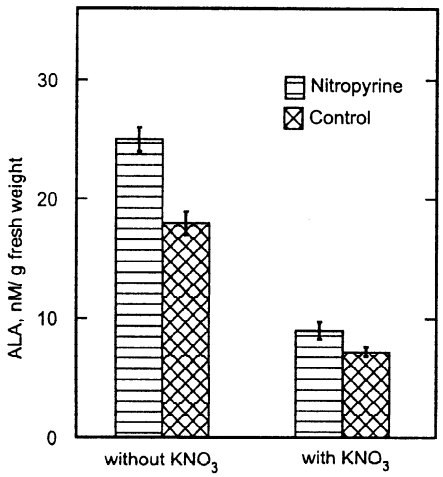

Fig. 1. δ-aminolevulinic acid (ALA) amounts (nM/g fresh weight) in green wheat plants, treated with NITROPYRINE®.

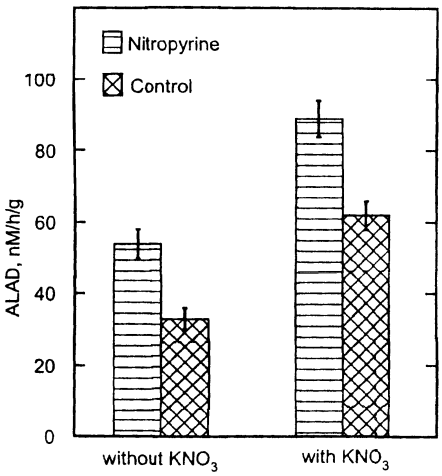

Fig. 2. δ-aminolevulinic acid dehydratase (ALAD) activity (nM/h/g fresh weight) in green wheat plants, treated with NITROPYRINE®.

The data in Fig. 2 shows activity of the enzyme ALAD, which catalyses the condensation of two molecules of ALA giving one molecule of porphobilinogen. The plants were treated with NITROPYRINE® in a background of an additional fertilizing with KNO_3, the controls were not treated and grown in condition of normal potassium and nitrogen content in the soil. The enzyme activity in treated plants increased, which was observed in the case of normal and higher nitrogen level in the soil, the activity

being about 1,5 times higher than in the control plants, non-treated with NITROPYRINE®. The enrichment of the soil with KNO_3 led to a considerable increase of the ALAD activity, also in the non-treated plants, but the treated plants in the presence of KNO_3 showed higher enzyme activity – 54,0 nM/h/g and 89,1 nM/h/g respectively. The control plants showed activity of ALAD as follows – 32,9 nM/h/g (without KNO_3) and 62,3 nM/h/g (with additional KNO_3). ALAD activity increased considerably after the soil fertilizition, probably due to the low initial content of nitrogen in the latter.

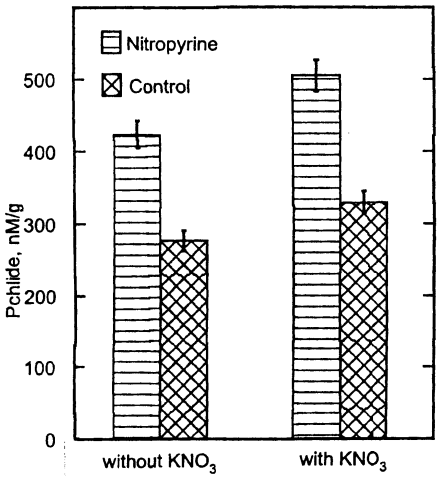

Fig. 3. Influence of NITROPYRINE® on protochlorophylide (Pchlide) content in etiolated wheat plants.

Fig. 3 shows the amount of Pchlide in treated and non-treated with NITROPYRINE® wheat plants. Increased Pchlide accumulation was observed after NITROPYRINE® treatment both in presence and absence of KNO_3, being about 50% higher than the control plants. The measurement of the etiolated leaves, grown in the soil, enriched with KNO_3 showed the amount of Pchlide – 505 nM/g, and 330 nM/g in the control plants. In the normal soil corresponding amounts were in the same range – 424 nM/g and 276 nM/g of Pchlide.

Results concerning chlorophyll accumulation are in a good agreement with the results of accumulation of the chlorophyll precursor Pchlide. Etiolated plants, treated with NITROPYRINE® after irradiation, demostrated higher chlorophyll accumulation. Those results are presented in Fig. 4 and Fig. 5. Figure 4 shows data of the chlorophyll content in wheat plants, grown in normal soil. The data show stimulating effect of NITROPYRINE® treatment for the chlorophyll accumulation, which was better pronounced for chlorophyll *b* (about 122%), compared with chlorophyll *a* (about 117%). There was also some increase in the level of accumulated carotenoids (113%). The results of the treatment with NITROPYRINE® on the background of higher potassium and nitrogen level is shown in Fig. 5. A small difference can be detected in plants grown in normal soil – the amounts of accumulated pigments in the treated plants were about 14–16% more than in the control plants.

Discussions

The results presented here show that treated green and etiolated wheat plants with NITROPYRINE® demonstrated another implementation of this combination of enzyme

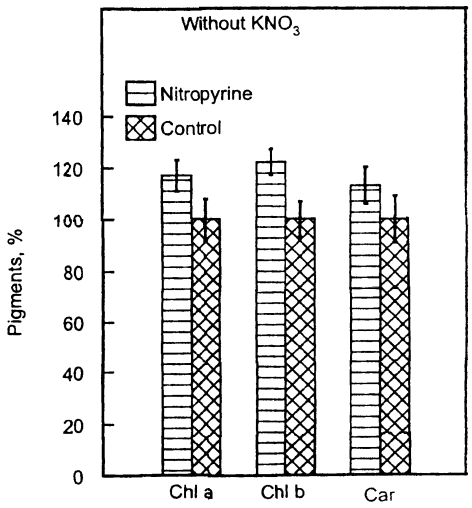

Fig. 4. Influence of NITROPYRINE® on pigments accumulation: Chl *a*, Chl *b* and carotenoids in wheat plants without KNO_3 fertilizing.

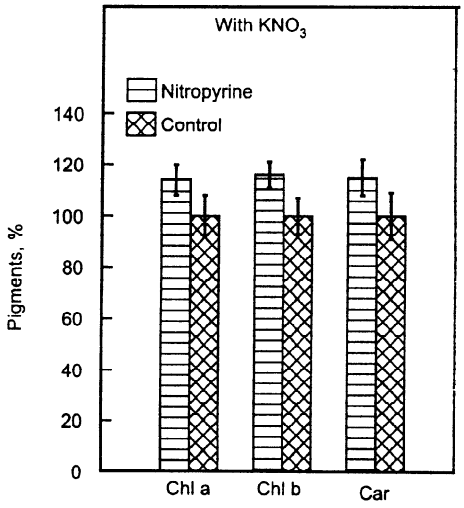

Fig. 5. Influence of NITROPYRINE® on pigments accumulation: Chl *a*, Chl *b* and carotenoids in wheat plants with KNO_3 fertilizing.

cofactors. So far, the influence of NITROPYRINE® on the nitrate metabolism was previously shown, and especially in some plants with a tendency of abnormal nitrates accumulation, as salad, cucumber, garlic etc. (Zhelyazkov, Minkov, 1994, Zhelyazkov, Minkov, Patent No 60 673/1996). The presumption, that the preparation influences the NADPH-dependent enzymes has its extension in this work, showing that it can influence also the chlorophyll synthesis. Our experiments show that as a whole the NITROPYRINE® has an increasing and stimulating effect on chlorophyll synthesis. This influence can be divided in several stages. It increases the accumulation of early chlorophyll precursors like ALA and porphobilinogen (Fig. 1, 2). Most probably the mechanism of this influence is activation of the enzymes of the mentioned synthetic stages. The increase in the activity of one of these enzymes (ALAD) is well documented (Fig. 2). Much lower ALA content in the plants grown in a soil fertilized with KNO_3 is a consequence of the increased activity of ALAD which catalyses the condensation of two molecules of ALA to porphobilinogen. That is why in the control and in the treated with Nitropirin® plants the content of ALA is about three times lower. Presumably, there could be also an influence, caused by an indirect action of the nitrate metabolism. Such influence can be due to the increased reduction of nitrates to ammonium ions and their incorporation in glutamate which is the immediate ALA precursor. There are some data that the glutamate level, as a substrate for ALA synthesis, is one of the limiting factors in the early stages of chlorophyll synthesis (Averina et al., 1989). At the same time it is well known that the chlorophyll in green plants is synthesized via the 5-carbon skeleton of the glutamate (Beale et al., 1975, Castelfranco, Jones, 1975, Flur et al., 1975, Wellburn, 1975, Beale, 1990, Jordan, 1991).

NITROPYRINE® also influences the later stages of chlorophyll synthesis, such as Pchlide formation (Fig. 3). It is the critical point of chlorophyll pathway up to which the synthesis is not light dependent and this is the chlorophyll precursor which accumulates in etiolated leaves (Virgin, 1981). It is well known that the next, light dependent stage of synthesis is strongly dependent on the activity of the enzyme NADPH-Pchlide-oxidoreductase (Apel et al, 1980, Griffiths et al, 1984). The level of the active enzyme complexes is strongly influenced by the NADPH level in the cell (Piene et al, 1993). The NITROPYRINE® most probably increases the level of NADPH and that might be the reason for its influence on the later stages of synthesis – the formation of chlorophyll *a* and *b*. An indirect evidence was the higher chlorophyll accumulation which is in a good agreement with the higher levels of Pchlide, together with the NADPH, creates the active complex $Pchlide_{657}$, capable to transform the precursors in chlorophyllide and chlorophyll. This effect might increase the total photosynthetic process in treated plants, which can be another practical effect of the NITROPYRINE®, apart from its regulatory effect on nitrates.

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