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PHENMEDIPHAM INHIBITION OF BIOLOGICAL NITROGEN FIXATION IN PEA PLANTS

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RESUMEN

INHIBICION POR FENMEDIFAN DE LA FIJACION BIOLOGICA DE NITROGENO EN PLANTAS DE GUISANTES

Inhibición de la fijación biológica de nitrógeno en plantas de guisante por fenmedifán. El perfil ontogénico de la actividad nitrogenasa en plantas noduladas de guisante *(Pisum sativum* L.) es similar al del transporte electrónico dependiente del Fotosistema II de membranas tilacoidales aisladas de las mismas. La aplicación foliar de fenmedifán, un inhibidor tipo diurón, produce un fuerte descenso del transporte electrónico vinculado al Fotosistema II, proporcional al herbicida aplicado. Se observa una inhibición similar de la actividad nitrogenasa, con pérdida casi total de actividad después de 10 días de la pulverización con fenmedifán 100 μM. Sin embargo, hay una recuperación del transporte electrónico y de la actividad nitrogenasa aún después del tratamiento con fenmedifán 300 μM. Tanto en plantas controles como tratadas el contenido foliar de nitrógeno es paralelo al nivel de fijación biológica del mismo. Así pues, el fenmedifán deprime la fijación de N_2 por defecto en el aporte de fotosintetizado a la raiz, al inhibir el transporte electrónico de la fotosíntesis. Como ocurre con otros herbicidas de aplicación foliar, y a causa de su precario transporte a la raiz, hay que desechar un posible efecto directo del fenmedifán sobre la fisiología de la bacteria, o sobre la implantación de la simbiosis *Rhizobium* –leguminosa.

Palabras clave: fenmedifán, fotosíntesis, fijación de N_2.

SUMMARY

The ontogeny pattern of nitrogenase activity of nodulated pea *(Pisum sativum* L., cv. Lincoln) plants is similar to that of the PS II-linked activity of thylakoid membranes. Foliar application of the DCMU-type inhibitor phenmedipham showed a sharp decrease of PS II activity, proportional to the applied herbicide. A similar inhibition of nitrogenase activity was observed, with an almost total lost of activity after 10 days of 100 μM phenmedipham spraying However, a recovery of electron transport and nitrogenase activity occurs even after 300 μM phenmedipham application. The N content shows a parallelism with the N_2 fixation rate, both in controls and phenmedipham treated plants.

So, phenmedipham disturbs N_2 fixation by a decreased photosynthate supply to the roots, via inhibition of the photosynthetic electron transport. As occurs with some other leaf applied herbicides, and because of their awkward translocation to the roots, a direct effect of phenmedipham on the bacterium physiology, or on the feasibility of the ***Rhizobium***-legume symbiosis, must be overlook.

Key Words: phenmedipham, photosynthesis, N_2 fixation.

INTRODUCTION

Phenmedipham [methyl 3-(3-methyl-phenylcarbamoyloxy) phenyl carbamate] is a herbicide used against weeds in sugar beet and some legume crops. Even though aryl-carbamates show anti-mitotic effects (Shaw and Swanson, 1953), SWEP and phenmedipham induce a photosynthetic electron transport inhibition by binding to the PS II 32000-34000 daltons polypeptide which shields the quinones Q_A and Q_B, hampering the normal electron flow (Tischer and Strotmann, 1977).

In vitro experiments with isolated spinach chloroplasts have shown a I_{50} value for phenmedipham of 0.2 μM in the PS II-linked electron transport chain (Barón *et al.*, 1986; Chueca *et al.*, 1982). *In vivo* experiments with herbicide-sprayed spinach plants showed a rise of this value to 10 μM, which was 150 μM when phenmedipham was root applied. This electron transport inhibition produced a decay of CO_2 assimilation, with a I_{50} 0.05 μM in *in vitro* experiments with isolated choroplasts and 5 μM after *in vivo* foliar application (Díaz *et al.*, 1982).

Nitrogen fixation demands high energy supplies. N_2 reduction to NH_4^+ requires 6 reduction equivalents, and 2-3 additional ones used in the concomitat H_2 production. In addition, 12 ATP/N_2 are used to reduce the nitrogenase and to break down the interatomic bonds in the N_2 molecule. Energy is also required to the growth and functioning of nodules. In the *Rhizobium*-leguminosae association all this energy is provided by the host plant, with an additional cost for the photosynthate translocation to the roots (Pate and Harridge, 1978). Thus, a photosynthesis shortage can produce a double effect: a direct one on plant development, and a second indirect by inhibition of nitrogenase activity. In this work we study the effect of leaf applied phenmedipham on the N_2 fixation of nodulated pea plants.

MATERIALS AND METHODS

Pea (*Pisum sativum* L., cv. Lincoln) seeds were soaked in water (0 time), and geminated in trays containing autoclaved vermiculite.

Each seed was inoculated with 1 mL of water suspension (10^8 cells/mL) of *Rhizobium leguminosarum* strain 128 C53. After 8 days germination in a growth chamber, at 80% RH and 600 μmoles m^{-2} s^{-1} quantum flux (Grolux Sylvania), with 16 hours photoperiod and a day/night temperature of 22° C/17° C, seedlings were removed and each root re-inoculated with 1 mL of bacterial suspension. Seedlings were then hydroponically grown in 1.5 liter opaque metacrylate pots. Six cotyledon-excised seedlings were grown per pot, provided with the N-free medium of Rigaud and Puppo (1975), adjusted to pH 7.0 with KOH. Hydroponic media were continuously aerated, the evaporated water daily replaced, and the nutrient solution renewed every 10 days. Growth conditions were similar to those in vermiculite.

After 21 days growth, plants of a set of pots were homogeneously leaf sprayed with 25 mL per pot of acetone-water (1:3 v/v) solutions of 70, 100 and 300 μM phenmediphan (min. 99%, Serva Feinbiochemica, Heidelberg). Previous experiments showed that the acetone-water mixture did not produce any effect on the seedlings. Plants were collected at about 10, 15, 21, 26, 31, 36 and 39 days after seed imbibition. A complete experiment was made of 32 pots, and each measured determined on 4-8 plants selected at random. Dry weight of plant was determined after drying the fresh ones to constant weight at 60° C. Total N was determined in dry plants by Kjeldahl.

Thylakoid membranes were prepared from 4 g of washed leaves by a modification of the original method of Cockburn *et al.* (1968), with sorbitol-pyrophosphate as the homogeneization medium and sorbitol-Hepes as the suspending one. The changes consisted of a 0.4 M sorbitol concentration in both media and a pH 6.7 in the suspending solution. The final pellet of membranes was recovered in the suspending medium to get a chlorophyll concentration about 1 mg mL^{-1}. Chlorophyll content of membranes was determined according to Arnon (1949). The rate of the PS II-dependent electron transport $H_2O \rightarrow [Fe(CN)_6]^{3-}$ was measured in 50 mM Hepes-NaOH pH 7.6, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.32 M sorbitol, 0.5 mM $[Fe(CN)_6]^{3-}$, and thylakoid membranes equivalent to 60 μg chlorophyll, in a final volume of 3 mL. Determinations were carried out at 28° C in an Aminco DW-2a spectrophotometer equipped with red cross illumination (Corning 2-58 filter), and a blue filter (Corning 4-96) as a phototube protector, following the $[Fe(CN)_6]^{3-}$ decrease at 420 nm. The PS II-dependent electron transport was also measured by the Hill reaction $H_2O \rightarrow$ DPIP (dicholorophenolindophenol). The reaction mixture was as in the ferricyanide test, but with 66 μM DPIP instead of $[Fe(CN)_6]^{3-}$. To circunvent any effect of phenmedipham on the water splitting system, we have tested the transport chain DPC (diphenylcarbazide) $\rightarrow$ DPIP; in this case 2 mM DPC was added to the preceeding reaction mixture. The measuring conditions were as above, but reading the dissapearance of oxidized DPIP at 600 nm.

Nitrogenase activity was measured on detached root systems excised at

the cotyledonary node. Root systems were incubated with 4 mM C_2H_2 (10% acetylene in air), and sampled after 10 and 20 minutes for C_2H_4 determination by gas chromatography. Electron transport determinations were made in triplicate, whereas those of nitrogenase activity and total N were repeated 6 times. For every set of values we obtained the mean ± standard error.

RESULTS AND DISCUSSION

The *Rhizobium*-legume association shows a close dependence between biological N_2 fixation, plant photosynthetic activity and photosynthate translocation to the roots (Gordon *et al.*, 1987; Sheehy, 1987). Rates of N_2 fixation follow those of plant photosynthesis, when modified by dark-light transitions (Chetverikov and Timiryazev, 1985), by changes in the ambient CO_2 concentration (Murphy, 1986), or by photorespiratory inhibitors (Bedmar and Olivares, 1980). Some photosynthesis-inhibiting herbicides decrease N_2 fixation in cyanobacteria via a direct effect on nitrogenase activity (Gadkari, 1988). In addition, some pesticides disturb the *Rhizobium*-legume symbiosis, but concerned with direct effects on the bacterium physiology, or with the symbiosis feasibility after the herbicide addition to the root environment (Bollish *et al.*, 1985; Pati *et al.*, 1984). However, only few reports are focused on the relationship between a herbicide-induced drop of photosynthetic activity and low N_2 fixation rates (Bethlenfalvay *et al.*, 1979; De Felipe *et al.*, 1987).

Figure 1 (a, b, c) shows in the controls a gradual decrease of the PS II-linked electron transport during days 10 to 15-20, followed of a rise with a maximum about day 25, and a final progressive decrease. Mahon (1982) found a similar pattern for the CO_2 exchange rate (CER) in 6 genotypes of pea under field experimental conditions; the CER values declined over the growing season, with an increase about week 8. The initial drop of photosynthetic electron flow can be explained because the fast increase of foliar surface in the early ontogeny of the plant, which can thereby obtain sufficient photosynthate supplies. The quick rise of nitrogenase activity 20 days after seed imbibition (Fig. 4d) would claim for higher photosynthate supplies to the roots, which, in turn, induces an increase of photosynthetic activity on a chlorophyll basis. The final drop is a probably consequence of a progressive senescence.

Just after phemedipham application to the leaves, a sharp decrease of the PS II-linked electron transport was observed, with an inhibition proportional to the concentration of applied herbicide, which is about 70-80% at 300 μM phenmedipham (Fig. 1 a, b, c). However, 10 days after application of 300 μM phenmedipham a recovery of photosynthetic activity was observed, which matchs the control values about 40 days after seed imbibition. Shorter recovery times appear in plants trea-

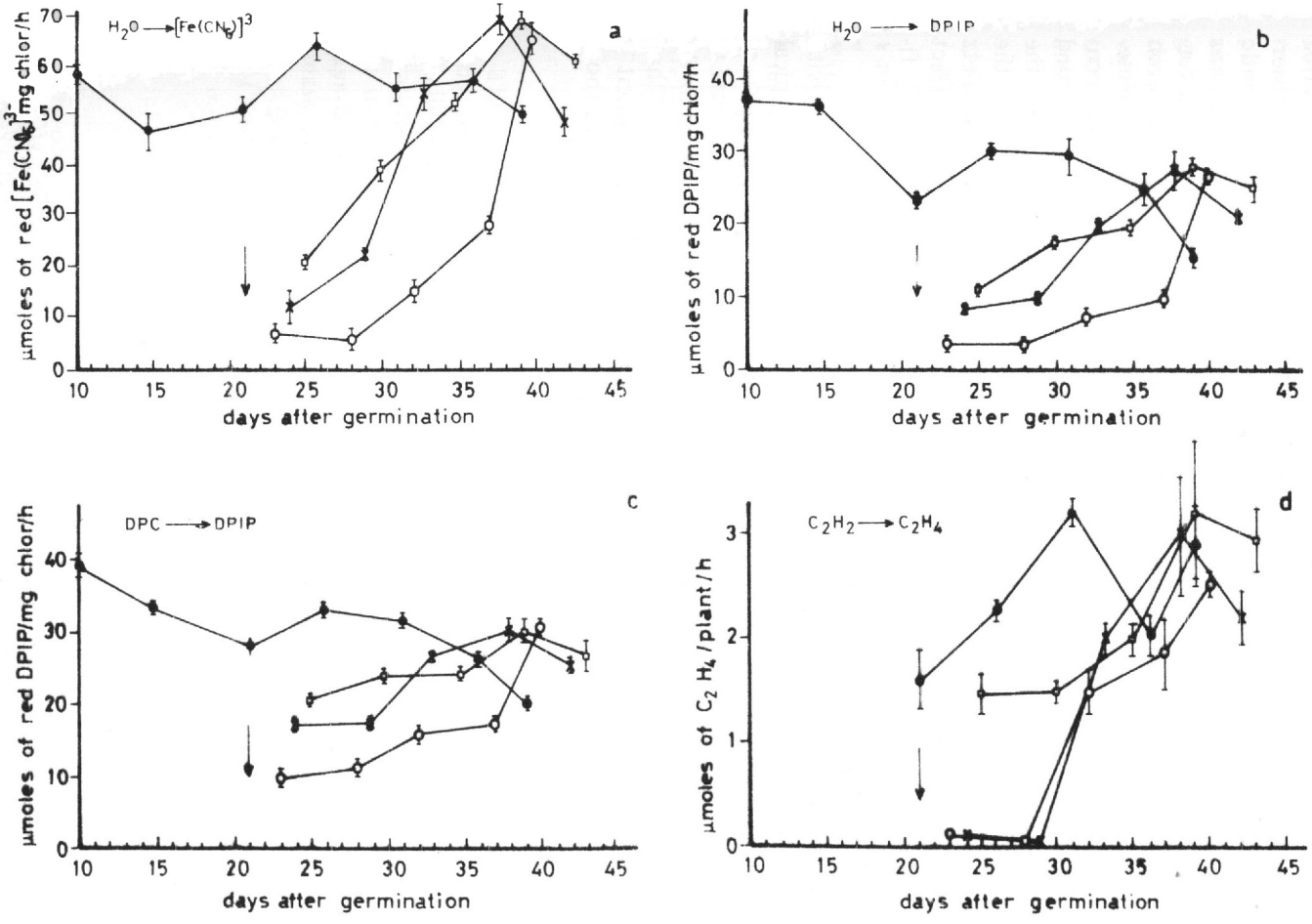

FIG. 1.—*PS II-dependent electron transport rates (a, b, c) of chloroplast membranes, and nitrogenase activity (d) of detached root systems, in controls and phenmedipham treated pea seedlings during plant ontogeny. (●) controls; (□) 70 µM phenmedipham; (x) 100 µM phenmedipham; (○) 300 µM phenmedipham. Arrows show the date of herbicide application. Values are the mean of 3 (electron transport) or 6 (nitrogen fixation) determinations ± standard error.*

ted with lower phenmedipham concentrations. Concerning the DPIP reductive electron transport chains, the Hill reaction $H_2O \rightarrow$ DPIP showed lower rates of DPIP reduction than the DPC $\rightarrow$ DPIP with diphenylcarbazide as electron donor. This can be due either to the shorter chain of the latter, or to an additional effect of phenmedipham on the water splitting system of photosynthesis, as it has been described for DCMU (Renger, 1973).

A sharp inhibition of nitrogenase activity also appears after phenmedipham application, with an almost total lost of activity about 10 days after herbicide spraying (Fig. 1d). The nitrogenase inhibition, and the subsequent recovery, parallels on time to the electron transport rate, which means a close relationship between both processes. Bethlenfalvay *et al.* (1979) also found a fast inhibition of both CER and nitrogenase activity after bentazon application to *Phaseolus vulgaris* plants. On the contrary, it was not observed any effect on the photosynthetic machinery after treatment of *Lupinus* plants with lindex, a trade mark formulation of cyanazine and linuron (De Felipe *et al.*, 1987); its deleterious effect on N_2 fixation was concerned with a herbicide-disturbed photosynthate translocation to the roots. This is in contrast with the earlier work of Rennie and Dubetz (1984), who did not find any effect of linuron on nodulation and N_2 fixation in soybean cultures.

Net values of nitrogenase activity for control plants show the typical ontogenic pattern, with a peak at anthesis (Pate, 1977). This occurs in pea cv. Lincoln after 30 days growth, with a maximum slightly higher than 3 μmoles of C_2H_4 produced plant^{-1} hour^{-1}, in the same range than those

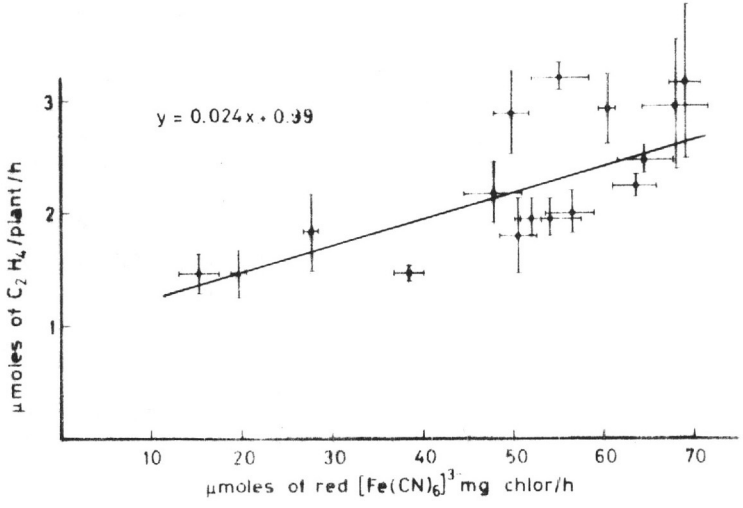

FIG. 2.—*Regression line obtained when the nitrogenase activity (μmoles of C_2H_4/plant/hour) was plotted against the PS II-dependent electron transport rate (μmoles of reduced ferricyanide/mg chlorophyll/hour), both in controls and phenmedipham treated plants. Values are the mean of 3 (electron transport) or 6 (nitrogen fixation) determinations ± standard error.*

found in other species (Waters *et al.*, 1980). Since the electron transport rate is not a real measure of the net photosynthesis, is not possible to obtain from our data any physiological correlation between photosynthetic activity and N_2 fixation. Nevertheless, if the rate values of electron transport $H_2O \rightarrow [Fe(CN)_6]^{3-}$ are plotted against those of C_2H_4 production, both in the controls and the herbicide-treated plants, the regression equation $y = 0.024 x + 0.99$ was obtained, with a correlation coefficient $r = 0.67$ (Fig. 2). More accurate correlations have been described (Dejong and Phillips, 1981) plotting the evolved C_2H_4 versus net photosynthesis, a more reliable parameter of sugar synthesis.

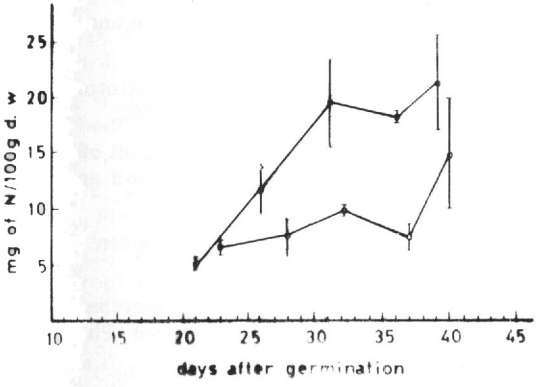

FIG. 3.—*Nitrogen content during ontogeny of controls (●), and pea plants leaf sprayed with 300 µM phenmedipham (○). Values are the mean of 6 determinations ± standard error.*

The N content through the ontogeny of the plant followed a close parallelism with that of biological N_2 fixation, including the physiologically unclear transitional drop about days 35-37 (Figure 3). A similar decrease in the N content 52-56 days after germination has been described in *Phaseolus vulgaris* (Hungria and Neves, 1986). In general, phenmedipham treatment produced a significant decrease of plant N, with about 50% control values after 30-35 days of plant development; the lack of significance in the N drop after 40 days growth is due to the limited sampling at that time.

CONCLUSIONS

As occurs with some other leaf applied herbicides, and because of their difficult translocation to the roots, phenmedipham does not show any direct effect on the establishment of *Rhizobium*-legume symbiosis. Nevertheless, its feature of photosynthetic electron transport inhibi-

tor determines a shortage in sugar synthesis, with a decreased photosynthate supplies to the roots, and a concomitant low N_2 fixation capability of bacterium.

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