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Influence of the insecticide phoxim on symbiotic and non-symbiotic nitrogen fixation determined by the acetylene reduction method

Indflydelse af insekticidet phoxim på symbiotisk og ikke-symbiotisk kvælstofbinding målt ved acetylenreductionsmetoden

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Summary

The influence of the insecticide phoxim on symbiotic and non-symbiotic nitrogen fixation was determined by means of the acetylene reduction method. 10 and 100 ppm phoxim significantly reduced the symbiotic nitrogen fixation of alfalfa grown in a sand-agar medium in test tubes after 8 days of incubation. The nitrogen fixation in media with 10, 100 and 1000 ppm phoxim was 21 days after treatment 56, 48 and 7 % of the control respectively. The non-symbiotic nitrogen fixation in soil iinoculated with *Azotobacter macrocytogenes* was significantly reduced by 10, 100 and 1000 ppm phoxim after 5 days, and after 14 days the nitrogen fixation was 82, 55 and 8 % of untreated respectively. The experiments showed that the acetylene reduction method is suitable to measure the effect of pesticides on biological nitrogen fixation.

Introduction

The influence of pesticides on biological nitrogen fixation has been determined by the effect on the number of nitrogen-fixing microorganisms in the treated soil (*Helweg,* 1973; *Kecskes,* 1970; *Kulkarni,* 1974; *van Schreven,* 1970) or by the number of surviving nitrogen-fixing microorganisms in growth media containing different concentrations of the pesticides (*Diatloff,* 1970; *Jensen,* 1969a; *Jordan,* 1969). Most experiments show only a limited effect of pesticides on the number of nitrogen-fixing microorganisms in soil whereas somewhat greater effects have been determined when pesticides are mixed into the growth media.

There is however a possibility, that the nitrogen fixation might have been decreased without any decrease in the number of microorganisms. The object of the present experiments has therefore been to determine the effect of a pesticide on symbiotic nitrogen fixation by its influence on the activity of the nitrogenfixing enzyme, nitrogenase, measured by the acetylene reduction method.

The nitrogen-fixing enzyme, nitrogenase, which reduces free nitrogen to ammonia, also reduces acetylene to ethylene. By measuring the amount of ethylene formed in different biological systems incubated in the presence of acetylene, the nitrogen-fixing capacity can be determined (*Dilworth,* 1966; *Hardy et al.* 1968; *Postgate,* 1972 and *Schöllhorn et al.,* 1967). The insecticide phoxim was used as a model.

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Materials and methods

Reagents. Technical phoxim (diäthoxy-thiophosphoryloxyiminophenylacetonitril) (83.3 % + 10 % butanol) was used in the experiments. The soil was a sandy soil of pH(H_2O) 7.1, 2.3 % humus, 6.9 % clay and 13.4 % silt. Technicical acetylene was used for incubation and 99.95 % ehylene for calibration.

Symbiotic nitrogen fixation. Alfalfa plants were grown aseptically in test tubes in which 25 ml of agar was covered with 15 g of sand (*Jensen,* 1955b). The plants were inoculated with a *Rhizobium meliloti* strain 142 from our culture collection at the two leaf stage, and were grown for two months in the greenhouse before pesticide addition. The pesticide was mixed with 1 g of sand before addition, the phoxim treated sand was poured down into each test tube through a narrow tube, to avoid contact with the leaves; pesticide concentration was calculated as ppm of total amount of sand per tube. 1 ml of water was added to each tube to ensure contact between the treated sand and the underlying original amount of sand. All treatments were replicated 4 times.

Non-symbiotic nitrogen fixation. Phoxim was added to air-dried soil in ethanol solution; the same amount of ethanol was added to each treatment. The solvent was allowed to evaporate and the soils were mixed thoroughly. 2 g of sucrose was added per 100 g of soil and the soil inoculated with 4 ml of a suspension of *Azotobacter macrocytogenes (Jensen,* 1955a) grown to OD_{660} about 1 in Burks modified medium (*Strandberg,* 1967). The nitrogenase activity in the soil became very low after 8 days of incubation if sucrose had not been readmitted.

Soil samples of 25 g were incubated at 25°C in 100 ml Erlenmeyer flasks with cotton stoppers. Water was added to about 45 % of water holdning capacity and evaporated water was replaced during the 14 days incubation period. All treatment were replicated 4 times.

As reported by *Ben-Bassat et al.,* (1972); *Ilag et al.,* (1968) and *Smith et al.,* (1971), some biological systems produce ethylene. Soil with pesticide was therefore incubated and analyzed for ethylene production without any addition of acetylene, but no ethylene production was ever found.

Determination of acetylene reduction. To determine nitrogenase activity the incubation flasks were mounted with rubber stoppers with a silicone membrane in a Quickfitt tube in order to add and remove air samples. 9 % acetylene was added and the flasks were incubated for 3-5 hours. The amount of ethylene produced was determined on Hewlett-Packard gaschromatograph, model 5750 with flame ionization detector; column: Porapak N 80 – 100 mesh, carrier: He. A small amount of methane, which was a contaminant of the acetylene was used as internal standard. After each incubation with acetylene, the flasks and tubes were carefully washed with moistened air to remove acetylene and ethylene; even small amounts of acetylene inhibit normal nitrogen fixation (*Schöllhorn et al.,* 1967) and ethylene inhibits the nodulation (*Grobbelaar et al.,* 1970) and is also a plant inhibitor (*Apelbaum et al.,* 1972 a, b). The activity of the nitrogenase enzyme was calculated as mol ethylene produced per minute and was found to be proportional to time up to 5 hours of acetylene incubation. A standard curve to determine the amounts of ethylene produced was prepared with 99.95 % ethylene.

Results and discussion

Effect on symbiotic nitrogen fixation. Table 1 shows a significant inhibitory effect of both 10 and 100 ppm phoxim on the symbiotic nitrogen fixation of alfalfa after 8 days of incubation with pesticide. The inhibition lasted for more than 2 weeks and 1000 ppm phoxim almost completely stopped the nitrogen fixation.

Effect on non-symbiotic nitrogen fixation. Table 2 shows the effect of phoxim on nitrogen fixation in soil inoculated with Azotobacter macrocytogenes. Only 1000 ppm phoxim had

Table 1. Influence of the insecticide phoxim on symbiotic nitrogen fixation.
Tabel 1. Indflydelse af insektmidlet phoxim på symbiotisk kvælstofbinding.

Concentration	Incubation time (days)			
	0	8	13	21
Control	100a	100a	100a	100a
10 ppm	84a	44b	46b	56b
100 ppm	90a	56b	39b	48b
1000 ppm	84a	–	–	7c

Influence of 10, 100 and 1000 ppm phoxim on symbiotic nitrogen fixation as measured on nodulated alfalfa grown in sand-agar culture. The nitrogen fixation is determined by the acetylene reduction method and calculated as percentage of untreated. Values that are significantly different at $p < 0.05$ are signed by different letters in each column.

Indflydelse af 10, 100 og 1000 ppm af insektmidlet phoxim på symbiotisk kvælstofbinding hos podede lucerneplanter dyrket i sandagar substrat. Kvælstofbindingen måltes ved acetylenreduktionsmetoden og er udregnet i % af ubehandlet. De værdier, som er signifikant forskellige ved $p < 0.05$ er mærket med forskellige bogstaver i den enkelte kolonne.

Table 2. Influence of the insecticide phoxim on non-symbiotic nitrogen fixation in soil.
Tabel 2. Indflydelse af insektmidlet phoxim på ikke symbiotisk kvælstofbinding i jord.

Concentration	Incubation time (days)				
	1	3	5	10	14
Control	100a	100a	100a	100a	100a
10 ppm	93a	96a	79b	82b	88b
100 ppm	100a	86a	29c	66c	55c
1000 ppm	71b	41b	19c	13d	8d

Influence of 10, 100 and 1000 phoxim on non-symbiotic nitrogen fixation determined in soil inoculated with *Azotobacter*. The nitrogen fixation is measured by the acetylene reduction method and calculated as percentage of untreated. Values that are signed by different letters in each column are significantly different at $p < 0.05$.

Indflydelse af 10, 100 og 1000 ppm phoxim på ikke symbiotisk kvælstofbinding i jord podet med *Azotobacter*. Kvælstofbindingen er målt ved acetylenreduktionsmetoden og udregnet i % af ubehandlet. De værdier, som er signifikant forskellige ved $p < 0.05$ er mærket med forskellige bogstaver i den enkelte kolonne.

a significantly inhibitory effect after 1 day of incubation, but after 5 days both 10, 100 and 1000 ppm phoxim show an inhibitory effect as compared to untreated. The effect lasted for more than two weeks and for 1000 ppm the inhibition seemed to enhance during the time of incubation.

Discussion and conclusion

The determined effect on symbiotic nitrogen fixation can be due either to an effect on plant and bacteria metabolism or an influence on the function of the nitrogenase enzyme. The inhibition of nitrogen fixation of Azotobacter in soil can be due either to an influence on cellmetabolism or to a blocking of the function of the nitrogenase enzyme. The results are from laboratory experiments and they should not without precautions be related to field conditions, since other environmental factors might give different results. This is especially true for the Rhizobium experiments, since the pesticidal effect in the sand-agar medium is expected to be much greater than in soil.

The methods mentioned seem a useful supplement to other methods used to determine the effects of pesticides on soil microorganism. The acetylene reduction method is quick and exact and allows repeated determinations on the same sample. Furthermore it is based on an important microbial process.

Sammendrag

Indflydelsen af insektmidlet phoxim på symbiotisk og ikke-symbiotisk kvælstofbinding blev målt ved hjælp af acetylenreduktionsmetoden. Denne metode udnytter, at det kvælstofbindende enzym nitrogenase, der reducerer frit kvælstof til ammoniak, også kan reducere acetylen til ethylen, som kan måles gaschromatografisk.

Indflydelsen på symbiotisk kvælstofbinding blev målt på podede lucerne planter, dyrket i sand-agar kultur i reagensglas. Både 10 og 100 ppm phoxim i vækstmediet reducerede efter 8 dages inkubering kvælstofbindingen hos podede lucerneplanter (se tabel 1). Efter 21 dages inkubering med 10, 100 og 1000 ppm phoxim

var kvælstofbindingen reduceret til henholdsvis 56, 48 og 7 % af ubehandlede planter inkuberet i samme periode.

Phoxims indflydelse på den ikke symbiotiske kvælstofbinding måltes i jord podet med *Azotobacter macrocytogenes*. Både 10, 100 og 1000 ppm phoxim reducerede kvælstofbindingen efter 5 dages inkubering (se tabel 2). Efter inkubering i 14 dage var kvælstofbindingen reduceret til henholdsvis 88, 55 og 8 % af ubehandlet jord inkuberet i samme periode.

Resultaterne viser, at acetylenreduktionsmetoden er velegnet til måling af pesticiders indflydelse på den biologiske kvælstofbinding, og kan supplere de øvrige metoder, som anvendes til undersøgelse af pesticiders indflydelse på jordbundens mikroflora.

Resultaterne stammer fra laboratorieforsøg og gælder ikke uden forbehold under markforhold, både fordi koncentrationerne ved normal anvendelse sjældent når op på 10 ppm og fordi virkningen på kvælstofbindingen hos podede lucerneplanter er målt i et sand agar medium, hvor pesticidets skadevirkning må formodes at være væsentligt større end i jord.

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