GASEOUS HYDROGEN CYANIDE AS AN AGENT TO CONTROL NEMATODES IN PLANT MATERIALS*

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At present there is a lack of chemical agents on the market, which could be employed for protecting seed material, planting stock and wood against quarantine phyto-parasitic nematode species *Ditylenchus dipsaci* and *Bursaphelenchus xylophilus*. The object of this research consisted in the development of a new experimental system that would allow testing gaseous hydrogen cyanide for its possible use as an alternative fumigant to control phyto-parasitic nematodes. In this work the nematode species *Caenorhabditis elegans* was used as a model for testing. At the HCN concentration of 7 $g.m^{-3}$ and the time of exposure of 270 minutes 100% mortality of the model species was achieved. Our future research activities should be aimed at establishing of the optimum ranges of temperature and relative humidity to be maintained in the fumigation chamber with a view to find the optimum process parameters, the concentration of HCN, air temperature and relative humidity in particular. It is expected that the use of hydrogen cyanide gas under favourable conditions will provide reliable elimination of phyto-parasitic nematodes in seed material, planting stock and wood.

gaseous hydrogen cyanide; plant protection; *Caenorhabditis elegans*; model organism; phytoparasitic nematodes

INTRODUCTION

Quarantine phyto-parasitic nematodes belong to the most significant plant pests. The current situation in this field is caused by the absence of efficient methods of chemical control. Nowadays, in the Czech Republic there is only one approved agent – Basamid late (containing Dazomet as an active ingredient) – of the required type. Therefore, it is in the interest of growers to extend the spectrum of applicable active substances that could be used to protect agricultural crops against this group of pests.

The nematodes *Ditylenchus dipsaci* (stem and bulb eelworm) and *Bursaphelenchus xylophilus* (pine wood nematode) belong to the phyto-parasitic nematodes of special importance. The first of them is deemed a very serious pest of vegetables and fodder crops. In the conditions prevailing in the Czech Republic the stem and bulb eelworm cause damage, particularly to the vegetables of the onion family. Heavy infestations will frequently lead to the total loss of the yield. The basic method of protection from the stem and bulb eelworm consists in the use of sound seed material. In case the growers want to be sure of the absence of the stem and bulb worm in seed, they have to resort to seed treatment. A sulphide-based pesticide (Sulka) has been used for the purpose up to now, however its performance is relatively low and, moreover, the term of its registration validity has already expired. That is why only its quantities currently in stock may be used until exhaustion. The growers, therefore, have to rely on the treatment of seed with warm water, the method, that is relatively efficient, but its application to a higher amount of seed is difficult. Moreover, if it is not done properly, the whole treated quantity may suffer damage.

The harmfulness of the Pine Wood Nematode is a problem of global relevance. The species appears to be one of the worst pests of woody plants, particularly of those of the *Pinus* genus. The Pine Wood Nematode comes from North America, where it inhabits the local species of pines, without causing any significant harm to them. After it had been introduced into Japan and, later, into other East Asian countries, calamitous damage occurred to the domestic species of the *Pinus* genus (Mamiya, 1988). In 1999 the presence of pine wood nematode was discovered in Europe in Portugal (Mota et al., 1999) and in Spain in 2008 (Albelleira et al., 2011). Even in spite of extraordinary phytosanitary measures have been taken, the current situation, particularly in Portugal, does not support any assumption of successful eradication of the pine wood nematode in continental Europe. One of the basic extraordinary measures taken against the propagation of pine wood nematode consists in the heat treatment of timber coming from the areas at risk of occurrence of the pest. Other and significant problems in the field stem from the lack of suitable

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active substances that could be used as nematocides, particularly since the absolute ban was put on the use of methylbromide by the European Commision (Regulation (EC) No 2037/2000).

Therefore, it is in the best interest of growers to extend the spectrum of active substances applicable to the protection of agricultural crops and other plant material from quarantine phyto-parasitic nematodes. Agents considered auspicious in this respect include gaseous hydrogen cyanide (HCN, marketed under its trade name 'Uragan D2' by Draslovka Kolín). The use of the substance offers significant advantages that may be gained by its application in practice, particularly the fact that the substance occurs naturally in the environment (even in minimum concentrations), features very high penetration properties, is highly reactive and, therefore, easily and quickly degradable, while leaving no undesirable residues. A problem can be seen in the extreme toxicity of the substance at higher concentrations. That is why its application should be carried out by qualified personnel only in official sealed systems. The substance is deemed very suitable for the treatment of plant material stored in large quantities, whether it comprises timber, seed crop or planting stock. In other words, there is a real chance to employ gaseous hydrogen cyanide as an agent for protecting plants and wood against infestation by the stem and bulb eelworm or the pine wood nematode.

At present time, the investigation of the effects of gaseous hydrogen cyanide on the stem and bulb eelworm and pine wood nematode is carried out within the framework of the NAZV project of the Ministry of Agriculture No. QI111B065. This paper presents the first results obtained by testing of gaseous hydrogen cyanide as affected the viability of the free-living model nematode *Caenorhabditis elegans* in a newly designed experimental system.

MATERIAL AND METHODS

An experimental system was designed to test the effect of gaseous hydrogen cyanide on nematodes, making it possible to evaluate the effects of treatment on the mortality and survival of nematodes.

Nematodes

The nematode species *C. elegans* (Fig. 1.) was selected as a model organism for testing the effects of gaseous hydrogen cyanide on nematodes. The species is commonly used in the studies of nematodes for the purpose. In order to obtain a sufficient number of nematodes, *C. elegans* nematodes were grown and reproduced using the procedure of Stiernagle (1999) in vitro on Petri dishes of 60 mm diameter, being fed with *Escherichia coli* (op-50 genotype) bacteria. First, the dishes with substrate were prepared for nematode cultivation, in which 20 gravid female nematodes were transferred using sterile nematologic needles. The cultures prepared in that way were then incubated for seven days at the temperature of 23°C. Then, the nematodes were separated from the cultures employing the modified Baermann funnel method (Hooper, 1986) lasting four hours. The resulting dispersion of nematodes at various stages of development was placed in embryonal dishes and the larvae of the second and third stages of development were selected under a stereo-microscope and transferred in glass vials filled with physiologic solution. The nematodes prepared in this manner were immediately used for the following experiments.

System for testing the nematocidic activity of gaseous HCN

Sterile cultivation substrate (supplied by Stender) was used as a carrier of nematodes in the model system. On one side, it adequately simulated the natural life conditions of *Caenorhabditis elegans* allowing their problem-free survival, and, on the other side, it served as a suitable material for HCN penetration. The substrate was sterilised in advance in an autoclave at the temperature of 121°C for 25 minutes to eliminate any possible antagonistic organisms or naturally occurring nematodes. The substrate (5 g) was placed on the bottom of a 50 ml vial with a modified lid. Lid modification consisted in its perforation and covering its neck with the Uhelon fabric of 80 µm mesh size allowing free access of HCN to the vial. Nematodes were placed in the prepared substrate by means of a glass capillary and an automatic pipette. As determined, the number of nematodes per vial was within the range of 300 ± 20 individuals.

Fumigation

The fumigation of the prepared vials filled with nematodes was carried out by a specialised facility

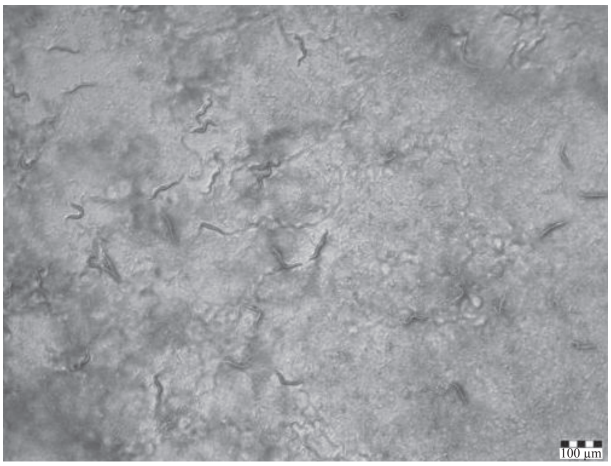

Fig. 1. Nematodes *C. elegans* in culture

(Draslovka Kolín) using a prototype experimental fumigation chamber. The chamber allows the continuous, non-invasive withdrawal of individual samples through an air lock antechamber in preset time intervals. The samples were taken from the fumigation chamber stepwise in the following intervals elapsing from the treatment start: 60, 120, 180, 240, 270, 300, 330, and 360 minutes; 6 parallel samples were taken for each time of exposure. The concentration of gaseous hydrogen cyanide in the chamber was monitored and maintained at the required level of 7 $g.m^{-3}$ (this concentration was chosen according to amount of hydrogen cyanide used for treatment of plant material against insect pests) during the trial. A control (blank) experiment was carried out likewise but the samples were kept outside the chamber for the time of fumigation to see if non-hydrogen cyanide treatment mortality occurs to ensure that false positive results will be not recorded.

Evaluation

After the fumigation, chamber was cleared from the HCN containing atmosphere by ventilation; nematodes from the samples were separated by the modified Baermann method.

The design of vials allows the direct extraction of nematodes, which markedly facilitates the sample handling. It is then only required the container to be filled with distilled water and placed upside down in the Baermann funnel. The mesh size of the fabric covering the vial neck allows the live nematodes to migrate into the funnel, while the dead ones will be kept in the vial. The control variant was evaluated in the same manner.

After 12 hours of extraction the suspension of nematodes was transferred into an embryonal dish and the counts of nematodes in individual samples were determined by stereomicroscope.

The measured data were evaluated by statistical methods using the analysis of variance. In order to detect statistically significant differences among the results of individual fumigation times and the data of untreated control samples the Tukey's HSD test (Statistica Cz 10 software of StatSoft, Inc.) was employed.

RESULTS AND DISCUSSION

Samples taken at any time of fumigation showed statistically significant reduction in the number of nematodes ($p \leq 0.05$) when compared with an untreated control. As soon as after 60 minutes the exposure to HCN resulted in the reduction in the count of live nematodes by about 50%. Mortality of *C. elegans* showed a continuous growth and after 270 minutes of exposure it achieved 100% (Fig. 2). Any comparison of our results with published data is, however, very difficult, as there is a lack of information in the literature concerning the effect of hydrogen cyanide gas on nematodes. On the other hand, based on the obtained results we can state that the use of gaseous hydrogen cyanide appears to be a highly effective to cause the high death rate of the model nematode *C. elegans.* The relatively fast effect of the substance seems to be advantageous, taking into account a fact, e.g., that at exposure to Phospine the full (100%) mortality of *C. elegans* was achieved as late as after 24 hours (Z u r y n et al., 2008). Gaseous hydrogen cyanide affects at a very low concentration compared to other agents. For instance, the treatment by means of sulfonyl fluoride at the concentration of 60 $g.m^{-3}$ for 48 hours did not result in any reduction in the count of pine wood nematodes (S o m a et al., 2001) and the concentration of about 100 $g.m^{-3}$ of sulfuryl fluoride was required to cause 100% mortality of the same species (D w i n n e l et al., 2003). It is also interesting to compare the effect of HCN with that of previously employed methyl bromide. To achieve the full (100%) mortality of pine wood nematode in wood blocks the above-mentioned agent should be applied at the concentration of 240 $g.m^{-3}$ (D w i n e l l, 1994).

It should be admitted that there are not too many alternative agents that could be used to control phytoparasitic nematodes in replacement of methyl bromide, whose production and importation have been prohibited only recently. Out of the substances already tested in the protection of wood and other plant materials against insects or fungal diseases a few chemicals can be taken into account, including, e.g., carbon disulfide, carbonyl sulfide, ethyl formate, ethylene oxide, propylene oxide or the mentioned fosfane. The use of those compounds, however, entails numerous risks consisting mostly in the presence of after-treatment harmful residues, hazardous to human health, poor penetration into plant materials or environmental hazards (B a n k s, 2003). In this respect gaseous hydrogen cyanide seems to be one of the most promising substances. Of course, its application, too, is connected with certain risks stemming particularly from its high acute toxicity.

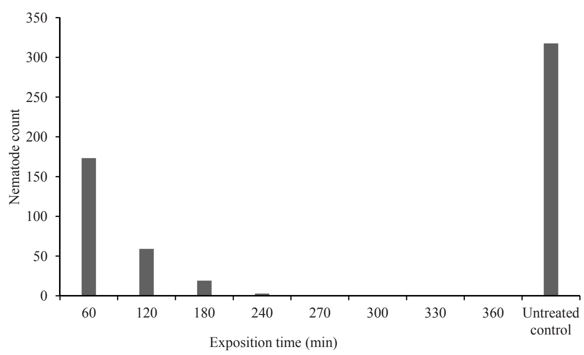

Fig. 2. Effect of the action of gaseous hydrogen cyanide on the *C. elegans*

Nevertheless, in comparison with the above-mentioned chemicals, taking into account its high performance, these risks are acceptable.

CONCLUSION

Within the framework of this research an original experimental system was proposed to test the effects of gaseous hydrogen cyanide on the model nematode of *C. elegans* species. The obtained data indicate the high efficiency of the tested substance, manifested in the high death rates of the model organism, achieved by treating it using relatively low concentrations of hydrogen cyanide in air and short exposure times. In the future the research will be aimed at the investigation of the direct action of gaseous hydrogen cyanide action on phyto-parasitic nematodes. Research activities will be focused particularly on the temperature and relative humidity of process atmosphere and the concentration of hydrogen cyanide to establish the optimum combination of process parameters for reliable elimination of nematodes in seed material, planting stock and wood.

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Plynný kyanovodík jako prostředek pro ošetření rostlinného materiálu vůči háďátkům

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V současné době na trhu není dostatek chemických prostředků pro ochranu osiva, sadby a dřeva proti karanténním fytoparazitickým háďátkům druhů háďátko zhoubné (*Ditylenchus dipsaci*) a háďátko borovicové (*Bursaphelenchus xylophilus*). Cílem tohoto výzkumu byl vývoj nového experimentálního systému pro modelové testy účinnosti plynného kyanovodíku (HCN) jako alternativního fumigantu proti fytoparazitickým háďátkům. K pokusům byl použit modelový druh háďátka *Caenorhabditis elegans*. Při koncentraci 7 g.m^{-3} a expozici trvající 270 min. byla zjištěna 100% mortalita modelového druhu háďátka. Dalším cílem výzkumu bude optimalizace teploty a vlhkosti ve fumigační komoře se záměrem nalézt optimální kombinaci těchto parametrů a koncentrace kyanovodíku pro spolehlivou eradikaci fytoparazitických háďátek v osivu, sadbě i dřevu.

plynný kyanovodík; ochrana rostlin; *Caenorhabditis elegans*; modelový organismus

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