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EFFECT OF HYDROSTATIC PRESSURE ON THE VIABILITY OF EPN NATIVE ITALIAN STRAINS *STEINERNEMA FELTIAE* AND *HETERORHABDITIS BACTERIOPHORA* (1)

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Anifantis A.S., Marziale R., Pascuzzi S., Ravlic J., Grujić N., Tarasco E. - Effect of hydrostatic pressure on the viability of EPN native Italian strains *Steinernema feltiae* and *Heterorhabditis bacteriophora*.

The use of entomopathogenic nematodes (EPNs) as a non-chemical treatment system is one of the most modern techniques promoted by the 2008/128/EC standards for the biological control of plant pathogens. The distribution of EPNs could be carried out with the same sprayers used for the distribution of pesticides. This research is focused on the effects of the mechanical stress due to hydraulic compression on EPNs Italian strains, *Steinernema feltiae* and *Heterorhabditis bacteriophora*, to assess their residual strength and vitality under laboratory tests. Liquid suspensions of nematodes were subjected to 8 different levels of hydrostatic pressure from 5 to 40 bar. The hydraulic compression test was conducted through a hydrostatic compression system specifically designed for the experimental test. Although correlation curves show an increment of 14% in nematode mortality, the statistical analysis did not show any significant effect of static pressure on the vitality and infectivity of EPNs, that makes them suitable for field application with sprayers. These preliminary positive results on the effects of compression on the viability of EPNs are preparatory to subsequent field tests under high pressure.

KEY WORDS: non-chemical treatments sprayers, biological control equipment, entomopathogenic nematodes, hydrostatic compression.

INTRODUCTION

The most innovative frontiers in reducing the environmental impact of agricultural crops involve the use of precision agriculture for the estimation of the quantity of pesticides to be used also through unmanned aerial vehicle (UAV) systems integrated with smart sprayer equipment (ANIFANTIS *et al.*, 2019), the automatic detection of pathologies, punctual treatments, etc. However, these techniques involve the use of sophisticated technologies and highly qualified operators at the service of agricultural enterprises and the progress made so far does not allow their dissemination in small and medium-sized enterprises with low investment capital. Other areas of research focus on crops in a protected environment as they ensure a significant increase in productivity, greater disease control and reduced use of chemicals, especially for hydroponic crops. However, although the benefits are considerable, economic and environmental expenditure is very high despite all the present technologies used to reduce energy consumption (ANIFANTIS, 2017; ANIFANTIS *et al.*, 2016, 2018, 2019; RUSSO *et al.* 2014) on the one hand and the use of plastics (ANIFANTIS *et al.*, 2012; PRZYWARA *et al.*, 2019; RUSSO *et al.*, 2016) on the other. Certain biological pathogen or pest control techniques are preferable to all other techniques from an environmental point of view (REF). Biological control techniques are more sustainable than chemical treatment techniques because they reduce the

use of chemical pesticides and environmental degradation (REF). Entomopathogenic nematodes (EPNs) are the most promising biocontrol agents among nematodes (REF). Although the first species of EPN has been described almost 100 years ago, their flooding application only started about 30 years ago. Increased understanding of the nematode biology, host range, epizootiology, advances in production technology, formulation, storage and application resulted in an exponential increase of interest in the market (ARTHURS *et al.*, 2004). Beside research advances, their rapid expansion was supported also by exemption or ease of registration based on reports of no effect on humans, mammals and plants (EHLERS, 2005). Their successful biocontrol attributes are based on the unique partnership of host seeking nematodes and lethal insect-pathogenic bacterium carried inside the nematode's intestine (POINAR, 1993). Half a century had to pass from description of the first species to their mass production due to the development of cheap broad-spectrum insecticides that shifted attention away from biocontrol agents. Since the 1980s, application of EPNs has experienced growth due to the development of relatively cheap, large scale liquid culture production technology and pressure on pesticide use due to the development of resistance, surpassing residue levels in food and negative impacts on the environment. EPNs of Steinernematidae and Heterorhabditidae families are parasites of insects (POINAR, 1990) and their infectious activity varies with the species and

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strain and is affected by abiotic and biotic factors, which influences their infectivity potential, survival, motility, development and reproduction (MOLYNEUX, 1985; KAYA, 1990; ZERVOZ *et al.*, 1991; MASON and HOMINICK, 1995; WILSON AND GAUGLER, 2004; TORRINI *et al.*, 2016; EL KHOURY *et al.*, 2018). One of the limiting abiotic factors is the mechanical distribution in the field which is commonly carried out with the same sprayers used for the distribution of pesticides; there is a real possibility that such an operation could induce stress due to sudden changes in pressure and mechanical stresses generated within the rapid changes in pressure and flow rate that occur in common sprayer machines (NILSSON and GRIPWALL, 1999; ŁACZYŃSKI *et al.*, 2004; BULGACOV *et al.*, 2018).

Studies on reducing the viability of EPNs, however, do not provide unique answers. In fact, while some studies (FIFE *et al.*, 2003, 2004; ADE *et al.*, 2010; LANZONI *et al.*, 2014) report that pressure does not affect the vitality of *Heterorhabditis bacteriophora* Poinar, *H. megidis* Jackson & Klein (Rhabditida, Heterorhabditidae) and *Steinernema carpocapsae* Weiser (Rhabditida, Steinernematidae), others underline a 10% reduction in vitality of *S. feltiae* Filipjev as a result of piston pump distribution (NILSSON and GRIPWALL, 1999). Moreover, other authors

indicate a linear decrease in the vitality of *H. bacteriophora* in comparison to the duration of the hydraulic agitation (ŁACZYŃSKI *et al.*, 2004).

This work aims to assess the effects of the increase of hydraulic compression, as a mechanical parameter, on the strength and vitality of Italian native strains of *S. feltiae* and *H. bacteriophora*.

MATERIALS AND METHODS

NEMATODES

Native strains of *S. feltiae* (ItS-MF1) and *H. bacteriophora* (ItH- LU1) (TARASCO *et al.*, 2015) were used in the experiment. Nematodes were cultured in last-instar *Galleria mellonella* L. (Lepidoptera, Pyralidae) larvae at a temperature of 24°C. To obtain fresh infective juveniles (IJs), 10 wax worms on a 100 x 10 mm Petri dish with one 90 mm filter paper were treated with ca. 2,000 IJs in 1.5 ml of water. Two weeks after treatment, wax worms were put on modified White traps (WHITE, 1927) for the recovery of new generations of IJs. Collected IJs were kept at 13 °C and used within 5 days after harvesting. For each test, a sample of 140 ml of suspension containing

Fig. I - Flow chart of the experimental test of hydrostatic compression effects on nematode mortality.

300 nematodes was taken. The liquid suspensions of nematodes were subjected to different levels of hydrostatic pressure for 20 seconds. Each experiment was repeated 3 times. Nematodes were counted in Petri dish capsules with a gridded bottom using a dissection microscope. Only active nematodes have been counted (GREWAL, 2002) and relative vitality determined as a percentage of the alive individuals on the total number of nematodes. After each hydraulic compression test, the nematodes were released on filter papers in Petri dishes with 5 *Galleria* larvae to check and evaluate their infectivity (Fig. I).

EVALUATING THE EFFECT OF STATIC PRESSURE

The hydraulic compression test was conducted through a compression system specifically designed for the experimental test (Fig. II, 1). The experimental test bench (Fig. II, 2) consists of a water tank (S), a manually operated hydraulic piston pump with a maximum working pressure of 50 bar (P), two gate valves designed to manage the overpressure and the flow rate (V1, V2), a cylinder with the internal volume of 140 ml, seat of the compression test (C), three gate valves to separate the water to the nematodes solution and maintained the pressure water

Fig. II - Experimental test bench: (1) Implemented; (2) Pipe and instrument diagram.

to the nematodes solution and maintained the pressure level for the time required by the test (V3, V4, V5), a hydraulic pressure gauge with an accuracy of 2 bar (M). Eight different levels of hydrostatic pressure were tested: 5-10-15-20-25-30-35-40 bars

The compression test took place with the following steps and starting from an initial condition with all valves opened:

- 1) Closing V2, V4 and filling the hydraulic circuit up to V5 level with the water.
- 2) Closing V3 and opening V4 and removal of residual water in cylinder C.
- 3) Closing V4 and filling the solution (140 ml) water and nematodes through V5.
- 4) Closing V5 and opening V3 with cylinder compression up to the desired pressure and keeping the cylinder at a higher geodetic altitude than the hydraulic circuit (nematodes tend to float).
- 5) Closing V4 and holding at test pressure the cylinder C.
- 6) Opening V5 and emptying the cylinder C and sampling vitality of nematodes.

- 7) Opening of all valves and flushing of the circuit for the next test.

Data were pooled and analyzed using a general linear model procedure (ANOVA - analysis of variance) and significant differences among means were separated by HSD Tukey's test (STATISTIX 9.0, 2008). All comparisons were set at 0.05 level of significance. Three mortality tests were conducted for each of the eight levels of pressure for both EPNs species. Mortality was calculated as the ratio of rigid to curved nematodes in percent.

RESULTS

The results reported in Table 1 represent the means of ten samples for each test at a known pressure. ANOVA analysis showed that static pressure of up 40 bars, even prolonged for 20 seconds, does not cause significant damage to the EPNs examined. There is a slight increase in mortality of nematodes as the pressure increases but it is not statistically relevant. Moreover, the data did not show any effect of static pressure on the infectivity of EPNs

Table 1 - Nematode mortality of Italian EPN native strains *S. feltiae* and *H. bacteriophora* after 20 seconds of exposure to 8 levels of hydrostatic compression (from 5 to 40 bar).

<i>H. BACTERIOPHORA</i>								
Pressure [bar]	5	10	15	20	25	30	35	40
1st Mortality test	4	4	6	6	6	7	10	11
2nd Mortality test	4	6	5	7	5	9	9	9
3rd Mortality test	3	4	8	5	8	7	8	9
<i>S. FELTIAE</i>								
Pressure [bar]	5	10	15	20	25	30	35	40
1st Mortality test	3	4	8	7	13	12	11	13
2nd Mortality test	5	4	10	11	9	11	15	12
3rd Mortality test	3	5	5	9	9	13	10	13

These data confirmed for the EPN native Italian strains *S. feltiae* and *H. bacteriophora* that pressure does not affect their vitality and infectious activity as similar

nematodes (FIFE *et al.*, 2003, 2004; ADE *et al.*, 2010; CHOJNACKI *et al.*, 2010; FURTADO MOREIRA *et al.*, 2013).

Although ANOVA analysis, showed that the increase

of static pressure does not cause significant damage to the EPNs examined, there is a statistical correlation between increased pressure and nematodes viability. As shown in Fig. III, the higher pressure induces a different mortality increase for the two nematode species. In fact, the mortality

of *S. feltiae* increases faster than *H. bacteriophora*. Moreover, the correlation coefficient of Pearson is very high, approximately up to 95% for both species, even using a linear interpolation curve. For these reasons the pressure-dependent mortality of *H. bacteriophora* and *S. fel-*

Fig. III - Nematodes mortality of Italian EPN native strains *S. feltiae* and *H. bacteriophora* after 20 seconds of exposure to 8 levels of hydrostatic compression (from 5 to 40 bar). No significant differences ($P < 0.05$) between the strains and among the pressure levels were detected.

tiae can be described by equation 1 and equation 2, respectively. Although the two equations have a different angular coefficient and therefore species *S. feltiae* is more sensitive to pressure than species *H. bacteriophora*, both curves meet at about 3% mortality, this is mainly due to external factors, different from pressure, leading to low mortality. On the other hand, the increase in mortality with increasing pressure triples for the first species and fivefold for the second species when it reaches 40 bar.

Linear interpolation equations between nematode mortality and pressure increase:

$$M = 0.16 P + 3, \quad (1)$$

$$M = 0.28 P + 2.8, \quad (2)$$

Where M is the mortality (%) and P is the level of hydrostatic pressure to which the nematodes were tested. As shown in equations 1 and 2 the linear mortality curves increase with a slope of 9° and 16° respectively, so for the species *S. feltiae* the mortality is almost double compared to the species *H. bacteriophora*. However, the mortality growth gradient with respect to pressure is rather low and therefore, this leads to the conclusion that both species have excellent resistance even at higher pressures, especially the species *S. feltiae*, and that they can be used for more demanding applications such as those using low drift nozzles at high pressure.

Further experiments will be conducted to estimate the dynamic effects and rapid transient pressure variation effects, typical of hydraulic machines, on the viability of nematodes.

DISCUSSION

In the present work the effects of increased hydrostatic pressure on the viability of nematodes have been analyzed. Although HSD Tukey's statistical survey has shown that the effects of pressure on nematode mortality are statistically meaningless, as also shown by some previous studies on nematodes, correlation studies indicate that there is a strong link between pressure increase and nematode mortality as shown by other studies. However, this increase, fortunately, is very small, around 6-12%, suggesting that EPNs should also be used in sprayers with high pressure and liquid vein fragmentation (ŠIMA *et al.*, 2003; ŁACZYŃSKI *et al.*, 2006). Thanks to the pressure increase it is possible to reach more hidden areas and increase the leaf coverage coefficient. Further experiments will be conducted to estimate the dynamic effects and rapid transient pressure variation effects, typical of hydraulic machines, on the viability of EPNs. Furthermore, subsequent studies will be carried out in the field with the use of high-pressure sprayers and high concentration EPNs solutions

in order to maximize biological control through this innovative technique.

REFERENCES

- ADE G., LANZONI A., MARTELLI R., PEZZI, F.; UGUZZONI, A., 2010 - *Effects of mechanical distribution on the viability of the entomopathogenic nematode Steinernema carpocapsae*. - Atti Giornate Fitopatologiche, vol. 2, pp. 87-94.
- ANIFANTIS A.S., 2017 - *Performance assessment of photovoltaic, ground source heat pump and hydrogen heat generator in a stand-alone system for greenhouse heating*. - Chemical Engineering Transactions, vol. 58, pp. 511-516, DOI:10.3303/CET1758086 (ScopusID:2-s2.0-85026389376).
- ANIFANTIS A., CANZIO G., CRISTIANO G., DE LUCIA B., RUSSO G., VECCHIETTI L., IMMIRZI B., MALINCONICO M., SANTAGATA G., 2012 - *Influence of the Use of Drip Irrigation Systems and Different Mulching Materials on Ornamental Sunflowers in Greenhouse Cultivation*. - Acta Horticulturae, 952: 385-392. ISSN: 0567-7572. DOI: 10.17660/ActaHortic.2012.952.48 (ScopusID: 2-s2.0-84863700751).
- ANIFANTIS A.S., PASCUZZI S., SCARASCIA MUGNOZZA G., 2016 - *Geothermal source heat pump performance for a greenhouse heating system. An experimental study*. - Journal of Agricultural Engineering, 47(3), 544, pp. 164-170. DOI:10.4081/jae.2016.544 (ScopusID: 2-s2.0-84990966946).
- ANIFANTIS A.S., COLANTONI A., PASCUZZI S., 2017 - *Thermal energy assessment of a small scale photovoltaic, hydrogen and geothermal stand-alone system for greenhouse heating*. - Renewable Energy, 103: 115-127. DOI: 10.1016/j.renene.2016.11.031 (ScopusID: 2-s2.0-84995899642).
- ANIFANTIS A.S., COLANTONI A., PASCUZZI S., SANTORO F., 2018 - *Photovoltaic and hydrogen plant integrated with a gas heat pump for greenhouse heating: A mathematical study*. - Sustainability (Switzerland), 10(2), 378: 378-389. doi:10.3390/su10020378 (ScopusID: 2-s2.0-85041453767).
- ANIFANTIS A. S., CAMPOSEO S., VIVALDI G. A., SANTORO F., PASCUZZI S., 2019 - *Comparison of UAV photogrammetry and 3D modeling techniques with other currently used methods for estimation of the tree row volume of a super-high-density olive orchard*. - Agriculture (Switzerland), 9 (11); doi:10.3390/agriculture9110233 (ScopusID: 2-s2.0-85074324465).
- ARTHURS S., HEINZ K.M., PRASIFKA J.R., 2004 - *An analysis of using entomopathogenic nematodes against above-ground pests*. - Bulletin of Entomological Research, 94: 297-306.
- BULGAKOV V., PASCUZZI S., SANTORO F., ANIFANTIS A.S., 2018 - *Mathematical Model of the Plane-Parallel Movement of the Self-Propelled Root-Harvesting Machine*. - Sustainability (Switzerland), 10(10): 3614; DOI:10.3390/su10103614.
- CHOJNACKI J., DULCET E., GRIEGER A., 2010 - *Influence of Static Pressure on Viability of Entomopathogenic Nematodes – Steinernema feltiae*. - World Academy of Science, Engineering and Technology International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering, 4(5): 273-275.
- EHLERS R.-U., 2005 - *Forum on Safety and Regulations*. In: Nematodes as Biocontrol Agents, P. S. Grewal, R.-U. Ehlers & D. I. Shapiro-Ilan (Eds.), Wallington, United Kingdom: CABI Publishing, pp.107-114.
- EL KHOURY Y., ORESTE M., NOUJEIM E., NEMER N., TARASCO E., 2018 - *Effect of temperature on the pathogenicity of Mediterranean native entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from natural ecosystems*. - Redia, 101; 123-127. <http://dx.doi.org/10.19263/REDIA-101.18.16>
- FIFE J.P., DERKSEN R.C., OZKAN H.E., GREWAL P.S., 2003 - *Effect of pressure differential on the viability and infectivity of entomopathogenic nematodes*. - Biological Control, 27: 65-72.
- FIFE J.P., OZKAN H.E., DERKSEN R.C., 2004 - *Physical effect of conventional spray equipment on a biological pesticide*. - Aspects of Applied Biology, International Advances in Pesticide Application, 71: 495-502.
- FURTADO MOREIRA G., SIMÕES DE PAULA BATISTA E., NEVES CAMPOS E. B., LEMOS R.E., DA COSTA FERREIRA M., 2013 - *Spray Nozzles, Pressures, Additives and Stirring Time on Viability and Pathogenicity of Entomopathogenic Nematodes (Nematoda: Rhabditida) for Greenhouses*. - PLoSOne, 8(6): e65759.
- GREWAL S.G., 2002 - *Formulation and application technology*. In: Entomopathogenic Nematology (R. Gaugler, Editor) CAB International., pp: 265-287.
- KAYA H.K., 1990 - *Soil ecology*. In: Entomopathogenic nematodes in biological control (R. Gaugler & H.K. Kaya Eds). Boca Raton, FL: CRC Press, pp: 93-115.
- LANZONI G., ADE R., MARTELLI P., RADEGHIERI F., PEZZI A., 2014 - *Technological aspects of Steinernema carpocapsae spray application alone or mixed with Bacillus thuringiensis aizawai in spinach crop*. - Bulletin of Insectology, 67 (1): 115-123.
- ŁACZYŃSKI A., DE MOOR A., MOENS M., SONCK B., RAMON H., 2004 - *An application technique for biological plant protection products containing entomopathogenic nematodes*. - Aspects of Applied Biology, International Advances in Pesticide Application, 71: 489-493.
- ŁACZYŃSKI A., DE MOOR A., DIERICKX W., MOENS M., DARIUS P., SONCK B., RAMON H., 2006 - *The Effect of hydraulic spraying on the viability of the nematode Heterorhabditis bacteriophora*. - Short communication. Crop Protection, 25(10): 1135-1141.
- MASON J.M., HOMINICK W.M., 1995 - *The effect of temperature on infection, development and reproduction of Heterorhabditid*. - Journal of Helminthology, 69: 337-345.
- MOLYNEUX A., 1995 - *Survival of infective juveniles of Heterorhabditis spp. and Steinernema spp. (Nematoda: Rhabditida) at various temperatures and their subsequent infectivity for insects*. - Revue de Nematologie, 8: 165-170.
- NILSSON U., GRIPWALL E., 1999 - *Influence of application technique on the viability of the biological control agents Verticillium lecanii and Steinernema feltiae*. -

- Crop Protection, 18: 53-59.
- POINAR G.O. JR., 1990 - *Taxonomy and biology of Steinernematidae and Heterorhabditidae*. In: Entomopathogenic Nematodes in Biological Control. (R. Gaugler & H. K. Kaya Eds.). Boca Raton, FL: CRC Press., pp. 23-61.
- POINAR G.O. JR., 1993 - *Origins and phylogenetic relationships of the entomophilic Rhabditis, Heterorhabditis and Steinernema*. - Fundamental and Applied Nematology, 16(4): 333-338.
- PRZYWARA A., KACHEL M., KOSZEL M., LESZCZYŃSKI N., KRASZKIEWICZ A., ANIFANTIS A.S., 2019 - *The influence of digestate on the static strength of spring rapeseeds (Brassica napus var. arvensis)*. - Sustainability (Switzerland), 11(7), 2133. ISSN: 2071-1050.
- RUSO G., ANIFANTIS A.S., VERDIANI G., SCARASCIA MUGNOZZA G., 2014 - *Environmental analysis of geothermal heat pump and LPG greenhouse heating systems*. - Biosystems Engineering, 127: 11-23. ISSN: 1537-5110. DOI: 10.1016/j.biosystemseng.2014.08.002 (ScopusID: 2-s2.0-84906735097).
- RUSO G., VERDIANI G., ANIFANTIS A.S., 2016 - *Re-Use of Agricultural Biomass for Nurseries Using Proximity Composting*. - Contemporary Engineering Sciences, 9(24): 1151-1182. DOI:10.12988/ces.2016.68135 (ScopusID: 2-s2.0-85010304552).
- ŠIMA T., NOZDROVICKÝ L., DUBEŇOVÁ M., KRÍŠTOF K., KRUPÍČKA J., 2013 - *Effect of crop residues on nitrous oxide flux in the controlled traffic farming system during the soil tillage by LEMKEN Rubin 9 disc harrow*. - Agronomy Research, 11: 103 -110.
- TARASCO E., CLAUSI M., RAPPAZZO G., PANZAVOLTA T., CURTO, G., SORINO R., ORESTE M., LONGO A., LEONE D., TIBERI R., 2015 - *Biodiversity of entomopathogenic nematodes in Italy*. - Journal of Helminthology, 89: 359-366.
- TORRINI G., LANDI S., TARASCO E., ROVERSI P. F., 2016 - *Evaluation of Steinernema carpocapsae survival and infectivity after cryopreservation*. - BioControl, 61(4): 461-469.
- ZERVOS S., JOHNSON S.C., WEBSTER J.M., 1991 - *Effect of temperature and inoculum size on reproduction and development of Heterorhabditis heliothidis and Steinernema glaseri (Nematoda: Rhabditoidea) in Galleria mellonella*. - Canadian Journal of Zoology, 69: 1261-1264.
- WHITE G.F., 1927 - *A method for obtaining infective nematode larvae from cultures*. - Science (Washington, D.C.), 66: 302-303.
- WILSON M., GAUGLER R., 2004 - *Factors limiting short-term persistence of entomopathogenic nematodes*. - J. Appl. Entomol., 128(4): 250-253.