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Field Application of a Lysosomal Assay as Biomarker of Copper Oxychloride Exposure, in the Snail *Helix aspersa*

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In agriculture throughout the world, great emphasis is placed on the protection of crops through the control of plant diseases. Copper oxychloride ($Cu_2Cl(OH)_3$) is a broad-spectrum fungicide applied to the foliage of a variety of fruits and vegetables in South Africa, especially in orchards and vineyards. It is applied at a rate of 1.25 – 7.5 kg ha^{-1} with several applications per season (Krause et al. 1996). This fungicide may be a source of copper contamination in the agricultural environment and may also affect non-target organisms such as snails and earthworms. It is therefore necessary to find accurate and effective methods of monitoring the effects of this fungicide on non-target biota in an agricultural environment. The use of cellular and subcellular biomarkers in invertebrates may be a possible method to employ for this purpose.

On the subcellular level, lysosomes have become increasingly popular in biomarker studies, since they have the ability to bioconcentrate a wide range of environmental contaminants, including lipophilic xenobiotics and metals (Moore 1990). The use of lysosomal stability, to provide an index of cellular condition that correlates significantly with physiological condition, has been proposed by Allison and Young (1969) and Bayne et al. (1979). According to Moore (1990) the lysosomal membrane permeability is increased as a result of accumulated contaminants and this leads to a loss of the acid hydrolase content into the cytosol, eventually causing cellular damage. Lowe et al. (1995) proposed that the efflux of lysosomal contents into the cytosol can be measured by a neutral red retention (NRR) time assay. According to Seglen (1983), the efficiency of neutral red retention in the lysosome is dependent on the efficiency of membrane bound proton pumps. Svendsen and Weeks (1995) stated that any event impairing this proton pump system will result in a lowered neutral red retention time. The NRR time assay reflects a normal physiological process that has become compromised following damage to the membranes (Lowe et al. 1995) and can, according to Svendsen and Weeks (1995), serve as an early warning system, since it can indicate contamination even at low levels.

The NRR time assay has been used successfully as a biomarker of copper exposure, using invertebrates such as earthworms and aquatic molluscs as models (e.g. Svendsen and Weeks 1995; 1997; Weeks and Svendsen 1996; Harreus et al.

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1997; Ringwood et al. 1998; Nicholson 1999; Reinecke and Reinecke 1999 and Scott-Fordsmand et al. 2000). This assay has also been used as a biomarker of experimental exposure to the fungicide copper oxychloride: Snyman et al. (2000) determined lysosomal leaking in hemocytes of the common garden snail *Helix aspersa*. These authors found that snails exposed to 80 and 240 $\mu g\ g^{-1}$ dry mass copper oxychloride respectively, for a period of six weeks, exhibited significantly elevated whole body copper concentrations and significantly shorter NRR times (10.2 ± 3.5 and 2.7 ± 2.8 minutes respectively) than control snails. Moreover, these authors found that NRR times decreased progressively over time, as body copper burdens and concomitant lysosomal damage increased.

The aim of the present study was to validate the laboratory findings described above and in further details in Snyman et al. (2000), through a field study in the vineyards of the Western Cape, South Africa. The ultimate goal was to determine the field application of hemocytic lysosome response in *Helix aspersa*, as a biomarker of stress due to copper oxychloride exposure.

MATERIALS AND METHODS

Snails of similar weight (6.8 ± 0.7 g) were collected from two vineyards in the Western Cape, South Africa. One of these sites represented a vineyard where copper oxychloride had been applied, and one site served as control, i.e. a vineyard with no history of pesticide use. Animals from the treated site were collected in December 1999 and February 2000, i.e. one week after copper oxychloride application and two months thereafter, respectively. No fungicide was applied during the period between the two sampling dates. Snails from the control site were collected once during December 1999.

An Eosin Y test was performed, in order to confirm that the process of hemocyte extraction and subsequent handling did not seriously damage the cells. This was done according to the method described by Svendsen and Weeks (1995).

Immediately after field collection, the neutral red retention (NRR) times of individual snails were determined. Hemolymph extraction from the visceral hemocoel was done using the method of Svendsen and Weeks (1995). A small hole was pierced in the shell of the animal, using a sterilized needle, and 20 µl hemolymph was then drawn from the visceral hemocoel, into an equal volume of temperature-adjusted snail Ringer (5.0 g NaCl + 0.08 g KCl + 0.6 g $CaCl_2$ dissolved in 1 liter of distilled water). Two drops hemolymph/Ringer solution were then placed on a microscope slide and 20 µl neutral red working solution added to each drop. The working solution was prepared by mixing 2.5 ml snail Ringer and 10 µl neutral red stock solution (20 mg neutral red powder dissolved in 1 ml DMSO). Each hemolymph sample was viewed under a light microscope. The total number of hemocytes, as well as the number of hemocytes with fully red-stained cytosols (i.e. neutral red leakage from the lysosomes), was counted at two-minute intervals. Only the most abundant cell type, namely the smaller,

hyaline, agranular hemocytes with pseudopodia were counted. The point, expressed in minutes, at which 50 % of the total number of cells in a sample had fully stained cytosols, was taken as the neutral red retention time.

Subsequent to NRR time measurements, each animal was wet weighed, frozen and dried for 24 hours at 60 °C. The whole animal, including the shell, was then ground with a pestle and mortar and a 1 g subsample used for metal analysis. Samples were digested in 10 ml 55 % nitric acid at a temperature of 40 °C for a period of 2 hours, after which the temperature was increased to 120 °C for 1 hour. After cooling, samples were filtered through 0.45 μm filter paper. Finally, the samples were diluted to 20 ml with distilled water. Body copper concentrations were determined with a Varian AA-1275 flame atomic absorption spectrophotometer and expressed as $\mu g\ g^{-1}$ dry mass.

The data were analyzed statistically for differences in NRR times and body copper concentrations, using ANOVA. Regression analysis was done to determine the relationship between NRR times and body copper concentrations. All calculations were done with the Jandel Scientific Sigmastat 2.0 computer program. The data were graphically portrayed using the Microsoft Excel 97 computer program.

RESULTS AND DISCUSSION

The Eosin Y test indicated a cell viability of more than 90 %. Extraction and handling of the hemocytes therefore did not influence the results of the NRR time assay to a great extent.

Figure 1 illustrates the mean neutral red retention times (minutes) of hemocytes of *Helix aspersa,* collected from the two field sites. Significant differences ($p<0.05$) in neutral red retention times were found between the control and treated (first and second sampling) sites, as well as between the first and second sampling at the treated site. Snails from the control site had the longest mean neutral red retention time (27.6 ± 4.8 minutes) and snails from the treated site (second sampling) the shortest (12.0 ± 3.6 minutes). A mean NRR time of 22.8 ± 4.2 minutes was measured for snails collected from the treated site on the first sampling date.

Figure 1 also shows the mean whole body copper concentrations ($\mu g\ g^{-1}$ dry mass) in *Helix aspersa*, collected from the two field sites. Significant differences ($p<0.05$) in whole body copper concentrations were found between the control and treated (first and second sampling) sites, as well as between the first and second sampling dates at the treated site. Snails from the control site had the lowest mean whole body copper concentration (47.0 ± 14.4 $\mu g\ g^{-1}$ dry mass) and those from the treated site (second sampling) the highest (274.6 ± 113.7 $\mu g\ g^{-1}$ dry mass).

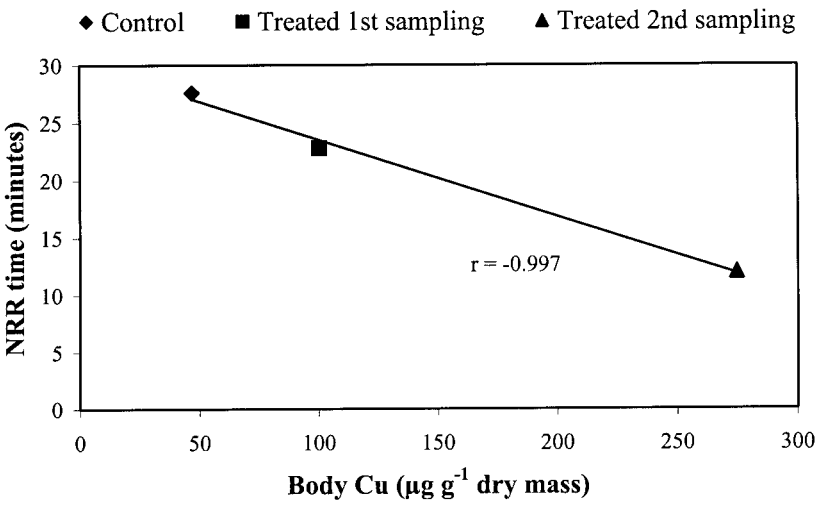

Figure 1. Relationship (r = -0.997) between mean neutral red retention (NRR) times (minutes) and mean whole body copper concentrations (µg g^{-1} dry mass) of *Helix aspersa*, collected from an untreated vineyard (Control) and from a treated vineyard, 1 week after application (Treated 1st sampling) and 2 months after application (Treated 2nd sampling) of copper oxychloride (n = 10 for Control and Treated 1st sampling, n = 6 for Treated 2nd sampling).

Regression analysis revealed a strong significant ($p<0.001$) negative correlation (r = -0.997) between mean NRR times and mean whole body copper concentrations, measured for animals from the two field sites (Figure 1).

In this field survey, a clear inverse relationship between increasing body copper concentrations and decreasing NRR times was found for *Helix aspersa*. Snails collected from the control site exhibited significantly longer NRR times and significantly lower whole body copper concentrations than snails from the treated vineyard. Snails collected from the treated vineyard, one week after copper oxychloride application, already exhibited significantly shorter NRR times and significantly higher whole body copper concentrations than snails from the control site. This may be an indication that the ability to regulate body Cu may have already been affected, resulting in an increased internal dose. It was also found that snails collected two months after copper oxychloride application had significantly shorter NRR times and significantly higher whole body copper concentrations, compared to snails collected only one week after application of the fungicide. This may provide further evidence, in addition to the experimental results of Snyman et al. (2000), that exposure time and actual body Cu burdens are important factors influencing lysosomal membrane response as measured by NRR times in *H. aspersa*.

The results of this survey provide evidence for the field validation of the laboratory results of Snyman et al. (2000) and indicate that the lysosomal responses in hemocytes of *Helix aspersa,* and the measuring thereof by the neutral red retention time assay, can indeed be considered a useful biomarker of stress resulting from both experimental and field exposure to copper oxychloride. It renders further support for the findings of Svendsen & Weeks (1995; 1997), Weeks & Svendsen (1996), Harreus et al. (1997), Ringwood et al. (1998), Nicholson (1999) and Scott-Fordsmand et al. (2000), who have also found the NRR time assay to be a reliable biomarker of exposure to copper.

The NRR time assay has been shown to be an early warning of stress induced by the fungicide copper oxychloride and can, in conjunction with other cellular and physiological parameters and toxicological endpoints, certainly improve the reliability and accuracy of interpretations regarding cause and effect. The assay can serve as a useful tool to determine the bioavailability of copper in vineyards after spraying events. The extent to which this biomarker could also serve as a tool for predicting higher level effects of ecological relevance, requires further studies.

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