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Exposure of the cyanobacterium *Nostoc muscorum* from Portuguese rice fields to Molinate (Ordram®): Effects on the antioxidant system and fatty acid profile

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ABSTRACT

Herbicide contamination of aquatic ecosystems is a serious global environmental concern. Several herbicides enhance the intracellular formation of reactive oxygen species, and can lead to the damage of macromolecules and to a decrease of oxidant defenses in a wide range of non-target microorganisms including cyanobacteria. The effects of molinate (a thiocarbamate herbicide used for controlling grassy weeds in rice fields) on the activities of antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and glutathione *S*-transferase were evaluated in *Nostoc muscorum*, a freshwater cyanobacterium with a significant spread in Portuguese rice fields. These were determined in *N. muscorum* cultures acutely (72 h) exposed to concentrations ranging from 0.75 to 2 mM of molinate. This study also analyzed the effects of molinate on: (1) the nonenzymatic antioxidant contents (reduced and oxidized glutathione, carotenoids, and proline), (2) the oxidative cell damage measured in terms of lipid peroxidation (MDA level) and electrolyte leakage (intactness of plasma membrane), and (3) the total fatty acid profile. The results showed that the activities of all antioxidant enzymes decreased dramatically with the rising concentration of molinate after 72 h. Time-dependent and concentration-dependent increase in MDA and enhanced cell membrane leakage were indicative of lipid peroxidation, formation of free radicals and oxidative damage. Compared to control, 72-h herbicide exposure increased lipid peroxidation by 5.4%, 19% and 28% with 0.75, 1.5 and 2 mM of molinate, respectively. Similarly, herbicide stress induced an increase in electrolyte leakage (5.8%, 29.5% and 30.2% above control, with 0.75, 1.5 and 2 mM of molinate, respectively). The increased production of proline at higher molinate concentrations (the values rose above control by 45%, 95% and 156% with 0.75, 1.5 and 2 mM, respectively) indicated the involvement of this osmoprotectant in a free radical scavenging mechanism. Moreover, a radical decline in both glutathione pool, carotenoids and saturated fatty acids were also observed. The results of the present study lead us to conclude that: (1) both enzymatic and nonenzymatic antioxidative defense system of *N. muscorum* are dramatically affected by molinate, (2) the herbicide induces peroxidation, (3) it contributes to an increase of the unsaturation level of cell membrane fatty acids. These evidences should be taken in account when using *N. muscorum* as an environmental indicator species in studies of herbicide biotransformation and biomarker response as well as in environmental monitoring programmes.

1. Introduction

On a worldwide basis, rice is the single most important food crop for over half of mankind. Although European Union (EU) is ranked only 17th among the world's main rice producers, periodic and relatively stable production estimates at EU-27 level are of interest for the European Commission. The 400,000 ha of land in EU that is used to grow rice is mainly in Italy (218,000 ha), Spain (96,000 ha), Portugal (22,000 ha), Greece (22,000 ha), and France (17,000 ha) (Confalonieri et al., 2010). The rice-growing areas are often near ecologically important coastal wetlands, such as Po River Basin in Italy (Padovani et al., 2006), Natural Park of La Albufera in Spain (Peña-Llopis et al., 2001)], Ile de Camargue Basin in France (Comoretto et al., 2008)], Axios River Basin in Greece (Konstantinou et al., 2006), and Lower Mondego River Basin in Portugal (Marques et al., 2008).

The use of pollutants such as inorganic fertilizers and pesticides in the Mediterranean region has expanded enormously over the past 20–40 years. Consequently, surface waters and ground-

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waters have become increasingly contaminated with nitrates and pesticide residues. Stoate et al. (2009) reviewed the data published by pesticide-monitoring programs that studied European agricultural areas. Some studies reported pesticide contamination exceeding the 0.1 μg L^{-1} EU limit in the surface waters and groundwaters of Portugal in areas occupied by intensive agriculture, rice fields included. Molinate (*S*-ethyl hexahydro-1*H*-azepine-1-carbothioate; CASN 2212-67-1), the active ingredient in Ordram®, is a selective thiocarbamate herbicide which is widely used for weed control in rice fields. In central Portugal's rice fields, Castro et al. (2005) detected molinate levels as high as 3.9 μg L^{-1} and 15.8 μg L^{-1} in underground water and receiving subsidiary waters of Mondego River, respectively. The maximum value of 48 μg L^{-1} obtained in the Sado River (southern Portugal) by Cerejeira et al. (2003) posed a real environmental threat. Molinate was the most frequently detected pollutant amongst several pesticides in rice field groundwaters of the Low Sado region, with maximum concentration levels of 60 μg L^{-1}. Molinate and its degradation metabolites were classified as very toxic to aquatic organisms and capable to causing long-term adverse effects on aquatic environments (Aizawa, 2001; Castro et al., 2005; Silva et al., 2006).

In the years 2006–2008, 26, 21 and 7 tons of molinate were sold in Portugal, respectively (M. Vieira, Portuguese Ministry of Agriculture, pers. commun., 2010). The year by year decrease in sales of molinate is a consequence of an increasing concern about its negative effects to the environment. The risk associated with molinate is high because it is: (i) normally applied under flooded conditions, (ii) highly soluble in water (880 mg L^{-1} at 20 °C), (iii) hydrolyzed in soil to form ethyl mercaptan, carbon dioxide and dialkylamine, and (iv) photodecomposed to secondary products in rice field water (Soderquist et al., 1977; Ampong-Nyarko and De Datta, 1991; Kamrin and Montgomery, 2000; Tomlin, 2000). Sometimes, water contamination with molinate is so persistent that preventive measures have been taken to enable detoxification or its prohibition.

One of the major environmental concerns about herbicide contamination is its bioaccumulation in the ecosystem's primary producers and its subsequent propagation through the trophic chain. Cyanobacteria are one of the main diazotrophic components of the primary microbiota producers in rice fields and significantly contribute to building-up soil fertility. The species *Nostoc muscorum* is common in rice fields worldwide and it comprises a large proportion of the total number of cyanobacteria in Portuguese paddy fields (Galhano et al., 2010). Moreover, some genera of N_2-fixing cyanobacteria (*e.g. Nostoc* and *Anabaena*) could be present in rice field ecosystems as primary producers and as a component of symbiotic associations (Whitton, 2000). Therefore, it is important to emphasize that the use of herbicides may have a great impact on these symbiosystems, possibly decreasing rice field productivity.

There are reports on the effects of molinate on mammals (Campbell et al., 2008), amphibians (Kang et al., 2009), fish (Peña-Llopis et al., 2001), hexapods (Faria et al., 2006), decapods (Ekanem et al., 2009), cladocerans (Sancho et al., 2003), higher plants (Hsieh et al., 1998), and marine bacteria (Phyu et al., 2005). However, reports on effects on plants and cyanobacteria are scarce.

Oxidative damage in living organisms, including plants and cyanobacteria, is characterized by the enhanced production of reactive oxygen species (ROS) *viz.* superoxide anion ($O_2^{\bullet -}$), and peroxides, including hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$) and singlet oxygen free radical ($^1O_2$) (Halliwell and Gutteridge, 1999; Srivastava et al., 2005; Pospíšil, 2009; Demidchik, 2010). In oxygenic photosynthetic organisms such as cyanobacteria, ROS are inevitably generated by photosynthetic electron transport, especially when the intensity of light-driven electron transport outpaces the rate of electron consumption during CO_2 fixation (Latifi et al., 2009). Because cyanobacteria in their natural habitats are often exposed to changing external conditions, such as temperature (Liu et al., 2005; Srivastava et al., 2006), irradiation (Singh et al., 2002; Latifi et al., 2009), heat (Hossain and Nakamoto, 2003; Sakthivel et al., 2009), ultraviolet radiation (Gupta et al., 2008; Zeeshan and Prasad, 2009), desiccation/drought (Rajendran et al., 2007; Higo et al., 2008), salinity (Srivastava et al., 2005; Bhadauriya et al., 2009), and metals (Choudhary et al., 2007; Fatma et al., 2007), their ability to perceive ROS and to rapidly initiate antioxidant defenses is crucial for their survival (Latifi et al., 2009). Every cyanobacterial cell possesses a complex array of enzymatic antioxidant defense system which comprises mainly the enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione *S*-transferase (GST) (Srivastava et al., 2005; Wiktelius and Stenberg, 2007). SOD catalyzes the dismutation of superoxide free anions ($O_2^{\bullet -}$) to O_2 plus H_2O_2, and are rather considered the first line of defense against damage by $O_2^{\bullet -}$. CAT decomposes H_2O_2 into H_2O and O_2 [rapid H_2O_2 decomposition reduces the likely formation of highly reactive hydroxyl radical ($OH^\bullet$) through Fenton and Haber–Weiss reactions]. APX plays a crucial role in H_2O_2 detoxification as it catalyzes the GSH-dependent reduction of H_2O_2 to monodehydroascorbate and H_2O, thus protecting membrane lipids against oxidation [these reductions of peroxides imply the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG)]. NADPH-dependent GR helps to maintain a high ratio of GSH:GSSG through GSH replenishment, which in turn also acts on H_2O_2 removal in the ascorbate–glutathione cycle. The major function of GST is generally considered to be to act as detoxifying enzyme (Srivastava et al., 2005; Wiktelius and Stenberg, 2007; Latifi et al., 2009; Blokhina and Fagerstedt, 2010). In addition to ascorbate and α-tocopherol, glutathione (both in reduced and oxidized forms) and carotenoids constitute the nonenzymatic antioxidative pathway (Srivastava et al., 2005, 2006).

To our knowledge, there is limited understanding of molinate-induced molecular mechanisms which trigger oxidative stress by generating ROS after herbicide exposure in cyanobacteria. Therefore, we postulated that molinate, like most environmental stresses, induces ROS production and causes oxidative damage in cyanobacteria. To test this hypothesis, an attempt was made to study the effects of molinate causing cellular damage in *N. muscorum* by measuring enzymatic (SOD, CAT, APX, GR, GST) and nonenzymatic (GSH, GSSG, total carotenoids) antioxidants. To support our hypothesis, molinate-induced changes on other parameters were also determined *viz.* proline content, lipid peroxidation, electrolyte leakage and fatty acid methyl ester (FAME) profile. These parameters were chosen because: (1) intracellular proline content has been reported to be an important index for stress tolerance capacity due to its function as a hydroxyl radical and singlet oxygen scavenger (Fatma et al., 2007), (2) ROS causes lipid peroxidation wherein the lipids in the cell membranes are damage (Choudhary et al., 2007), (3) when ROS concentrations reach a certain threshold, it activates a programmed cell death response (PCD) in the cells and this is quantified by measuring the amount of ion leakage (Jambunathan, 2010), (4) FAME profile is considered to be a useful biomarker to assess the effect of pesticides on aquatic microbial communities (Littlefield-Wyer et al., 2008), and (5) functional integrity of membrane lipids, fatty acid unsaturation degree, and causes of free fatty acid liberation and regulation of lipid peroxidation, are issues closely connected to ROS metabolism (Blokhina and Fagerstedt, 2010). Considering the importance of *N. muscorum* as natural biofertilizer in rice fields, and the frequent use of herbicides against weeds, the aim of the present investigation is not only important to the scientific community but also to rice environment and rice industry.