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Allelopatic effects of cyanobacteria extracts containing microcystins on *Medicago sativa–Rhizobia* symbiosis

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ABSTRACT

The eutrophication of water leads to massive blooms of cyanobacteria potentially producers of highly toxic substances: cyanotoxins, especially microcystins (MC). The contamination of water used for irrigation by these toxins, can cause several adverse effects on plants and microorganisms. In this work, we report the phytotoxic effects of microcystins on the development of symbiosis between the leguminous plant *Medicago sativa* (Alfalfa) and rhizobia strains. The exposure of rhizobial strains to three different concentrations 0.01, 0.05 and 0.1 μg MC ml^{-1} led to decrease on the bacteria growth. The strains of rhizobia Rh L1, Rh L2, Rh L3 and Rh L4 reduced their growth to, respectively, 20.85%, 20.80%, 33.19% and 25.65%. The chronic exposure of alfalfa seeds and seedlings to different MC concentrations affects the whole stages of plant development. The germination process has also been disrupted with an inhibition, which reaches 68.34% for a 22.24 μg MC ml^{-1}. Further, seedlings growth and photosynthetic process were also disrupted. The toxins reduced significantly the roots length and nodule formation and leads to an oxidative stress. Thus, the MCs contained in lake water and used for irrigation affect the development of symbiosis between *M. sativa* and *Rhizobia*.

1. Introduction

Eutrophication has become increasingly widespread in aquatic ecosystems; this phenomenon leads to excessive blooms of cyanobacteria (Codd, 1995). Several species of cyanobacteria causing blooms are known for their production of various types of toxins namely hepatotoxins. Of about 30% of the potentially toxic cyanobacteria species, representing 40% of all globally known toxic species have been documented in various aquatic ecosystems of Morocco (Oudra et al., 2008). *Microcystis* is the most common bloom forming genus in Algeria and Morocco (Douma et al., 2009). The most common and important hepatotoxins produced are the microcystins (MC), which are usually produced by *Microcystis aeruginosa* (Dittmann and Weigand, 2006). They include more than 70 structural variants (Zurawell et al., 2005) being MC-LR the most prevalent one (Dawson, 1998). MC have been shown to be potent inhibitors of protein phosphatases 1A and 2A for animals and higher plants (Hastie et al., 2005). Those proteins are involved in several physiological and molecular processes (Sheen, 1993; Takeda et al., 1994).

Several studies have reported that submerged and emerged aquatic plants can uptake an MC-LR (Pflugmacher et al., 1998, 2001; Yin et al., 2005). This toxin causes plant growth inhibition and photosynthetic disorders (Pflugmacher, 2002). The exposure of *Lepidium sativum* to 1 μg MC-LR l^{-1} induced a significant reduction of root growth (Gehringer et al., 2003). In *Lemna gibba*, toxic *Microcystis* extract cause changes in the peroxidase activity and phenolic compounds as well as decrease in plant growth and chlorophyll contents (Saqrane et al., 2007). As for terrestrial plants, crop irrigation by water containing cyanobacteria can lead to an exposure of aerial parts of plants to cyanobacteria and their toxins (Abe et al., 1996).The absorption of cyanobacterial toxins, in sufficient concentrations, by terrestrial plants may induce morphological (Koś et al., 1995; McElhiney et al., 2001; Pflugmacher et al., 2006) and physiological disturbances (M-Hamvas et al., 2003; Gehringer et al., 2003; Chen et al., 2004).

These toxins may influence the germination of seeds as well as the early stages of plants development (Chen et al., 2004; Saqrane et al., 2008), the length of primary roots and the photosynthesis process (Koś et al., 1995; Abe et al., 1996; McElhiney et al., 2001; Pflugmacher et al., 2006). They can also induce leaf necrosis

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(Babica et al., 2006) and act on the metabolism of plants (Smith and Walker, 1996). The exposure to an MC has inhibited the growth and development of both rice (*Oryza sativa*) and rape (*Brassica napus*) seedlings. The MC also inhibits the germination rate of seeds and seedling length in addition to an oxidative stress demonstrated by the activities of peroxidase and superoxide dismutase enzymes (Chen et al., 2004). The translocation of MC-LR and LF through roots to shoots for seedlings of 11 agricultural plants was also reported (Peuthert et al., 2007).

Spray irrigation of lettuce plants (*Lactuca sativa*) with water containing *Microcystis* leads to persistence of colonies and singles cells of cyanobacterium on the plants leaves after irrigation (Peuthert et al., 2007) (Codd et al., 1999). Moreover plants could accumulate an MC in their leaves and roots at concentrations ranging 0.07–1.2 μg MC g^{-1} fresh weight (Codd et al., 1999) (Mohamed and Al Shehri, 2009). This study suggested that ground waters and plants should be continuously monitored in order to protect the public health against exposure to such potent hepatotoxins.

The uptake of cyanotoxins in seedlings of several agricultural plants has a significant negative impact on the various stages of plants development. This can cause economic losses by the reduction in yield and productivity, in addition to the impact on human and animal health, following the consumption of contaminated plants. The effects of cyanotoxins, on both aquatic and terrestrial plants, have been studied. On the other hand, the investigation of their potential toxic effects on legumes plants is rare (McElhiney et al., 2001; Pflugmacher et al., 2006).

These plants such as beans, lentils, alfalfa are of great value, because of their importance for human and animal nutrition and their role with symbiotic rhizobia in atmospheric nitrogen fixation. Crop yields are greatly improved in nodulated legume plants; the rhizobia legumes constitute natural fertilizers. Rhizobia are bacteria Gram-negative; they have ability to associate with roots plants and form a specialized organ called the nodule, located on the roots or rarely on the stems (Zakhia and De Lajudie, 2001). Symbiotic fixation of atmospheric nitrogen is the main pathway of atmospheric nitrogen in the nitrogen cycle. The reaction is catalyzed by nitrogenise enzyme it consists on the reduction of nitrogen (N_2) into ammonia (NH_3) available form. Most of the symbiotic N_2 in soil occurs in the symbiosis between rhizobia and plant of the legume family.

McElhiney et al. (2001) have reported that the exposure of *Phaseolus vulgaris* to an MC-LR had no effect on seedlings growth. In the opposite, the development of root system decreased by 30% compared to a bean plant growing without toxins (McElhiney et al., 2001).

Our work investigated the phytotoxic effects of cyanotoxins (MC) produced by *M. aeruginosa* from Lalla Takerkoust lake (Marrakech, Morocco), on the leguminous plant *M. sativa* and on symbiotic bacteria (rhizobia). According to our knowledge, the effects of cyanotoxins on rhizobial growth and their symbiotic association with alfalfa were not previously reported.

2. Material and methods

2.1. Detection, identification and quantification of an MC in cyanobacteria bloom material

The cyanobacterial bloom material was collected, in September 2005, from Lalla Takerkoust reservoir, with a 27 μm Nitex phytoplankton net. The sample was freezed and stored at −25 °C until an MC quantification by the HPLC-PDA analysis. After de-freezing, the biomass was sonicated (3 Hz × 10 min) and centrifuged (4000 g × 12 min). The supernatant was retained and used to expose the seeds and seedlings.

The aqueous extract of natural cyanobacterial bloom was used, in this report, in order to take off natural conditions during bloom event.

MC were detected, identified and quantified using a High Performance Liquid Chromatography (HPLC) system as described in Oudra et al. (2002). In brief, the detection of an MC was performed with a Merck Hitachi HPLC system with photodiode array detection system (HPLC-PDA) composed of L-7100 pump, an L-7200 auto-sampler, a D-7000 interface and an L-7450 photodiode array detector set at 238 nm. The separation of MCs was achieved using an analytical LiChrospher R 100, 5 μm ODS column (LiChroCART-250-4 cartridge system, Merck, Germany). The mobile phase was composed of Milli-Q Plus water (Millipore) and acetonitrile, both containing 0.05% of tri-fluoro-acetic acid and a flow of 1 mL min^{-1}. A 28–70% acetonitrile gradient during 30 min was used. The UV-spectrum for each separated fraction was checked and the MC variants were identified by their characteristic UV-spectrum (UVmax absorbance at 238–240 nm). Values of an MC reported in this work represent the total amount of the variants found.

2.2. Effects of MC on the growth of rhizobia

2.2.1. Isolation and purification of rhizobial strains

Four strains of rhizobia (Rh L1, Rh L2, Rh L3 and Rh L4) were isolated from root nodules of alfalfa plants collected from the Marrakech region. Nodules of alfalfa plant were disinfected with sodium hypochlorite (4°) and rinsed several times in sterile physiological water. The nodule was crushed in a tube containing 1 mL of sterile physiological water. The suspension was seeded on Petri dishes containing Yeast Extract Mannitol (YEM) medium agar (Vincent, 1970) with Red Congo. After incubation during 48 h at 28 °C, colonies of rhizobia characterized by a sticky aspect and off white color (without absorption of Congo red) were isolated and purified on YEM medium. The strains were stored at −25 °C in glycerol 25%.

2.2.2. Evaluation of the effect of different MC concentrations on strains of rhizobia

One milliliter of each rhizobial culture in YEM broth medium containing about 10^9 CFU/ml served as inoculums and was added to flasks containing 100 ml of YEM broth medium. Thereafter, 1 ml of an MC solution was added to the medium in order to achieve final concentrations of 0.01, 0.05 and 0.1 μg MC/ml. The control flask was the YEM broth medium added with 1 ml of sterile distilled water.

The flasks were incubated at 28 °C, in darkness and under continuous agitation (250 rpm). The rhizobia growth was followed by counting colonies forming units per ml (CFU/ml) on YEM medium agar.

2.3. Effects of MC on seeds germination

To evaluate the effect of an MC on the germination, seeds of alfalfa (*M. sativa*) were sterilized with sodium hypochlorite 12° for 5 min, followed by three washes in sterile distilled water. Four parallel exposures of seeds were performed and prepared on three replicates (20 seeds in each Petri dish). The control seeds were exposed to sterile distilled water. The germination boxes were placed in the incubator at 25 °C in the dark under sterile conditions. During the germination process, 2 ml of the aqueous extract were added regularly to prevent dryness. The rate of germination was determined.

2.4. Effects of MC on the seedlings of M. sativa

Seedlings were grown in sand and watered daily with distilled water. Exposure of alfalfa began 10 days after sowing and lasted one month. Toxin concentration in the water used for watering of seedlings was 0, 2.22, 11.12 or 22.24 μg MC ml^{-1}. The aqueous extract of toxins was supplemented with nutrient solution. At the end of the experiment, several parameters were evaluated: crop yield, length of the stem and of the root, number of nodules, chlorophyll fluorescence and the evaluation of plants defense reaction through the quantification of phenolics content and peroxidase activity, enzyme involved in the scavenge of the reactive oxygen species generated in stress conditions.

2.5. Evaluation of M. sativa crop yield

In order to evaluate the effect of an MC on crop production, the plant biomass was weighed and the results expressed in grams fresh weight per m^2 per day.

2.6. Effect of MC on M. sativa-rhizobia symbiosis (roots length and number of nodules)

After the exposure period, *M. sativa* seedlings were used to determine the effect of MC on the roots nodules formation. Plants were rinsed with distilled water, and then the length and the number of nodules were determined by visual inspection.

2.7. Chlorophyll fluorescence evaluation

The photosynthetic activity of plants, based on pigment content and fluorescence, is an important process, which provides energy for plants development.