Journal of Genetic Engineering and Biotechnology xxx (2017) xxx–xxx

HOSTED BY

Contents lists available at ScienceDirect

Journal of Genetic Engineering and Biotechnology

journal homepage: www.elsevier.com/locate/jgeb

Original Article

Cytotoxic and molecular impacts of allelopathic effects of leaf residues of *Eucalyptus globulus* on soybean (*Glycine max*)

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ARTICLE INFO

Article history:
Received 12 February 2017
Received in revised form 3 August 2017
Accepted 20 August 2017
Available online xxxx

Keywords:
Eucalyptus globulus
Glycine max
Comet assay
Cysteine proteases

ABSTRACT

Eucalyptus trees litter plays a crucial role in structuring plant populations and regulating crop quality. To help characterize the allelopathic impact of Eucalyptus plantations and understand the interactions between tree litter and understorey plant populations, we performed two different genomic approaches to determine soybean (*Glycine max*) crop plant response to biotic stress induced by leaf residues of Eucalyptus globulus trees. For assessing cell death, a qualitative method of DNA fragmentation test (comet assay) was employed to detect cleavage of the genomic DNA into oligonucleosomal fragments and help to characterize the apoptotic event among the experimental samples. In addition, quantitative method of genome analysis at the transcriptional level also was conducted to investigate the expression responses of soybean genome to allelochemicals. Expression of specific genes, which are responsible for the breakdown of proteins during programmed cell death PCD (cysteine proteases and their inhibitors), was examined using semi-quantitative RT-PCR (sqPCR). Results of both conducted analyses proved significant genetic effects of *Eucalyptus* leaf residues on soybean crop genome, revealed by steady increase in DNA damage as well as variation in the transcript levels of cysteine proteases and inhibitors. Further detailed studies using more sensitive methods are necessary for a comprehensive understanding of the allelopathic effects of *Eucalyptus* plantations on crops.

1. Introduction

Allelopathy has been recognized as an important ecological mechanism that influences the type of existing vegetation in an ecosystem, plant biodiversity, the dominance and succession of plants, as well as crop management and productivity [1]. Recent reports have proved allelopathic effects revealed by forest trees on vegetation suppression and soil sickness [2,3]. The forest tree, *Eucalyptus globulus* is one of the most widely cultivated trees, owing to its fast growth, wider adaptability and high productivity. Nevertheless, it spread into areas of natural vegetation and has been listed among the exotic pest plants. Regarding the ecological impact of *Eucalyptus*, it has been demonstrated to reduce the diversity of associated species and the productivity of understorey crops [4]. Allelochemicals are naturally released from intact living or dead *Eucalyptus* tissues and accumulated in soil rhizosphere at high concentrations, generating allelopathic impacts. *Eucalyptus* species have been evaluated for their allelopathic effects on different plant species [4,5,3]. Secondary metabolites including certain phenolic acids and volatile oils released from the leaves, bark and roots of certain *Eucalyptus spp*. have been identified as harmful biological exudates to other plant species. The potential mechanisms underlying *Eucalyptus* allelopathic effects on the growth of neighboring crops have been explored in many species, including weeds and crops [6,7].

Screening bioassays are crucial tools in identifying allelopathic potential of plant species. In addition to the traditional bioassays, methods based on molecular tools have been employed to explore

Abbreviations: PCD, programmed cell death; sqRT-PCR, semiquantitative reverse transcription polymerase chain reaction; CPE, papain–like cysteine proteases; VPE, vacuolar processing enzyme (legumain-like cysteine proteases); CC, cystatins; EUGL, Eucalyptus ground leaves.

Peer review under responsibility of National Research Center, Egypt.

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http://dx.doi.org/10.1016/j.jgeb.2017.08.005
1687-157X/

the allelopathic potential of a particular plant as well as the mechanisms of allelochemicals action in cells and genomes. Recently, this approach associating molecular DNA markers with classical bioassays have been used for better exploring and understanding allelopathy. Nevertheless, cytogenetic and molecular analyses have been reported as consistent data, suggesting their complementary use. Although allelopathy is an environmentally friendly method for weed control, the inducible genetic variation and the molecular mechanism for allelopathy on the plant species need to be elucidated. In this context, test plants in allelopathic research, should be sensitive and have an effective response in a short time, even when low concentrations of allelochemicals are used. Soybean [*Glycine max* (L.) Merr.] has been cited in literature as good candidate in allelopathy investigations [8,3]. Meanwhile, it is one of the most important agricultural crops for oil and protein. Several genetic studies on soybean germplasm also have provided in-depth insights into functional genes and genetic mechanisms related to plant responses to biotic and abiotic stresses [8,3].

Genotoxic damage can have long-term effects in natural ecosystems, however, there are few reports on the potential genotoxicity of *Eucalyptus*. For DNA damage assessment, the single cell gel electrophoresis assays (Comet assays) have been used to evaluate the genotoxicity of environmental agents in animals and terrestrial plants [9–11]. The comet assay on plants has become a valuable method for assessment of the environmental and experimental genotoxic impact. As the assay is specific and non-invasive, it has been reported as ideal to complement other test systems for DNA damage detection. Comet assay is a very sensitive and simple technique for measuring primary DNA damage events, such as single-strand and double-strand breaks, the generation of alkali-labile sites and excision repair sites and changes in chromosomal structure [11].

Genome analysis at the transcriptional level might be employed to provide evidence about the allelochemicals mode of action, and the mechanisms of defense against them as well. In this research, soybean (*Glycine max*) provides example of the expression responses of plant genome to environmental stresses. In this regard, specific genes or groups of genes that can be linked to a molecular target site could be tested. Among these, cysteine proteases are involved in a variety of processes in response to both biotic and abiotic stress [12] and responsible for the breakdown of proteins during cell death. Most of plant cysteine proteases are belonging to the papain (C1) or legumain (caspases) (C13) families, which involved in programmed cell death PCD [13–15]. Legumains are widely existed in plants and located in the vacuoles or cell wall [16]. They are known as vacuolar processing enzymes (VPE), and reveal caspase-like activity [14]. On the other hand, inhibitors of cysteine proteases (Cystatins), have crucial role in regulation of normal physiological processes, and involved in defense mechanisms against biotic and abiotic stress [17–19]. Rapid identification of soybean cysteine proteases and their inhibitors has been facilitated by the soybean genome database. This information has provided a more comprehensive analysis of the changes in transcripts encoding the cysteine protease–cystatin system proteins in soybean plants during development.

The present work aimed to evaluate the allelopathic interactions between *Eucalyptus* leaves residue and understorey plant populations, particularly crop plants. Comet assay was used to detect the DNA damage and apoptotic effect on soybean cells. Additionally, genome analysis of 12 proteases genes and their specific inhibitors were carried out at the transcriptional level. Therefore, it will be easily to verify that soybean crop is more or less affected by allelopathic interaction with *Eucalyptus*. Such information should be beneficial when planning for sowing these important crops near or beneath of eucalypt trees.

2. Materials and methods

2.1. Plant material and experimental design

Fresh mature leaves of *Eucalyptus globulus* trees were collected from *Eucalyptus* plantations, Qarwa district, Taif province, Saudi arabia. The leaves were washed, air dried, and ground to fine powder. Soybean (*Glycine max*) seeds were obtained from the Agricultural seed store. Pot experiment was conducted under natural conditions in plastic pots, containing mixture of clay-sandy (2:1, w/w) soil. Soybean seeds were planted in pots containing mixture of soil and *Eucalyptus* ground leaves (EUGL) in a percentage of 0 (control), 10, 20, 30, 40, 50, (w/w, residue/soil). Pots maintained in a growth chamber under controlled temperature (20 °C ± 2) and photoperiod of 10–14 h (light/Dark). The pots were divided into six groups including the control and the five different concentrations of *Eucalyptus* leaf residue. Each treatment was replicated 3 times in a completely randomized experimental design. Each pot was planted with 5 seeds of soybean at 3 cm depth. They were irrigated with water, and harvested after 3 weeks for further analyses.

2.2. DNA fragmentation test (comet assay)

The comet assay was carried out following the protocol described by Juchimiuk et al [20]. Individual soybean leaves were placed in 200 μl of cold 400 mMTris-HCl buffer, pH 7.5. To obtain low frequency of DNA damage in control cells, the leaf was gently sliced to release nuclei into the buffer under yellow light. Each slide previously coated with dried normal melting point (NMP) 1% agarose; was covered with a mixture of equal volumes of nuclear suspension and low melting point agarose (LMP) at 40 °C. The slide was coverslipped and placed on ice for at least 5 min, after then coverslip was removed. LMP agarose (0.5%) was placed on the slide; coverslip was mounted again and then removed after 5 min on ice. Slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) and incubated for 15 min. Electrophoresis was performed at 16 V, 300 mA for 30 min at 4 °C. Subsequently, slides were submerged in neutralization buffer (400 mM Tris-HCl, pH 7.5) and stained with ethidium bromide (20 μg/ml) for 5 min. They were dipped in ice-cold distilled water, covered with coverslip and viewed under a fluorescence microscope with computerized image analysis system (Komet Version 3.1. Kinetic Imaging, Liverpool, UK). Images of 250 randomly selected cells (50 cells from five replicate slides) were analyzed for each treatment. The integrated intensity profiles for each cell were computed, and the comet cell components were estimated to evaluate the range of derived parameters. To quantify the DNA damage tail length (TL) and tail moment (TM) were evaluated. Tail length (length of DNA migration) is related directly to the DNA fragment size and presented in micrometers. It was calculated from the centre of the cell. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail.

2.3. RNA isolation and RT-PCR assay

Total RNA was extracted from soybean leaf tissues according to MacRae [21]. To generate c-DNA of cysteine proteases and specific inhibitors genes, specific primers were supplied by Macrogen Inc. (Korea) according to Du Plessis [22]. Five genes of papain like cysteine proteases (*CP 1-5*), 3 genes of legumain–like proteases (*VPE1-3*) and four genes of cystatins (*CC1-4*), were selected to generate the gene expression profiling. Total RNA was reverse transcribed using the Access RT-PCR System (Promega) and a PXE 0.5 thermocycler (Thermo Scientific) following the manufacturer's instruc-

Please cite this article in press as: Abdelmigid HM, Morsi MM. Cytotoxic and molecular impacts of allelopathic effects of leaf residues of *Eucalyptus globulus* on soybean (*Glycine max*)Eucalyptus globulus on soybean (*Glycine max*) –>. Journal of Genetic Engineering and Biotechnology (2017), http://dx.doi.org/10.1016/j.jgeb.2017.08.005