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2 MicroRNAs: an emerging class of root exudate component of wheat response to polluted soil

3

4 S. Firmin¹, D. Houben¹, J. Fontaine², A. Lounès-Hadj Sahraoui², I. Trinsoutrot- Gattin³, M-P.
5 Faucon¹

6

7 1 AGHYLE, Unilasalle, 19, rue Pierre Waguet BP 30313 F-60026 Beauvais Cedex, France

8 2 Unité de Chimie Environnementale et Interactions sur le Vivant (UCEIV), Université du

9 Littoral Côte d'Opale, 50, Rue Ferdinand Buisson, 62228 Calais Cedex, France

10 3 AGHYLE, Unilasalle, SFR NORVEGE Fed4277, 3rue du Tronquet , 3 Rue du Tronquet, F-
11 76134 Mont-Saint-Aignan, France

12

13 *Address for correspondence: S. Firmin. AGHYLE, Institut Polytechnique UnilaSalle. 19
14 Rue Pierre Waguet 60000 Beauvais, France. (Email : stephane.firmin@unilasalle.fr)

15

16 Keywords : root exudation, miRNAs , oxidative stress, wheat, bisphenol A

17

18 ABSTRACT

19

20 MicroRNAs (miRNAs) are recognized as key molecular regulators in most of intracellular processes
21 essential for plant adaptation to environmental changes. microRNAs can mediate regulation of gene
22 expression involved in development, acquisition of nutrients, defense and interactions with soil
23 microorganisms to enhance stress tolerance of plants. Accumulating evidences indicate that
24 microRNAs are also transferred within cells, organs or whole plant and released in soil as stress signals
25 of the plant-microbiome communication. However, little information is available about how mobile
26 microRNAs are conveyed from plants to the rhizosphere. To understand whether this intriguing
27 phenomenon occurs through root secretion, morphological changes, peroxidase (POX), superoxide
28 dismutase (SOD) and catalase (CAT) antioxidant enzymes activities, as well as the expression profiles
29 of developmental and oxidative stress responsive microRNAs were analyzed in root tissues and exudates
30 collected from wheat (*triticum aestivum*) exposed to bisphenol A (BPA) at low and high concentrations
31 (0.1 and 5000 mg. kg⁻¹ soil). BPA contamination significantly affected aerial and root biomass, and

32 miR164, miR167 and miR398 expression in roots of the group that received the highest dose.
33 The presence of miR156, miR164, miR167 was detected in root exudates of controls and
34 contaminated plants while BPA exposure increased miR 167 levels in exudates at high dose.
35 Collectively, this experiment provided first results reporting microRNAs regulatory network
36 participation in the BPA stress response of wheat. The identification of microRNAs as integral
37 component of the root exudates will extend our knowledge about the molecular signaling in
38 plant - soil microbiome interactions.

39

40 Main

41

42 Interactions between host and microbiomes in soil can exert through a complex and continuous
43 dialogue materialized by root exudation of bioactive molecules that select and modulate the
44 structure, functions, and activities of soil microbiota communities. Recently, research on new
45 metabolites obtained from plant exudates focused on plant protection mechanisms against
46 pathogens and leads to the discovery of extracellular DNA secreted by border cells to neutralize
47 root infection (Baetz and Martinoia 2014) . Other nucleic acids as circulating microRNAs may
48 probably be secreted from root to influence surrounding microbiota in soil when grown under
49 abiotic stress. There is accumulating investigations showing that environmental stresses affect
50 production or degradation of miRNAs in root tissue and promote their movement throughout
51 the entire plant and its surface (Pandey et al. 2018 ; Daniela et al. 2018) but whether microRNAs
52 are transferred from root cell to the rhizosphere remains unknown. Here, we bridge this gap of
53 knowledge by demonstrating physiological changes and expression of miRNAs related to stress
54 tolerance in roots and root exudates from wheat grown on BPA contaminated soil.

55 BPA has emerged as a serious environmental concern owing to its extensively use for consumer
56 goods production, release into soils with effluents and wastes, and negative impact on soil

57 microbial communities and plants (Zaborowska et al. 2023). We used the same experimental
58 set-up as described in Yacoumas et al., (2020) to generate abiotic stress in wheat model exposed
59 to increasing doses of BPA and to investigate root expression and transfer to exudates of several
60 conserved miRNAs that have been reported to modulate growth and ROS responsive genes
61 under drought and chemical exposure (Shi et al., 2018; Li et al., 2020; Geng et al., 2020). In
62 accordance with previous works, BPA exposure at high concentration level significantly
63 affected wheat biomass (Kim et al. 2018) and oxidative stress response measured by the activity
64 of antioxidant enzymes. Unlike POX, SOD and CAT activities were enhanced by the soil
65 contamination at 500 mg. kg⁻¹ BPA after the 7 weeks of cultivation (figure 1). No evidence of
66 BPA effects was observed on root growth and enzymatic antioxidant defense systems at
67 environmentally relevant concentration, confirming that homeostasis of ROS played an
68 important role in limiting root growth of highly exposed plants.

69 In the present study, we identified 5 miRNAs in root samples from BPA treated and untreated
70 plants, respectively. Among them, 3 miRNAs (miR164, miR167, miR398) were appeared to be
71 differentially expressed in wheat exposed to high BPA dose (figure 2). In addition, to our
72 knowledge, this is the first time that BPA induced down expression of plant miRNAs has been
73 described. This reduction of root miRNA content was only observed in plants suffering from
74 severe growth delay, implying that the miRNAs participate directly or indirectly, to the
75 coordination of the complex network of signaling molecules involved in developmental process
76 and stress adaptive response. Several down-regulated miRNAs such as mir164 and mir167
77 identified in our study have been previously reported to target transcripts encoding for auxin
78 signaling and transcriptional regulators of root growth and redox status in developing wheat,
79 rice, barley, soya and sugar cane (Shriram et al. 2016).

80 BPA stress among other abiotic stress that generate ROS production in plants was shown to
81 alter the expression of redox sensitive miRNAs regulated by oxidative stress. These responsive

82 miRNAs included miRNAs families such as miR398 and miR164 which were already known
83 to regulate key antioxidant enzymes activities by controlling mRNA stability of these enzymes
84 under oxidative stress conditions (Li et al. 2017).

85 The turnover of root miRNAs may have important role inside root cells of stressed plants but
86 their modified expression might also account for secondary side effects such as a plausible
87 extracellular trafficking and elimination of root miRNAs through secretion mechanisms. To
88 explore this possibility, we performed molecular profiling analysis of the exudation solutions
89 and showed that root exudates collected from control and BPA exposed plants were enriched
90 for miRNAs. Of five miRNAs found in root tissue, all except miR398 and miR159 were
91 expressed in exudate samples and detected with fewer than 30 cycles of PCR, suggesting they
92 were relatively abundant (figure 2). Using root miRNA that does not vary considerably between
93 individuals to perform data normalization of miRNA expression analysis, we found that root
94 system facilitates export of miRNAs in exudates of highly stressed plants. MiR167 displayed
95 higher expression in exudate of plants exposed to high BPA dose and correlate with its relative
96 abundance in root tissue. These findings suggest that the accumulation of these compounds in
97 root exudates are likely to be the result of endogenous production and transport from root cells
98 toward to the surrounding environment. Further investigations are needed to understand exactly
99 how root tissue transfers miRNAs to the root rhizosphere interface and how exudation is
100 controlled by BPA exposure.

101 Moreover, it remains unresolved under what circumstances plants benefit from miRNAs
102 exudation such as the precise role of miRNAs exuded in improving plant tolerance to soil
103 contamination. MiRNAs are not simply repressors of gene expression within the plant but also
104 an external messenger, whose movement outside the plants can spread post transcriptional gene
105 silencing to microorganisms living in close relation with them (Song et al. 2019). Although
106 this mechanism is probably similar from the cross-kingdom exchange of miRNAs recently

107 described between plants to plants and plants to pathogenic fungi interactions. Indeed, miR167,
108 miR156 and miR164 have been identified and predicted among 78 miRNAs in wheat to target
109 transcripts of several fungal species using computational approaches (Mathur et al. 2020).
110 MiR156 was also reported to shuttle from plants overexpressing miR156 to neighboring plants
111 sharing the same hydroponic medium (Brosnan and Mitter 2021). Therefore, it is reasonable to
112 think that exuded miRNAs which are evolutionary conserved from species to species could play
113 a more important role than just waste elimination from the root system and influence the
114 composition and activity of the soil microbiome to their advantage.

115 In conclusion, our results showed that plant miRNAs exist in root exudates of wheat model.
116 Abiotic stress conditions reduced expression level of several oxidative stress and
117 developmentally regulated miRNAs in root resulting to increased exudation of miR167. The
118 relative abundance of this emerging family of metabolites know affecting plant–microbiome
119 interactions provides new insight on complexity and function of root exudation and holds great
120 potential for improve our understanding on soil ecosystem functioning and find new strategies
121 for enhancing the ability of plants to adapt and survive under harsh conditions caused by soil
122 degradation.

123

124 METHODS

125 1. Soil properties and contamination

126 The soil used in the present study was collected in the 10-20 cm depth horizon of cultivated soil
127 below wheat crop in the farm of UniLaSalle Institute in Beauvais, France (N 49°28'13.88", E
128 2°4'0.45"). The physicochemical properties of the soil were described previously and was
129 classified as haplic luvisol (Yacoumas et al., 2020). The soil was sieved through a 5mm stainless
130 mesh and mixed with river sand (22% ; v:v). Then a sub-sample of soil was spiked with 50 ml

131 solution of bisphenol A dissolved in acetone. After evaporating acetone in the room temperature
132 overnight, the spiked soil was homogenized and mixed with unspiked soil using a roller mixer
133 to reach environmental relevant and roots growth phytotoxic concentrations of 0,1 and 5000
134 mg BPA. kg⁻¹ dry soil (Kim et al. 2018). A solvent control soil was prepared with BPA free
135 acetone solution using similar procedure.

136

137 2. Greenhouse experiment and plant cultivation

138 A greenhouse experiment was conducted from April to May 2020 according to the protocol
139 detailed by Yacoumas et al 2020. Wheat seed (*Triticum aestivum* cultivar pakito, RAGT
140 semences Rodez, France) were sterilized with 10% (v/v) H₂O₂ for 30 min, washed with distilled
141 water, soaked in saturated CaSO₄ for 6 h, and then germinated at 17°C for 7 days in the dark
142 between two layers of filter paper moistened with saturated CaSO₄. Six uniform seedlings with
143 visible 1-2cm roots were selected and planted using polypropylene pots filled with 2L of
144 uncontaminated soil (control), solvent control soil (acetone) or soil contaminated with BPA at
145 0,1 and 5000 mg.kg⁻¹ dry soil. All of the treatments were performed in four replicates. Pots
146 were randomly positioned in a greenhouse with controlled conditions (photoperiod 16h light/8 h
147 dark, light intensity of 10 Watts. m⁻², day/night temperature of 20 °C). Plants were watered
148 three times per week with tap water. Half of seedlings were removed after one week of
149 exposure. Other seedlings (3 per pot) were grown and harvested after 6 additional weeks of
150 culture. Roots were gently rinsed with tap water to eliminate adhering soil and cleaned with
151 adsorbing paper. Root exudates were collected then, roots with their leaves were weighted for
152 the measurement of biomass. Fresh leaves and root tissues were sampled separately and
153 immediately frozen in liquid nitrogen for molecular and biochemical analysis.

154 3. CAT, SOD and POX activity analysis

155 Root and leaves sample (300 mg) of frozen tissues was used for the determination of total
156 proteins, SOD, POX, and CAT activities. Plant tissue samples were ground and homogenized
157 in a buffer solution. Next, the homogenized tissue was centrifuged and supernatants were
158 analyzed using a UV-light spectrophotometer microplate reader according to the protocol
159 described by Firmin et al., (2015) and Labidi et al., (2017). Total SOD and CAT activity were
160 determined using commercial kits (SOD kit, Sigma Aldrich; catalase assay kit Gaithersburg,
161 USA). SOD activity was measured by monitoring the absorbance at 550 nm due to the inhibition
162 of nitroblue tetrazolium (NBT) reduction during 5 min. CAT activity was detected by recording
163 the decrease of absorbance at 540 nm due to H₂O₂ reduction. Total POX activity was quantified
164 by monitoring 2,2'-azino-bis(3)-ethylbenzothiazoline-6-sulfonate (ABTS) oxidation induced
165 absorbance at 405 nm using a calibration curve made with horseradish peroxidase (Sigma-
166 Aldrich, Saint-Quentin Fallavier, France). Activity values were normalized to proteins
167 concentration (BCA protein assay kit, Sigma Aldrich)

168

169 4. Total RNA extraction

170 Total RNA was isolated from wheat roots and exudates using miRNA miRNeasy Mini
171 extraction Kit (Qiagen, France) according to the manufacturer's protocol. Exudate samples
172 were purified prior extraction using membrane filtration followed by differential
173 ultracentrifugation. Total RNA was then quantified and assessed for quality using a Nanodrop
174 2000 (Thermo Fisher Scientific). RNA samples were stored at -80 °C until further analysis.

175

176 5. Real-time quantitative RT-PCR

177 The expression of miRNAs was measured by quantitative reverse transcription PCR. In brief,
178 cDNA was synthesized using 1 µg of total RNA, miScript Nucleics Mix and the miScript

179 Reverse Transcriptase Mix in HiFlex Buffer incubated at 37°C for 60 min and 95°C for 5 min
180 according to the protocol of miScript II RT Kit (Qiagen). Quantitative Real time -PCR was
181 performed by using a StepOne plus apparatus (Thermo Fisher Scientific) with PCR kits. The
182 PCR primers sequences presented in supplementary table S1 were obtained commercially from
183 Eurofins (Ebersberg, Germany). MiRNA quantification was carried out using MiScript SYBR
184 Green PCR kit (Qiagen) consisting of QuantiTect SYBR Green PCR Master Mix and miScript
185 Universal Primer. Reaction mixtures were incubated at 95°C for 15min, followed by 40 cycles
186 of 94°C for 15 s, 55°C for 30 s, 70°C for 30s. The expression levels of miRs were measured in
187 terms of threshold cycle (Ct) value and normalized to TEF1 in each root sample, which was
188 used as an internal control, whereas miR156 was selected as the housekeeper for gene
189 expression of miRs in exudates.

190

191 6. Statistics

192 Parameters were analyzed by Kruskal-Wallis test and pairwise comparison using R statistics
193 3.3.2 software (R Core team, 2017). Differences between the treatments were declared
194 significant at the p-value of 0.05 ($p < 0.05$).

195

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198 for their technical assistance with the greenhouse experiment and molecular analysis.

199 Data availability statement

200 The datasets generated during and/or analysed during the current study are available from the
201 corresponding author on reasonable request.

202

203 Conflict of Interest statement

204 The authors declare that they have no conflict of interest

205

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Figure 1 : Phenotype and root morphology of wheat-grown on the soil, in the absence and in the presence of 0,1 or 5000 mg BPA /kg of dry soil.

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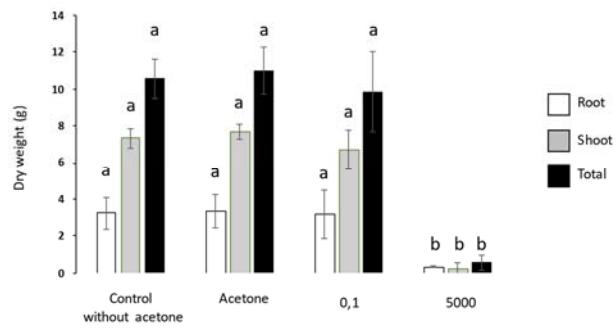


Figure 2 : Root, shoot and total plant dry weights (g) of wheat-grown on the soil, in the absence and in the presence of 0,1 or 5000 mg BPA /kg of dry soil. The values are expressed as Mean \pm SD; N=5.

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2

Table 1 : Catalase (CAT), Superoxyde dismutase (SOD), Peroxydase (PO) activity of wheat-grown on the soil in the absence and in the presence of 0,1 or 5000 mg BPA /kg of dry soil. The values are expressed as Mean \pm SD; N=3.

	CAT ($\mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$)		SOD (IU $\mu\text{g}^{-1} \text{protein}$)		PO (IU $\mu\text{g}^{-1} \text{protein}$)	
	Root	Leave	Root	Leave	Root	Leave
Control without acetone	0,003 \pm 0,002 a	3,22 \pm 0,56 a,b	0,29 \pm 0,24 a	3,05 \pm 1,92 a	3,92 \pm 1,78 a	2,75 \pm 0,60 a
Acetone	0,003 \pm 0,002 a	2,94 \pm 0,72 b	0,29 \pm 0,14 a	3,01 \pm 2,15 a	6,98 \pm 2,70 a	2,90 \pm 0,41 a
0.1 mg BPA / kg	0,002 \pm 0,002 a	2,94 \pm 0,50 a,b	0,25 \pm 0,12 a	3,08 \pm 2,41 a	5,96 \pm 0,58 a	3,49 \pm 0,71 a
5000 mg BPA /kg	0,022 \pm 0,008 b	2,15 \pm 0,65 a,b	0,64 \pm 0,24 b	3,11 \pm 1,86 a	7,07 \pm 1,46 a	3,14 \pm 0,74 a

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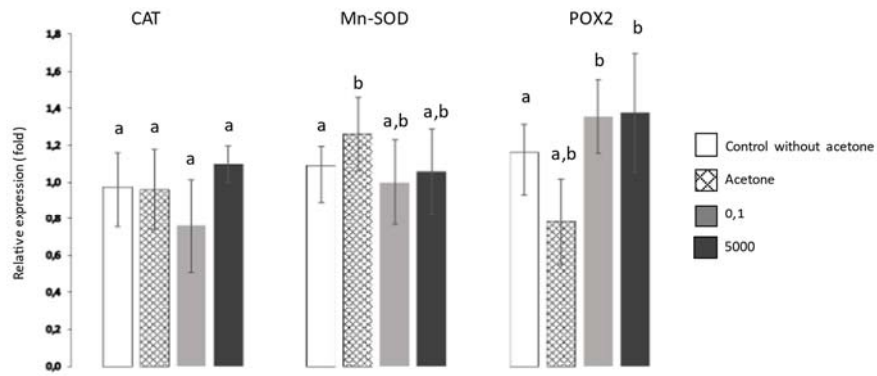


Figure 3 : Expression level of antioxidant enzyme mRNAs in root of wheat grown on the soil in the absence and in the presence of 0,1 or 5000 mg BPA /kg of dry soil. The values are expressed as Mean \pm SD; N=3 using TEF1 for normalization.

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4

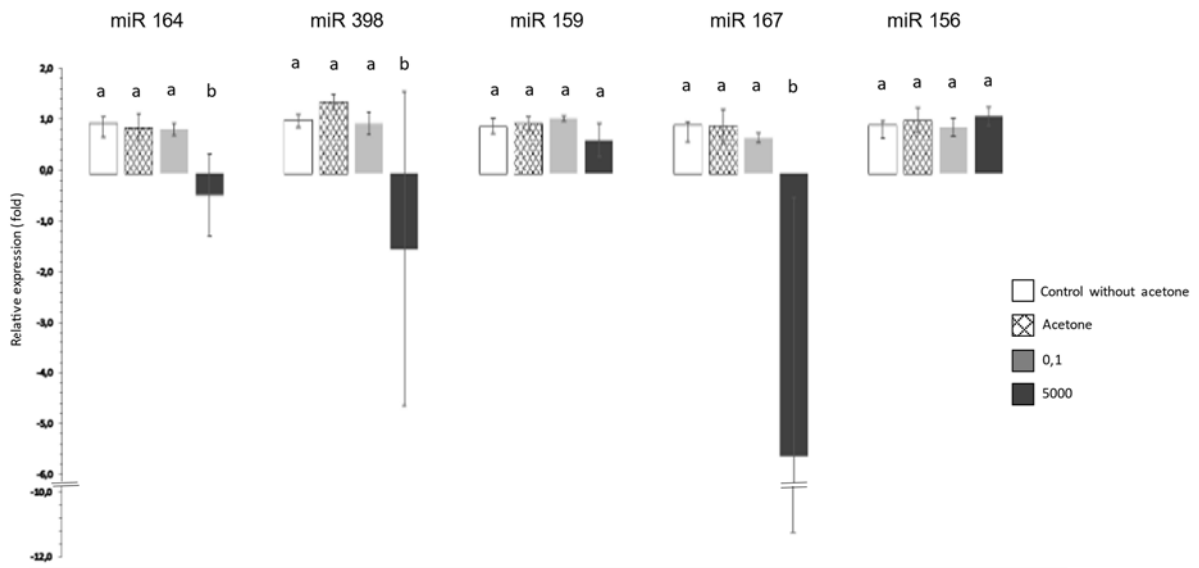


Figure 4 : Expression level of microRNAs in root of wheat grown on the soil in the absence and in the presence of 0,1 or 5000 mg BPA /kg of dry soil. The values are expressed as Mean \pm SEM; N=3 using TEF1 for normalization.

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277

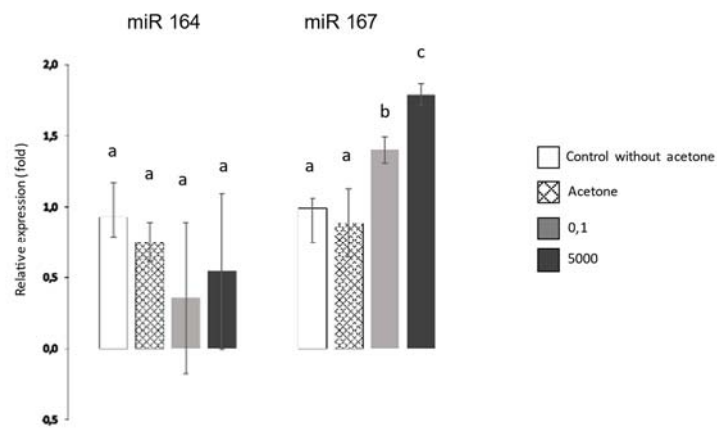


Figure 5 : Expression level of microRNAs in root exudate of wheat grown on the soil in the absence and in the presence of 0,1 or 5000 mg BPA /kg of dry soil. The values are expressed as Mean \pm SEM; N=3 using miR 156 for normalization.

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