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1 **Inhibitory effects of extracellular self-DNA: a general biological process?**

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17

18 **Summary**

- 19 • Self-inhibition of growth has been observed in different organisms, but an underlying
20 common mechanism has not been proposed so far. Recently, extracellular DNA has
21 been reported as species-specific growth inhibitor in plants and proposed as an
22 explanation of negative plant-soil feedback. In this work the effect of exDNA was
23 tested on different species to assess the occurrence of such inhibition in organisms
24 other than plants.
- 25 • Bioassays were performed on six species of different taxonomic groups, including
26 bacteria, fungi, algae, plants, protozoa and insects. Treatments consisted in the
27 addition to the growth substrate of conspecific and heterologous DNA at different
28 concentration levels.
- 29 • Results showed that treatments with conspecific DNA always produced a
30 concentration dependent growth inhibition, which instead was not observed in the
31 case of heterologous DNA.
- 32 • Reported evidence suggests the generality of the observed phenomenon which opens
33 new perspectives in the context of self-inhibition processes. Moreover, the existence
34 of a general species-specific biological effect of exDNA raises interesting questions
35 on its possible involvement in self-recognition mechanisms. Further investigation at
36 molecular level will be required to unravel the specific functioning of the observed
37 inhibitory effects.

38

39

40 **Key words:** autotoxicity, exDNA, self-recognition, exDNA functions, heterologous DNA.

41

42 **Introduction**

43 Self-inhibition or autotoxicity has been reported for several organisms including bacteria
44 (Andersen et al. 1974; Trinick and Parker 1982), fungi (Bottone et al. 2011), algae (Inderjit
45 and Dakshini 1994), plants (Singh et al. 1999) and animals (Akin 1966).

46 The mechanism has been mostly ascribed to the release and accumulation of different toxic
47 compounds in the growth environment, but a specific class of chemicals accounting for both
48 toxicity and species-specificity has never been identified. On the other hand, theoretical and
49 modelling studies on species coexistence have suggested the involvement of a general
50 mechanism to explain species-specific inhibition (Freitas and Fredrickson 1978; Bever 1994;
51 Mazzoleni et al. 2010).

52 The recent observations by Mazzoleni et al. (2014) of inhibitory effects by extracellular self-
53 DNA in plants provided new perspectives for understanding litter autotoxicity and negative
54 plant-soil feedbacks. The authors reported significant evidence that fragmented extracellular
55 DNA (exDNA) has a concentration dependent and species-specific inhibitory effect on
56 plants' growth. These findings suggested an unexpected functional role of exDNA in intra-
57 and inter-specific plant interactions at ecosystem level.

58 While the molecular mechanisms behind these phenomena certainly deserve in-depth
59 investigations, more basic questions arise: does extracellular self-DNA act as inhibitor on
60 biological systems other than plants? Could this be the general mechanism behind the
61 observed phenomena of self-inhibition and autotoxicity?

62

63 **Materials and Methods**

64 In order to test the occurrence of species-specific inhibition by exDNA, a set of laboratory
65 experiments was performed on six species selected across different taxonomic groups.
66 Systematic experiments included exposures to self DNA and to heterologous DNA from
67 *Arabidopsis thaliana* as a model organism, plus a control with distilled water. Extraction of
68 genomic DNA from each species was performed using standard Qiagen® (Valencia, CA,
69 USA) extraction kits and DNA purity was spectrophotometrically assessed at 260 nm on a
70 NanoDrop™ 1000 (Thermo Scientific, Wilmington, DE, USA) and visually verified on
71 1.5% agarose gel using Sybr® Safe (Invitrogen). The extracted DNA was fragmented by
72 sonication according to Mazzoleni et al. (2014) in order to obtain fragments mainly
73 distributed in the range between 50 and 1000 bp, with similar size distribution for all DNA
74 samples. The organisms were exposed to increasing concentrations of self-DNA while
75 heterologous DNA was applied at the maximum concentration tested for self-DNA. Other
76 experiments were preliminary performed to assess possible different effects from different
77 sources of heterologous DNA. The specific experimental settings and treatment
78 concentrations were adapted to the growth requirements of the different species as reported
79 below. *Bacillus subtilis* was selected as target Gram-positive bacterium. It was pre-grown on
80 Luria Broth (LB) at 37 °C with agitation (200 rpm). An inoculum was prepared with 10 ml of
81 preculture and 4 ml of LB. Treatments included self-DNA at three concentration levels (40,
82 200, and 400 µg/ml) and heterologous DNA (400 µg/ml) from *A. thaliana*, *Aspergillus niger*,
83 *Escherichia coli*, and *Sarcophaga carnaria*. All cultures were incubated with agitation (200
84 rpm) at 37 °C, with three replicates for each treatment and the control. After 24 hrs of
85 incubation, 0.5 ml were taken from each tube and serial dilutions in LB were prepared, from
86 which 100 µl were placed on LB agar plates. Plates were incubated at 37 °C until appearance
87 of colony-forming units (CFU).

88 *Trichoderma harzianum* was used as target fungus in a bioassay on spore germination.
89 Fungal spores were produced by pure cultures on potato dextrose agar (PDA). Spores were
90 diluted to a concentration of $1 \times 10^6 \text{ ml}^{-1}$. Treatments included extracellular self-DNA (8, 80,
91 and 800 $\mu\text{g/ml}$) and heterologous DNA (800 $\mu\text{g/ml}$) from *A. thaliana*, *Aspergillus niger*,
92 *Bacillus subtilis* and *Sarcophaga carnaria*, with three replicates for each treatment. The
93 germination bioassay was performed in ELISA plates (96 wells, 100 μl each), each well
94 coated with 10 μl of liquid 10% PDB substrate, DNA at treatment concentration, fungal
95 spores, and sterile distilled water. Spore germination and germ tube elongation of the conidia
96 were assessed by spectrophotometric analysis and optical microscopy after 20 hrs of
97 incubation at 24 °C.

98 The green microalga *Scenedesmus obliquus* was maintained in Chu's n° 10 medium (Chu
99 1942). The cultures were incubated at 25°C under 270 $\mu\text{moles photons m}^{-2} \text{ sec}^{-1}$ light
100 intensity with 16:8 hrs light photoperiod. Treatments of *S. obliquus* were carried out with
101 self-DNA (50 and 500 $\mu\text{g/ml}$) in the culture medium and heterologous DNA (500 $\mu\text{g/ml}$)
102 from *A. thaliana*, with two replicates for each treatment. Algal growth was assessed by cell
103 counts at the optical microscope after serial dilutions, and growth curves were built for each
104 treatment, until reaching stationary phase (7 days).

105 *Acanthus mollis* seedlings were treated with self-DNA (2, 20, and 200 $\mu\text{g/ml}$) and
106 heterologous DNA (200 $\mu\text{g/ml}$) from *A. thaliana*, *Quercus ilex* and *Sarcophaga carnaria*,
107 with three replicates for each treatment. Bioassays were done in vitro by using surface sterile
108 seeds (n=20 in each plate) placed in 9 cm Petri dishes over sterile filter papers imbibed with 4
109 ml of test solutions. Seedling root length was measured.

110 Plasmodia of the ameboid protozoan *Physarum polycephalum*, a slime mold widely used in
111 bioassays were maintained in the dark at 24 °C on 1% agar plates and were fed with oat
112 flakes. Laboratory stocks were subcultured onto new 1% water agar plates and fed oat flakes.

113 Mature cultures (15 days) on Petri plates were used to produce slime mold biomass for total
114 DNA extraction. Tip portions (17 ± 5 mm²) of the plasmodia were taken from stock cultures 8
115 hours after feeding time and placed on agar substrates at the conditions of maintenance, with
116 three replicated plates for each treatment and the untreated control. Extracted self-DNA (290,
117 580, and 1060 µg/ml) and heterologous DNA (1060 µg/ml) from *A. thaliana* were applied on
118 0.2 g of oat flakes placed at the centre of each plate. Pictures of plasmodial growth patterns
119 were taken from each plate every 24 hrs for 96 hrs and used to calculate spreading area size
120 following Takamatsu et al. (2009).

121 The dipteran *Sarcophaga carnaria* was grown in pure culture on 12 x 12 cm² plates (2 cm
122 height) at 10 °C, fed with ground meat. Treatments included self-DNA (10, 100, and 1000
123 µg/ml) and heterologous DNA (1000 µg/ml) from *A. thaliana* mixed with 1 g of food. Three
124 replicated plates, each containing 10 larvae, were prepared for each treatment, plus the
125 untreated control. All plates were incubated in the dark at 10 °C. Development, survival, and
126 time required for the formation of pupae were monitored every 3 days during a 21-days
127 incubation period.

128 A generalized linear mixed model (GLMM) was used to analyse the results of the bioassays.
129 Since different metrics were used to assess the performance of target species, data were
130 expressed as percent of untreated controls. Tested effects on species performance included
131 the target species (6 levels) as random effect, and treatment (3 levels: heterologous DNA,
132 self-DNA and untreated control) and 2nd order interaction as fixed effects. Since the
133 experimental design was not fully balanced with respect to concentration levels of DNA
134 treatment, a further GLMM was tested to assess the effect of DNA concentration, limited to
135 samples treated with self-DNA. Also in this model the target species (6 levels) and its
136 interaction with self-DNA concentration were included as random effects. In both GLMMs
137 pair-wise differences were tested for statistical significance using post-hoc Duncan tests.

138

139 **Results**

140 The experiments produced consistent results for all target species with evident effects of
141 inhibition by self-DNA (Figure 1). The effect of all treatments was highly significant with
142 different responses to either heterologous or self-DNA without differences between species
143 (Table 1a). The application of heterologous DNA did not produce any significant growth
144 reduction compared to control, with the exception of *B. subtilis* which showed some
145 inhibition also in this case (Table 2). This was consistent with results from preliminary tests
146 with different heterologous DNA sources, showing the absence of inhibitory effects in all
147 cases, with the exception of the tested bacterium, which was inhibited at variable levels by
148 heterologous DNA (Table 3).

149 On the contrary, treatments with conspecific DNA always resulted in a concentration
150 dependent growth reduction (Table 1b), showing an inhibitory effect on all tested species
151 (Table 2), consistent with the observations on plants by Mazzoleni et al. (2014). At lower
152 self-DNA concentration the inhibitory effect was reduced with different responses for
153 different species (see significant interactive term in Table 1b).

154

155 **Discussion**

156 Species-specific inhibitory effects of exDNA has been recently reported for higher plants
157 (Mazzoleni et al. 2014). Here we extend such results to a set of organisms from different
158 taxonomic groups.

159 Extracellular DNA has been found both in soil and marine sediments in large amounts
160 (Steffan et al. 1988). Its long persistence in soil has been related to chemical stability and
161 protection against enzymatic degradation by absorption to both mineral and organic
162 components (Levy-Booth et al. 2007). Such accumulation of DNA molecules mainly derives

163 from degradation of organic matter, though release by excretion from living cells is also
164 reported (Nielsen et al. 2007).

165 Extracellular DNA has been proposed to serve different functions (Vlassov et al. 2007). It has
166 been proposed to be a major source for the transfer of genetic information (Weinberg and
167 Stotzky 1972; Graham and Istock 1978; Nielsen et al. 2007). It has been reported to play a
168 role in the formation of microbial biofilms (Whitchurch et al. 2002; Steinberger and Holden
169 2005), in the protection from pathogen attack in root cap “slime” (Wen et al. 2009; Hawes et
170 al. 2011) and in extracellular traps (Brinkmann et al. 2004; Goldmann and Medina 2012).
171 Extracellular DNA has also been considered as a relevant source of nutrients for plants
172 (Paungfoo-Lonhienne et al. 2010) and microbes (Finkel and Kolter 2001; Palchevskiy and
173 Finkel 2006; Pinchuk et al. 2008).

174 The role of exDNA as species-specific inhibitor has been recently reported for higher plants
175 (Mazzoleni et al. 2014), providing a novel explanation for negative plant-soil feedbacks such
176 as inhibition of plant recruitment, growth and reproduction in soils previously occupied by
177 conspecifics (Bever et al. 1997, van der Putten 2003; Kulmatiski et al. 2008; Mangan et al.
178 2010). The same effect could be the explanation of the frequently reported interspecific
179 facilitation but rare occurrence of intraspecific facilitation in terrestrial ecosystems
180 (Bonanomi et al. 2010). Further studies are needed to clarify the interplay between DNA
181 persistence in the environment and related ecosystem diversity.

182 The experiments presented in this paper confirmed the occurrence and the concentration
183 dependency of the inhibition by extracellular self-DNA in bacteria, fungi, algae, plants,
184 protozoa and insects. The possible bias in these results by the presence of residual chemicals
185 from DNA extraction can be excluded because the heterologous DNA, not producing
186 inhibitory effects, was extracted with the same method and applied at the same high
187 concentration of self-DNA.

188 The range of target species, including prokaryotes and both unicellular and multicellular
189 eukaryotes, highlights the widespread occurrence of self-DNA inhibitory effect. An
190 interesting evidence of self-inhibition in vertebrates was reported on *Rana pipiens* (Richards
191 1958, 1962), clearly showing a significant reduction of tadpoles growth in water previously
192 occupied by conspecifics, unaffected by the presence of unrelated species and only slightly
193 inhibited by phylogenetically related ones (Akin 1966). Richards (1958) suggested that "alga-
194 like" pathogens could be the cause of the observed growth inhibition, but the involvement of
195 such pathogens in small tadpoles inhibition was later falsified (West 1960). Akin (1966)
196 suggested the involvement of an unknown self-inhibiting agent. Other works related this
197 inhibition to the production of some "proteinaceous" compounds by large tadpoles (Rose and
198 Rose 1961, Runkova et al. 1974, Stepanova 1974, Steinwascher 1978). Notably, Richards
199 (1962) showed that growth inhibition could be removed after physical and chemical
200 treatments like filtration, centrifugation, heating, sonication, freezing and thawing, ultraviolet
201 light and low pH. We propose that all these observations can coherently be ascribed to the
202 species-specific inhibitory effects of exDNA accumulated in the growth medium.

203 A distinct topic where the specificity of action of exDNA could play an important role is self-
204 recognition. Callaway and Mahall (2007) reviewed the evidence regarding how plants are
205 able to distinguish self from non-self conspecific individuals. In particular, Dudley and File
206 (2007) demonstrated kin recognition at root level in *Cakile edentula* without proposing an
207 explanatory mechanism. Considering the high specificity of the information stored in DNA,
208 we speculate that it can potentially mediate recognition not only at species level, but also
209 within species to distinguish kin from unrelated individuals.

210 In this work, we presented phenomenological evidence supporting the hypothesis of the
211 general occurrence of an inhibitory effect of extracellular self-DNA and of its possible
212 involvement in recognition signalling processes. Are these functions of exDNA going to be a

213 new paradigm? The reported findings certainly suggest intriguing questions and ideas, which
214 may open new research scenarios. For example, in ecology, experiments can be planned to
215 investigate the relevance of this effect in the regulation of species coexistence and
216 competition, in the interactions with natural enemies, in relation with nutrient depletion and
217 symbiont community changes, and its general occurrence in natural conditions. Moreover, a
218 more comprehensive experimental design should address the relationship between inhibition
219 and phylogenetic distance among target species and exDNA sources.

220 In a broader context of life sciences, other issues can be considered. The reported species-
221 specificity of DNA inhibition seems consistent in eukaryotes (both unicellular and
222 multicellular organisms), but this should be further investigated on a larger number of taxa.
223 On the other hand, the effect on prokaryotes appears less certain considering that
224 heterologous DNA also produced a performance reduction in the only observed case of
225 *Bacillus subtilis*. This definitely requires further experimental work on more species.

226 Finally, the investigation of the molecular mechanisms behind the observed inhibitory
227 phenomenon is certainly a major challenge to be faced. It has been widely demonstrated that
228 exDNA can be uptaken by living cells in both prokaryotes and eukaryotes, such as higher
229 plants (Paungfoo-Lonhienne et al. 2010) and mammalian (Groneberg et al. 1975) where it
230 can be transported to the nucleus (Wienhues et al. 1987) and possibly integrated into the
231 genome of the guest cell (Doerfler et al. 1995). Indeed, cells present mechanisms of
232 protection from exDNA uptake. Bacterial restriction enzymes cleave foreign nucleic acids
233 while protecting their own genome by methylation (Wilson 1988). More sophisticated
234 processes of specific clearance of exDNA are found in vertebrates (e.g. Stenglein 2009). The
235 above mentioned mechanisms refer to the recognition of exogenous DNA, whereas little is
236 known about the processes involved in specific responses to self-DNA, for which the
237 mechanisms of viral, retroviral transposons, or other types of parasitic DNA could be taken

238 into account. Future studies are needed to clarify the inhibitory effects of extracellular self-
239 DNA at both cellular and molecular levels, including the processes of recognition, uptake,
240 and transport in both prokaryotes and eukaryotes.

241

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350

351 **Tables**

352 **Table 1.** Summary of the general linear mixed model (GLMM) testing for main and
 353 interactive effects of target species and treatments on species performance in the bioassays.

a) Model I: self and heterologous DNA						
	Effect type	SS	df	MS	<i>F</i>	<i>P</i>
Target species	Random	2134.7	5	426.9	1.53	0.2656
Treatment	Fixed	88928.9	2	44464.4	159.60	< 0.0001
Target species x Treatment	Random	2822.9	10	282.3	7.66	< 0.0001
b) Model II: concentration of self-DNA						
	Effect type	SS	df	MS	<i>F</i>	<i>P</i>
Target species	Random	18277.5	5	3655.5	6.55	0.0077
Concentration	Fixed	21909.3	2	10954.7	20.13	0.0005
Target species x Concentration	Random	5095.7	9	566.2	14.91	< 0.0001

354

355

356 **Table 2.** Performance of target species exposed to extracellular heterologous DNA from
 357 *Arabidopsis thaliana* and self-DNA at different concentration levels. Data are mean \pm
 358 standard deviations of different growth metrics for different species, expressed as % of
 359 untreated controls. Within each target species, asterisks indicate significant difference
 360 between exposure to heterologous and self-DNA at high concentration (Duncan post-hoc
 361 tests for the effect of treatment from GLMM model I in Table 1). Different letters indicates
 362 significantly different groups for the effect of self-DNA concentration (Duncan post-hoc tests
 363 from GLMM model II in Table 1). Values not significantly different from the controls are
 364 reported in italic fonts.

Target species	H DNA		self-DNA	
	high	high	mid	low
<i>Bacillus subtilis</i>	58.2 \pm 7.4 *	7.7 \pm 5.6 <i>a</i>	6.0 \pm 2.6 <i>a</i>	41.4 \pm 6.5 <i>b</i>
<i>Physarum polycephalum</i>	93.9 \pm 7.5 *	0.7 \pm 0.2 <i>a</i>	18.4 \pm 3.9 <i>b</i>	44.7 \pm 7.5 <i>c</i>
<i>Scenedesmus obliquus</i>	95.8 \pm 6.7*	14.1 \pm 6.7 <i>a</i>	-	60.6 \pm 3.4 <i>b</i>
<i>Trichoderma harzianum</i>	93.3 \pm 9.0 *	9.1 \pm 3.0 <i>a</i>	53.0 \pm 10.0 <i>b</i>	67.0 \pm 16.0 <i>c</i>
<i>Acanthus mollis</i>	94.8 \pm 8.7 *	26.8 \pm 1.4 <i>a</i>	81.7 \pm 3.7 <i>b</i>	98.1 \pm 5.4 <i>c</i>
<i>Sarcophaga carnaria</i>	96.1 \pm 4.0 *	12.5 \pm 4.0 <i>a</i>	11.7 \pm 3.0 <i>a</i>	44.2 \pm 8.0 <i>b</i>

365 **Table 3.** Performance of target species exposed to extracellular heterologous DNA from
 366 different sources. Data are mean \pm standard deviations of different growth metrics for
 367 different species, expressed as % of untreated controls. Values not significantly different
 368 from the controls are reported in italic fonts.

Target species	Source of heterologous DNA				
	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Aspergillus niger</i>	<i>Sarcophaga carnaria</i>	<i>Quercus ilex</i>
<i>Bacillus subtilis</i>	51 \pm 13%	-	62 \pm 24%,	42 \pm 13%	-
<i>Trichoderma hartianum</i>	-	108 \pm 14%	91 \pm 11%	98 \pm 9%	-
<i>Acanthus mollis</i>	-	-	-	102 \pm 11%	94 \pm 19%

369

370 **Figure Legends**

371

372 **Figure 1.** Effects of exposure to heterologous DNA from *Arabidopsis thaliana* and self-DNA
373 on different organisms. All species show significant concentration dependent inhibitory
374 effects by self-DNA. See Materials and Methods for details on experimental conditions.

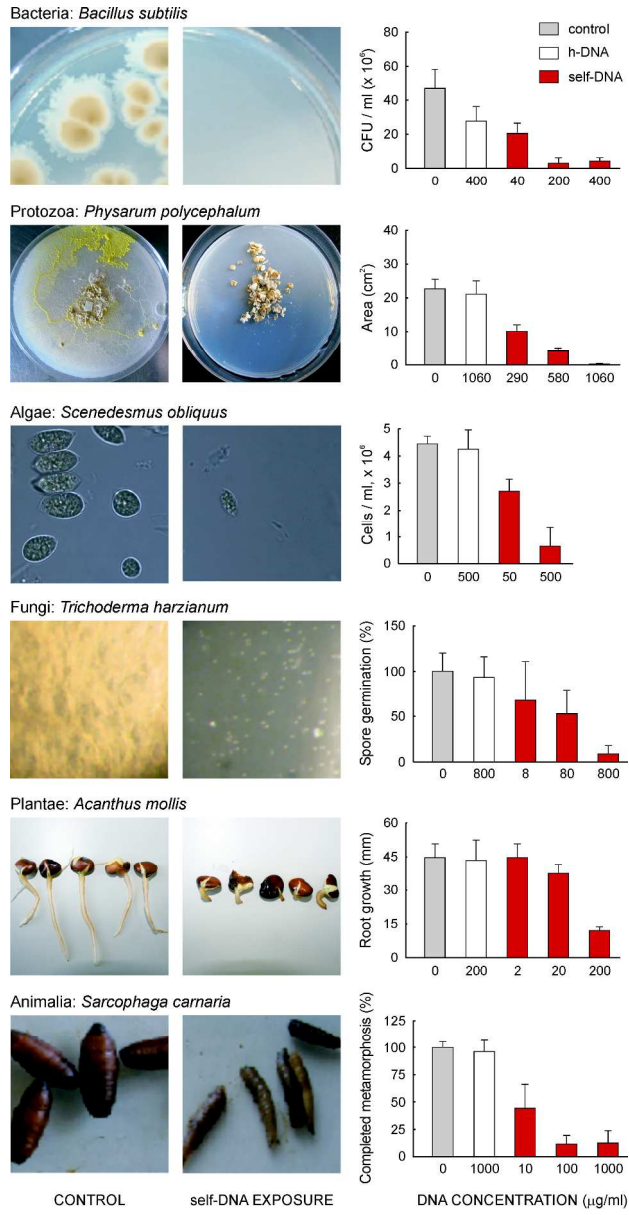


Figure 1