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Fast and Reproducible 96-Well Plate-Based Method for the Evaluation of the Antigerminative Potential of Plant Extracts and Phytotoxic Compounds

G. Flieller, L. Riffault-Valois, M. Bergaentzlé, S. Ennahar

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1 **A fast and reproducible 96-well plate-based method for the evaluation of the**
2 **antigerminative potential of plant extracts and phytotoxic compounds**
3

4 Flieller G., Riffault-Valois L., Bergaentzlé M., Ennahar S.

5 Université de Strasbourg, CNRS, IPHC UMR 7178, Chimie Analytique des Molécules
6 BioActives et Pharmacognosie, F-67000 Strasbourg, France

7
8 Corresponding author: RIFFAULT-VALOIS Ludivine - ludivine.valois@unistra.fr

9
10 **ABSTRACT**

11 With the aim of evaluating the antigerminative activity of plant extracts, a miniaturized assay
12 using 96 well plates (WP assay) was developed and compared to the long-established assay
13 using Petri dishes (PD assay). The WP assay yielded results comparable to those of the PD
14 assay using an ethanolic extract of the Himalayan balsam and lawsone as a standard. It also
15 allowed the needed volume of the test solution to be cut by half and the number of required
16 cress seeds to be cut by more than 1.5. The WP assay was then successfully applied to various
17 extracts of Himalayan balsam, molecules (2,4-D, glyphosate and 2-MNQ) and target seeds
18 (radish, lettuce, and wheat). By being adapted to 96 well plates format, the antigerminative WP
19 assay is a promising alternative to the PD assay. Besides, its convenience and low resource
20 consumption make it ready for accelerated and high throughput screening, as well as
21 automation.

22 **Key words:** allelopathy; antigerminative test; screening; lawsone; *Impatiens glandulifera*
23 Royle

27 1. INTRODUCTION

28 Weeds are undesirable plants that grow and compete for resources with plants of interest
29 causing economic loss to farmers and a decrease in crop quality ¹. In order to control weed
30 proliferation, synthetic chemicals, called herbicides, are used worldwide. Despite their low cost
31 and high efficiency ¹, chemicals such as 2,4-D or glyphosate can be highly toxic to the
32 environment and cause groundwater and air pollution ^{2,3}. Also, many have a wide spectrum of
33 activity, thereby impacting non-targeted species like fish, soil microorganisms ⁴ and humans ².
34 Less harmful substitutes are therefore needed to limit the negative impact of herbicides.
35 Bioherbicides, constituted of living organisms or their bioactive metabolites, may represent an
36 interesting natural alternative with limited environmental impact ¹. Such products have been
37 marketed in the USA, Canada and Europe since the early 80s and their use is on the rise across
38 the world ⁵. In 2012, 11 bioherbicides of microbial origin were registered, mainly in North
39 America and Japan, but not all of them are available to purchase ¹.

40 Plants can also be a source of bioherbicides. They have in fact the capacity to interact
41 with each other in a positive or negative way using various signaling molecules, a phenomenon
42 called allelopathy. These signaling molecules, called allelochemicals, can be released through
43 root exudation, evaporation, leaf leaching ⁶, and decomposition ⁷. They are chemically diverse
44 and include alkaloids, terpenes, flavonoids and fatty acids ⁸. Due to this broad chemical nature,
45 they can influence plant growth in different ways: germination alteration, nutrient uptake,
46 hormone production, and radicle growth inhibition ⁵. Some allelochemicals are known to be
47 antigerminative compounds such as naphthoquinones, especially juglone ⁹ and plumbagin ¹⁰. It
48 is also the case for some flavonoids such as kaempferol-3-*O*- β -D glucoside which inhibits
49 *Echinochloa colonum* L. root growth ¹¹. These allelochemicals could potentially be used as
50 regular herbicides at different stages of weed development (preplant, pre or post-emergence
51 herbicides) ¹². Other active substances include organic acids such as pelargonic and caprylic

52 acids, which have been authorized as bioherbicides in France ¹³. They inhibit the growth of
53 seedlings (like *Lepidum sativum* L.) by disrupting their cell membranes ¹⁴.

54 Methods have been developed that are aimed at identifying active compounds released
55 from plants and evaluating their impact on the germination and growth of targeted plants. Most
56 studies use the same dicotyledonous target plants: *Lactuca sativa* L. ^{15,16}, *L. sativum* L. ^{17,18} or
57 *Raphanus sativus* L. ^{19,20}. These are good model species with seeds large enough to be easily
58 manipulated, short germination times (between 2 and 4 days) and homogeneous germination
59 rates. Moreover, *L. sativa* and *R. sativus* are known to be very sensitive to inhibition by
60 allelochemicals ^{15,21}. In some instances, target monocotyledonous species, such as *Triticum*
61 *aestivum* L. ¹⁷ or *Avena sativa* L., are also investigated ¹⁹.

62 There are four types of methods used in allelopathic studies, depending on the nature of
63 the sample tested (plant, extract, or pure compound): sandwich, filter paper, dish pack and plant
64 box methods. With liquid samples such as plant extracts and pure molecules, the filter paper
65 method in Petri dishes (PD assay) is generally used to evaluate the allelopathic potential by
66 measuring the germination rate of seeds or growth parameters of seedlings (root and hypocotyl
67 lengths). In most instances, 9 cm Petri dishes are used with a sample volume from 2 ¹⁹ to 10
68 mL ²¹ and a number of seeds from 10 ²⁰ and 30 ²¹. Some studies however use smaller containers
69 like 3.5 cm Petri dishes ²² or 96 well plates ²³ with lower amounts of sample solutions.

70 Given the wide range of methods used and the lack of data on method validation, results
71 from different studies are difficult to compare, which limits our understanding of the overall
72 activity of allelochemicals. In addition, no consensus seems to exist as to the mathematical
73 processing of germination data. Germination is usually expressed as percentage, and the
74 number of germinated seeds and data analysis are typically based on a normal linear model.
75 However, the original variable (the number of germinated seeds) is discrete and follows a
76 binomial distribution with only two possible results (germinated or not germinated). The use of

77 generalized linear models (GLMs) seems therefore to be more appropriate for statistical
78 analysis of germination data ²⁴.

79 Considering the parameters which can be different from one study to another (samples
80 volumes, number of seeds...), it appears that there is a need for the development of a
81 standardized antigerminative test using the least amount of sample volume and number of target
82 seeds. It should thereby be adapted to the screening of the activity of large numbers of test
83 plants to discover new allelochemicals which could be used to replace the harmful herbicides.

84 Using 96 well plates, the present study aims at developing a simple and standardized
85 method (WP assay) which yields reproducible antigerminative data with reduced numbers of
86 seeds, sample volumes as well as time and space necessary per test. A naphthoquinone,
87 lawsone, was used as a standard bioherbicidal molecule for test development. Cress (*L. sativum*
88 L.) seeds were chosen as target, because of their easy handling (macroscopic size), short time
89 of germination (maximum of germination reached in a few days) and stable germination rate
90 between used batch. This test was then firstly implemented on an ethanolic extract of the
91 Himalayan balsam (*Impatiens glandulifera* Royle [IGR]), and then applied to 5 other IGR
92 extracts of different polarity. This plant is considered as invasive in Europe and known to have
93 antigerminative properties ^{25,26}. Its activity may be attributed to the presence of phenolic acids,
94 flavonoids ²⁷ and naphthoquinones comprising lawsone ²⁸. In addition to lawsone, other
95 molecules were also tested on cress seeds including two herbicides (2,4-Dichlorophenoxyacetic
96 acid [2,4-D] and glyphosate) and another naphthoquinone, 2-Methoxy-1,4-naphthoquinone (2-
97 MNQ). Moreover, to evaluate the phytotoxicity of these compounds, three additional species
98 were used as targets: one monocotyledonous, wheat, and two dicotyledonous, radish and
99 lettuce. Seeds from these species have different shapes and sizes, but also different culture
100 conditions (light or obscurity). This diversity allowed a good understanding of the activity of
101 the compounds and extracts tested in this study.

102 MATERIALS AND METHODS

103 *Chemical and reagents*

104 Petri dishes (50 mm diameter) and filter paper (47 mm diameter, Whatman™) were purchased
105 from VWR (Rosny Sous Bois, France) and 96 well plates with lids from Thermo Fisher
106 Scientific (Illkirch Graffenstaden, France). Ethanol (EtOH), ethyl acetate (EtOAc), and heptane
107 were purchased from Fisher (Illkirch Graffenstaden, France), 2-propanol (*i*-PrOH) was
108 purchased from Carlo Erba (Val-de-Reuil, France) and H₂O was obtained using an ultrapure
109 water System from Merck Millipore (Molsheim, France). Lawsons, 2-MNQ, glyphosate and
110 Tween 20 were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). 2,4-D was
111 purchased from Duchefa Biochemie (Haarlem, the Netherlands).

112 1.1. *Plant material*

113 Garden cress (*L. sativum* L.), lettuce (*L. sativa* L.), radish (*R. sativus* L.) and wheat (*T. aestivum*
114 L.) seeds were purchased locally. Aerial parts (stems and leaves) of IGR were collected in Bas-
115 Rhin, France. An herbarium specimen was prepared, and the species identity was confirmed by
116 an expert from the herbarium of the University of Strasbourg. After harvest, plant material was
117 stored at -80°C to prevent degradation. It was then roughly grinded in liquid nitrogen to
118 facilitate the freeze drying which followed. The dry plant was then grinded to a homogeneous
119 fine powder using a coffee mill and stored in dark glass bottles at ambient temperature until
120 use.

121

122 1.2. *Extraction*

123 IGR powder (3.5 g) was suspended in 35 mL of solvent (heptane, *i*-PrOH/H₂O [50/50; v/v],
124 EtOAc, EtOH, EtOH/H₂O [60/40; v/v] or H₂O) and put under stirring at 610 rpm for 40 min.
125 Then the solution was centrifuged at 5,000 rpm for 10 min. The supernatant was collected, and
126 the pellet was subjected to two more extractions with fresh solvent. The supernatants were

127 pooled together, and the solvent was evaporated using a rotary evaporator. The dry IGR extracts
128 were weighed and dissolved to a final concentration of 40 mg/mL in *i*-PrOH/H₂O (50/50; v/v)
129 for heptane, *i*-PrOH and EtOAc extracts, in EtOH/H₂O (60/40; v/v) for EtOH and EtOH/H₂O
130 (60/40; v/v) extracts, and in H₂O for the aqueous extract. These stock solutions were stored at
131 4°C until use.

132

133 1.3. *Antigerminative bioassay*

134 1.3.1. *Solutions preparation:*

135 Working solutions at 0.25, 1, 2.5, 5, 10 and 20 mg/mL were prepared in EtOH/H₂O (60/40;
136 v/v), *i*-PrOH/H₂O (50/50; v/v) or H₂O from the extract's stock solution. Lawsone, 2,4-D and 2-
137 MNQ working solutions were prepared in EtOH and glyphosate ones in H₂O at 0.03, 0.3, 1.5,
138 3, 6 and 9 mM.

139 The final concentrations (after solvent evaporation and Tween20 addition) in the PD assay and
140 in the WP assay were 0.083, 0.33, 0.83, 1.66, 3.33 and 6.66 mg/mL for IGR ethanolic extract,
141 and 0.01, 0.1, 0.5, 1, 2 and 3 mM for lawsone. For the application to the standards (2,4-D, 2-
142 MNQ, glyphosate and lawsone) the concentrations used were 0.01, 0.1, 0.5, 1, 2 and 3 mM on
143 the different seeds. The IGR extracts were tested at 1.66 and 6.66 mg/mL on cress seeds.
144 Lawsone was used as positive control at 0.46 mM for the different extracts.

145

146 1.3.2. *PD assay*

147 Petri dishes were used with one layer of filter paper soaked with 0.5 mL of IGR ethanolic
148 extract, lawsone solutions or solvents. The solvents (EtOH/H₂O [60/40; v/v] and EtOH) were
149 used as negative controls. Petri dishes containing the soaked filter paper were placed under a
150 hood for 45 min to allow the solvents to evaporate. Twenty cress seeds were then placed in each
151 dish along with 1.5 mL of a Tween 20 solution at 0.05% in H₂O (v/v). As germination control,

152 the seeds were just in contact with the Tween solution. Each experimental condition was tested
153 in triplicate (60 seeds total) and the overall experiment was repeated over three different days
154 for the lawsone solutions.

155

156 1.3.3. *WP assay*

157 Each well of the plate was filled with 20 μ L of IGR extract, lawsone, glyphosate, 2,4-D and 2-
158 MNQ solutions or solvents per well. No filter paper was used. The solvents (EtOH/H₂O [60/40;
159 v/v], *i*-PrOH/H₂O [50/50; v/v], EtOH) were used as negative controls. 96 well plates were
160 placed in a Speed Vac centrifugal vacuum evaporator (Thermo Scientific, Savant SPD121P) at
161 35°C for 30 min. One cress seed was added per well and covered with 60 μ L of a Tween 20
162 solution at 0.05% in H₂O (v/v). As germination control, seeds were just in contact with the
163 Tween solution. For the validation part using cress seeds, each experimental condition was
164 tested over 20 wells and in triplicate (60 seeds in three plates with 20 wells per condition) and
165 the overall experiment was repeated over three different days for the lawsone. Lower numbers
166 of seeds were used for method optimization (determination of the least usable number of seeds)
167 in triplicates with, respectively, 10, 12, or 15 seeds for lawsone solutions and 12 seeds for IGR
168 ethanolic extract solutions.

169 For the application of the method to other standards like 2,4-D, glyphosate and 2-MNQ using
170 different type of seeds (cress, lettuce, radish, and wheat), only 12 seeds were used per condition
171 in triplicate. For the application to different types of IGR extracts, 12 cress seeds were used per
172 condition in triplicate.

173

174 1.3.4. *Incubation and data collection*

175 Dishes and plates were closed, sealed with paraffin tape (Parafilm) and kept at 25°C for 48h in
176 a culture chamber (Panasonic Versatile Environment Test Chamber MLR 352) with a

177 photoperiod of 16 h light / 8 h dark using 3LS light (60 PPFD Photosynthetic Photon Flux
178 Density) for cress and lettuce seeds. Radish and wheat seeds were incubated in the dark.
179 At the end of incubation time, seeds were photographed with a Lumix DMC-GH3 camera
180 (Panasonic) mounted on a stereomicroscope (Leica) and using an enlargement factor of 1.6.
181 Seeds were considered germinated when the radicle length exceeded 1 mm.

182

183 1.4. *Statistical analysis*

184 Germination rates were expressed as mean values and standard errors were calculated for
185 all treatments using MS Excel. All statistical analyses were carried out using the R software (R-
186 4.0.3)²⁹, considering the significance level of $\alpha=0.05$ for all applied tests. The repeatability and
187 the intermediate precision (day to day precision) of the methods were evaluated using the within
188 day and between days standard deviations obtained by Analysis of Variance. To compare the
189 results obtained with different methods and concentrations, a post-hoc multiple comparison was
190 performed using a generalized linear model for binary data and computing the Tukey's honest
191 significance test (multcomp R package).

192

193 2. RESULTS and DISCUSSION

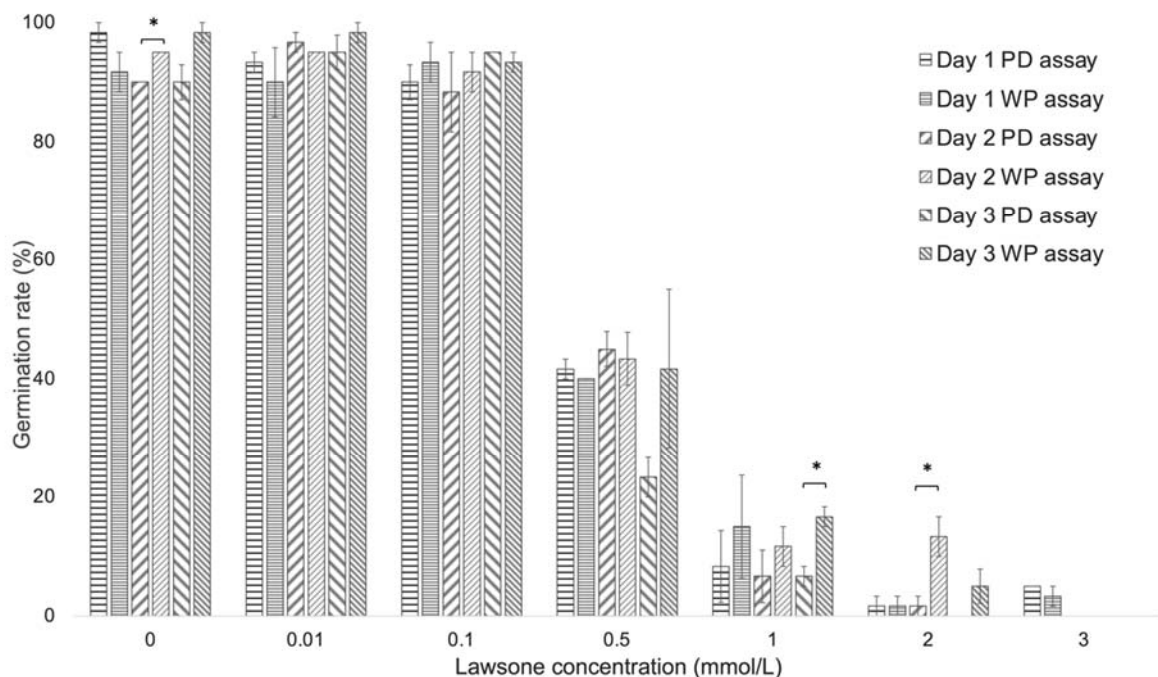
194 A miniaturized antigerminative assay (WP assay) was developed using 96 well plates instead
195 of Petri dishes as in the most widely used assay. Both assays were submitted to a validation
196 procedure evaluating their selectivity, repeatability, intermediate fidelity using lawsone as
197 bioherbicide molecule and cress seeds as target. The trueness of the WP assay was evaluated
198 by comparing the results to the ones obtained with the reference method (the PD assay with 20
199 seeds). It was then optimized by decreasing the number of seeds needed. Repeated tests were
200 carried out with an ethanolic extract of IGR with the aim of evaluating the selectivity,
201 repeatability, and trueness of the WP assay in the case of a complex mixture as opposed to a

202 single molecule like lawsone. Other molecules known to have herbicidal activity (2,4-D, 2-
203 MNQ and glyphosate) and 6 extracts of IGR were also tested on cress seeds. Seeds of lettuce,
204 radish, and wheat were also used as targets to evaluate the phytotoxicity of lawsone and 2,4-D.
205

206 2.1.Method validation:

207 2.1.1. Selectivity of the methods:

208 First, the selectivity of the PD and WP assays was checked. Regardless of the assay, in the
209 absence of lawsone, the germination rate was nearly 100 %. In the presence of lawsone, an
210 antigerminative effect was observed, as the germination rate dropped to 50 % at 0.5 mM and to
211 almost 0 % at 3 mM (Fig.1). The inhibition of the germination of the cress seeds could be
212 clearly attributed to the activity of lawsone.



213

214 Figure 1 - Cress seed germination rates over 3 days in response to increasing concentrations of
215 lawsone as assessed by the PD assay and the WP assay. Data are expressed as means +/-
216 Standard Error to the Mean (SEM) from three replicates. * Indicates a significant difference
217 between the values obtained with each method according to t-test at $\alpha = 0.05\%$.

218 2.1.2. Precision of the methods:

219 The precision of the methods was assessed using the coefficient of variation of the repeatability
220 (CV_R) and of the intermediate precision (CV_{IP}). With both methods, the results were similar,
221 with few interday variations, as shown by the obtained CV_{IP} (Table 1). The WP assay appeared
222 however to be more precise than the PD assay. The CV_R and CV_{IP} were lower at the smallest
223 lawsone concentrations (between 0 and 0.10 mmol/L) than at concentrations of 0.50 mM and
224 above. This could be attributed to differences in germination rates between individual seeds
225 which impact the coefficients of variation of repeatability and intermediate precision.

226 For the concentration on the middle of the range (0.50 mmol/L), the germination rate was more
227 variable because of the seeds' sensitivity to lawsone. Germination is a complex phenomenon
228 which requires many steps to be complete and the activation of particular "switches"³⁰. It can
229 be hypothesized that at this concentration, the quantity of antigerminative molecules in the
230 extract is neither too low to allow seed germination, nor too high to block it, but somewhere
231 between. The fact that a seed germinates depends on its sensitivity to the allelochemical(s) and
232 the balance between a state of germination and its absence is delicate.

233

234 Table 1 – Coefficients of variation of the repeatability (CV_R) and of the intermediate precision
235 (CV_{IP}) depending on the antigerminative assay and the lawsone concentration used.

Lawsone concentration (mmol/L)	PD assay		WP assay	
	CV_R	CV_{IP}	CV_R	CV_{IP}
0.00	3.59	5.96	3.92	4.75
0.01	3.92	3.92	6.36	6.83
0.10	7.97	7.97	5.39	5.39
0.50	12.86	33.51	33.70	33.70
1.00	105.75	105.75	65.27	65.27
2.00	212.13	212.13	70.71	107.04
3.00	0.00	173.21	150.00	212.13

236

237

238 2.1.3. Trueness:

239 The newly developed WP assay was compared to the PD assay using a t-test evaluating its
240 trueness. The results showed differences between some values for day 2 at 0 mM and 2 mM
241 and for day 3 at 1 mM for example (Fig.1). Nonetheless, the order of magnitude of the values
242 remained the same for a specific concentration of lawsone and no inappropriate values were
243 observed. These differences can be considered negligible for the rest of the method
244 development and applications.

245 The WP assay was validated using a standard molecule: lawsone. Using 96 well plates
246 instead of Petri dishes simplified the experimental process. Seed germination in 96 well plates
247 could be easily visualized without touching the seeds, whereas in Petri dishes the radicles were
248 sometimes intertwined which made this more tedious. Moreover, the volume of test solution
249 needed per concentration was reduced: 1.2 mL per 60 seeds for the WP assay compared to 1.5
250 mL for the PD assay. To further reduce this volume and facilitate even more the test procedure,
251 the reduction of the number of seeds tested was investigated.

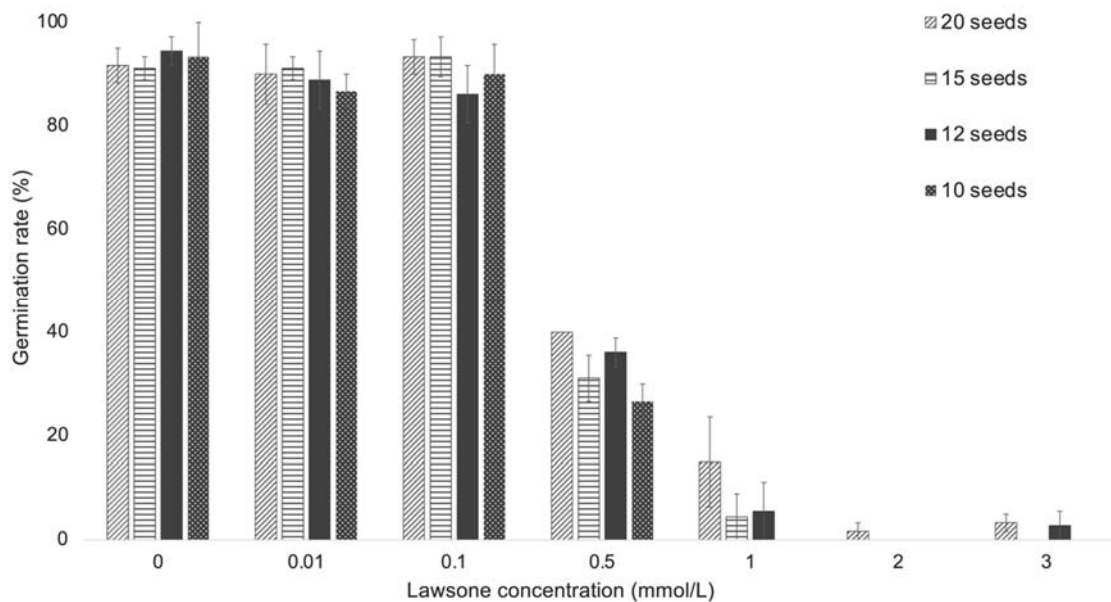
252

253 2.2. WP assay optimization:

254 To optimize the WP assay by decreasing the required amounts of solvents and samples, as
255 well as the time needed for the experiment and data processing, an investigation was carried
256 out to determine the lowest number of seeds necessary to obtain significant data. Starting from
257 the previously used number of 20 seeds, initial tests were done computationally to eliminate the
258 seed numbers that will give a different result than expected.

259 First, a computational experiment was performed using the data set obtained when
260 performing the PD assay based on 20 seeds, considering each seed either germinated or non-
261 germinated, for the six extract concentrations. Among the data set, a random draw was done 10
262 times, each time independent from the other, for a fixed number of seeds. Four amounts of seeds

263 were tested: 15, 12, 10 and 5 chosen randomly among the 20. For each draw, the result
 264 (expressed in germination rate) was compared to the one obtained with the previous experiment,
 265 using 20 seeds in a Petri dish. The differences between the gathered results were expressed as
 266 p-value. It was noticed that the highest number of differences was obtained when 5 seeds were
 267 randomly chosen. This number decreased when more seeds were chosen: 8 inadequate values
 268 out of 60 were obtained when using 5 seeds; 3 when using 10 seeds; 1 when using 12; and none
 269 when using 15. These results were confirmed experimentally and compared to the one obtained
 270 with 20 seeds with lawsone using 15, 12 and 10 seeds per 96 well plate in triplicate (Fig.2). The
 271 number of 5 seeds was not tested as the estimated number of inappropriate values was too high.
 272 No differences were noticed between the 4 different amounts of seeds tested. The selectivity of
 273 the method was not lost, with a decrease in seed germination rates in line with the increase of
 274 the lawsone concentration. Moreover, the germination rates obtained were similar to those
 275 obtained with 20 seeds.



276
 277 Figure 2- Impact of a decreasing number of seeds on the accuracy of the measured seed
 278 germination rate in response to lawsone concentration using the WP assay. The data are
 279 expressed as means +/- SEM from three replicates.

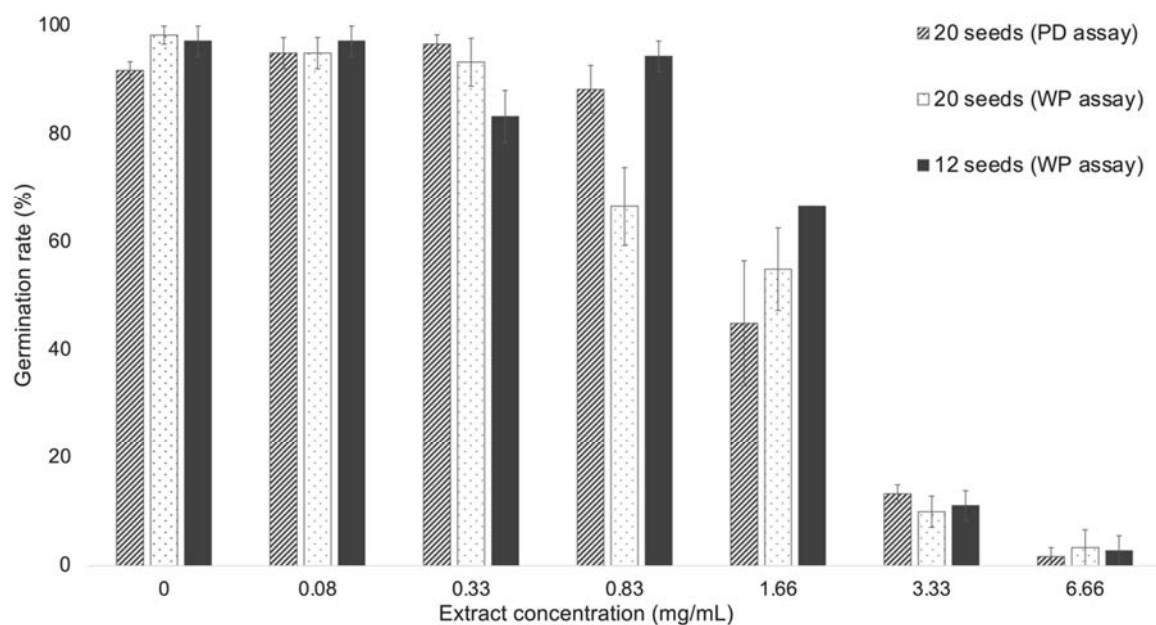
280 In light of these results, a seed number of 12 was selected, as it represents a substantial
281 reduction compared to the initial number of 20 seeds, while having no significant impact on the
282 accuracy of determined germination rates. In addition, this number allows an optimal use of the
283 96 well plates per experiment. Seed number reduction also led to a decrease of the volume of
284 solvents needed per triplicated assay: from 1.5 mL with 60 seeds (20 μ L per seed) to 720 μ L
285 with 36 seeds. Compared to the PD assay, the optimized WP assay uses half the amount of
286 samples and 1.5 times less seeds.

287

288 2.3.WP assay verification to an ethanolic extract of *Impatiens glandulifera* aerial parts:

289 2.3.1. Selectivity:

290 After being validated using a standard molecule, the developed WP assay was tested using
291 a complex mixture of molecules in the form of an ethanolic extract of IGR. The germination of
292 cress seeds exposed to extract concentrations ranging from 0.01 to 6 mg/mL was monitored.
293 The results obtained with PD assay and with WP assay using 20 seeds were compared with the
294 ones obtained with the optimized conditions using 12 seeds (Fig.3). A decrease in germination
295 rate was observed in parallel with the increase in IGR ethanolic extract concentration regardless
296 of the assay and the number of seeds used.



297

298 Figure 3 - Comparison of the cress seed germination rate using the Petri dish and the 96 well
 299 plate methods with 20 or 12 seeds in response to increasing concentrations of ethanolic extract
 300 of IGR. Data are expressed as means +/- SEM from three replicates.

301

302 2.3.2. Repeatability:

303 As the experiments were done during the course of one day, only the repeatability could be
 304 evaluated. For the PD assay the standard deviation (SD) was between 2 and 10 except with an
 305 extract concentration of 1.67 mg/mL where it was 20. For the WP assay with 12 seeds the
 306 standard deviation was below 9 (Table 2).

307 Globally the order of magnitude of the means for each concentration was the same. The biggest
 308 variabilities are observed, as with the lawsone, when the germination rate is around 50 % (for
 309 1.67 mg/mL when 20 seeds were used). It was due to the seed differences in allelochemicals
 310 sensitivity.

311

312 Table 2- Germination rates of cress seeds as a function of the concentration IGR's ethanolic
 313 extract. Rates were measured using the PD assay with 20 seeds and the WP assay with 20 seeds
 314 and with 12 seeds. Data are expressed as means +/- SD from three replicates and SD is shown
 315 in brackets.

Extract concentration (mg/mL)	PD assay (20 seeds)	WP assay (20 seeds)	WP assay (12 seeds)
0.00	91.82 (2.77)	98.33 (2.89)	97.22 (4.81)
0.08	95.00 (5.00)	95.00 (5.00)	97.22 (4.81)
0.33	93.33 (2.90)	96.67 (7.64)	83.33 (8.33)
0.83	88.33 (7.64)	66.67 (12.58)	94.44 (4.81)
1.67	55.00 (20.00)	45.00 (13.22)	66.67 (0.00)
3.33	10.00 (2.90)	13.33 (5.00)	11.11 (4.81)
6.67	3.33 (2.90)	1.67 (5.77)	2.78 (4.81)

316

317

318 2.3.3. Trueness:

319 The trueness was evaluated by comparing the results obtained with the WP assay (using 12
 320 and 20 seeds) to those obtained with the PD assay (considered as reference). Overall, no
 321 differences were observed in the results obtained with both assays and numbers of seeds. The
 322 effect of the extract is observed from a concentration of 1.67 mg/mL regardless of the assay
 323 (Fig.3).

324 The newly developed WP assay allowed a quick evaluation of antigerminative activity
 325 compatible with higher scale studies of large numbers of plant extracts by reducing the amount
 326 of material and solvents needed. This represents a major benefit of the WP assay as compared
 327 to other techniques such as the paper filter test including some evaluating the potential of
 328 *Impatiens* species which may use up to 9 mL of aqueous plant extract per concentrations point
 329 for 75 seeds (3 mL per replicate for 25 seeds of *Hieracium murorum* or *Scrophularia nodosa*)
 330 ²⁶ and even 21 mL of essential oil per concentrations point for 30 seeds of *R. sativus* L., *L.*
 331 *sativum* L., *L. sativa* L., and *T. aestivum* L. (7 mL per replicate with 10 seeds of target plant ¹⁷).

332 To our knowledge, the smallest sample volume ever to be reported in an antigerminative
333 test is 50 μ L (10 μ L sample diluted in 40 μ L of water), which has been used per each well in a
334 96 well plates assay²³. Using macroscopic seeds (size superior to 2 mm) like those of *L. sativum*
335 L., the scale reduction of antigerminative tests has however never been attempted. The WP
336 assay will allow the screening of the antigerminative potential of large numbers of samples and
337 can be adapted to various target species.

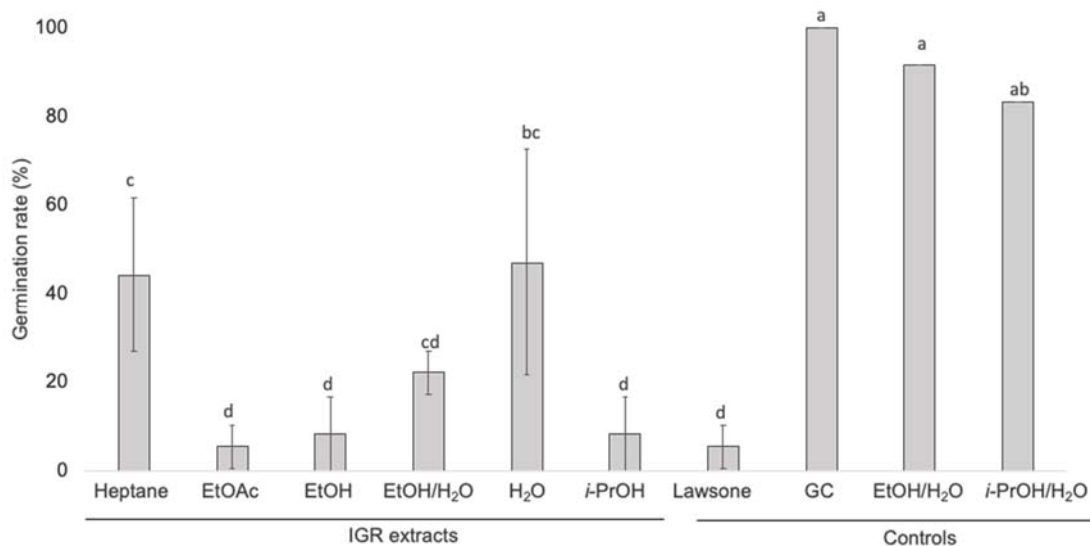
338

339 2.4. Application to different extracts and molecules:

340 2.4.1. Application to different extracts of IGR

341 In order to evaluate more precisely the antigerminative potential of IGR aerial parts, extracts
342 were prepared using solvents of different polarities. Their activities were then tested using the
343 newly developed assay on cress seeds at 1.66 mg/mL and 6.66 mg/mL, and compared with
344 lawsone as a standard molecule. No differences were showed between the controls whatever
345 the solvent tested with a germination rate around 100% (Fig.4).

346 All IGR extracts led to complete inhibition of the germination of cress seeds at 6.66 mg/mL
347 (data not shown). However, at 1.66 mg/mL, germination was possible (Fig.4). The activity of
348 EtOAc, EtOH, *i*-PrOH and EtOH/H₂O (60/40; v/v) extracts was similar to lawsone with a
349 germination rate under 20%, whereas extracts using heptane and H₂O showed a lower activity
350 with a germination rate higher than 40%. This activity may be linked to the presence of
351 naphthoquinones²⁶, a class of molecules already known to have antigerminative properties and
352 which could be preferentially extracted by MeOH, EtOAc³¹ or hydroalcoholic mixture
353 (EtOH/H₂O [35/65; v/v])²⁸ depending of the studied naphthoquinones.



354

355 Figure 4 – Comparison of the antigerminative activity of extracts from IGR aerial parts at 1.66
 356 mg/mL on the germination of cress seeds. The data are expressed as means +/- SEM from three
 357 replicates. Different letters indicate a significant difference according to Tukey Test $\alpha = 0.05\%$.

358

359 Thanks to the WP assay, different extracts having various polarity indices were tested at
 360 two concentrations simultaneously and their activity can be compared with each other or with
 361 a chosen standard. This allows a faster screening, targeting and selection of the most active
 362 extracts for further investigation, without using large amounts of samples.

363

364 2.4.2. Application to different molecules:

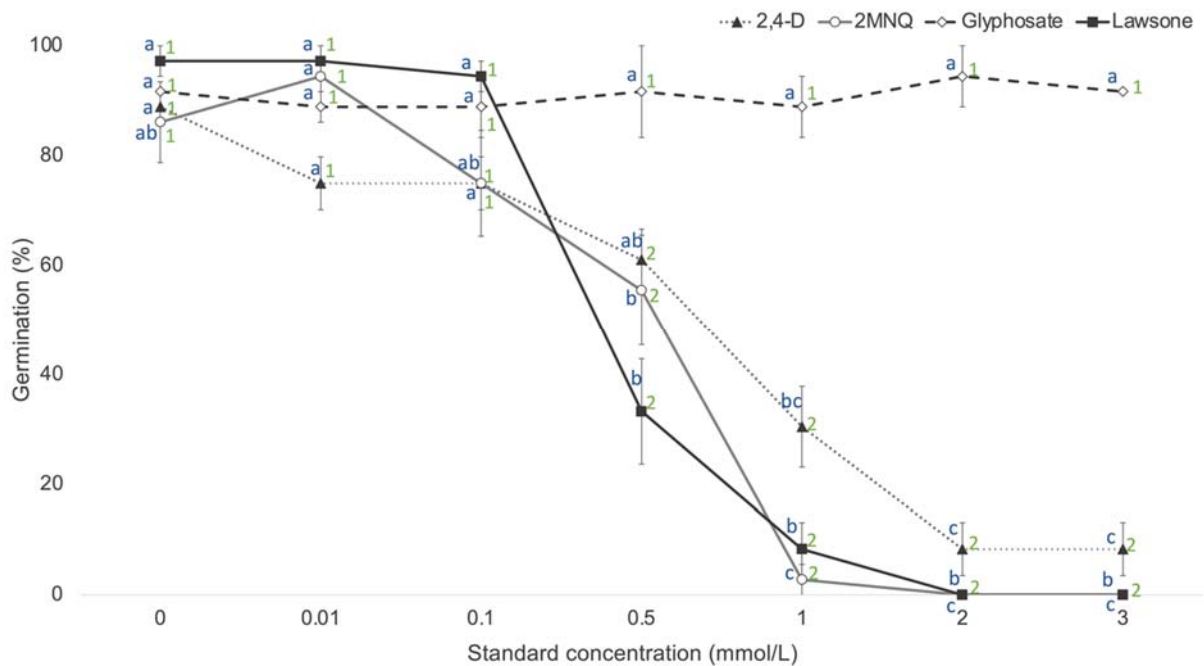
365 The WP assay can be used to evaluate the antigerminative properties of a large array of
 366 samples, including various extracts, but also molecules of different natures. In addition to
 367 lawsone, three other molecules were tested against cress germination: glyphosate and 2,4-D,
 368 commercialized herbicides, and 2-MNQ, a naphthoquinone. The concentration range was from
 369 0 mM to 3 mM.

370 Glyphosate showed no effect on cress seed germination regardless of the concentration
371 used (Fig.5). This was also the case in a study by Piotrowicz-Cieślak *et al.*, 2010³², who tested
372 glyphosate concentration from 1 μ M to 2,000 μ M of glyphosate on cress seeds. However,
373 glyphosate affected the radicle length which suggests that it acts as a post-emergence herbicide
374 and not as an antigerminative one.

375 With 2,4-D on the other hand, the germination rate decreased as the concentration of the
376 herbicidal molecule increased. The activity of the molecule started over 0.5 mM, with a
377 germination rate near 60% and it decreased progressively as the concentration of 2,4-D
378 increased. At the higher concentrations (2 and 3 mM) the germination rate of cress seeds was
379 still near 10%.

380 With 2-MNQ, a decrease in cress germination was observed at 0.5 mM, and this activity
381 increased with increasing concentrations. The germination rate was near 0% when the
382 concentration of 2-MNQ reached 2 mM.

383 The activities of the two naphthoquinones tested (lawsone and 2-MNQ) were similar,
384 with no noticeable effect at concentrations below 0.1 mM and no germination observed at
385 concentrations higher than 2 mM. The 2,4-D seemed to have a similar profile of activity than
386 the 2 naphthoquinones even if the germination rate seemed a little higher for the same
387 concentration, no significant differences were seen.



388

389 Figure 5 – Germination rates of cress seeds in response to increasing concentrations of lawsone,
 390 2,4-D, 2-MNQ and glyphosate using the WP assay. The data are expressed as means +/- SEM
 391 from three replicates. Different letters (in blue) indicate a significant difference according to
 392 Tukey Test $\alpha = 0.05\%$ between the germination rate of cress seeds according to increasing
 393 concentration of standard. Different numbers (in green) indicate a significant difference
 394 between the germination rate of cress seeds at one considered standard concentration according
 395 to Tukey test $\alpha = 0.05\%$.

396

397 2.5. Utilization of different target seeds:

398 With the aim of measuring the specificity of action of molecules tested using the WP assay,
 399 three additional plant seeds were used as targets. Wheat was chosen as monocotyledonous
 400 species, and lettuce and radish as dicotyledonous ones. Results were compared with those
 401 obtained for cress seeds using lawsone and 2,4-D at concentrations ranging from 0 to 3 mmol/L.

402

403

404 2.5.1. Lawsone:

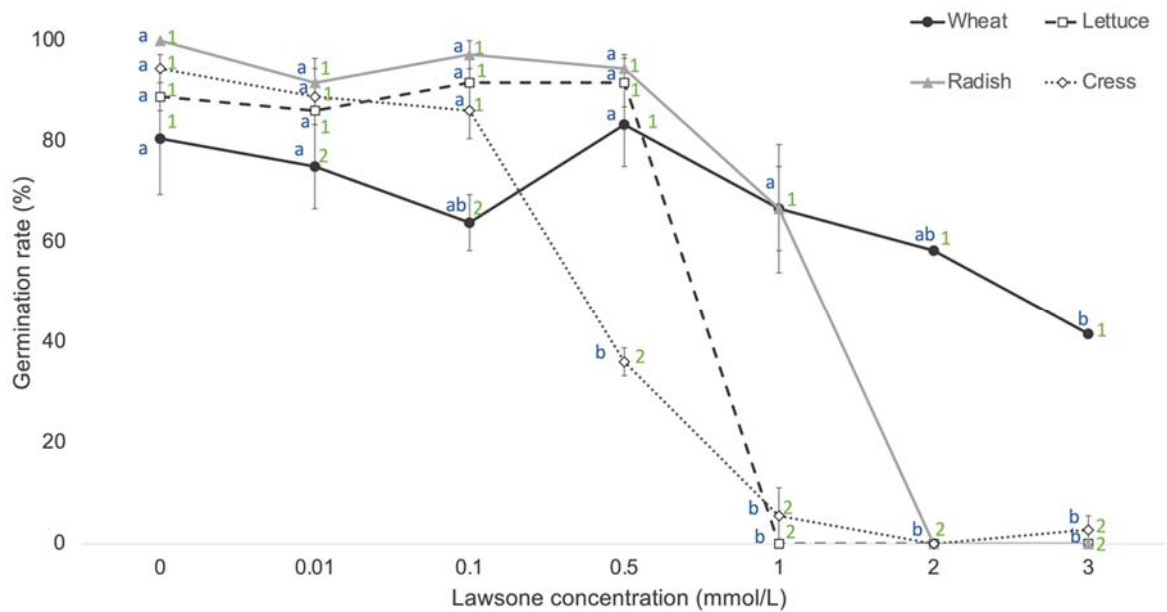
405 For the dicotyledonous plants (radish and lettuce), the germination rate decreased as the
406 concentration of lawsone increased. However, no significant effect on the germination was
407 observed at concentrations of 0.1 mM and below (Fig.6). No effect was observed at 0.5 mM, a
408 concentration which was effective against cress seeds. With lettuce, as with cress, a
409 concentration of 1 mM yielded total inhibition of the germination, whereas with radish 2 mM
410 were necessary to obtain a such effect. These results were similar to those obtained by Campos
411 *et al.*, 2019 ³³, who observed no effect of lawsone below 0.11 mM on both radish and lettuce
412 using a PD assay. The same trend was observed by Kocacë Aliskan and Terzi, 2001 ³⁴, with
413 juglone (another naphthoquinone) showing no inhibition of radish and wheat germination at a
414 concentration of 1 mM, whereas cress germination was totally inhibited. These results suggest
415 that cress seeds are more sensitive to naphthoquinones compared to those of radish and lettuce.

416 Wheat on the other hand was less sensitive to lawsone than the other seeds tested.
417 Indeed, no clear effect on the germination rate was observed when the lawsone concentration
418 was below 2 mM. In fact, a higher variability of the germination rate of wheat seeds was
419 observed with lawsone concentrations below 1 mM making it difficult to clearly establish an
420 antigerminative effect at these concentrations. However, the germination rate dropped to 40%
421 with a lawsone concentration of 3 mM, which was significantly lower than observed for
422 concentrations below 0.01 mM.

423 The four seeds tested showed different sensitivity levels to lawsone. The most sensitive
424 one was cress with an antigerminative effect from 0.5 mM, followed by lettuce then radish.
425 Wheat seeds showed the lowest sensitivity to lawsone, with an effect from 3 mM and a
426 germination rate still at 40 %.

427

428



429

430 Figure 6 – Germination rates of wheat, lettuce, cress, and radish seeds in response to increasing
 431 concentration of lawsone from 0 to 3 mM using the WP assay. The data are expressed as means
 432 +/- SEM from three replicates. Different letters (in blue) indicate a significant difference
 433 according to Tukey Test $\alpha = 0.05\%$ between the germination rate of a species according to
 434 increasing concentration of lawsone. Different numbers (in green) indicate a significant
 435 difference between the germination rate of the four species at one considered concentration
 436 according to Tukey test $\alpha = 0.05\%$.

437

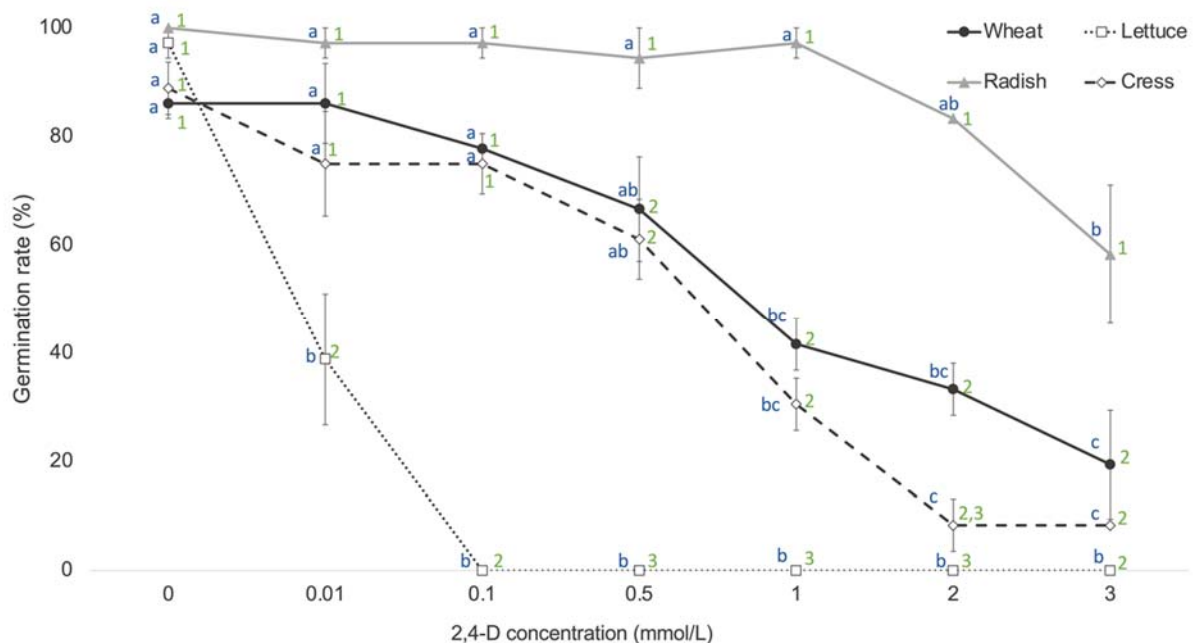
438 2.5.2. 2,4-D:

439 With 2,4-D, a decrease in the germination rate of the four seeds tested was observed when the
 440 concentration increased (Fig.7). For radish, the percentage of germination was significantly
 441 affected from a concentration of 3 mM of 2,4-D reaching 60% of seeds germinated. The
 442 germination of lettuce seeds was inhibited from 0.01 mM of 2,4-D with 50% of germination
 443 and totally blocked from 0.1 mM. For wheat and cress, the inhibition of germination was
 444 equivalent and visible only from 1 mM of 2,4-D. In a study by Hamner C.L et al., 1946³⁵, a
 445 concentration of 0.4 mM of 2,4-D still allowed a germination rate of wheat between 90 % and
 446 80 %. At 3 mM, the germination rate of lettuce, wheat and cress was statistically the same.

447 The four seeds had very different sensitivity profiles to 2,4-D with lettuce being the most
 448 affected by 2,4-D, then wheat and cress with the same germination rate regardless of the
 449 concentration used. Finally, radish was the less affected by 2,4-D with a germination rate at
 450 60% even at 3 mM of 2,4-D.

451 Using the same target species, it could be shown that the ranking of seeds by sensitivity was
 452 not the same between lawsone and 2,4-D. However, lettuce and cress seeds seemed to be the
 453 most sensitive to both molecules, which explains their use as targets in antigerminative tests.

454



455

456 Figure 7 - Germination rates of wheat, lettuce, cress, and radish seeds in response to increasing
 457 concentration of 2,4-D from 0 to 3 mM using the WP assay. The data are expressed as means
 458 +/- SEM from three replicates. Different letters (in blue) indicate a significant difference
 459 according to Tukey Test $\alpha = 0.05\%$ between the germination rate of a species according to
 460 increasing concentration of 2,4-D. Different numbers (in green) indicate a significant difference
 461 between the germination rate of the four species at one considered concentration according to
 462 Tukey test $\alpha = 0.05\%$.

463

464 In conclusion, the newly developed antigerminative WP assay using 96 well plates was
465 evaluated comparatively to the commonly used PD assay. Both assays were validated with the
466 evaluation of their repeatability and intermediate precision using lawsone as a standard
467 molecule, then applied to a complex mixture: an ethanolic extract of IGR. The obtained data
468 were processed using statistical tests adapted to the analysis of antigerminative activity. The
469 WP assay yielded results similar to those of the PD assay. It was further optimized with the use
470 of only 12 target seeds and a subsequent decrease in the required sample amount by 1.5
471 compared to the PD assay. The WP assay allowed comparing the antigerminative activity of
472 six IGR extracts, demonstrating that four of them were as active as lawsone at 0.46 mM. It also
473 showed that 2-MNQ and 2,4-D had a similar effect on cress seeds while glyphosate didn't
474 impact the germination rate. The difference of sensitivity of four target plant species to two
475 phytotoxic compounds was also evaluated, showing clearly different behaviours. Thus, it was
476 demonstrated that this bioassay can be applied for testing both extracts of different polarities
477 and pure molecules on various target seeds which can be monocotyledonous or dicotyledonous.
478 This led to a significant improvement in the performance of the assay for an easy and quick
479 evaluation of antigerminative activity of extracts and pure molecules.

480

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482

483 **CONTRIBUTIONS**

484 GF wrote the main manuscript text. SE, MB and LRV reviewed the manuscript. GF and LRV
485 designed and implemented the experimental procedures. MB and GF developed the method
486 validation procedure. All authors reviewed the final version of the manuscript.

487

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