

1 ***Plasmodiophora brassicae* in its environment-effects of temperature and light on resting**

2 **spore survival in soil**

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14

15 **Abstract**

16 Clubroot caused by *Plasmodiophora brassicae* is an important disease on cruciferous crops
17 worldwide. Management of clubroot has been challenging, due largely to the millions of resting
18 spores produced within an infected root that can survive dormant in the soil for many years. This
19 study was conducted to investigate some of the environmental conditions that may affect the
20 survival of resting spores in the soil. Soil samples containing clubroot resting spores (1×10^7
21 spores g^{-1} soil) were stored at various temperatures for two years. Additionally, other samples
22 were buried in soil, or kept on the soil surface in the field. The content of *P. brassicae* DNA and
23 the numbers of viable spores in the samples were assessed by quantitative polymerase chain
24 reaction (qPCR) and pathogenicity bioassays, respectively. The results indicated that $4^{\circ}C$, $20^{\circ}C$
25 and being buried in the soil were better conditions for spore survival than were $-20^{\circ}C$, $30^{\circ}C$ and
26 at the soil surface. Most of the spores kept on the soil surface were killed, suggesting the
27 negative effect of light on spore viability. Additional experiments confirmed that ultraviolet (UV)
28 light contributed a large negative effect on spore viability as lower pathogenicity and less DNA
29 content were observed from the 2- and 3-hour UV light treated spores compared to the untreated
30 control. Finally, this work demonstrated that DNA-based quantification methods such as qPCR
31 can be poor predictors of *P. brassicae* disease potential due to the presence and persistence of
32 DNA from dead spores.

33

34 **Keywords:** Clubroot, qPCR, Pathogenicity, UV light, Equipment sanitation, Canola

35

36 **Introduction**

37 Clubroot, caused by the protist *Plasmodiophora brassicae* Woronin, is an important threat to
38 Canadian canola (*Brassica napus* L.) production [1]. In the Canadian Prairies, clubroot was first
39 identified on canola in 2003 in a dozen fields near Edmonton, Alberta [2]. The disease has spread
40 throughout much of the canola producing areas of Alberta [3,4], and has also been confirmed in
41 canola fields in Saskatchewan [5], Manitoba [6], Ontario [7] and North Dakota [8].

42

43 Many strategies have been proposed for clubroot management and amongst those, crop rotation,
44 along with the use of clubroot-resistant cultivars, was demonstrated to be effective [9]. However,
45 in fields with high resting spore populations, the selection for virulent, but rare, *P. brassicae*
46 pathotypes can lead to a shift in the population. The once rare virulent pathotype(s) becomes
47 predominant and new sources of resistance must then be found. Shifts toward virulent pathotypes
48 have been reported in Alberta since 2014 [10,11]. As a result of these findings, crop rotation (2-3
49 year break from a host crop), coupled with deployment of cultivars with genetic resistance, have
50 been strongly recommended as the most reliable and sustainable clubroot management method
51 [12,13].

52

53 The environmental effects on *P. brassicae* and clubroot have been well documented [14-18].
54 However, these reports have measured the effects of environmental and soil parameters on the
55 germination, infection, severity of disease and root gall decay. Few studies have evaluated the
56 effects of the soil environment on resting spore viability. The resting spores, which start the
57 initial infection, have been reported to survive in the soil for up to 20 years, with a half-life of
58 about 4 years [19]. More recently, there is evidence that, rather than a half-life, the resting spores

59 experience a sharp decline in viability during the first two years without a host [12,20] followed
60 by a slow decline of spore viability over the next 10 to 20 years. Regardless of the kinetics of
61 spore degradation, little is known about which environmental parameters have the greatest
62 effect(s) on spore survival. During the prolonged surviving time, the viability of spores may be
63 impacted by soil type, soil pH values, water content, temperature and light [14]. Other factors
64 may include the number of freeze-thaw cycles, rapid temperature shifts, microbial activity,
65 population size, premature signals to exit dormancy, etc [14]. There is almost no information on
66 how these soil environment parameters may affect the long-term viability of *P. brassicae* resting
67 spores. In one study, the soil pH was found to play an important role in spore viability, and that
68 soil temperature and moisture had much less effect [21]. However, the duration of the
69 environmental exposure was only 30-days leaving us without an understanding of long-term
70 effects of the soil environment on resting spore survival.

71
72 Since *P. brassicae* resting spores generally reside underground and are non-motile, most spores
73 produced during one growing season remain in situ as dormant inoculum able to survive adverse
74 environmental conditions or prolonged absences of host plants. Thus, in locations where resting
75 spores have been present for many years, it is understood that while some spores survive, many
76 others die beside the living ones. With this in mind, studies on clubroot diagnostics and clubroot
77 management using the amount of DNA assessed by polymerase chain reaction (PCR) or
78 quantitative PCR (qPCR) to represent the population of living spores in plant or soil samples
79 could result in inaccurate conclusions. This inaccuracy results from the inability to ensure that
80 only DNA from living cells is amplified by PCR, and thus the contribution of DNA from dead
81 spores to quantification experiments is unknown. To address this complication, we evaluated the

82 survival of resting spores using qPCR analysis side-by-side with pathogenicity bioassays. The
83 results from the two approaches were compared and any inconsistency was further investigated.
84 The objectives of this study were 1) to assess the viability of resting spores after being stored in
85 different conditions for two years, 2) to identify any differential effects of environmental
86 conditions on the survival of spores, 3) to illustrate the difference between DNA content and the
87 biological potential (number of living spores) by using the same samples assessed by qPCR and
88 pathogenicity bioassays, and 4) improve our understanding of *P. brassicae* survival, biomass
89 assessments, and clubroot epidemiology.

90

91 **Materials and methods**

92 **Ethics statement**

93 No specific permission was required for the fields from which the *P. brassicae* populations were
94 derived. All of the field studies were carried out in a closed and protected green house or a
95 growth chamber in Crop Diversification Centre North (CDCN). This study did not involve
96 endangered or protected species.

97

98 ***Plasmodiophora brassicae* populations**

99 Two *P. brassicae* populations, one pathotype 3H and the other composed mainly of pathotype
100 5X, were used exclusively in this study. The pathotype 3H population was the Led09 strain [22]
101 and was determined to be pathotype 3 by its reactions on the Williams' differential hosts [23],
102 and pathotype H according to reactions on the Canadian Clubroot Differentials [24]. The
103 pathotype 5X population was collected from a canola field in Sturgeon County, Alberta in 2016
104 and later was found to be pathotype 5X by using the specific PCR primers developed by Zhou et

105 al. [25]. These two populations were maintained on the susceptible canola cultivar Westar in a
106 greenhouse at CDCN. In the greenhouse, galls were collected from inoculated plants 42 days
107 after inoculation. Galls were stored at -20°C for one month, and then chopped into 1 cm³ cubes,
108 which were mixed at a ratio of 50% pathotype 3H to 50% pathotype 5X. This mixture was used
109 to prepare resting spores for soil inoculation immediately or after being stored at -20°C for two
110 years.

111

112 **Preparation of soil samples**

113 A 2 kg black soil sample was collected from a research field plot at CDCN. The soil was
114 autoclaved twice for one hour at 121°C, 15 psi and air dried at room temperature for three days.
115 The dried sample was ground with a coffee grinder and then aliquoted into 1.5-mL
116 microcentrifuge tubes with 1 g soil per tube. The tubes were stored at -20°C.

117

118 **Soil inoculation with resting spores**

119 Resting spores were prepared from samples of the gall mixture according to Zhang et al. [26].
120 The spore suspension was adjusted to 2×10^8 mL⁻¹ and from which dilutions were prepared. For
121 soil inoculation, 50 µL of a spore suspension was added into one of the 1.5-mL tubes containing
122 1 g soil, which ensured that all inoculated soil samples had the same water content (50 µL g⁻¹ soil)
123 despite different spore concentrations. After inoculation, the tubes were kept at room temperature
124 for one hour to allow the inoculum to be absorbed evenly in the soil sample. The tubes were then
125 vortexed for 10 seconds and immediately used in the subsequent experiments. For simplicity,
126 these tubes will be referred to as ‘original-tubes’ throughout the rest of this paper.

127

128 **Treatments of resting spores in different environmental conditions**

129 The original-tubes were prepared at the final concentration of 1×10^7 spores g⁻¹ soil. One
130 hundred of the original-tubes were sealed in a 1-L glass media bottle (Fig. 1A), and each bottle
131 was kept in one of the following conditions: 1) buried in soil at 10 cm beneath the soil surface in
132 a field at CDCN, 2) on the surface of bare soil in the same field, 3) in an incubator set at 30°C, 4)
133 in a growth chamber set at 20°C, 5) in a 4°C refrigerator and 6) in a -20°C freezer. All bottles
134 except the one on soil surface were wrapped with double-layers of aluminum foil to ensure
135 darkness and to protect the bottle. After approximately two years (23.5 months), tube samples
136 were collected from each treatment and the soil in the tubes were subjected to qPCR analyses for
137 measurement of *P. brassicae* DNA, and plant inoculation bioassays for measurement of
138 pathogenicity.

139

140 **Autoclave treatment**

141 Original-tubes were prepared at the final concentration of 1×10^7 spores g⁻¹ soil. With their caps
142 open, the tubes were autoclaved with the dry cycle mode at 121°C, 15 psi for 0.5, 1, 2 or 3 hours.
143 After autoclave, the tubes were kept at 4°C for no more than 24 hours before subjected to qPCR
144 analyses.

145

146 **Ultraviolet light treatment**

147 For UV light treatment, original-tubes were prepared at the final concentration of 1×10^6 spores
148 g⁻¹ soil. Then the soil from each tube was transferred into a 5-cm petri dish. With the lid closed,
149 the petri dish was shaken by hand for 30 seconds to make sure that the soil and the inoculum
150 were well-mixed. After removing the lid, the petri dish was placed in a PCR workstation (Model

151 P-048-02; CBS Scientific, San Diego, CA) and treated with UV-light for 1, 2 or 3 hours. The
152 petri dishes were 15 cm beneath the 30-watt UV lamp, which resulted in a 400 μ W/cm² UV
153 intensity being applied to the samples, as calculated according to the manual of the PCR
154 workstation. After UV treatment, the petri dishes were kept under fluorescent light in an
155 alternative biosafety hood until the complement of the 3-hour UV treatment. Then the soils of all
156 treatments were transferred back to the 1.5-mL tubes and kept at 4°C for no more than 24 hours
157 before being subjected to qPCR analyses and pathogenicity bioassays.

158

159 **DNA extraction from soil samples**

160 Before sampling for DNA extraction, the 1-g soil sample in each 1.5-mL tube was homogenized
161 by transferring the soil into a Ziploc bag and rubbing the bag by hand. From each bag 100 mg of
162 soil was collected. Using the DNeasy PowerSoil kits (Qiagen Canada, Toronto, ON), genomic
163 DNA was extracted from these 100-mg samples with a QIAcube instrument (Qiagen). The
164 resultant DNA from each soil sample was eluted in 50 μ L elution buffer included in the
165 PowerSoil kit. DNA concentration was not measured.

166

167 **Quantitative PCR analysis**

168 A pair of qPCR primers (CrqF2/CrqR2) was manually designed according to the rDNA sequence
169 of a *P. brassicae* strain (GenBank accession number: KX011135). In this study, all PCR analyses
170 were probe-based with the combination of this primer pair and the fluorescently labeled probe
171 PB1 [27]. The sequences of CrqF2, CrqR2 and PB1 are: CTAGCGCTGCATCCCATATC,
172 TGTTTCGGCTAGGATGGTTC and 6-FAM/CCATGTGAA/ZEN/CCGGTGAC/3IABkFQ,
173 respectively. The primers and probe were synthesized by Integrated DNA Technologies (IDT;

174 Coralville, IA). All qPCRs were conducted in PrimeTime Gene Expression Master Mix (IDT)
175 using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Canada, Montreal, QC). Each
176 15 μ L qPCR reaction contained 0.5 μ M of each primer, 0.18 μ M probe and 2 μ L template DNA.
177 The qPCR program consisted of 40 cycles of denaturation at 95°C for 10 sec (3 min for the
178 initial denaturation) and annealing/extension at 60°C for 30 sec.

179

180 **Pathogenicity bioassays**

181 The conical part of the 1.5-mL tubes containing 1 g inoculated soil was cut off. The tubes were
182 sited upside-down on a floating tube rack (Fig. 1B) and 500 μ L of water was added to each tube.
183 Seedlings of canola cultivar Westar, generated from seeds on moist filter paper under continuous
184 light for 96 hours, were transferred into the tubes with one seedling per tube. The tubes were
185 incubated in a growth chamber maintained at 24°C/18°C (day/night) with a 16-h photoperiod.
186 After five days, the cap of the tube was opened and the tube was transplanted into a 1.9-L square
187 pot filled with Sunshine mix #4 potting soil (SunGro Horticulture, Agawam, MA). The pots were
188 kept on individual plates to prevent cross-contamination in the greenhouse maintained at
189 24°C/18°C (day/night) with a 16-h photoperiod and watered from the bottom every second day
190 with tap water at pH 6.4 (adjusted with HCl). After 42 days, the plants were separated into
191 classes using a 0-to-3 scale [28], where 0 = no clubbing, 1 < one-third of the root with symptoms
192 of clubbing, 2 = one-thirds to two-thirds clubbed, and 3 > two-thirds clubbed (Fig. 1C).

193

194 **Experimental design**

195 The initiation of the resting spore storage treatments and the construction of the qPCR standard
196 curve were conducted in May 2017. Other experiments were conducted in or after April 2019.

197 All qPCR analyses and pathogenicity assays were conducted twice with similar results. Data
198 from both experiments of the pathogenicity assay on the 2-year-stored spores were included. For
199 other pathogenicity assays and all qPCR analyses, data from only one experiment was included.
200 In each qPCR analysis, template DNA was extracted from three biological samples (three 100
201 mg soil samples from alternative tubes) for each treatment. For each DNA, qPCR was conducted
202 with three technical repeats. The averaged data from these three technical repeats was treated as
203 one data point in the statistical analysis. In each pathogenicity assay, each treatment was tested
204 on five biological repeats (tubes). After transplanting, the pots in each experiment were kept on
205 the greenhouse benches as randomized complete blocks.

206

207 **Statistics**

208 Data from each experiment was subjected to analysis of variance (ANOVA) using Microsoft
209 Excel. Based on the ANOVA results, differences between treatments were assessed with the
210 Fisher's LSD test ($P \leq 0.05$) using the Excel add-in DSAASTAT developed by Dr. Onofri at the
211 University of Perugia, Italy (<http://www.casaonofri.it/repository/DSAASTAT.xls>).

212

213 **Results**

214 **Accuracy of the quantitative PCR assay**

215 A qPCR standard curve was constructed based on the quantification cycle (Cq) values of DNA
216 templates extracted from soil samples containing a 10-fold dilution series (from 1×10^7 to $1 \times$
217 10^3 spores g^{-1} soil) of *P. brassicae* resting spores (Fig. 2). The regression equation of the
218 standard curve is $Y = -3.0650X + 40.7583$ with $R^2 = 0.9968$. The calculated primer efficiency is

219 112%. For any soil sample, if the Cq value was obtained, its spore concentration or the
220 equivalent of spore concentration could be calculated using the regression equation.

221

222 **Quantitative PCR analysis of spores stored in different conditions for two years**

223 After stored in different environmental conditions for two years, soil samples inoculated with *P.*
224 *brassicae* resting spore were analysed by qPCR. Based on the Cq values, the equivalent spore
225 concentration was calculated (Fig. 3). Compared to the fresh prepared 1×10^7 spores g⁻¹ soil
226 control, soil samples stored at -20°C, 4°C or being buried in soil maintained a similar amount of
227 *P. brassicae* DNA. In contrast, DNA in the soil samples stored at 20°C, 30°C or on soil surface
228 was less than the control. In addition, DNA in the soil sample kept on soil surface was also less
229 than the sample stored at -20°C or 4°C. These results indicated that 20°C, 30°C and the soil
230 surface conditions are not favorable to the survival of *P. brassicae* resting spores and among
231 them the soil surface conditions are most harmful. Furthermore, the DNA amount in a canola
232 gall, before and after being stored at -20°C for two years was not different. When the mean Cq of
233 the fresh prepared 1×10^7 spores g⁻¹ soil control (M = 19.48, SD = 0.69; Fig. 2) was compared to
234 that of the soil sample prepared and assessed two years ago (M = 19.11, SD = 0.58; Fig. 1) by a
235 Student's t-test, no significant difference was found (t = 0.7109, p = 0.5164).

236

237 **Pathogenicity assay of spores stored in different conditions for two years**

238 The pathogenicity of spores in the soil samples was evaluated based on the clubroot severity on
239 the susceptible canola cultivar Westar (Fig. 4A). In the two pathogenicity experiments, soil
240 samples stored at 20°C, 4°C and being buried in soil showed similar pathogenicity to the fresh
241 prepared 1×10^7 spores g⁻¹ soil control, while the remaining treatments showed a reduction in

242 pathogenicity. The soil sample stored at 4°C showed a similar pathogenicity in one experiment
243 and lower pathogenicity in the other experiment, compared to the fresh prepared 1×10^7 spores
244 g^{-1} soil control. No clubroot symptoms were observed from the soil sample kept on soil surface
245 in both experiments. Interestingly, the soil sample stored at -20°C showed pathogenicity lower
246 than the samples stored at 4°C, 20°C or buried in the soil.

247

248 Difference in pathogenicity among the soil samples can be attributed to the fact that the numbers
249 of viable spores in these samples were different. To estimate the disease potential of viable
250 spores in these soil samples, pathogenicity bioassays on soil samples containing a 5-fold dilution
251 series (1×10^7 to 1×10^3 spores g^{-1} soil) of resting spores were conducted in parallel with the
252 pathogenicity assays of the stored samples. The results showed that a spore concentration $\geq 5 \times$
253 10^3 spores g^{-1} soil could cause clubroot with same severity as the spore concentration at 1×10^7
254 spores g^{-1} soil (Fig. 4B). Thus, we could conclude that the concentration of living spores in the
255 soil samples kept on soil surface, at 30°C or at -20°C were lower than 5×10^3 spores g^{-1} soil
256 while the concentration in the sample on soil surface was lower than 1×10^3 spores g^{-1} soil.

257

258 **Pathogenicity assays vs. quantitative PCR**

259 Comparing the results from the qPCR analysis to those of the pathogenicity bioassays indicated
260 that qPCR should not be used to assess the numbers of living spores or disease potential. When
261 spores die, their DNA can remain in the sample and produce qPCR amplicons, although more or
262 less of this DNA would degrade depending on the environmental conditions. This is illustrated
263 by the qPCR and pathogenicity bioassay results of the soil sample stored at -20°C. The qPCR
264 data indicated that the amount of DNA in the -20°C sample was equivalent to $1 \times 10^{6.39}$ spores

265 g^{-1} soil, which is the highest mean among all treatments except the fresh prepared 1×10^7 spores
266 g^{-1} soil control (Fig. 3). However, pathogenicity bioassays indicated that less than 5×10^3 living
267 spores g^{-1} soil were present in this sample. The difference between the qPCR result and the
268 pathogenicity bioassay result of this sample was greater than those of the samples on the soil
269 surface, or at 20°C or 30°C. This could be explained by the assumption that most spores in
270 samples incubated at -20°C were dead, but -20°C provided an environment that better
271 maintained the integrity of DNA released from the dead cells. To test the contribution of dead
272 spores to the DNA content in the soil sample, an autoclave experiment was conducted in which
273 soil samples containing 1×10^7 spores g^{-1} soil were autoclaved and then analyzed by qPCR.
274 After autoclaved for 0.5 or 1 hour, *P. brassicae* DNA could still be detected (Fig. 5) with mean
275 Cq values at 31.28 ± 0.55 and 32.84 ± 0.60 , respectively. After autoclaved for more than 2 hours,
276 the mean Cq values of the samples reached to 36 and some technical repeats failed to generate a
277 Cq value. This result indicated that autoclaving for 2 hours is required to destroy all *P. brassicae*
278 DNA released from dead spores. The 2-hour autoclaving may not be necessary for preparing
279 negative control material for pathogenicity bioassays because the widely used 1-hour autoclave
280 is sufficient to kill all the resting spores. However, in experiments with PCR or qPCR being used
281 to quantify spore numbers based on DNA, autoclaving the sample for more than 2 hours is
282 recommended.

283

284 **Ultraviolet light treatment**

285 The pathogenicity difference between the sample buried in soil and that kept on the soil surface
286 indicated that radiant sunlight may be able to kill resting spores. UV light, as a component of
287 sunlight, is known to be harmful to life forms due to its ability to damage DNA [29]. To test the

288 effect of UV light on the survival of the resting spores, soil samples containing 1×10^6 spores g⁻¹
289 soil were treated with UV light and subjected to qPCR analyses and pathogenicity bioassay.
290 After treatment with UV light for one or two hours, a decrease in the quantity of DNA was
291 observed (Fig. 6A). When the treatment time was extended to 3 hours, only 50% ($10^{5.66-5.94}$) of
292 DNA, compared to the non-treated control, could be identified from the soil sample, which
293 indicated that more than half of the resting spores were killed by the 3-h UV light treatment. This
294 result was supported by the pathogenicity bioassays (Fig. 6B), in which 2- and 3-hour UV light
295 treated samples showed significantly reduced pathogenicity compared to the non-treated control.

296

297 **Discussion**

298 **Assessment of *Plasmodiophora brassicae* with quantitative PCR**

299 In this study we used primer pair CrqF2/CrqR2 and probe PB1 for qPCR assessment of *P.*
300 *brassicae* spores. The probe has been used with the primer pair DC1F/DC1mR [27,30], which
301 was derived from Rennie et al. [31] but with the reverse primer modified. We redesigned the
302 primers to avoid potential primer dimers as predicted by the Multiple Primer Analyzer
303 (<https://www.thermofisher.com>). Compared to other published primers, this primer pair showed
304 similar specificity and sensitivity to *P. brassicae* when used in SYBR green based qPCR (Feng,
305 unpublished data). Since the sequence of the probe PB1 doesn't create more specificity, we
306 believed that this primer pair would be as efficient as others when used in probe-based qPCR.

307

308 The efficiency of the primers used for the template DNA in this study was 112%. This could be
309 attributed to the PCR inhibitors present in the DNA template solution. For example, if the
310 standard curve was created from a dilution series of one genomic DNA preparation with

311 inhibitors (generally chemicals used for DNA extraction), primer efficiency larger than 100%
312 would be expected, due to the fact that the inhibitors in the original template solution were
313 diluted along with DNA dilution. However, in this study, the standard curve was created by
314 serial DNA preparations from soil samples inoculated with spore dilutions. Thus, we concluded
315 that at least some of the PCR inhibitors were derived from *P. brassicae* spores. The more spores
316 from which the DNA derived, the more inhibitors in the DNA solution.

317

318 It has not escaped our notice that the standard deviations of qPCR data from each treatment in
319 the UV light experiment (Fig. 6A) were smaller than those in the 2-year-storage experiments
320 (Fig. 3). We inferred that in the 2-year-storage experiments, stochastic factors had more time to
321 interact with the spore samples. Therefore, more variability was created within the different
322 tubes, although these tubes were kept in the same bottle.

323

324 In this study, we provided the evidence that using qPCR data to enumerate or estimate living
325 spores can, in some cases, dramatically overestimate the numbers of viable resting spores and
326 disease potential. Few studies have been conducted to evaluate differences between using qPCR
327 results and pathogenicity bioassay results to assess *P. brassicae* resting spore viability. PMA-
328 PCR has been used for the assessment of *P. brassicae* living spores [32,33], however while this
329 method has the potential to improve the measurement of viable spores via qPCR, the usefulness
330 of PMA-PCR on quantification of absolute spore numbers and the standardization of the
331 procedure, especially the step of photoactivation, still need to be improved. Additionally, the
332 effects of photoactivation on living spores may have deleterious consequences as shown in this
333 study with UV light experiments. Unfortunately, the results of the experiments reported herein

334 suggest that the best way to measure spore viability and disease potential is pathogenicity
335 bioassays, with vital staining of spores being a next best option [33].

336

337 **Survival of *Plasmodiophora brassicae* spores in different temperatures**

338 Among the four controlled temperatures, 4°C and 20°C were better than -20°C and 30°C on
339 maintaining spore viability (Fig. 4A). These results indicated that compared to 4°C and 20°C,
340 continuous freezing, or high temperature (>20°C) are more harmful to the spores. It has been
341 suggested that spores might be stored as dense suspensions at 3-4°C for 3 years without loss of
342 viability [34], which was supported by the results of the soil sample kept at 4°C. In contrast, it
343 has been a standard practice to store galls at -20°C for several years as stock inoculum [35],
344 which was not supported by the results of the soil sample kept at -20°C. Furthermore, in this
345 study, the spores buried in soil, which would have been continuously frozen during the two
346 winter seasons, still maintained full viability. We attributed the disagreement between our results
347 and those previously reported to the different water content in the samples. All our soil samples
348 contained 5% water before being stored in different conditions. Samples in the -20°C treatment
349 were frozen as soon as the treatment was started. In contrast, samples in the field treatment were
350 not frozen for several months until the winter came, which likely allowed the soil samples to lose
351 most of their water content. The tubes were sealed in bottles, but not with an air-tight seal.
352 Frozen as plant galls or in dry samples may minimize the occurrence of cell crystallization thus
353 more cells will survive. The samples buried in the soil might also experience high temperatures,
354 but it should have been only intermittently with short time periods in the two Edmonton summer
355 seasons.

356

357 **Survival of *Plasmodiophora brassicae* spores in field conditions**

358 In many plant pathogens, a compensation for being soil-borne is to produce larger numbers of
359 thick-walled, long-lived spores. The resting spores of *P. brassicae* have evolved to retain
360 viability in the soil environment despite exposure to many seasons of adverse weather, and
361 temperature/moisture extremes. Field studies indicated that *P. brassicae* resting spores have a
362 half-life of approximately 3.6 years and viable spores might exist for 20 years in the absence
363 of suitable hosts before spore populations were eroded to undetectable levels [19]. More recently
364 some experiments have suggested that the decay may not be linear, but rather a rapid decline in
365 viability occurs over the first year or two without a host, followed by a much slower decline over
366 the next 10 to 20 years [12,20]. In this study, we found that most spores can maintain full
367 viability and pathogenicity after being buried in the soil for two years. In contrast, most spores at
368 the soil surface totally lost their viability and pathogenicity after two years. However, since
369 spores were kept in 1.5-mL tubes and the tubes were kept in bottles, results from either treatment
370 cannot be used to predict the fate of spores in real field conditions. Nevertheless, the differences
371 between the two treatments provided evidence that spores buried in the soil had more chances to
372 survive than those on soil surface. According to Kim et al. [36], more than 97% of the total of *P.*
373 *brassicae* inoculum was present in the surface soil (0-5 cm depth) and few resting spores were
374 found below 40 cm. Cranmer et al. [37] indicated that genomic DNA of *P. brassicae* equivalent
375 to 10^5 spores g⁻¹ soil of resting spores could be detected below the plow layer. Thus, in field
376 conditions, pathogenic populations are influenced by the spore concentrations on the soil surface
377 and in the soil, with potentially drastic differences in their viability decline curves for the two
378 locations. In this study, spores buried in the soil remained pathogenic for more than 2 years but
379 those on the soil surface did not. Furthermore, the low viability of spores on soil surface

380 suggested that tillage between rotated crops might actually improve clubroot management by
381 exposing more spores to the damaging radiant sunlight present at the soil surface.

382

383 **Effect of ultraviolet light on spore viability**

384 It has been known for many years that UV light has various effects on fungi and other
385 microorganisms [38]. When DNA is exposed to UV radiation adjacent thymine bases will be
386 induced to form cyclobutane pyrimidine dimers by the condensation of two ethylene groups at C-
387 5 and C-6. Additionally, adjacent thymines can be linked between the C-4 residue and the C-6 of
388 its neighbor. In either case, a “kink” is introduced into the DNA. Therefore, by exposing
389 microorganisms to UV radiation, their DNA will be photo-damaged and will not be amplified by
390 DNA polymerase [39]. As another consequence, the DNA can't replicate and thus the cells die.

391

392 *Plasmodiophora brassicae* has developed the ability to form resting spores with mechanisms to
393 resist adverse thermal and aqueous conditions. Among many other factors, melanin may play a
394 role in resting spores for UV light resistance. Melanin is a broad term for a group of natural
395 pigments and localized in cell walls in most organisms [40,41]. In *P. brassicae*, the L-
396 Dopachrome biosynthesis pathway, which is involved in melanin synthesis, has been predicted
397 by a bioinformatic study [42]. However, in natural conditions, especially outside agriculture,
398 most resting spores of *P. brassicae* would be buried in the soil for their entire life. Thus, it is
399 likely that *P. brassicae* did not evolve a strongly expressed UV light resistance during evolution.
400 This was supported by the current study, in which the *P. brassicae* resisting spores were found to
401 be sensitive to UV light treatment.

402

403 To prevent the spread of clubroot, sanitation of field equipment is important. Currently bleach is
404 recommended as one of the most effective chemicals for inactivation of clubroot resting spores
405 (<https://www.canolawatch.org/2018/06/27/clubroot-disinfectants-bleach-is-best/>). However,
406 bleach is corrosive to metals, causes rubber to harden, stains and damages clothing and footwear.
407 Considering the effect of UV light on the viability of resting spores as demonstrated in the
408 current study, UV light treatment may be an alternative for equipment sanitation. Compared to
409 other treatments, UV light treatment has several advantages. Firstly the cost will be low because
410 no consumable materials are required; secondly, it will be easy to perform because no attention
411 or action is required during the treatment process; and finally, it is environment-friendly. On the
412 other hand, shortcomings include that UV light does not readily penetrate surfaces but functions
413 on direct surfaces only. Furthermore, it may require long exposure times for good efficacy. Thus,
414 we would leave this topic open and expect future applied or adaptive studies to take this further.

415

416 The observed damage by UV light also challenges the notion that the spread of *P. brassicae* can
417 be due to resting spores moving with wind-blown dust and soil erosion [43,44]. The results
418 presented herein on the damaging effects of exposure to sunlight or UV would suggest that most
419 spores moving with wind-blown soil occur at the soil surface, and likely inactivated after
420 prolonged exposures to sunlight.

421

422 **Inoculating canola with *Plasmodiophora brassicae*-infected soil**

423 As with other biotrophic plant pathogens, plant inoculation with *P. brassicae* can be challenging.
424 In diagnosis service activities and studies on the pathogenicity of multiple strains (e.g.
425 pathotyping, screening for resistant germplasms), inoculum sources are generally limited by the

426 amount of *P. brassicae*-containing soil or root galls. Using small amounts of inoculum allows for
427 more experimental replications to be set up, but often decreases the efficiency of infection. In
428 this study, we developed and optimized a simple procedure for canola seedling inoculation (Fig.
429 1B and C; termed tube-method), which is extremely useful when the inoculum source is limited.
430 Compared to the soil inoculation method [45], the tube-method uses much less inoculum;
431 compared to the sealing-dipping method [46], the tube-method has higher levels of consistency
432 among repeats and efficiency of infection. We recommend this inoculation method, regardless of
433 whether the original inoculum is soil or root gall, in *P. brassicae* pathogenicity assays conducted
434 in either labs or greenhouses.

435

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562

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577

578 **Figure Legends**

579 **Fig 1.** The tube-method for *Plasmodiophora brassicae* resting spore treatments and
580 pathogenicity assay. Tubes each containing 1 g soil inoculated with 1×10^7 resting spores in
581 glass bottle ready for environmental treatment (A) and methods for pathogenicity testing of the
582 treated soil samples (B and C). B, Canola seedlings inoculated with soil samples containing
583 resting spores. C, the 0-to-3 clubroot rating scale.

584

585 **Fig 2.** The standard curve generated from the mean of quantification cycle (Cq) values against
586 log10 of spore concentrations in soil samples containing tenfold serial dilutions of
587 *Plasmodiophora brassicae* resting spores. The regression equation, the R² score of the equation
588 and the efficiency of the primers (E), are indicated on the top of the curve. Each data point is
589 shown as mean \pm standard deviation (n=3).

590

591 **Fig 3.** qPCR analyses of soil samples containing 1×10^7 *Plasmodiophora brassicae* resting
592 spores g⁻¹ soil that have been stored in different conditions for two years. Using the mean Cq
593 value of each sample obtained from qPCR and the regression equation in Fig. 2, the value of Y
594 was calculated for each treatment and shown as the equivalent to log concentration. Means in the
595 plot topped by the same letter do not differ based on Fisher's LSD test at P ≤ 0.05 (n = 3).

596

597 **Fig 4.** Pathogenicity bioassays of soil samples inoculated with *Plasmodiophora brassicae* resting
598 spores. Means in the plot topped by the same letter do not differ based on Fisher's LSD test at P
599 ≤ 0.05 (n = 5). A, Two experiments on soil samples containing 1×10^7 spores g⁻¹ soil spores that
600 had been stored in different conditions for two years. Negative CK, an uninoculated soil sample
601 that has been stored at -20°C for two years; Positive CK, a fresh prepared soil sample containing

602 1×10^7 spores g⁻¹ soil spores. B, one experiment on fresh prepared soil samples containing a 5-
603 fold dilution series (1×10^7 to 1×10^3 spores g⁻¹ soil) of spores.

604

605 **Fig 5.** qPCR analysis of soil samples containing 1×10^7 *Plasmodiophora brassicae* resting
606 spores g⁻¹ soil that have been autoclaved for 0, 0.5, 1, 2 or 3 hours. Using the mean Cq value of
607 each sample obtained from qPCR and the regression equation in Fig. 2, the value of Y was
608 calculated for each treatment and shown as the equivalent to log concentration. Means in the plot
609 topped by the same letter do not differ based on Fisher's LSD test at P ≤ 0.05 (n = 3).

610

611 **Fig 6.** qPCR analysis (A) and pathogenicity assay (B) of soil samples containing 1×10^6
612 *Plasmodiophora brassicae* resting spores g⁻¹ soil that have been treated by UV light for 0, 1, 2 or
613 3 hours. Means in the plot topped by the same letter do not differ based on Fisher's LSD test at P
614 ≤ 0.05 (n = 3 for qPCR; n = 5 for pathogenicity assay). In the qPCR analysis, using the mean Cq
615 value of each sample obtained from qPCR and the regression equation in Fig. 2, the value of Y
616 was calculated for each treatment and shown as the equivalent to log concentration.

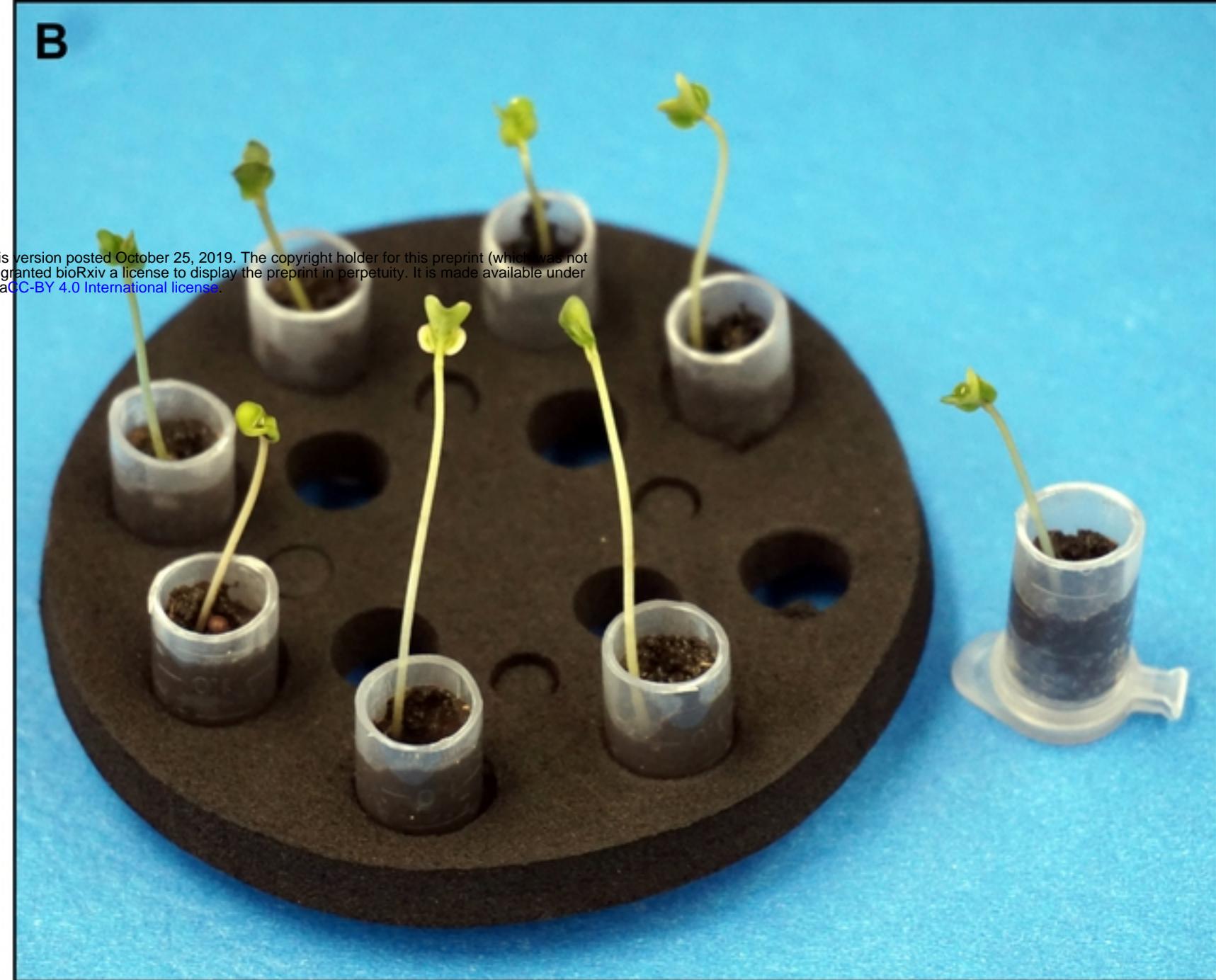
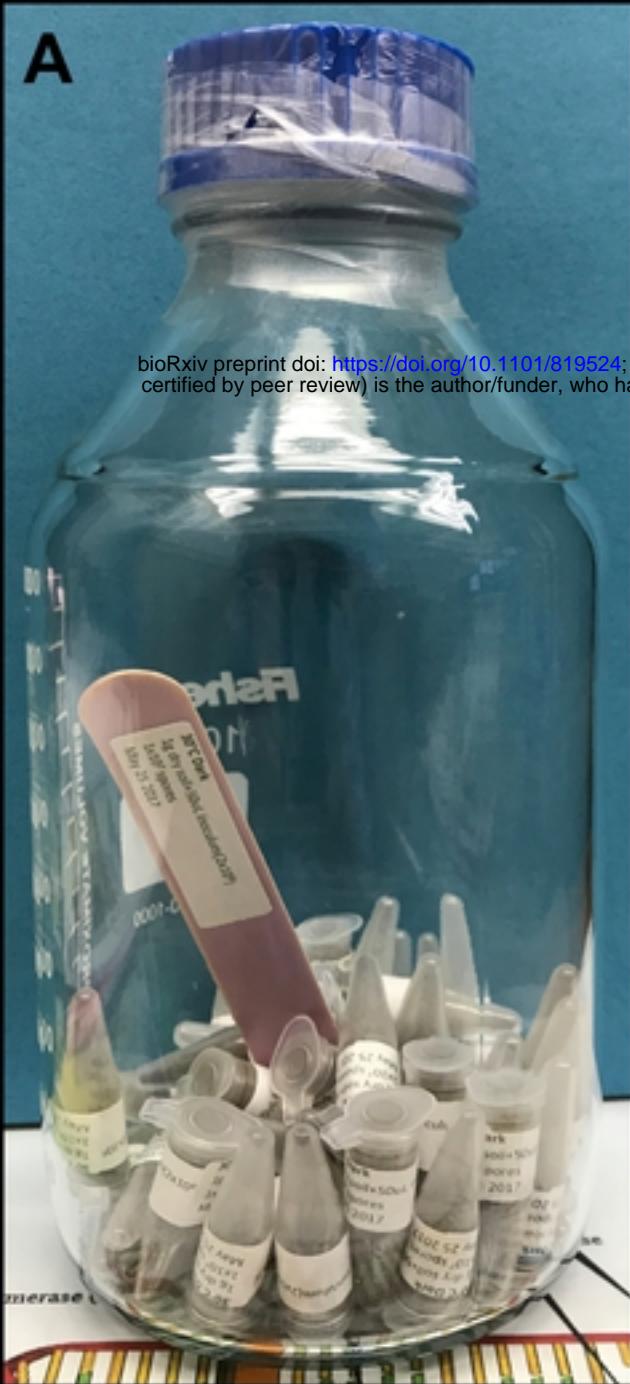


Figure 1

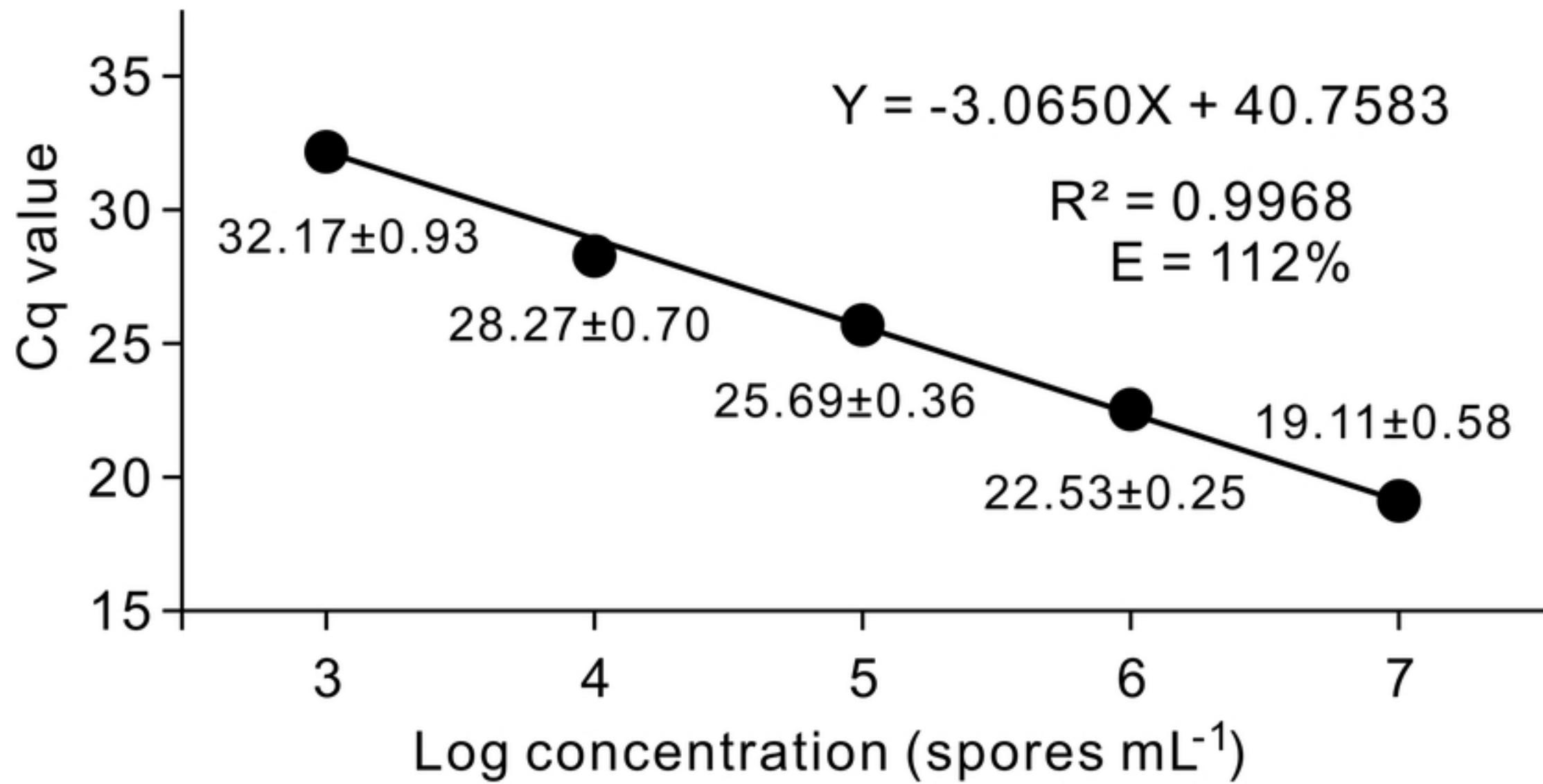


Figure2

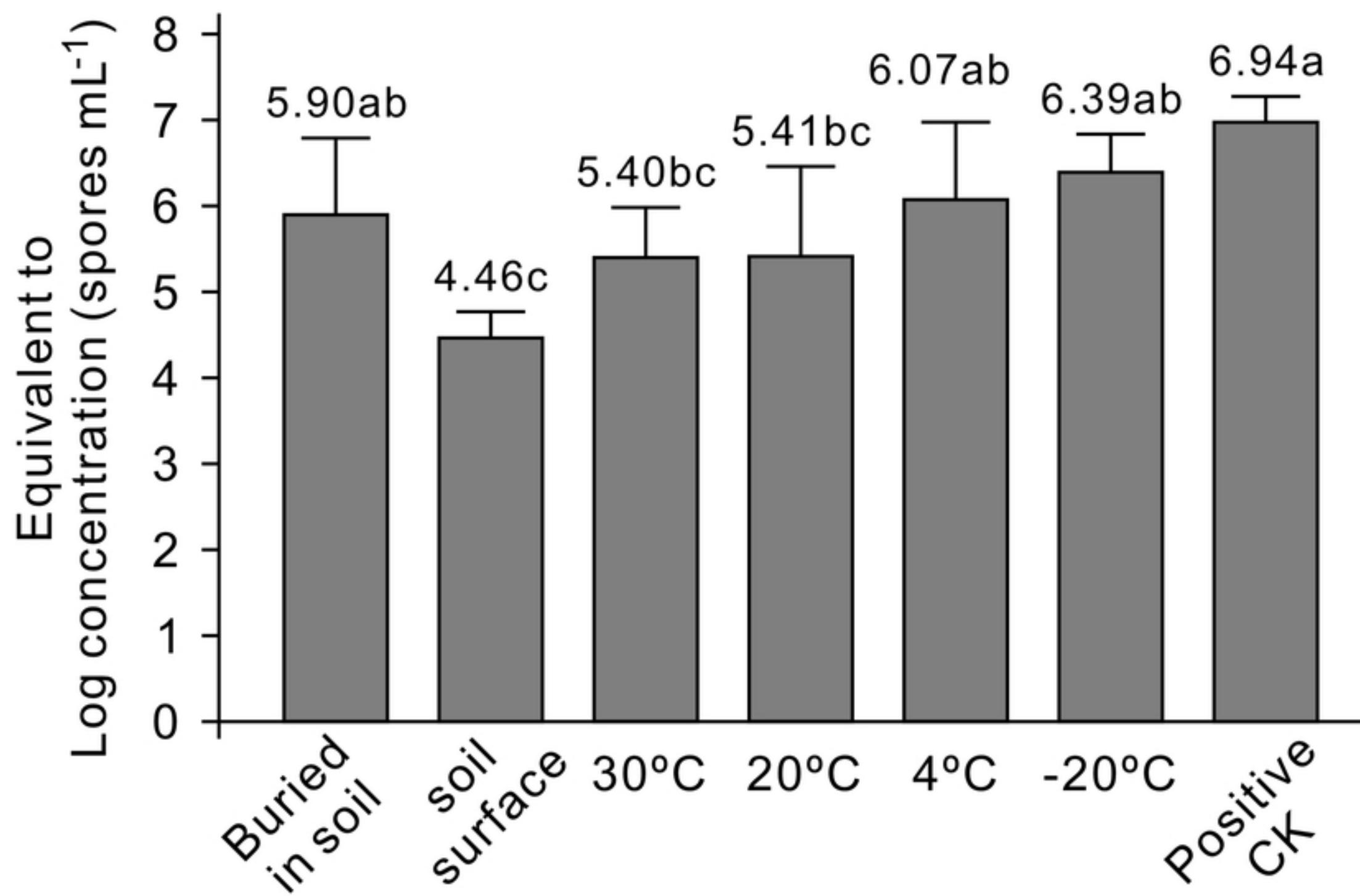
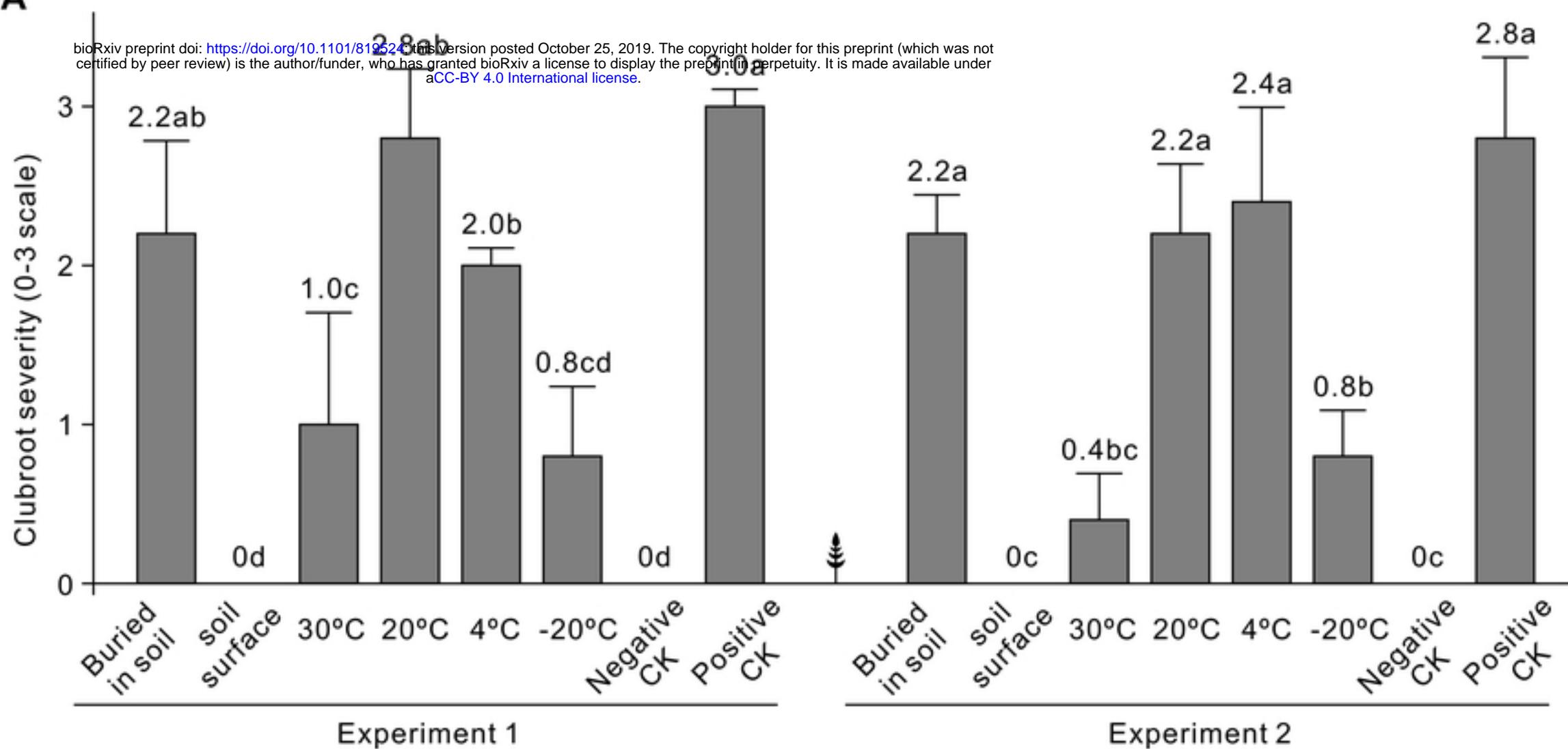
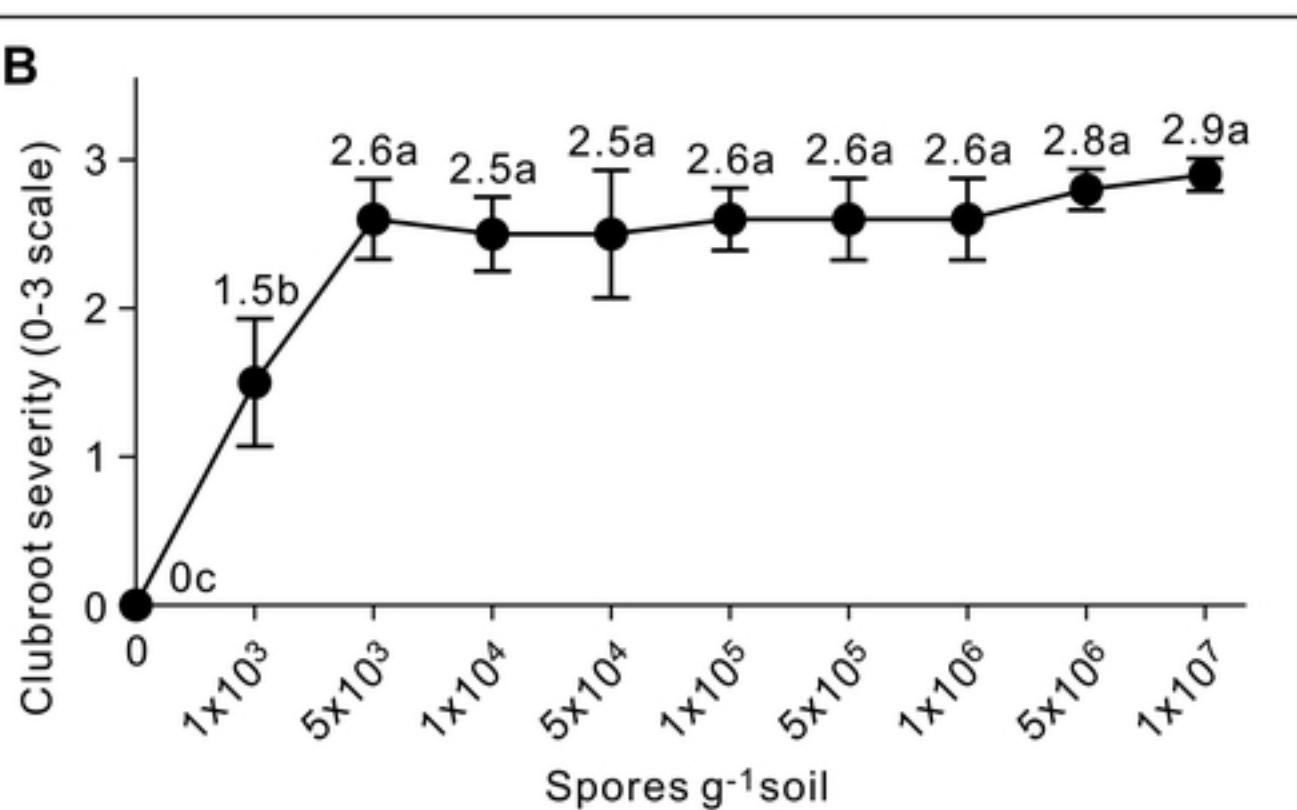


Figure3

A

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**B****Figure 4**

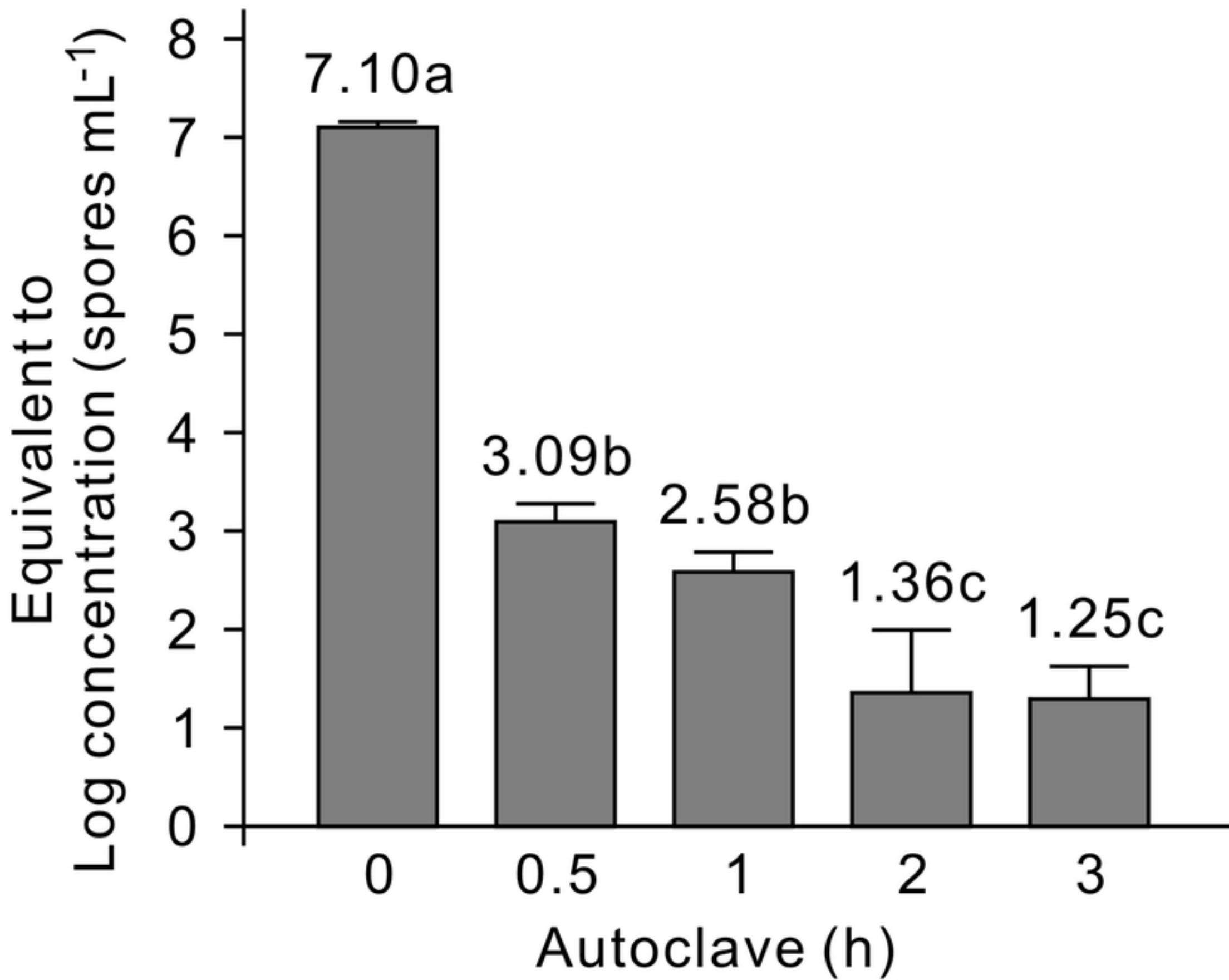


Figure5

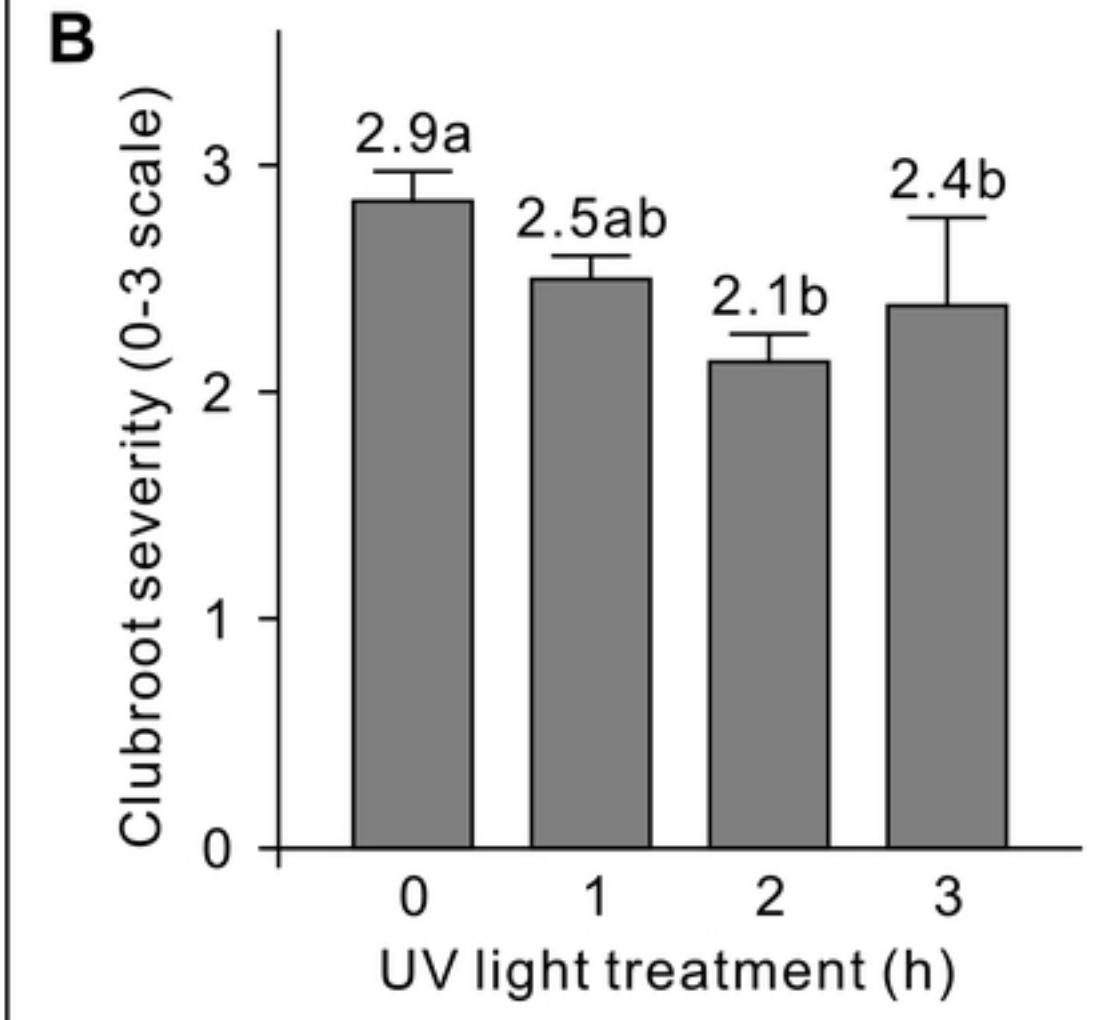
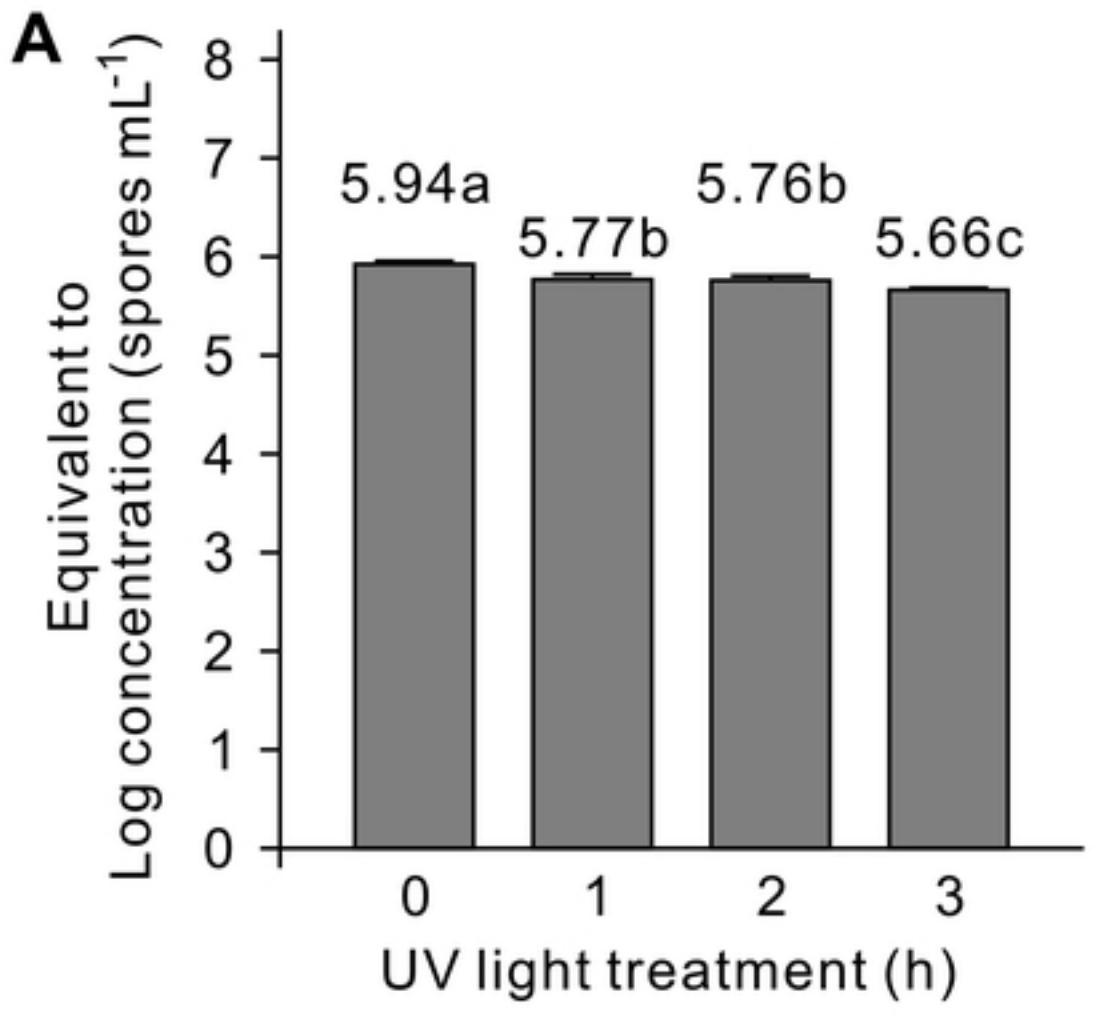


Figure 6