

# 1 Effective seed sterilization methods require optimization 2 across maize genotypes

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22 interactions, root-associated microbiota, seed microbiome, microbiome, microbiota

23

## 24 **Abstract**

25 Studies of plant-microbe interactions using synthetic microbial communities (SynComs) often require the removal  
26 of seed-associated microbes by seed sterilization prior to inoculation to provide gnotobiotic growth conditions. A  
27 diversity of seed sterilization protocols have been developed in the past and have been used on different plant  
28 species with various amounts of validation. From these studies it has become clear that each plant species requires  
29 its own optimized sterilization protocol. It has, however, so far not been tested if the same protocol works equally  
30 well for different varieties and seed sources of one plant species. We evaluated six seed sterilization protocols on  
31 two different varieties (Sugar Bun & B73) of maize. All unsterilized maize seeds showed fungal growth upon  
32 germination on filter paper, highlighting the need for a sterilization protocol. A short sterilization protocol with  
33 hypochlorite and ethanol was sufficient to prevent fungal growth on Sugar Bun germinants, however a longer  
34 protocol with heat treatment and germination in fungicide was needed to obtain clean B73 germinants. This  
35 difference may have arisen from the effect of either genotype or seed source. We then tested the protocol that  
36 performed best for B73 on three additional maize genotypes from four sources. Seed germination rates and fungal  
37 contamination levels varied widely by genotype and geographic source of seeds. Our study shows that consideration  
38 of both variety and seed source is important when optimizing sterilization protocols and highlights the importance of  
39 including seed source information in plant-microbe interaction studies that use sterilized seeds.

## 40 Introduction

41 Plant-associated microorganisms are critical for plant development, evolution, and resistance to biotic and  
42 abiotic stresses (Cordovez et al., 2019; Philippot et al., 2013; Turner et al., 2013). Past studies on plant-microbe  
43 interactions have focused on studies in natural soils, which provide realistic growth conditions for the plant, but at  
44 the same time make it difficult to understand specific interactions between plant and microbes due to the high  
45 diversity of microbial communities and the presence of many biotic and abiotic factors that can confound results. In  
46 recent years there has been growing interest in studying plant-microbe interactions using plants inoculated with  
47 fully-defined synthetic microbial communities (SynComs) grown under controlled gnotobiotic conditions. These  
48 SynCom-based studies have led to major discoveries on the dynamics of plant-microbe and microbe-microbe  
49 interactions, plant colonization and community assembly processes, and the molecular mechanism leading to plant  
50 phenotype changes induced by specific microbes (Vorholt et al., 2017, Carlstrom et al., 2019; Finkel et al., 2020;  
51 Wagner et al., 2021; Wippel et al., 2021). Ultimately, the hope is that development of plant specific SynCom  
52 inoculants can improve yields and crop protection in sustainable agriculture (Shayanthan et al., 2022).

53 SynCom experiments often rely on sterile or gnotobiotic seeds to be able to relate the effects of specific  
54 microorganisms or groups of microorganisms to plant and microbial community responses. Seeds usually carry  
55 diverse microorganisms both on their surface as epiphytes (Torres-Cortes, 2018), and inside the seed as endophytes  
56 (Samreen et al., 2021). For gnotobiotic SynCom studies, this natural seed microbiome needs to be removed.  
57 Furthermore, removal of seed endophytes is likely necessary for experimental investigation of their importance for  
58 plant-microbiome interactions; for example, testing the hypothesis that seed endophytes have outsized effects on  
59 seedlings (Newcombe et al. 2023, 2018).

60 Many seed sterilization protocols have been used to generate microbe-free seeds with varying success.  
61 These include chlorine gas (Leukel and Nelson, 2940), plasma treatment (Khamsen et al., 2016), gamma irradiation  
62 (Cuero et al., 1986), heat treatment (Watts et al., 1993; Bishop et al., 1997; Regalado González, et a., 2020)  
63 microwaving (Seaman and Wallen 1967), and sodium hypochlorite/bleach (Davoudpour et al., 2020; Lindsey et al.,  
64 2017). To help reduce seed endophytes, Watts et al (1993) proposed a pre-soak treatment prior to heat treatment.  
65 Some methods for eliminating seed contamination can negatively affect seed germination (Davoudpour et al., 2020).  
66 No consensus has emerged yet for the best sterilization protocol that balances eliminating microbes while  
67 maximizing germination. In a comprehensive analysis of seed microbiota from 50 plant species, Simonin et al.  
68 (2022) found high variation in seed community composition from the same species, possibly suggesting a variety or  
69 location effect. Most sterilization protocols are optimized for a single variety of the respective plant species and it is  
70 currently unclear if protocols for sterilization need to be adapted for different plant lineages and/or sources of seeds.

71 One important question when evaluating seed sterilization approaches is, how to assess the sterility of the  
72 resulting seeds. In theory, sterility implies the complete absence of viable microbes, however, currently no feasible  
73 approach exists to determine complete absence of viable microbes in sterilized seeds. Past studies have assessed  
74 sterility of seeds by directly placing seeds in or on a growth medium (Seamen and Wallan, 1967; Dawar et al. 2007;  
75 Cuero et al. 1986; Davoudpour et al. 2020; Scott et al. 2018; Watts et al. 1993; Lindsey et al. 2017), or by spreading  
76 the last wash solution on nutrient agar (Pal et al. 2022). Few studies (see Davoudpour et al., 2020) have gone beyond

77 traditional plate cultivation to determine the presence of viable microbes following sterilization using fluorescence  
78 microscopy. While some approaches do not depend on cultivation (e.g., Bergmann et al., 2022), most do. Because  
79 many bacteria and fungi are difficult or impossible to culture, their elimination is therefore challenging to test.

80 Here we evaluated and compared seed sterilization protocols for maize (*Zea mays* L.), which is one of the  
81 most important cereal and bioenergy crops (Wallace et al., 2014; Nuss and Tanumiharjko, 2010; Edgerton 2009;  
82 Farrell et al., 2006, Pacala and Socolow, 2004), and for which extensive genetic and molecular tools to study plant-  
83 microbe interactions are available, including a well-established SynCom (Niu et al., 2017; Salvato et al., 2022;  
84 Krumbach et al., 2021). We compared six sterilization protocols which combine variations of sodium  
85 hypochlorite/ethanol surface sterilization, prolonged heating of seeds to eliminate endophytes, pre-soaking to  
86 eliminate endophytes, and germination with fungicide. Since, based on prior studies, we knew that fungal  
87 overgrowth is one of the main indicators of contamination of maize seeds, we used fungal outgrowth after  
88 germination as the measure to assess sterility. We determined the impact of each protocol on fungal outgrowth and  
89 seed germination rates for two different varieties of maize: a commercial hybrid sweet corn (“Sugar Bun”) and the  
90 inbred maize variety B73. We then explore the impact of the most effective sterilization protocol for B73 on fungal  
91 outgrowth and germination rates in other inbred and hybrid varieties and seeds sourced from different geographical  
92 locations.

## 93 **Materials and Methods:**

### 94 **Experiment 1: Testing six sterilization protocols on two maize varieties**

95 For the first experiment in this study, we tested six sterilization protocols on two varieties of maize seeds: a  
96 commercial hybrid sweet corn variety (Sugar Bun 267T lot 68707, Johnny’s Selected Seeds, Waterville, Maine,  
97 USA) and an inbred field corn line (B73; produced by hand-pollination in a field at Central crops research station in  
98 Clayton NC, 2021). Before sterilization, seeds were visually checked and discarded if cracks or damage were visible  
99 (Gu et al., 2019). Each sterilization treatment was applied to 72 seeds of each variety.

100 We tested two different protocols (short and long) and two modifications of each of the two protocols (pre-  
101 soaking and germination with fungicide) (Fig. 1B). For the short sterilization protocol, quality-checked maize seeds  
102 were placed in a 50 ml conical tube (15-20 seeds/tube) and the tubes were filled to 45 ml with 70% ethanol and  
103 shaken by inversion for 3 min. Ethanol was discarded and each tube was filled with a 2% sodium hypochlorite  
104 solution and shaken by inversion for 3 min. The sodium hypochlorite solution was discarded and seeds were rinsed 5  
105 times with sterile deionized water.

106 For the long sterilization protocol, quality-checked maize seeds were placed in aluminum foil overnight  
107 (>12h) in an incubator at 50 °C. To avoid crowding and provide even dispersal of heat, several foil packages were  
108 spread evenly in the incubator, with each package containing 10-15 seeds. After the heat treatment, 15-20 seeds  
109 were placed in a 50 ml conical tube and the seeds were immersed in 95% ethanol (~45 ml) and shaken by inversion  
110 for 1 min. Ethanol was discarded and each tube was filled with 5% sodium hypochlorite + 0.01% Tween20 solution  
111 and placed in a shaker at 200 rpm for 20 min. The sodium hypochlorite + Tween20 solution was discarded and each

112 tube was filled with 70% ethanol and shaken by inversion for 3 min. Ethanol was discarded and seeds were rinsed 3  
113 times with sterile deionized water. After the third rinse, seeds were immersed in sterile deionized water for 1 h.

114 In addition, we examined the impact of two modifications for each of the protocols above. The first was to  
115 determine whether pre-soaking the seeds lowers the incidence of fungal endophytes as suggested previously (Watts  
116 et al., 1993). We evaluated the impact of pre-soaking by soaking the seeds for 3 min in sterilized deionized water  
117 prior to both the short (“short-pre-soak”) and the long (“long-pre-soak”) sterilization protocols.

118 Working next to a flame, we placed individual treated seeds into the wells of sterile 24-well polystyrene  
119 tissue culture plates (FabLab cat. No. FL7123). Wells were lined with sterile germination paper (Anchor Paper Co.,  
120 USA). Three 24-well replicate plates were prepared per treatment (total of 72 seeds for each treatment) with 200  $\mu$ l  
121 of sterile 0.5 X Murashige and Skoog basal salts liquid medium (Caisson Labs, Smithfield, UT, USA) per well. For  
122 the second modification, we included one treatment with the addition of a fungicide (Chlorothalonil; 1% Daconil in  
123 200  $\mu$ l of 0.5 X Murashige and Skoog) following the short (“short+”) and long (“long+”) sterilization protocols. The  
124 tissue culture plates were covered with lids, randomized, and incubated in a gravity convection incubator at 30 °C in  
125 the dark. Germination and fungal growth was recorded for each well at 7 days.

126 Germination was recorded as the appearance of the radicle emerging from the maize seed. Fungal  
127 contamination was recorded as any visible fungal growth. To determine the impact of contamination on germination,  
128 we recorded the proportion of seeds that did or did not germinate and were contaminated and not contaminated (Fig.  
129 1A).

130 We counted each plate of 24 seeds as one experimental replicate. The six treatments for the experiment are  
131 detailed in Figure 1B. We replicated each of the six treatments three times for each of the two maize varieties (Sugar  
132 Bun and B73) and converted the raw counts of seeds germinated or contaminated to a percentage for each plate. We  
133 then normalized percentages by transforming them using a central log ratio transform (Greenacre, 2021). We tested  
134 the distribution within each treatment for normality using the Shapiro-Wilk test and for homogeneity of variances  
135 using Levene’s Test. We performed a one-way ANOVA on the normalized data to determine the relationship  
136 between the treatments and the number of uncontaminated-germinated seeds.

137

### 138 **Experiment 2: Testing one of the best performing protocols on four genotypes from four different sources**

139 For the second experiment, we addressed how the best protocol for B73 in experiment 1 performed across  
140 common maize inbred and hybrid seeds sourced from different locations and years. Specifically, we applied the  
141 long sterilization protocol with germination in fungicide (long+) to B73, Mo17, and their two hybrids, B73 x Mo17,  
142 and Mo17 x B73. These two hybrids are from reciprocal crosses between identical lines- in each case the female  
143 parent is listed first. For these four genotypes we tested seed sourced from (i.e. produced by hand-pollination in)  
144 Central crops Research Station in Clayton, NC (CL) in 2021 and 2022 (CL21 and CL22), San Juan de Abajo,  
145 Nayarit, Mexico (MX; 2021-2022), and a greenhouse in North Carolina, USA (GH; 2021-2022). For the  
146 greenhouse we only had B73 and B73 x Mo17 seeds.

147 For the comparison of seed sources, we sterilized 24 seeds of each variety and from each source and  
148 recorded germination and contamination as described above. We used a comparison of independent proportions

149 using a mid-P approach to Fisher's exact test (StatsDirect, 2013) to determine differences in germination and  
150 contamination.

## 151 **Results**

### 152 **Experiment 1: Best and worst performing protocols differ between two seed varieties**

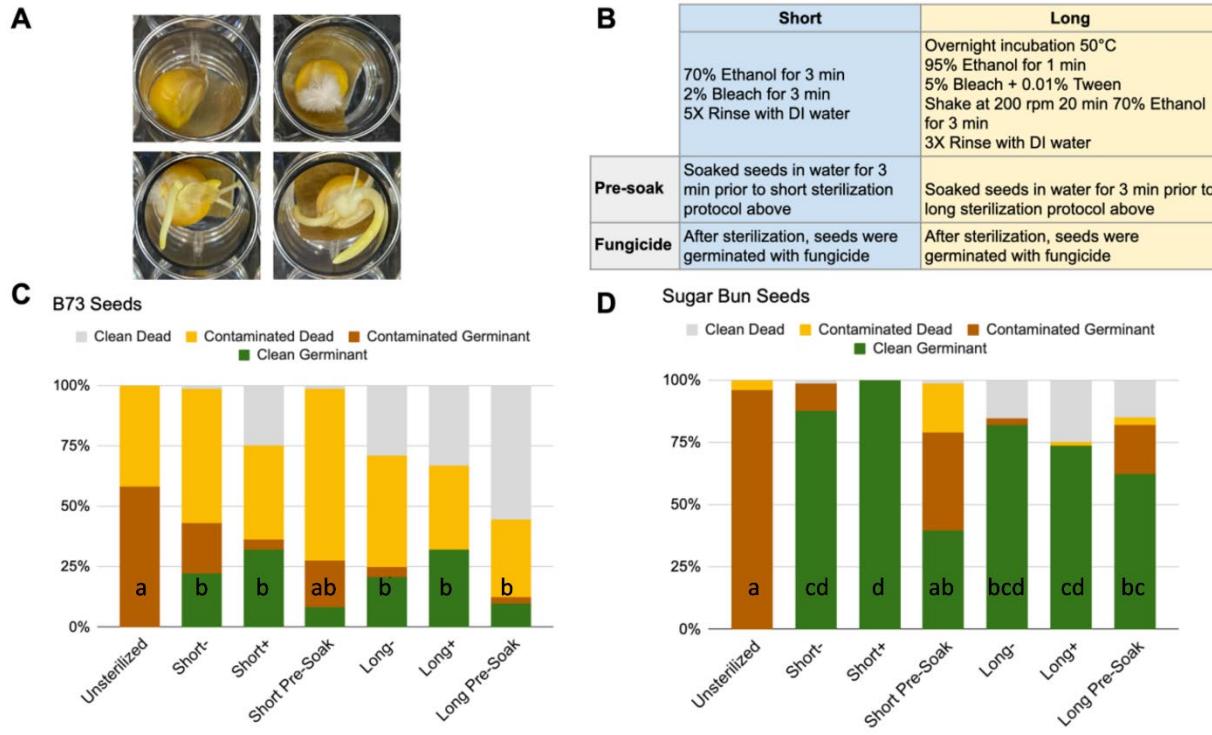
153 There was a significant effect of treatment on the number of clean germinants in both B73 ( $F(7, 16) = 5.45$ ,  
154  $p = 0.002$ ) and Sugar Bun ( $F(7, 16) = 5.45, p = 0.002$ ). All of the untreated (non-sterilized) seeds for both varieties  
155 showed fungal growth (Fig 1C & 1D). All treatments, except the short pre-soak, significantly increased the number  
156 of clean germinants compared to the untreated group for both seed varieties. While none of the sterilization  
157 protocols completely eliminated fungal contamination, the B73 seeds treated with the long sterilization with  
158 fungicide (long+) treatment and the Sugar Bun seeds treated with the short sterilization with fungicide (short+)  
159 treatment yielded only clean germinants (Fig.1).

160 Relative to the untreated seeds, the short sterilization protocol significantly increased the number of clean  
161 germinants for both B73 (from 0% to 22.2% clean germinants; Tukey HSD adjusted p-value <0.05) and Sugar Bun  
162 (from 0% to 87.5% clean germinants; Tukey HSD adjusted p-value <0.01). In both genotypes, the short sterilization  
163 protocol significantly reduced the contamination rate providing clean germinants.

164 Similar to the short sterilization protocol, the long sterilization protocol significantly increased the number  
165 of clean germinants for both B73 (from 0% to 20.8% clean germinants; Tukey HSD adjusted p-value <0.05) and  
166 Sugar Bun (from 0% to 73.6% clean germinants; Tukey HSD adjusted p-value <0.01). In both genotypes, the long  
167 sterilization protocol significantly reduced the contamination rate providing clean germinants.

168 The addition of fungicide during germination consistently increased the number of clean germinants for  
169 B73 in both the short (22.2% short sterilization to 31.9% short sterilization with fungicide) and long (20.8% long  
170 sterilization to 31.9% long sterilization with fungicide) treatments. However, these increases were not significant.  
171 For Sugar Bun seeds, fungicide addition following the short sterilization protocol increased the number of clean  
172 germinants from 87.5% to 100% (not significant). Fungicide addition following the long sterilization protocol  
173 decreased the number of clean germinants from 81.9% to 73.6% (not significant).

174 Pre-soaking the seeds in water prior to the short sterilization protocol did not significantly increase the  
175 number of clean germinants compared with untreated seeds in B73 seeds (8.3% clean germinants) or Sugar Bun  
176 seeds (25% clean germinants) and showed no improvement over the short or the long sterilization protocol alone.  
177



178

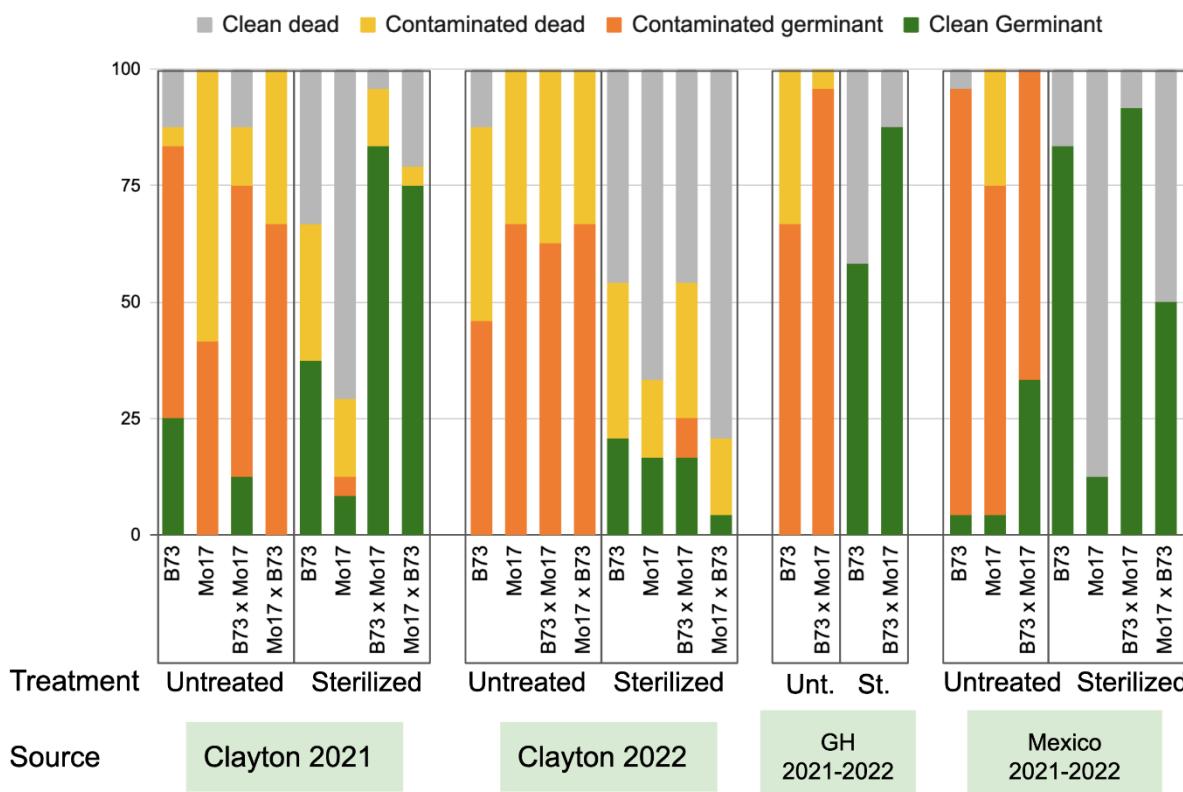
179 **Figure 1. Six seed sterilization protocols tested on two varieties of maize.** Examples of clean dead (top left),  
180 contaminated dead (top right), contaminated germinant (bottom left), and clean germinant (bottom right) B73 seeds  
181 (A). Overview of the six sterilization protocols used (B). Percentage of seeds that germinated and/or were  
182 contaminated with fungal growth for B73 (C), and Sugar Bun seeds (D) for each of the different protocol variations.  
183 Letters indicate significant differences for clean germinants (Tukey HSD,  $p$ -value  $< 0.05$ ).  
184

## 185 Experiment 2: Genotypes and seed sources differ strongly in fungal contamination and germination rates

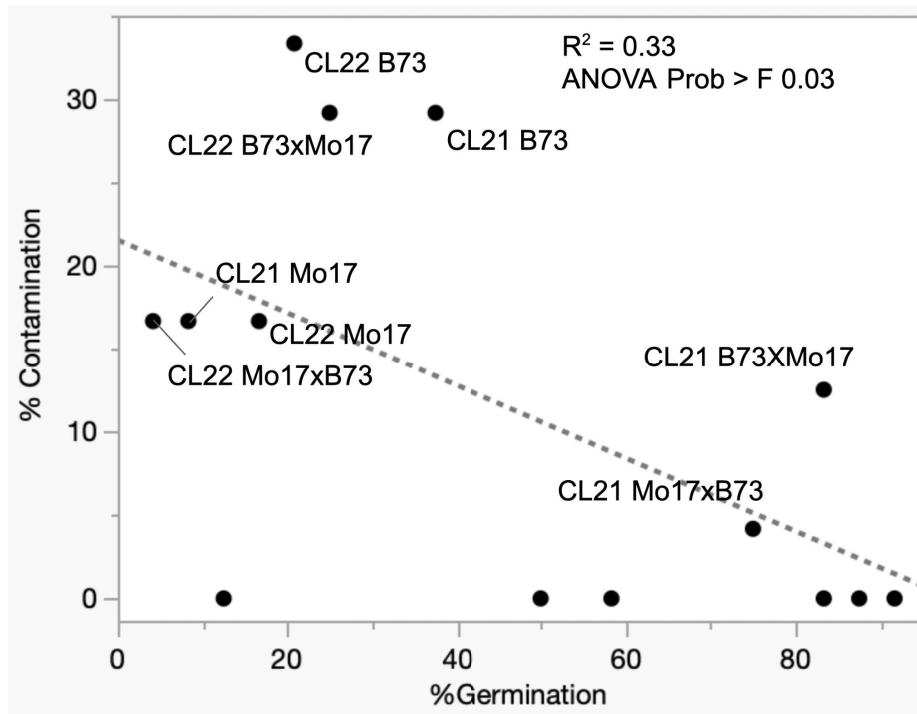
186 We tested the efficiency of the long sterilization protocol with fungicide on four genotypes (B73, Mo17,  
187 B73 x Mo17 and Mo17 x B73) from four different sources (i.e. either place or production year differed), Central  
188 crops Research Station in Clayton, NC (CL) in 2021 and 2022 (CL21 and CL22), San Juan de Abajo, Nayarit,  
189 Mexico (MX; 2021-2022), and a greenhouse in North Carolina, USA (GH; 2021-2022). Seed source had a large  
190 effect on the amount of fungal contamination and germination after sterilization. The long sterilization protocol with  
191 fungicide (long+) eliminated fungal contamination in all but three of the seeds that germinated across all varieties ( $N$   
192 = 157). Fungal contamination could not be completely removed from the NC field sourced (CL) seeds, however,  
193 most of the seeds that still showed fungal contamination after sterilization did not germinate. In contrast, none of the  
194 greenhouse (GH) and Mexico field-sourced (MX) seeds showed any contamination. The contamination rate  
195 following sterilization of CL seeds ranged from 4% to 33% and differed between genotypes (Fig. 2), however, there  
196 was no significant difference in seed contamination from year to year (CL21 and CL22) when comparing within  
197 genotypes.

198 From year to year (comparing CL21 and CL22), the inbred lines B73 (exact two sided (mid)  $P = 0.2259$ )  
199 and Mo17 (exact two sided (mid)  $P = 0.4272$ ) didn't show any difference in the proportion of germinating seeds.  
200 However, both hybrids, B73 x Mo17 (exact two sided (mid)  $P < 0.0001$ ) and Mo17 x B73 (exact two sided (mid)  $P$

< 0.0001) showed significantly lower germination rates in seeds harvested in 2022 than seeds harvested in 2021. The source of seeds did not significantly impact the proportion of germinated Mo17 seeds. Mo17 seeds from all sources only had a 12.5% (SD 4.2%) average germination rate. However the source location did significantly impact the proportion of B37 seeds that germinated. The proportion of B73 seeds that germinated was not significantly different between CL21 and GH (two sided (mid)  $P = 0.1648$ ), but is for CL22 and GH (exact two sided (mid)  $P = 0.0099$ ). The germinating proportion of CL seeds was significantly lower than MX seeds for both CL21 (two sided (mid)  $P = 0.0015$ ), and CL22 (two sided (mid)  $P < 0.0001$ ) seeds. There was no significant difference in the proportion of Mo17 x B73 seeds that germinated when comparing CL and MX sources. The proportion of germinated B73 x Mo17 seeds appears to be driven more by the year differences than source differences. The germination proportion of B73 x Mo17 seeds from the CL22 source (25%) was much lower than those from the CL21 source (83%). However, germination of CL21 B73 x Mo17 seeds did not differ from germination of GH or MX seeds (two sided (mid)  $P > 0.99$  for both comparisons). We found a weak negative relationship between fungal contamination and germination rate (Fig. 3).



214  
215 **Figure 2. Efficiency of the long sterilization protocol with fungicide on four genotypes from four sources.**  
216 Percent germination and contamination for 14 different combinations of genotype and source, sterilized and  
217 untreated. Labels are source (Clayton, GH= greenhouse, and Mexico), year or season harvested (2021 and 2022),  
218 and genotype (B73, Mo17, B73 x Mo17, and Mo17 x B73). For GH we only had B73 and B73 x Mo17 seeds. Each  
219 bar represents the results from 24 sterilized seeds.



221  
222 **Figure 3. Relationship between germination and contamination.** More contamination following the long+  
223 sterilization protocol is associated with lower germination. Only Clayton-sourced seeds were included as GH and  
224 Mexico seeds show no contamination.  
225

## 226 Discussion

227 The goals of our study were (1) to identify protocols that allow for the efficient sterilization of maize seeds,  
228 while maintaining good germination rates, and (2) to test if the same protocol would work best for seeds from  
229 different varieties and sources or if the protocol would need to be adapted to the specific seeds. We found that the  
230 best performing protocol, as well as seed sterilization success differed between maize varieties and seed sources.

231 All of the seeds from both B73 and Sugar Bun harbored fungi, which manifested as fungal outgrowth in  
232 untreated seeds, illustrating the importance of a seed sterilization protocol when exploring plant interactions with  
233 known microorganisms in SynComs. The sterilization approaches that we tested removed up to 100% of the fungal  
234 outgrowth from Sugar Bun seeds and up to 65.3% from the B73 seeds. The short sterilization protocol with  
235 germination in a dilute fungicide yielded 100% germination with no contamination for Sugar Bun seeds. However,  
236 none of the sterilization methods tested here were able to completely remove fungal contamination from B73 seeds  
237 that were sourced from a field in North Carolina. The long sterilization protocol with fungicide did, however,  
238 eliminate all contamination from germinants.

239 B73 was more sensitive to sterilization protocols than Sugar Bun. The short sterilization protocol inhibited  
240 germination of B73 by almost 30% while, except for sterilization following pre-soaking, there was no impact on  
241 Sugar Bun germination. Although the long sterilization protocol lowered the germination rate of B73, compared  
242 with the untreated control, we found that using the long protocol with germination in fungicide led to a ~32%

243 germination rate producing clean, uncontaminated seedlings. It also appears that the long sterilization protocol  
244 mostly inhibited germination in contaminated B73 seeds that may have germinated, as there was no difference in the  
245 proportion of clean germinants between the short and long protocols with fungicide.

246 By extending our study to different varieties, and different source locations, we were able to show that  
247 fungal contamination levels vary greatly between seed sources even after treatment with our best-performing  
248 sterilization protocol. We found that fungal contamination was much more prevalent and difficult to eliminate in  
249 field-grown seeds from North Carolina. Seeds that were grown in the greenhouse in North Carolina or field-grown  
250 in Mexico showed no contamination following sterilization. These differences may be due to the differences in the  
251 growth environments at these locations. The CL seeds were grown in an environment with many sources of fungal  
252 contamination and no chemical control (fungicide) was used. In Mexico, a fungicide was used and in GH, we  
253 hypothesize that there were fewer sources of contamination since it was contained in a greenhouse and grown during  
254 winter. We also found that there may be some negative interaction between the fungal contamination and  
255 germination of the hybrid maize varieties used in this study as indicated by the higher contamination and lower  
256 germination of CL22 seeds versus CL21 seeds.

257 Our study illustrates two potential strategies for ensuring gnotobiotic growth conditions for SynCom  
258 studies in maize roots. One strategy is to pre-germinate seeds following sterilization to allow choosing of clean  
259 seedlings for inoculation and transplantation into the experimental system as has been previously done for maize  
260 (Niu et al., 2017). The second strategy, recommended when inoculation can't wait until after germination, is to  
261 optimize a sterilization strategy for each variety, source, and year of seed used in the study to maximize reduction in  
262 fungal contamination while preserving a sufficient amount of germination.

263 Based on our results we can make the following recommendations for testing sterilization protocols on new  
264 varieties and sources of maize seeds. If pre-germination of seedlings is an option we would recommend to test both  
265 the short and long sterilization protocols without pre-soaking followed by germination in wells containing fungicide  
266 as at least one of these protocols will likely eliminate fungal contamination while retaining a good amount of  
267 germination. If the subsequent study will include fungal inoculants the protocols should also be tested without  
268 germination with fungicide to avoid impacts of residual fungicide on fungal inoculants. If germination without  
269 fungicide does not provide a sufficient number of clean germinants an alternative seed source/variety can be chosen,  
270 or alternatively the effect of residual fungicide on the fungal inoculant after transfer of seedlings to the gnotobiotic  
271 system can be tested to determine the size of the effect of residual fungicide if any on the inoculant.

272 One key limitation of our study and all previous seed sterilization studies is that testing for complete  
273 absence of live microbes from seeds is difficult if not impossible (see Introduction). We used fungal outgrowth after  
274 sterilization as the measure to evaluate success of the sterilization protocol. While we think that the elimination of  
275 fungal outgrowth likely indicates elimination of other, less visible microorganisms as well, we cannot exclude that  
276 some microorganisms remain viable in or on the seed and could grow up in the plant upon germination. For  
277 experiments where complete sterility is required, one potential route to obtain further evidence of sterility could be  
278 to characterize microbial content of larger seedlings using 16S rRNA gene amplicon sequencing for bacteria and  
279 archaea and ITS amplicon sequencing for fungi. While detection of these genes would not necessarily indicate

280 contamination, as traces of DNA from dead cells and kit contamination (Salters et al., 2014) could lead to detection,  
281 monitoring of microbial amplicon abundances in relation to plant amplicon abundances (e.g. plastid 16S rRNA  
282 gene) across a time course of growth would provide good indication of microbial growth if the microbial signal does  
283 not get diluted during plant growth. Additionally, crushing up the seeds after sterilization followed by plating on  
284 bacterial and fungal growth media could provide additional evidence for sterility of the seeds. If bacterial growth  
285 were detected, antibiotics targeting bacteria could be used in addition to fungicide during germination as has been  
286 done previously (Pal et al., 2022).

287 While we only evaluated protocols on maize seeds in this study, we do think that the observed variability in  
288 germination rate and contamination level after sterilization of different varieties and seed sources, will likely also be  
289 relevant for other plant species. Therefore, testing and optimization of sterilization protocols in other plant species  
290 should include multiple varieties and seed sources as well. Additionally, our data also show that for plant-  
291 microbiome studies reporting of the seed source (location and year it was harvested) is critical in addition to  
292 information on the variety to be able to assess if observed differences between studies/experiments are potentially  
293 due to differences in microbial content of seeds.

## 294 **Author contributions**

295 All authors contributed to the conception and design of this study. JJP, GP, PBK, SV and GH ran experiments. JJP  
296 and AA analyzed the data. JJP and MK wrote the first draft of the manuscript. All authors contributed to manuscript  
297 revision, read, and approved the submitted manuscript.

298

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309 **References**

- 310 Bergmann, G. E., Heitmann, S. J., Busby, P. E., & Leveau, J. H. (2022). Characterization of Seed Mycobiota Using  
311 Culture-Dependent and Culture-Independent Approaches. In *Microbial Environmental Genomics (MEG)* (pp. 65-  
312 78). New York, NY: Springer US.
- 313
- 314 Bishop, D. L., Levine, H. G., Kropp, B. R., & Anderson, A. J. (1997). Seedborne fungal contamination:  
315 consequences in space-grown wheat. *Phytopathology*, 87(11), 1125-1133.
- 316
- 317 Carlström, C. I., Field, C. M., Bortfeld-Miller, M., Müller, B., Sunagawa, S., & Vorholt, J. A. (2019). Synthetic  
318 microbiota reveal priority effects and keystone strains in the *Arabidopsis* phyllosphere. *Nature Ecology & Evolution*,  
319 3(10), 1445-1454.
- 320
- 321 Cordovez, V., Dini-Andreote, F., Carrión, V. J., & Raaijmakers, J. M. (2019). Ecology and evolution of plant  
322 microbiomes. *Annu. Rev. Microbiol.*, 73(1), 69-88.
- 323
- 324 Cuero, R. G., Smith, J. E., & Lacey, J. (1986). The influence of gamma irradiation and sodium hypochlorite  
325 sterilization on maize seed microflora and germination. *Food Microbiology*, 3(2), 107-113.
- 326
- 327 Davoudpour, Y., Schmidt, M., Calabrese, F., Richnow, H. H., & Musat, N. (2020). High resolution microscopy to  
328 evaluate the efficiency of surface sterilization of *Zea Mays* seeds. *Plos one*, 15(11), e0242247.
- 329
- 330 Dawar, S., Syed, F., & Ghaffar, A. (2007). Seed borne fungi associated with chickpea in Pakistan. *Pakistan Journal  
331 of Botany*, 39(2), 637.
- 332
- 333 Edgerton, M. D. (2009). Increasing crop productivity to meet global needs for feed, food, and fuel. *Plant physiology*,  
334 149(1), 7-13.
- 335
- 336 Farrell, A. E., Plevin, R. J., Turner, B. T., Jones, A. D., O'hare, M., & Kammen, D. M. (2006). Ethanol can  
337 contribute to energy and environmental goals. *Science*, 311(5760), 506-508.
- 338
- 339 Finkel, O. M., Salas-González, I., Castrillo, G., Conway, J. M., Law, T. F., Teixeira, P. J. P. L., ... & Dangl, J. L.  
340 (2020). A single bacterial genus maintains root growth in a complex microbiome. *Nature*, 587(7832), 103-108.
- 341
- 342 Greenacre, M. (2021). Compositional data analysis. *Annual Review of Statistics and its Application*, 8, 271-299.
- 343
- 344 Gu, R. L., Huang, R., Jia, G. Y., Yuan, Z. P., Ren, L. S., Li, L. I., & Wang, J. H. (2019). Effect of mechanical  
345 threshing on damage and vigor of maize seed threshed at different moisture contents. *Journal of Integrative  
346 Agriculture*, 18(7), 1571-1578.
- 347
- 348 Khamsen, N., Onwimol, D., Teerakawanich, N., Dechanupaprittha, S., Kanokbannakorn, W., Hongesombut, K., &  
349 Srisonphan, S. (2016). Rice (*Oryza sativa* L.) seed sterilization and germination enhancement via atmospheric  
350 hybrid nonthermal discharge plasma. *ACS applied materials & interfaces*, 8(30), 19268-19275.
- 351
- 352 Krumbach, J., Kroll, P., Wewer, V., Metzger, S., Ischebeck, T., & Jacoby, R. P. (2021). Metabolic analysis of a  
353 bacterial synthetic community from maize roots provides new mechanistic insights into microbiome stability.  
354 *BioRxiv*, 2021-11.
- 355
- 356 Leukel, R. W., & Nelson, O. A. (1940). *The use of chlorine gas as a seed disinfectant* (No. 576). US Department of  
357 Agriculture.
- 358
- 359 Lindsey III, B. E., Rivero, L., Calhoun, C. S., Grotewold, E., & Brkljacic, J. (2017). Standardized method for high-  
360 throughput sterilization of *Arabidopsis* seeds. *JoVE (Journal of Visualized Experiments)*, (128), e56587.
- 361

- 362 Niu, B., Paulson, J. N., Zheng, X., & Kolter, R. (2017). Simplified and representative bacterial community of maize  
363 roots. *Proceedings of the National Academy of Sciences*, 114(12), E2450-E2459.
- 364
- 365 Newcombe, G., Harding, A., Ridout, M., & Busby, P. E. (2018). A hypothetical bottleneck in the plant microbiome.  
366 *Frontiers in microbiology*, 9, 1645.
- 367
- 368 Newcombe, G., Marlin, M., Barge, E., Heitmann, S., Ridout, M., & Busby, P. E. (2023). Plant seeds commonly host  
369 *Bacillus* spp., potential antagonists of phytopathogens. *Microbial ecology*, 85(4), 1356-1366.
- 370
- 371 Nuss, E. T., & Tanumihardjo, S. A. (2010). Maize: a paramount staple crop in the context of global nutrition.  
372 *Comprehensive reviews in food science and food safety*, 9(4), 417-436.
- 373
- 374 Pacala, S., & Socolow, R. (2004). Stabilization wedges: solving the climate problem for the next 50 years with  
375 current technologies. *science*, 305(5686), 968-972.
- 376
- 377 Pal, G., Kumar, K., Verma, A., & Verma, S. K. (2022). Seed inhabiting bacterial endophytes of maize promote  
378 seedling establishment and provide protection against fungal disease. *Microbiological Research*, 255, 126926.
- 379
- 380 Philippot, L., Raaijmakers, J. M., Lemanceau, P., & Van Der Putten, W. H. (2013). Going back to the roots: the  
381 microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, 11(11), 789-799.
- 382
- 383 Regalado González, J. J., Berdion, V., Tossi, V. E., Iannone, L. J., Pitta-Alvarez, S. I., & Novas, M. V. (2020).  
384 Influence of *Epichloë* coenophiala, a seed-borne endophyte, on the micropropagation of tall fescue. *Mycological  
385 Progress*, 19, 1301-1308.
- 386
- 387 Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., ... & Walker, A. W. (2014).  
388 Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC biology*, 12,  
389 1-12.
- 390
- 391 Salvato, F., Vintila, S., Finkel, O. M., Dangl, J. L., & Kleiner, M. (2022). Evaluation of protein extraction methods  
392 for metaproteomic analyses of root-associated microbes. *Molecular Plant-Microbe Interactions*, 35(11), 977-988.
- 393
- 394 Samreen, T., Naveed, M., Nazir, M. Z., Asghar, H. N., Khan, M. I., Zahir, Z. A., ... & Choudhary, M. (2021). Seed  
395 associated bacterial and fungal endophytes: Diversity, life cycle, transmission, and application potential. *Applied  
396 Soil Ecology*, 168, 104191.
- 397
- 398 Seaman, W. L., & Wallen, V. R. (1967). Effect of exposure to radio-frequency electric fields on seed-borne  
399 microorganisms. *Canadian Journal of Plant Science*, 47(1), 39-49.
- 400
- 401 Schneider, K., & Gugerty, M. K. (2011). Agricultural productivity and poverty reduction: Linkages and pathways.  
402 *Libraries Test Journal*, 1(1), 56-74.
- 403
- 404 Shade, A., Jacques, M.-A., and Barret, M. (2017). Ecological patterns of seed microbiome diversity, transmission,  
405 and assembly. *Curr. Opin. Microbiol.* 37, 15–22. doi: 10.1016/j.mib.2017.03.010
- 406
- 407 Shayanthan, A., Ordoñez, P. A. C., & Oresnik, I. J. (2022). The role of synthetic microbial communities (syncom) in  
408 sustainable agriculture. *Frontiers in Agronomy*, 4, 58.
- 409
- 410 Simonin, M., Briand, M., Chesneau, G., Rochefort, A., Marais, C., Sarniguet, A., & Barret, M. (2022). Seed  
411 microbiota revealed by a large-scale meta-analysis including 50 plant species. *New Phytologist*, 234(4), 1448-1463.
- 412
- 413 StatsDirect Ltd. StatsDirect statistical software. <http://www.statsdirect.com>. England: StatsDirect Ltd. 2013.
- 414
- 415 Tian, F., Stevens, N. M., & Buckler IV, E. S. (2009). Tracking footprints of maize domestication and evidence for a  
416 massive selective sweep on chromosome 10. *Proceedings of the National Academy of Sciences*, 106(supplement\_1),  
417 9979-9986.

- 418  
419 Torres-Cortés, G., Bonneau, S., Bouchez, O., Genthon, C., Briand, M., Jacques, M. A., & Barret, M. (2018).  
420 Functional microbial features driving community assembly during seed germination and emergence. *Frontiers in*  
421 *plant science*, 9, 902.
- 422  
423 Trivedi, P., Leach, J. E., Tringe, S. G., Sa, T., & Singh, B. K. (2020). Plant–microbiome interactions: from  
424 community assembly to plant health. *Nature reviews microbiology*, 18(11), 607-62.
- 425  
426 Turner, T. R., James, E. K., & Poole, P. S. (2013). The plant microbiome. *Genome biology*, 14(6), 1-10.
- 427  
428 Vorholt, J. A., Vogel, C., Carlström, C. I., & Müller, D. B. (2017). Establishing causality: opportunities of synthetic  
429 communities for plant microbiome research. *Cell host & microbe*, 22(2), 142-155.
- 430  
431 Wagner, M. R., Tang, C., Salvato, F., Clouse, K. M., Bartlett, A., Vintila, S., Phillips, L., Sermons, S., Hoffmann,  
432 M., Balint-Kurti, P. J., & Kleiner, M. (2021). Microbe-dependent heterosis in maize. *Proceedings of the National*  
433 *Academy of Sciences*, 118(30), e2021965118.
- 434  
435 Wallace, J. G., Larsson, S. J., & Buckler, E. S. (2014). Entering the second century of maize quantitative genetics.  
436 *Heredity*, 112(1), 30-38.
- 437  
438 Watts, J. E., De Villiers, O. T., & Watts, L. (1993). Sterilization of wheat seeds for tissue culture purposes. *South*  
439 *African Journal of Botany*, 59(6), 641-642.
- 440  
441 Wippel, K., Tao, K., Niu, Y., Zgadzaj, R., Kiel, N., Guan, R., ... & Garrido-Oter, R. (2021). Host preference and  
442 invasiveness of commensal bacteria in the Lotus and *Arabidopsis* root microbiota. *Nature Microbiology*, 6(9), 1150-  
443 1162.
- 444  
445