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1 **Performance of carrot and onion seed primed with beneficial microorganisms in**
2 **glasshouse and field trials**

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26 **Abstract**

27 Beneficial microorganisms (*Clonostachys rosea* IK726, *Pseudomonas chlororaphis* MA342,
28 *Pseudomonas fluorescens* CHA0, *Trichoderma harzianum* T22 and *Trichoderma viride* S17a)
29 were successfully applied to carrot and onion seed during a commercial drum priming
30 process. Applied microorganisms were recovered above the target of at least 1×10^5 cfu g⁻¹
31 seed following subsequent application of pesticides to the seed according to standard
32 commercial practices of film-coating carrot and pelleting onion seed. Two glasshouse
33 experiments consistently showed that priming improved emergence of carrot seed and that *C.*
34 *rosea* IK726 further improved emergence time. Priming improved emergence of onion seed
35 in one glasshouse experiment, but had an unexpected negative effect on emergence in the
36 second experiment, possibly due to the proliferation of an unidentified indigenous
37 microorganism during priming, becoming deleterious in high numbers. In this experiment,
38 the application of beneficial microorganisms during priming negated this effect and
39 significantly improved emergence. For each crop, a series of field trials was also carried out
40 over three years, at two different sites each year. Although some positive effects of different
41 seed treatments were seen on emergence or yield in individual field trials, no consistent
42 effects were found for primed or microorganism-treated seed across all sites and years.
43 However, a combined analysis of data for all years and sites indicated that pesticide
44 application did consistently improve emergence and yield for both carrot and onion. This is
45 the first comprehensive study assessing glasshouse and field performance of carrot and onion
46 seed primed with beneficial microorganisms during a commercial process of drum priming in
47 the UK.

48

49 **Keywords** *Clonostachys rosea*, *Pseudomonas* spp., *Trichoderma* spp., Seed application,
50 Priming, Emergence, Growth promotion, Yield

51 **1. Introduction**

52 The use of chemical pesticides in horticulture and agriculture is becoming more restricted due
53 to environmental and health concerns and many active ingredients are being banned.

54 However, for sustainable crop production to continue, pathogens and pests still need to be
55 controlled in order to ensure healthy plant establishment and growth. The use of various
56 beneficial microorganisms or biological control agents has been extensively researched in
57 order to provide an alternative to chemical control, although there are still limited biological
58 control products on the market (Gerhardson, 2002). A viable option for the use of beneficial
59 microorganisms in horticulture and agriculture is application to seed. This specifically targets
60 the area where most benefit may be seen during seedling establishment, and beneficial
61 microbial colonization of the rhizosphere may further promote plant growth during the
62 growing season (Harman, 1991). Modes of action for beneficial microorganisms to promote
63 plant growth include direct parasitism of plant pathogens, competition for space or nutrients,
64 or production of antibiotics, enzymes or plant hormones (Whipps, 2001). Seed-applied
65 microorganisms can be considered either as a direct alternative to chemical seed treatment, or
66 as part of an integrated system, combining both microorganisms and pesticides (possibly at a
67 reduced dose). Produced commercially, growers would be able to buy microorganism-treated
68 seed in the same way that they currently purchase pesticide-treated seed.

69
70 Microorganisms have often been applied to seeds using experimental systems that are not
71 easily scaled up for commercial application. For example, application methods have included
72 suspensions, slurries, powders, peat carriers, or encapsulation in alginate (Fravel et al., 1998;
73 McQuilken et al., 1998; Walker et al., 2004). Bennett and Whipps (2008a) showed that
74 bacteria and fungi can be successfully applied to carrot and onion seed during the process of
75 drum priming. During priming, seeds are hydrated through the controlled addition of water to

76 start the physiological process of germination before the seed is planted. Following an
77 incubation period, and before the radicle emerges from the seed coat, the procedure is stopped
78 and the seed is dried back to a low moisture content. Priming ensures the entire seed batch is
79 at the same point in the germination process, so that once it is planted primed seed has a more
80 rapid and uniform emergence than unprimed seed (Rowse, 1996a; Rowse, 1996b).

81 Microorganisms can be added to the water used to hydrate the seed during drum priming,
82 frequently surviving and proliferating to high numbers on the seed (Wright et al., 2003b;
83 Bennett and Whipps, 2008a).

84

85 Previous work demonstrated the success of this application method using laboratory-scale
86 equipment and microorganisms applied to seed in this way survived on seed and in the
87 rhizosphere of carrot and onion, in glasshouse-based assays (Bennett and Whipps, 2008a).

88 This paper reports the application of beneficial microorganisms to carrot and onion seed
89 during a commercial-scale drum priming system and the performance of the primed
90 microorganism-treated seed in comprehensive glasshouse and field trials. The main aims
91 were i) to apply selected microorganisms to carrot and onion seed during commercial-scale
92 drum priming; ii) for each crop, to assess emergence and seedling fresh weight after 8 weeks
93 growth in two glasshouse experiments; and iii) for each crop, to assess emergence and yield in
94 a series of field trials carried out over three years, at two different sites each year. This is the
95 first report of extensive field testing of beneficial microorganisms applied to seed during the
96 commercially viable process of drum priming in the UK.

97

98 **2. Materials and methods**

99 *2.1 Beneficial microorganisms*

100 Beneficial microorganisms were selected for use in this study based on their known
101 biocontrol or plant growth promotion properties, or availability within a commercial product.
102 The bacterial isolates included *Pseudomonas chlororaphis* MA342, the active ingredient in
103 Cedomon[®], targeting cereal pathogens (Johnsson et al., 1998; Gerhardson, 2002), which was
104 obtained from Dr M. Hökeberg, BioAgri, Uppsala, Sweden; and *Pseudomonas fluorescens*
105 CHA0, which has activity against a wide range of soil-borne pathogens (Maurhofer et al.,
106 1994) and was obtained from Prof G. Défago, Federal Institute of Technology, Zurich,
107 Switzerland. The fungal isolates included *Clonostachys rosea* IK726, which has activity
108 against seed-borne pathogens and plant growth promotion properties (Jensen et al., 2002;
109 Jensen et al., 2004; Ravnskov et al., 2006) and was obtained from Dr D. F. Jensen, The Royal
110 Veterinary and Agricultural University, Copenhagen, Denmark; *Trichoderma harzianum* T22,
111 which was obtained from Dr G. Harman, Cornell University, Geneva, USA and is available
112 commercially as T-22[™] Planter Box and other formulations as a biocontrol agent; and
113 *Trichoderma viride* S17a, which was available from the culture collection at Warwick HRI,
114 University of Warwick, and has biocontrol activity against *Sclerotium cepivorum* (*Allium*
115 white rot pathogen) (Clarkson et al., 2002; Clarkson et al., 2006). Wild-type strains of all
116 isolates were used in this work. The minimum target application rate was 1×10^5 cfu g⁻¹ seed,
117 as evidence in the literature suggests that disease control activity or plant growth promotion
118 can be achieved when beneficial microorganisms are present above this rate on seed or roots,
119 or in soil (Adams, 1990; Raaijmakers and Weller, 1998).

120

121 2.2 Seed treatments

122 Bacterial suspensions were prepared at Germain's Technology Group (GTG), UK. Single
123 colonies grown on nutrient agar plates were used to inoculate sterile nutrient broth, which was
124 incubated overnight in rotary culture (25°C and 180 rpm). From the resulting master culture,

125 0.5ml aliquots were used to inoculate fresh flasks of nutrient broth (50ml). After incubation
126 in rotary culture (25°C and 180 rpm) for 4-5 hours, bacterial cell numbers were determined by
127 spectrophotometry of the suspension and reference to standard curves previously determined.
128 The required volume of bacterial suspension was then centrifuged (12000g for 10 minutes)
129 and the resulting pellet was resuspended in the volume of water pre-determined for seed
130 priming. Final cell numbers were calculated following spiral plating and counts. Fungal
131 isolates were grown on potato dextrose agar at 20°C , and following profuse sporulation the
132 spores were harvested by adding sterile distilled water to the plates and scraping the spores
133 into suspension. The suspension was filtered through a double layer of sterile lens tissue and
134 the concentration was determined by haemocytometer counts. Final numbers were
135 determined by spiral plating and counts.

136

137 The microorganism suspensions were individually applied to batches of carrot seed (cv.
138 Nairobi) and onion seed (cv. Hytech) through the commercial process of drum priming at
139 Elsoms Seeds Ltd, UK. This is a commercial scale version of the system previously
140 described by Bennett and Whipps (2008a). Other seed batches were also primed with water
141 only as a control, and further seed batches remained unprimed.

142

143 All seed batches (unprimed control, primed control, and primed with microorganisms) were
144 subsequently split and half were treated with standard pesticide seed-treatments following
145 commercial practice at GTG, UK. The other half remained untreated. For pesticide
146 application, carrot seed was film-coated with a mixture of Wakil XL (fungicide; a.i
147 cymoxanil, fludioxonil, metalaxyl) and Force ST (insecticide; a.i. tefluthrin), whereas onion
148 seed was pelleted with a mixture of HY-TL (fungicide; a.i thiram and thiabendazole) and
149 Force ST. In Year 1 (2004), Apron 35 (fungicide; a.i. metalaxyl) was also included in the

150 pesticide-treated onion pellets only. The full list of treatments is given in Table 1. Where
151 applicable, sub-samples of seed were taken to check the recovery of applied microorganisms
152 following the different treatments.

153

154 *2.3 Glasshouse experiment design and statistical analyses*

155 Separate experiments were conducted for carrot and onion. Within each experiment, there
156 were 12 seed treatments (Table 1), each planted in three soil types (light sandy loam (West
157 Winch, Norfolk); peat soil (Isleham, Cambridgeshire); sandy clay loam (Wellesbourne,
158 Warwickshire); see soil analysis details in Bennett and Whipps (2008a)). The soil had
159 previously been sieved to 5mm to remove stones, and the sandy clay loam was mixed 4:1
160 with vermiculite to improve its structure. Each experiment was arranged as a four replicate
161 randomized complete block design with 36 plots per replicate (one for each combination of
162 soil type and seed treatment). Each replicate combination contained 6 pots (sized 70 x 70 x
163 80 (deep) mm), into each of which were sown four seeds. Pots were watered from below as
164 necessary. Each experiment was carried out on two separate occasions, during the summer
165 months (March-September) of 2004 and 2005. No additional lighting was required in the
166 glasshouse during this period. The glasshouse temperature was maintained between 15-
167 25°C, with vents opening at 25°C.

168

169 Emergence was assessed daily until no further increase in numbers was seen, and the time to
170 50% of this emergence value was calculated. Seedlings were grown for 8 weeks, after which
171 time the final stand (as a percentage of seed sown) was determined for each replicate seed
172 treatment/soil-type combination. At harvest (8 weeks), all seedlings from the six pots in each
173 replicate seed treatment/soil-type combination were grouped together. Soil was washed off

174 the roots, they were blotted dry and the fresh weight of the seedlings was recorded. Using the
175 numbers of seedlings in the final stand, the mean weight per seedling was calculated.

176

177 Percentage values for the final stand data were arcsine transformed, and those for mean fresh
178 weight of seedlings were natural log transformed before analysis to satisfy the assumption of
179 homogeneity of variance. For each experiment separately, an analysis of variance was carried
180 out in GenStat for Windows, testing for the main effects of seed treatment, soil type and
181 pesticide application, and the interaction between these factors. All differences noted were at
182 the 5% significance level.

183

184 *2.4 Field trial design and statistical analyses*

185 There were two series of field trials, one each for carrot and onion. For each crop, trials were
186 conducted for three consecutive years, with one trial at Wellesbourne (Warwick HRI,
187 University of Warwick) and one at a different grower site each year (total of six trials per
188 crop). Each trial was arranged as a randomized complete block design, with four replicate
189 blocks, each containing 12 plots. Each plot was sown to one of the 12 seed treatments used in
190 the glasshouse experiments, using the same batches of treated seed (Table 1). For the onion
191 field trial in Year 3 (2006) only, *Trichoderma viride* S17a replaced *Clonostachys rosea* IK726
192 as one of the seed treatments.

193

194 In Year 1 (2004), each plot consisted of four rows of 3m length, drilled using a hand operated
195 cone-drill. In Years 2 (2005) and 3 (2006) each plot consisted of four 6m length rows, drilled
196 using a tractor-mounted Singulaire drill, with the inner two rows comprising the treated seed
197 and the outer two rows comprising untreated seed as guard rows. Onions were drilled in
198 March-April and harvested in August, whereas carrots were drilled in May and harvested in

199 September. The carrot crops at Wellesbourne were grown under horticultural fleece to avoid
200 infestation by carrot root fly and the fleece was removed when the window for infestation had
201 passed.

202

203 Emergence assessments were made 6-8 weeks after sowing, and seedlings were counted from
204 a set length along the rows. Due to changes in length of row assessed between years and
205 sites, all data were subsequently converted to a standard format before analysis to give
206 emergence counts per meter length of row. At harvest, the onion bulbs were lifted from the
207 ground and dried before yield assessments were made. Dried shoots were removed and the
208 number and weight of bulbs was recorded. The carrots had the leaves removed after harvest,
209 and the roots were washed before yield assessments were made with the number and weight
210 of carrots recorded. In Year 2, the carrots at the grower site were left in the ground and
211 protected with straw over winter and assessments were made when they were harvested the
212 following April. For both carrot and onion, as with the emergence data, all harvest data were
213 converted to a standard format before analysis to give the harvest count and harvest weight
214 per meter length of row. No transformations were considered necessary prior to analysis.

215

216 For all analyses, the treatment variability was sub-divided into a series of single degree-of-
217 freedom comparisons: priming (comparing the unprimed control with all primed treatments);
218 microorganism application (comparing the primed control with all primed seed treated with
219 microorganisms) and “microtype” (comparing the bacterial seed treatments with the fungal
220 seed treatments). Within “microtype”, the individual microorganism treatments were also
221 compared. All analyses also considered the interaction of these terms with the effect of
222 pesticide. A separate analysis was done for each site/year combination for each crop. Further
223 analyses considered the combined data for each variable across the two sites for each year

224 (separately for each crop). These analyses allowed the identification of any consistent
225 treatment effects between sites within the year. A final set of analyses considered the
226 combined data for each variable across all six trials (two sites in each of three years). These
227 analyses further included terms for the interaction between each of the treatment terms
228 considered in the individual trial analyses and the effect of year. These combined analyses
229 allow the identification of any consistent treatment effect between years, and of any strong
230 differences in treatment effects between years. In the combined analyses, plots allocated to *C.*
231 *rosea* IK726 were considered as missing values in Year 3 (as this treatment had been replaced
232 by *T. viride* S17a, which was not included in this final combined analysis).

233

234 **3. Results**

235 *3.1 Seed-applied microorganisms*

236 In all years, all beneficial microorganisms applied to carrot and onion seed were recovered in
237 excess of the target application rate of 1×10^5 cfu g^{-1} dry seed ($5 \log_{10}$ cfu g^{-1} seed), irrespective
238 of subsequent film-coating (carrot), pelleting (onion), or pesticide application (Table 2).

239

240 *3.2 Carrot glasshouse experiments*

241 In the first glasshouse experiment (Year 1), for treatments both with and without pesticides,
242 all primed seed treatments emerged faster than the unprimed treatment ($F_{5,105} = 115.21$, $P <$
243 0.001 ; Table 3). In addition, seed treated with either *C. rosea* IK726 or *T. harzianum* T22
244 emerged significantly faster than the primed control seed (Table 3). For all treatments, seed
245 in the peat soil emerged slower than in the other two soil types ($F_{2,105} = 102.43$, $P < 0.001$;
246 Table 3). The final stand was also affected by soil type ($F_{2,105} = 39.40$, $P < 0.001$; Table 3),
247 with the sandy clay loam soil producing the highest stands and the light sandy loam the
248 lowest. For all seed treatments, pesticide application improved the final stand ($F_{1,105} = 4.93$,

249 $P = 0.028$; Table 3). Neither the final stand nor the mean fresh seedling weight was affected
250 by priming or application of microorganisms. However, seedling fresh weight was influenced
251 by soil type ($F_{2,105} = 80.65$, $P < 0.001$; Table 3), with peat soil producing the heaviest
252 seedlings and sandy clay loam the lightest.

253

254 In the second glasshouse experiment (Year 2), for treatments both with and without
255 pesticides, all primed seed treatments again emerged faster than the unprimed treatment ($F_{5,105}$
256 $= 51.62$, $P < 0.001$; Table 3). In addition, seed treated with *C. rosea* IK726 emerged faster
257 than the primed control. For all treatments, seed in the peat soil emerged slower than that in
258 the other two soil types and seed in the light sandy loam emerged slower than that in the
259 sandy clay loam ($F_{2,105} = 67.87$, $P < 0.001$; Table 3). The final stand was greater in the sandy
260 clay loam soil than the other two soil types ($F_{2,105} = 19.09$, $P < 0.001$; Table 3), and pesticide
261 treatment again improved the final stand in all soils ($F_{1,105} = 9.17$, $P = 0.003$; Table 3).

262 Neither the final stand nor seedling fresh weight was affected by priming or microorganism
263 seed treatment. However, seedling fresh weight was influenced by soil type ($F_{2,105} = 34.15$, P
264 < 0.001 ; Table 3), with seedlings grown in peat soil weighing more than those grown in the
265 other two soils, and those grown in light sandy loam weighing more than those grown in
266 sandy clay loam.

267

268 3.3 Onion glasshouse experiments

269 In the first glasshouse experiment (Year 1), for treatments both with and without pesticides,
270 all primed treatments emerged faster than the unprimed control ($F_{5,105} = 4.49$, $P < 0.001$;
271 Table 4). For all treatments, seed in the light sandy loam emerged slower than that in the
272 other two soil types ($F_{2,105} = 6.92$, $P = 0.002$; Table 4). Neither the final stand nor seedling
273 fresh weight was affected by priming or application of microorganisms, although seedling

274 weight was affected by soil type ($F_{2,105} = 182.49$, $P < 0.001$; Table 4), with the light sandy
275 loam producing the heaviest seedlings and the sandy clay loam producing the lightest. In
276 addition, pesticide application resulted in a slight decrease in the mean fresh weight of
277 seedlings ($F_{1,105} = 5.33$, $P = 0.023$; Table 4).

278

279 In the second glasshouse experiment (Year 2), for treatments both with and without
280 pesticides, all microorganism treated seed, except that treated with *C. rosea* IK726, emerged
281 faster than the unprimed control, and seed treated with *P. chlororaphis* MA342 also emerged
282 faster than the primed control ($F_{5,105} = 2.96$, $P = 0.015$; Table 4). Seedlings emerged faster in
283 the peat soil than in the other two soil types ($F_{2,105} = 7.32$, $P = 0.001$; Table 4). The primed
284 control unexpectedly had a lower final stand than the unprimed control, but all microorganism
285 treatments improved the final stand compared to the primed control ($F_{5,105} = 4.75$, $P < 0.001$;
286 Table 4). Pesticide application also significantly improved the final stand ($F_{1,105} = 25.08$, $P <$
287 0.001 ; Table 4). Further analysis showed that whilst the primed control had the worst final
288 stand in the absence of pesticide application, this was not the case where pesticide had also
289 been included in the seed treatment ($F_{5,105} = 6.52$, $P < 0.001$; Table 5). The final stand was
290 also affected by soil type ($F_{2,105} = 10.67$, $P < 0.001$; Table 4), with the peat soil producing the
291 highest stands and light sandy loam the lowest. The seedling fresh weight was not affected by
292 either priming or microorganism treatment, but was influenced by soil type ($F_{2,105} = 641.03$, P
293 < 0.001 ; Table 4), with seedlings grown in peat soil weighing more than those grown in the
294 other two soil types. Again, pesticide application resulted in a slight decrease in the mean
295 fresh weight of seedlings ($F_{1,105} = 11.10$, $P = 0.001$; Table 4).

296

297 *3.4 Carrot field trials*

298 In Year 1, emergence results from both sites showed neither an overall benefit of priming nor
299 any differences between microorganism applications ($P > 0.05$, Table 6). Application of
300 pesticides increased emergence at Wellesbourne ($F_{1,33} = 85.04$, $P < 0.001$; Table 6), but not at
301 the grower site. Whilst treatment effects varied between sites, combined analysis of Year 1
302 data showed a consistent increase in emergence when pesticide was applied ($F_{1,77} = 31.21$, $P <$
303 0.001). At Wellesbourne only, and both with and without pesticides, a comparison of the
304 bacterial treatments showed that *P. fluorescens* CHA0 had lower emergence than *P.*
305 *chlororaphis* MA342 ($F_{1,33} = 10.22$, $P = 0.003$; Table 6). Similarly for the fungal treatments,
306 *C. rosea* IK726 resulted in a lower emergence than *T. harzianum* T22 in the absence of
307 pesticides only ($F_{1,33} = 4.88$, $P = 0.034$; Table 6). Whilst these effects were not significant at
308 the grower site, the combined (cross-site) analysis indicated a consistency of the latter effect
309 across sites ($F_{1,77} = 5.30$, $P = 0.024$).

310

311 In Year 1, results from both sites also showed no overall effect of priming or microorganism
312 application on the number of carrots at harvest ($P > 0.05$, Table 6). However, at
313 Wellesbourne only, *P. fluorescens* CHA0 reduced the number of carrots at harvest compared
314 to *P. chlororaphis* MA342, averaged across treatments both with and without pesticides ($F_{1,33}$
315 $= 4.44$, $P = 0.043$). For the fungal treatments, *C. rosea* IK726 resulted in fewer carrots at
316 harvest than *T. harzianum* T22, but only in the absence of pesticides ($F_{1,33} = 4.15$, $P = 0.05$;
317 Table 6). Again at Wellesbourne only, pesticide increased the number of carrots at harvest
318 ($F_{1,33} = 74.54$, $P < 0.001$), and whilst this was not significant at the grower site, the combined
319 (cross-site) analysis indicated a consistency of the effect of pesticide application across sites
320 ($F_{1,77} = 17.07$, $P < 0.001$). In Year 1, results from both sites showed no overall benefit of
321 priming, microorganism application or pesticides on the weight of carrots at harvest ($P >$
322 0.05 ; Table 6). However, at Wellesbourne only, on average the bacterial isolates resulted in a

323 greater weight of carrots at harvest than did the fungal isolates ($F_{1,33} = 4.37, P = 0.044$). This
324 effect was not consistent across sites.

325

326 In Year 2, there was no overall benefit of priming on emergence at either site ($P > 0.05$, Table
327 6). Microorganism application did influence emergence at Wellesbourne, where the primed
328 control seed emerged in greater numbers than microorganism-treated seed, although only in
329 the absence of pesticide ($F_{1,33} = 4.75, P = 0.037$; Table 6). Overall, pesticide increased
330 emergence at Wellesbourne ($F_{1,33} = 149.19, P < 0.001$), and although this was not significant
331 at the grower site, the combined analysis indicated a consistency of this effect across sites
332 ($F_{1,77} = 47.89, P < 0.001$). On average, the bacterial isolates resulted in a higher emergence
333 than the fungal isolates at Wellesbourne ($F_{1,33} = 5.49, P = 0.025$), although again this was not
334 consistent across sites.

335

336 Similarly, on average priming did not affect the number of carrots at harvest at either site in
337 Year 2 ($P > 0.05$, Table 6), but microorganism application did result in an overall decrease in
338 the number of carrots at harvest compared with the primed control, at Wellesbourne only
339 ($F_{1,33} = 7.28, P = 0.011$). Also at Wellesbourne only, bacterial seed treatments resulted in a
340 greater number of carrots at harvest than fungal seed treatments ($F_{1,33} = 7.18, P = 0.011$; Table
341 6). On average, pesticide increased the number of carrots at harvest at Wellesbourne ($F_{1,33} =$
342 $127.76, P < 0.001$), and although this effect was not significant at the grower site, the
343 combined (cross-site) analysis indicated that this effect was consistent across sites ($F_{1,68} =$
344 $36.29, P < 0.001$).

345

346 In Year 2, priming increased the weight of carrots at harvest at Wellesbourne only ($F_{1,33} =$
347 $9.83, P = 0.004$), but no overall benefit of microorganism application relative to the primed

348 control was found at either site ($P > 0.05$; Table 6). Pesticide increased the weight of carrots
349 at harvest at Wellesbourne ($F_{1,33} = 64.98$, $P < 0.001$) and, whilst this effect was not significant
350 at the grower site, the combined analysis indicated a consistency of this effect across sites
351 ($F_{1,68} = 9.56$, $P = 0.003$). At Wellesbourne only, the combination of microorganism
352 application with pesticide resulted in a greater weight of carrots, on average, than
353 microorganism treated seed without pesticide ($F_{1,33} = 4.73$, $P = 0.037$), but this effect was not
354 consistent across the two sites.

355

356 In Year 3, results from both sites indicated no overall effects of priming or microorganism
357 application on either emergence, or the number or weight of carrots at harvest ($P > 0.05$,
358 Table 6). However, at Wellesbourne, pesticide application increased emergence ($F_{1,33} =$
359 19.45 , $P < 0.001$), the number ($F_{1,29} = 17.86$, $P < 0.001$) and weight ($F_{1,29} = 6.83$, $P = 0.014$)
360 of carrots at harvest (Table 6). Although these effects were not significant at the grower site,
361 the combined (cross-site) analysis showed that pesticide consistently increased both
362 emergence ($F_{1,77} = 5.71$, $P = 0.019$) and the number of carrots at harvest ($F_{1,73} = 6.37$ $P =$
363 0.014).

364

365 Although significant effects were seen in some years (cross-site analyses) or at individual
366 sites, the final combined analysis showed that the effects of priming and microorganism
367 application were not consistent across all years and sites. However, the final combined
368 analysis also showed that pesticide treatment consistently improved both emergence ($F_{1,231} =$
369 71.60 , $P < 0.001$) and the number of carrots at harvest ($F_{1,218} = 54.82$, $P < 0.001$).

370

371 *3.5 Onion field trials*

372 In Year 1, results at both sites showed no overall benefit of priming or microorganism
373 application on either emergence or the number or weight of onions at harvest ($P > 0.05$, Table
374 7). However, pesticide application consistently increased emergence at both Wellesbourne
375 ($F_{1,33} = 6.77$, $P = 0.014$) and the grower site ($F_{1,33} = 7.44$, $P = 0.010$; Table 7). At the grower
376 site only, fungal seed treatments also increased the number of bulbs at harvest on average
377 compared to bacterial seed treatments ($F_{1,33} = 4.28$, $P = 0.046$). Further analysis of data from
378 the grower site showed that for treatments both with and without pesticide *C. rosea* IK726
379 increased the number of bulbs at harvest compared to *T. harzianum* T22 ($F_{1,33} = 6.71$, $P =$
380 0.014 ; Table 7). Although not significant at Wellesbourne, the combined (cross-site) analysis
381 showed that this effect was consistent across sites ($F_{1,77} = 4.07$, $P = 0.047$). In addition,
382 pesticide application consistently increased the number of onions at harvest for both sites
383 (Wellesbourne: $F_{1,33} = 9.44$, $P = 0.004$; grower: $F_{1,33} = 24.91$, $P < 0.001$; Table 7). Pesticide
384 application increased the weight of onions at harvest at the grower site ($F_{1,33} = 7.22$, $P =$
385 0.011), and although this was not significant at Wellesbourne, the combined analysis of data
386 from both sites showed that this effect was consistent across sites ($F_{1,77} = 5.98$, $P = 0.017$).

387

388 In Year 2 results at both sites showed no overall benefit of priming, microorganism
389 application or pesticide application on either onion emergence or the number of onions at
390 harvest ($P > 0.05$, Table 7). However, at Wellesbourne and with pesticides applied, the
391 primed control resulted in both greater emergence ($F_{1,33} = 12.89$, $P = 0.001$) and a higher
392 number of onions at harvest ($F_{1,33} = 11.80$, $P = 0.002$) than seed treated with microorganisms.
393 Although not significant at the grower site, the combined analysis similarly showed that
394 pesticide had a greater effect on the primed control than on the microorganism treated seed,
395 with respect to both emergence ($F_{1,77} = 6.97$, $P = 0.01$) and the number of onions at harvest
396 ($F_{1,77} = 7.48$, $P = 0.008$). At the grower site only, *P. fluorescens* CHA0 seed treatment

397 resulted in a greater number of bulbs at harvest than *P. chlororhysis* MA342 seed treatment
398 ($F_{1,33} = 4.48$, $P = 0.042$; Table 7), and *C. rosea* IK726 seed treatment resulted in a greater
399 number of bulbs at harvest than *T. harzianum* T22 ($F_{1,33} = 4.60$, $P = 0.039$; Table 7). These
400 effects were not consistent across sites.

401

402 In Year 2, results at both sites showed no overall effect of priming or microorganism
403 application on onion weight at harvest ($P > 0.05$; Table 7). Pesticide application increased the
404 onion weight at the grower site ($F_{1,33} = 8.89$, $P = 0.005$), and although not significant at
405 Wellesbourne, this effect was consistent in the combined (cross-site) analysis ($F_{1,77} = 6.24$, P
406 $= 0.015$). At Wellesbourne, treatment with *T. harzianum* T22 increased the weight of onions
407 compared to *C. rosea* IK726, but only in the absence of pesticides ($F_{1,33} = 8.15$, $P = 0.007$;
408 Table 7). This effect was not consistent across sites.

409

410 In Year 3, *T. viride* S17a replaced *C. rosea* IK726 as one of the two fungal seed treatments
411 (Table 1 and Table 7). At both sites, no overall effects of priming or microorganism
412 application were seen for emergence or for the number of bulbs at harvest ($P > 0.05$, Table 7),
413 but, at Wellesbourne only, emergence was improved by pesticide application ($F_{1,33} = 11.93$, P
414 $= 0.002$). Pesticide application also increased the number of bulbs at harvest ($F_{1,33} = 17.68$, P
415 < 0.001) at Wellesbourne, and, although this effect was not significant at the grower site, the
416 combined (cross-site) analysis indicated a consistency in this effect ($F_{1,76} = 7.37$, $P = 0.008$).
417 At the grower site only, microorganism treated seed had a greater emergence ($F_{1,33} = 8.59$, $P =$
418 0.006) and number of onions at harvest ($F_{1,32} = 16.12$, $P < 0.001$) than the primed control seed
419 with pesticide applied, whereas the opposite was true without pesticide applied (Table 7).
420 Although not significant at Wellesbourne, this effect was seen to be consistent in the
421 combined analysis (emergence: $F_{1,77} = 7.71$, $P = 0.007$; number of onions $F_{1,76} = 10.87$, $P =$

422 0.001). However, these results were largely influenced by the effects seen for *P. fluorescens*
423 CHA0 treated seed at the grower site in Year 3. Here, seed treated with *P. fluorescens* CHA0
424 plus pesticides had a greater emergence than the primed control seed plus pesticides, and a
425 greater emergence than seed treated with *P. fluorescens* CHA0 but without pesticides ($F_{1,33} =$
426 4.76, $P = 0.036$; Table 7). In addition, the primed control seed without pesticides had a greater
427 emergence than the seed treated with *P. fluorescens* CHA0 without pesticides. The same
428 pattern was found for with the number of onions at harvest at the growers site ($F_{1,32} = 4.90$, P
429 $= 0.034$; Table 7). Although not significant at Wellesbourne, the combined analysis indicated
430 a consistency of these effects across sites for emergence only ($F_{1,77} = 4.58$, $P = 0.035$). On
431 average, fungal seed treatments resulted in a greater number of bulbs at harvest than the
432 bacterial seed treatments at the grower site ($F_{1,32} = 9.89$, $P = 0.004$), and, whilst this effect
433 was not significant at Wellesbourne, the combined analysis indicated a consistency of this
434 effect across sites ($F_{1,76} = 4.79$, $P = 0.032$; Table 7).

435

436 In Year 3, results at both sites showed no overall benefit of either priming or microorganism
437 application for the weight of onions at harvest ($P > 0.05$, Table 7), but pesticide application
438 increased the weight at both sites (Wellesbourne: $F_{1,33} = 7.14$, $P = 0.012$; grower: $F_{1,32} = 6.43$,
439 $P = 0.016$). At the grower site only, seed treated with a microorganism treatment and
440 pesticide increased the weight of onions at harvest compared both to the primed control with
441 pesticide application, and also to microorganism treated seed without pesticide application
442 ($F_{1,32} = 5.58$, $P = 0.024$). Although not significant at Wellesbourne, some consistency in this
443 effect was seen in the combined site analysis ($F_{1,76} = 5.49$, $P = 0.022$). At the grower site
444 only, fungal treatments increased the weight of bulbs at harvest compared to the bacterial
445 treatments ($F_{1,32} = 6.32$, $P = 0.017$). Further analysis showed that *T. viride* S17a treated seed
446 increased the weight of bulbs at harvest compared to *T. harzianum* T22 at the grower site

447 ($F_{1,32} = 4.88$, $P = 0.034$), and whilst not significant at Wellesbourne, the combined analysis
448 showed some consistency in this effect across sites ($F_{1,76} = 4.70$, $P = 0.033$).

449

450 A final analysis of combined data from all years and sites was carried out, with plots treated
451 with *C. rosea* IK726 considered as missing values in Year 3 and *T. viride* S17a not included
452 in the analysis. This showed that pesticide application consistently increased emergence
453 ($F_{1,217} = 17.30$, $P < 0.001$), the number of bulbs ($F_{1,217} = 32.96$, $P < 0.001$) and the weight of
454 bulbs at harvest ($F_{1,217} = 19.34$, $P < 0.001$). The combined results also showed that *C. rosea*
455 IK726 seed treatment consistently resulted in a greater number of onions at harvest ($F_{1,217} =$
456 5.37 , $P = 0.021$) and a greater weight of onions ($F_{1,217} = 7.93$, $P = 0.005$) than *T. harzianum*
457 T22 seed treatment across sites and years, but that neither of these treatments was
458 significantly different from the primed control. Other effects were inconsistent across years
459 and sites.

460

461 **4. Discussion**

462 Seed priming is an established technique for improving emergence, particularly under
463 unfavorable conditions such as in cold or wet soil (McQuilken et al., 1998; Halmer, 2004).

464 The application of beneficial microorganisms to primed seed has potential to further improve
465 establishment of crops, and may provide disease control during the growing season if they
466 become established on the roots. Previously, the microorganisms used in this work have been
467 applied to seed using a laboratory scale drum priming system (Bennett and Whipps, 2008a).

468 In the current work, microorganisms were successfully applied to carrot and onion seed
469 during a commercial scale process of drum priming, and survived on seed following the
470 standard commercial practices of film-coating carrot and pelleting onion seed, both with or
471 without pesticide application. The performance of the treated seed was then tested in

472 glasshouse experiments in three different soil types, and in a series of field trials conducted
473 over three years.

474

475 Consistent results were found with two consecutive glasshouse experiments for primed,
476 microorganism treated carrot seed. In both years, primed carrot seed emerged faster than the
477 unprimed seed, confirming the benefits of priming for rapid and uniform seedling
478 establishment. In addition, *C. rosea* IK726 treated seed emerged faster than the primed
479 control in both years. Although this microorganism did not improve the final stand compared
480 to the primed control, the faster emergence confirms the potential for this fungal isolate to
481 provide benefit in the early establishment of carrot seedlings. As pesticide application also
482 consistently improved emergence in the current work, it suggests the presence of pathogens or
483 deleterious microorganisms either on the seed or in the soil. Other research has shown that *C.*
484 *rosea* IK726 primed onto carrot seed controls seed-borne fungal pathogens such as *Alternaria*
485 spp., thus improving early establishment (Jensen et al., 2004) and this isolate in particular has
486 potential to be further developed as a commercial biocontrol agent (Jensen et al., 2007). As
487 the positive effects of priming and application of *C. rosea* IK726 were seen consistently
488 across three different soil types, which themselves produced highly variable effects on
489 emergence and seedling fresh weight, this shows a robust effect of the fungal seed treatment.

490

491 For the glasshouse experiments using primed, microorganism treated onion seed no consistent
492 effects were seen for the consecutive years. In the first experiment, primed seed emerged
493 significantly faster than the unprimed seed, illustrating the positive effects of priming on
494 seedling establishment for this crop. However, in this experiment, no further beneficial
495 effects of microorganism seed treatment were seen. Unexpectedly, in the second experiment
496 the primed control seed performed poorly and did not significantly improve emergence over

497 the unprimed control, and in fact resulted in a lower final stand. A possible reason for this
498 may be that a deleterious microorganism (either indigenous to the seed or accidentally
499 introduced during priming) was present in low numbers on the seed initially and proliferated
500 during priming, resulting in an increase in numbers to the extent that a negative effect on plant
501 health was seen. Previous research has shown that indigenous microorganisms on seed,
502 including potentially deleterious ones, increase during priming (Nascimento and West, 1998;
503 Tylkowska and van den Bulk, 2001; Wright et al., 2003a; Jensen et al., 2004; Olszewski et al.,
504 2005). An indication that such a biological factor was involved in the poor establishment of
505 primed control seed was also seen when pesticide application negated the effect, resulting in a
506 greater final stand. Importantly though, the application of beneficial microorganisms during
507 priming also negated the effect and significantly increased emergence and final stand
508 compared to the primed control seed in this experiment. In this case, the beneficial
509 microorganisms performed as well as the pesticide application in improving establishment of
510 onion seedlings. Although the two onion experiments provided contrasting results, together
511 they suggest that in the absence of deleterious or pathogenic microorganisms priming alone is
512 enough to improve onion seedling establishment, whereas in the presence of potentially
513 deleterious or pathogenic microorganisms the addition of beneficial microorganisms during
514 priming will provide the best chance for successful crop establishment. The positive effects
515 of beneficial microorganism application in the second glasshouse experiment were consistent
516 across the different soil types, which again in themselves showed variable effects on
517 emergence and seedling weight, showing a robust effect of the microbial seed treatments.

518

519 Although previous research in glasshouse and laboratory experiments has shown positive
520 effects of beneficial microorganism application to primed seed, there is little reported on the
521 performance of primed microbial treated seed in field trials. Those that have been tested

522 under field conditions include pearl millet (Niranjan et al., 2004); sweet corn (Harman et al.,
523 1989; Callan et al., 1991; Mathre et al., 1999); peas (Harman et al., 1989) and faba bean (El-
524 Mougy and Abdel-Kader, 2008). In many cases the seed was first coated with the beneficial
525 microorganism, sometimes using a 'sticker', and then subsequently soaked for priming. In
526 the current work, beneficial microorganisms were applied to seed in a large-scale commercial
527 priming system before being field-tested.

528

529 The inherent variability of the field sites meant that if consistent effects of the seed treatments
530 were seen across sites (or years) this would indicate a robust treatment that would provide
531 improved establishment or yield under a variety of conditions. Unlike in the glasshouse
532 experiments, priming did not produce a consistent positive effect across all field trials. Other
533 research has also shown inconsistencies in the effects of priming in the field with respect to
534 emergence and yield in other crops (Giri and Schillinger, 2003; Subedi and Ma, 2005). In this
535 work, as emergence was assessed daily in the glasshouse experiments, and only after 6-8
536 weeks in the field trials, it may be that any early effects of improved emergence time were
537 missed in the field. Also, seed priming is typically found to be more beneficial under cold
538 soil conditions (McQuilken et al., 1998; Halmer, 2004), where faster germination and
539 establishment allow escape from seedling diseases. Environmental conditions, including soil
540 temperature at the time of establishment, were not recorded in this work and it may be that the
541 conditions were good enough that no further benefit of priming was seen in this case. The
542 negative effect of priming seen with the onion seed in the glasshouse experiment in Year 2
543 was not clearly shown in the field trial, despite the same seed batches being used for both the
544 glasshouse and field experiments. Reasons for this are unclear, although it may be that the
545 effects were more apparent in the glasshouse because of more controlled conditions and a
546 shorter assessment time. Alternatively, in the field situation the indigenous soil microbial

547 communities may have had a suppressive effect on any deleterious microorganisms present on
548 the seed.

549

550 The current work particularly focused on the effect of microorganisms applied during
551 priming. Again, the effects seen in the glasshouse experiments were not obviously repeated
552 in the field trials, and although some positive effects were seen in some years or at some sites,
553 the selected microorganisms were inconsistent overall in their effects for both the carrot and
554 onion crops. Although for onions the final combined analysis across all years and sites
555 indicated that *C. rosea* IK726 increased the number and total weight of onions at harvest
556 compared to *T. harzianum* T22, this effect was not significantly different to the primed
557 control. However, the final combined analyses of field trial data showed that pesticides
558 improved establishment and increased yield for both carrot and onion crops for all years and
559 sites.

560

561 The lack of consistent positive effects of the applied microorganisms is not clear, although
562 factors such as dose rate may be important. Evidence suggested that the inoculum dose of
563 microorganisms on the seed may have been too high, possibly resulting in a negative effect on
564 establishment or yield. During priming, the microorganisms increased in number to a
565 maximum carrying capacity on the seed, which was above the target of 1×10^5 cfu g^{-1} seed.
566 These numbers may have been too high, having a deleterious rather than a beneficial effect on
567 the crop. Secondary metabolites or plant growth promoting hormones produced by
568 rhizosphere bacteria or fungi that promote plant growth in low concentrations may become
569 inhibitory in high concentrations (Maurhofer et al., 1992; Barazani and Friedman, 2001). For
570 example, on occasions in this work the primed control without pesticide performed better than
571 the microorganism treated seed without pesticide, suggesting a possible negative effect of the

572 microorganisms, which may be related to dose. Although not a consistent effect across all
573 years and sites, a trend was also noticed where on several occasions microorganism-treated
574 seed with pesticides had better emergence or resulted in higher yield than microorganism-
575 treated seed without pesticides. One possibility is that the addition of the pesticide may have
576 limited the proliferation of the microorganisms in the rhizosphere, keeping their numbers
577 within the range required to provide benefit to the plant. Earlier research showed that *C.*
578 *rosea* IK726 and *T. harzianum* T22 in particular proliferate in the rhizosphere of carrot and
579 onion (Bennett and Whipps, 2008a). Also, some evidence in this work showed that seed
580 treated with microorganisms and pesticides not only performed better than the microorganism
581 treated seed without pesticides, but also better than the primed control seed with pesticides,
582 indicating potential for combined seed treatments. Combining pesticide and microorganism
583 application to obtain a consistently effective dose in an integrated way is a challenge for
584 future work.

585

586 It is likely that microorganisms applied during priming may show more positive effects in
587 situations of specific disease control. In this work, positive effects of microorganism
588 application were particularly seen in the glasshouse onion experiment in Year 2, where the
589 primed control performed poorly, potentially due to the proliferation of a deleterious
590 indigenous microorganism on the seed. Other work has shown the benefit of microorganisms
591 applied during priming in situations of disease control. For example, bio-priming with
592 *Pseudomonas aureofaciens* AB254 improved stands of sweet corn under disease pressure
593 from *Pythium* spp., particularly in wet soils in the field (Callan et al., 1991; Mathre et al.,
594 1999), and also improved emergence of tomato seedlings grown in soilless media inoculated
595 with *Pythium ultimum*, compared to the primed control (Warren and Bennett, 2000); solid
596 matrix priming with strains of *Trichoderma* spp. improved plant stands in soil infested with

597 *Fusarium graminearum* or *Pythium ultimum* (Harman et al., 1989); *Pseudomonas fluorescens*
598 bio-primed onto pearl millet improved plant growth and induced resistance to downy mildew
599 caused by *Sclerospora graminicola* (Niranjan et al., 2004); and antagonistic microorganisms
600 including *T. harzianum*, *T. viride*, *T. hamatum*, *Bacillus subtilis*, *B. cereus* and *P. fluorescens*
601 reduced root rot disease when bio-primed onto faba beans in glasshouse and field trials (El-
602 Mougy and Abdel-Kader, 2008). Microorganism treated primed seed may be most beneficial
603 when used under conditions where pathogens are known to cause specific problems.

604

605 Although this work has shown that the technology for targeting beneficial microorganisms to
606 seed is commercially viable, it has also highlighted the problems with obtaining consistently
607 positive effects when using microbial seed treatments in a field situation. The challenge
608 exists to find microorganisms that are best suited to the crops of interest, which consistently
609 impart some benefit in terms of growth promotion or disease control under field conditions as
610 pesticides currently do. It may be viable to use microorganism primed seed under more
611 controlled conditions, *eg* used in module-raised crops. Another avenue for exploration is the
612 use of combinations of microorganisms, which may have different modes of action, or may
613 provide synergistic effects. It has been shown that combinations of bacteria and fungi can be
614 simultaneously primed onto carrot and onion seed under laboratory conditions (Bennett and
615 Whipps, 2008b), but the performance of seed primed with more than one microorganism has
616 yet to be tested in field situations. These aspects require further investigation.

617

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628

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