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Article Title: Performance of carrot and onion seed primed with beneficial microorganisms in glasshouse and field trials

Year of publication: 2009

Link to published version:
http://dx.doi.org/10.1016/j.biocontrol.2009.08.001

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

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Abstract

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

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

1. Introduction

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

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

start the physiological process of germination before the seed is planted. Following an incubation period, and before the radicle emerges from the seed coat, the procedure is stopped and the seed is dried back to a low moisture content. Priming ensures the entire seed batch is at the same point in the germination process, so that once it is planted primed seed has a more rapid and uniform emergence than unprimed seed (Rowse, 1996a; Rowse, 1996b). Microorganisms can be added to the water used to hydrate the seed during drum priming, frequently surviving and proliferating to high numbers on the seed (Wright et al., 2003b; Bennett and Whipps, 2008a).

Previous work demonstrated the success of this application method using laboratory-scale equipment and microorganisms applied to seed in this way survived on seed and in the rhizosphere of carrot and onion, in glasshouse-based assays (Bennett and Whipps, 2008a). This paper reports the application of beneficial microorganisms to carrot and onion seed during a commercial-scale drum priming system and the performance of the primed microorganism-treated seed in comprehensive glasshouse and field trials. The main aims were i) to apply selected microorganisms to carrot and onion seed during commercial-scale drum priming; ii) for each crop, to assess emergence and seedling fresh weight after 8 weeks growth in two glasshouse experiments; and iii) for each crop, to assess emergence and yield in a series of field trials carried out over three years, at two different sites each year. This is the first report of extensive field testing of beneficial microorganisms applied to seed during the commercially viable process of drum priming in the UK.

2. Materials and methods

2.1 Beneficial microorganisms

Beneficial microorganisms were selected for use in this study based on their known biocontrol or plant growth promotion properties, or availability within a commercial product. The bacterial isolates included *Pseudomonas chlororaphis* MA342, the active ingredient in Cedomon®, targeting cereal pathogens (Johnsson et al., 1998; Gerhardson, 2002), which was obtained from Dr M. Hökeberg, BioAgri, Uppsala, Sweden; and *Pseudomonas fluorescens* CHA0, which has activity against a wide range of soil-borne pathogens (Maurhofer et al., 1994) and was obtained from Prof G. Défago, Federal Institute of Technology, Zurich, Switzerland. The fungal isolates included *Clonostachys rosea* IK726, which has activity against seed-borne pathogens and plant growth promotion properties (Jensen et al., 2002; Jensen et al., 2004; Ravnskov et al., 2006) and was obtained from Dr D. F. Jensen, The Royal Veterinary and Agricultural University, Copenhagen, Denmark; *Trichoderma harzianum* T22, which was obtained from Dr G. Harman, Cornell University, Geneva, USA and is available commercially as T-22™ Planter Box and other formulations as a biocontrol agent; and *Trichoderma viride* S17a, which was available from the culture collection at Warwick HRI, University of Warwick, and has biocontrol activity against *Sclerotium cepivorum* (*Allium* white rot pathogen) (Clarkson et al., 2002; Clarkson et al., 2006). Wild-type strains of all isolates were used in this work. The minimum target application rate was $1x10^5$ cfu g^{-1} seed, as evidence in the literature suggests that disease control activity or plant growth promotion can be achieved when beneficial microorganisms are present above this rate on seed or roots, or in soil (Adams, 1990; Raaijmakers and Weller, 1998).

2.2 Seed treatments

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

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

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

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

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

2.3 Glasshouse experiment design and statistical analyses

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

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

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

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

2.4 Field trial design and statistical analyses

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

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

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

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

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

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

3. Results

3.1 Seed-applied microorganisms

In all years, all beneficial microorganisms applied to carrot and onion seed were recovered in excess of the target application rate of $1x10^5$ cfu g^{-1} dry seed (5 $\log_{10}$ cfu g^{-1} seed), irrespective of subsequent film-coating (carrot), pelletting (onion), or pesticide application (Table 2).

3.2 Carrot glasshouse experiments

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

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

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

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

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

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

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

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

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

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

3.5 Onion field trials

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

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

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

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

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

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

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

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

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

4. Discussion

Seed priming is an established technique for improving emergence, particularly under unfavorable conditions such as in cold or wet soil (McQuilken et al., 1998; Halmer, 2004). The application of beneficial microorganisms to primed seed has potential to further improve establishment of crops, and may provide disease control during the growing season if they become established on the roots. Previously, the microorganisms used in this work have been applied to seed using a laboratory scale drum priming system (Bennett and Whipps, 2008a). In the current work, microorganisms were successfully applied to carrot and onion seed during a commercial scale process of drum priming, and survived on seed following the standard commercial practices of film-coating carrot and pelletting onion seed, both with or without pesticide application. The performance of the treated seed was then tested in

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

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

For the glasshouse experiments using primed, microorganism treated onion seed no consistent effects were seen for the consecutive years. In the first experiment, primed seed emerged significantly faster than the unprimed seed, illustrating the positive effects of priming on seedling establishment for this crop. However, in this experiment, no further beneficial effects of microorganism seed treatment were seen. Unexpectedly, in the second experiment 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

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

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

The lack of consistent positive effects of the applied microorganisms is not clear, although factors such as dose rate may be important. Evidence suggested that the inoculum dose of microorganisms on the seed may have been too high, possibly resulting in a negative effect on establishment or yield. During priming, the microorganisms increased in number to a maximum carrying capacity on the seed, which was above the target of 1×10^5 cfu g^{-1} seed. These numbers may have been too high, having a deleterious rather than a beneficial effect on the crop. Secondary metabolites or plant growth promoting hormones produced by rhizosphere bacteria or fungi that promote plant growth in low concentrations may become inhibitory in high concentrations (Maurhofer et al., 1992; Barazani and Friedman, 2001). For example, on occasions in this work the primed control without pesticide performed better that 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 contol (Warren and Bennett, 2000); solid
596 matrix priming with strains of *Trichoderma* spp. improved plant stands in soil infested with

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

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

Acknowledgements

We acknowledge funding from the Horticulture LINK program, Elsoms Seeds Ltd., Germains Technology Group UK, the Horticultural Development Council, and the Department for Environment, Food and Rural Affairs. We thank Dr Richard Walker (formerly of GTG, UK)

and Rae Cook (Elsoms Seeds Ltd.) for preparing seed treatments for all trials. We also thank other members of the project consortium for their contribution, in particular Robin Wood (Elsoms Seeds Ltd.), Dr Graham Kinsey (GTG, UK) and Dr Peter Halmer (formerly of GTG, UK). We acknowledge the assistance provided by Horticultural Services staff at Warwick HRI, University of Warwick, for the field trials at the Wellesbourne site, and staff at Elsoms Seeds Ltd. for field trials at the grower sites.

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