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# A commercial iron fertilizer increases the survival of *Fusarium oxysporum* f. sp. *orthoceras* propagules in a wheat flour–kaolin formulation

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**Abstract**
*Fusarium oxysporum* f.sp. *orthoceras,* a potential biocontrol agent against the parasitic weed *Orobanche cumana* Wallr., has proven to be efficacous under greenhouse conditions when formulated as wheat–kaolin granules. To help minimize the loss of viable fungal propagules during the formulation process, the addition of a commercial fertilizer containing the iron chelate of EDDHA to the formulation was investigated. The proportion of surviving propagules was significantly increased after adding the fertilizer. However, growing conidia in fertilizer-amended liquid medium did not reduce losses in viability during formulation. The efficacy of the formulated fungus and the storability at room temperature for the first 3 months was not affected by the iron fertilizer. The protective effect could neither be obtained with the chelator EDDHA alone nor with FeEDTA or $FeSO_4$.

**Keywords:** Fusarium oxysporum, Orobanche *spp., FeEDDHA, iron chelate, formulation, mycoherbicide*

## Introduction

Sunflower broomrape (*Orobanche cumana* Wallr.) is one of the major constraints to sunflower production in the Mediterranean region and southeast Europe (Parker 1994). Because of the complex relationship between parasite and host, broomrape control is extremely difficult and only partially effective. *Fusarium oxysporum* Schlecht. f.sp. *orthoceras* (Appel & Wollenw.) Bilai (FOO) was identified as a host-specific and effective biocontrol agent against *O. cumana* (Bedi 1994; Thomas et al. 1998, 1999). Mass production systems and granular formulation techniques have been developed to improve the application and storage of the fungus as a bioherbicide (Müller-Stöver et al. 2002, 2004). The soil application of wheat–kaolin granules (Pesta) has given a high level of control of *O. cumana* in the greenhouse, reducing shoot emergence of the parasite by up to 80% compared to the untreated control. A reduction of about 90% of the initial colony forming units (cfu) occurs during formulation especially due to the drying process when microconidia are used as inoculum, although there are

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certain additives that may reduce these losses (Shabana et al. 2003). During investigations of the possibility of increasing the efficacy of the formulated material by adding micronutrients to the Pesta dough, we added a commercial fertilizer containing the iron chelate of ethylenediaminedi(*o*-hydroxyphenyl) acetic acid (FeEDDHA) and observed a significantly higher number of viable colony forming units in the formulation compared to other treatments. Since conservation of viable inoculum is critically important, the objective of this study was to confirm the stabilizing effect of the fertilizer, to determine the dosage required and to investigate its effects on storability and efficacy of the formulated material. Furthermore, the chelator alone and other iron compounds were examined for their ability to stabilize fungal propagules during the formulation process.

## Materials and methods

### *Fungal strains*

An isolate of FOO was obtained from Dr J.S. Bedi (Punjab Agricultural University, Ludhiana, India) in 1995 (Thomas et al. 1998). Stock cultures were maintained on Special Nutrient-poor Agar (SNA, Nirenberg 1976). For long-term preservation, the isolate was stored on SNA amended with 5% (v/v) glycerol at $-80^{\circ}$C at the Institute of Plant Production and Agroecology in the Tropics and Subtropics, University of Hohenheim, Germany.

### *Inoculum production*

Microconidia to be formulated were grown in liquid culture using 250-mL Erlenmeyer flasks containing 100 mL autoclaved malt extract medium (20 mL malt extract [Biomalt, Kirn, Germany], 0.5 g $KH_2PO_4$, 0.5 g $MgSO_4$, 2 g yeast extract, 0.2 g chloramphenicol per l $H_2O$ deion.). One agar plug (1 cm diameter) from a fungal culture on SNA was used to inoculate each flask. The flasks were incubated on a rotary shaker (150 rpm) at ambient laboratory conditions ($20 \pm 3^{\circ}$C) for 5 days. The content of the flasks was homogenised for 5 s in a blender and mycelial fragments were separated by filtration through four layers of cheesecloth. Conidial density of the liquid culture was determined with a haemacytometer.

*Preparation of Pesta formulations of* Fusarium *and assessment of shelf life.* To prepare standard Pesta granules, 32 g durum wheat flour, 2 g sucrose, 6 g kaolin and a 23-mL suspension of microconidia were blended (modified after Connick et al. 1991). This standard preparation was compared to other preparations containing wheat flour, sucrose and liquid inoculum with the addition of:

a. 0.1, 0.5 or 1 g of the commercial fertilizer Flory 72-Fe (FeEDDHA 6% Fe [Planta Düngemittel GmbH, Regenstauf, Germany]), made up to 6 g with kaolin, respectively;
b. 1.0, 1.5 or 2 g of Flory 72-Fe made up to 6 g with kaolin, respectively;
c. 1 g Flory 72-Fe and 1 g Flory 72-Fe + 2 g yeast extract made up to 6 g with kaolin, respectively; and
d. Fetrilon (FeEDTA, 13% Fe [Compo GmbH, Münster, Germany] 1 g or 0.1 g) or $FeSO_4 \times 7$ $H_2O$ (0.3 or 0.03 g), made up to 6 g with kaolin, respectively.

To test whether the observed effect on propagule survival was due to an increased pH, the pH of the inoculum (pH 4.4) was raised by the addition of NaOH to the same value that was obtained by the addition of 1 g Flory 72-Fe (pH 7.4) prior to formulation.

Furthermore, the addition of EDDHA at a rate of 100 mg (dissolved in 2 N NaOH and pH adjusted to 6) per 40g-batch of formulation was compared to standard granules and to a preparation containing 100 mg of Flory 72-Fe.

Each mixture was kneaded and passed through a pasta maker. The resulting dough sheets (1–1.5 mm thick) were air-dried on aluminium foil, milled in a laboratory mill (Grindomix GM200, Retsch, Germany) and sieved to a particle size of 0.5–2 mm. The average water content of the final preparations was 7–8%. Cfu $g^{-1}$ formulated material was assayed by placing 0.1 g of the preparation into test tubes with 10 mL sterile $H_2O$ and three glass beads (0.6 cm diameter) and vortexing with repeated pulses to disintegrate the particles. One hundred-μL aliquots of appropriate dilutions were plated on half-strength PDA amended with 100 ppm chloramphenicol (three plates per each of three samples) and cfu $g^{-1}$ formulation were determined after incubation for 3 days at room temperature ($20 \pm 3^{\circ}C$). The formulations were stored in the refrigerator at 4°C in sealed zipper-top plastic bags ($14 \times 8$ cm). For assessment of shelf life of Pesta formulations, cfu $g^{-1}$ within the formulated material was assayed as described above after 3 months of storage at room temperature.

*Effect of Flory 72-Fe in the fungal growth medium on the subsequent survival of propagules in the formulation process.* To determine the potential stabilizing effect of Flory 72-Fe on conidia of FOO in the growth medium, 0 or 1 g of the fertilizer was added after autoclaving per each of two flasks containing 100 mL of malt extract medium, respectively. Conidia harvested were washed twice with deionised $H_2O$ and adjusted to the same conidial density ($1.7 \times 10^8$ $mL^{-1}$) in each treatment before formulation.

*Greenhouse experiments*

Sunflower and its parasite *O. cumana* were grown in a greenhouse at $25/15(\pm 5)^{\circ}C$ (day/night) with 13 h supplemental light (1000 W) in plastic pots ($13 \times 13 \times 13$ cm) containing pasteurized soil up to two-thirds of the pot depth. One g of standard Pesta granules ($3.5 \times 10^6$ cfu $g^{-1}$) or granules amended with Flory 72-Fe ($1.6 \times 10^6$ cfu $g^{-1}$) and 30 mg *O. cumana* seeds were mixed into the top 5 cm of the soil in each pot. Pots containing only *O. cumana* seeds served as a negative control. All pots were then filled with an additional 5 cm of soil. The experiment was arranged in a completely randomized design with five replicates per treatment. Three sunflower seeds (*Orobanche*-susceptible cultivar HA 89) were sown into each pot and plants were thinned to one plant per pot after 14 days. At the end of the experiment, emerged *O. cumana* shoots were counted, the soil was washed from sunflower roots and the total number of parasite shoots was determined. The experiment was repeated with susceptible sunflower cultivar Albena, standard Pesta granules containing $2.6 \times 10^6$ cfu $g^{-1}$ and granules amended with Flory 72-Fe containing $2.7 \times 10^6$ cfu $g^{-1}$.

*Statistical analysis*

All experiments were repeated once. Statistical analysis was applied to the combined data of repeated experiments when they had homogenous variances after Levene's test

(Levene 1960). Pairs of means were compared with Fisher's $t$-test. For multiple mean comparisons, analysis of variance (ANOVA) followed by Tukey's honest significant difference test (HSD) was used. All tests of significance were conducted at $P \leq 0.05$. When data were not normally distributed or showed heterogeneity of variances, they were square-root or log-transformed before analysis. Percentage data were arcsine-transformed before analysis (Gomez and Gomez 1984). Statistical analyses were performed using STATISTICA software (StatSoft Inc. 1997).

## Results and discussion

The preparations containing Flory 72-Fe yielded a significantly higher concentration of cfu $g^{-1}$ after the formulation process compared to the standard Pesta granules (Table I). Even 0.1 g Flory 72-Fe per 40-g batch of formulated material caused a significantly greater yield of cfu $g^{-1}$ than the standard granules, but significantly lower than preparations with 1 and 0.5 g, respectively. However, increasing the dosage to more than 1 g did not bring any further benefit regarding the number of viable cfu after formulation.

In previous experiments, we could double the survival (cfu) of microconidia per g of Pesta formulation compared to the preparation containing only sucrose by adding yeast extract (Shabana et al. 2003). The addition of 1 g Flory 72-Fe per 40 g batch of Pesta granules resulted in an at least 5-fold increased number of viable cfu and was more effective in protecting the fungal propagules than yeast extract. However, adding both yeast extract and Flory 72-Fe did not give any additional benefit (Table II).

No difference in efficacy regarding *Orobanche* control was observed between the standard Pesta granules and the granules amended with Flory 72-Fe in the pot experiment, under the soil and environmental conditions used. Both formulations significantly decreased the total number of *O. cumana* shoots (by 69 and 64%) and the number of emerged shoots (by 70 and 64%), respectively (Table III). After 3 months storage at room temperature, granules without fertilizer had lost about 80% of their initial cfu, whereas the granules supplemented with Flory 72-Fe lost about 64%. These differences were not statistically significant. Thus, the addition of Flory 72-Fe

Table I. Effect of Flory 72-Fe added in different dosages to the dough on the survival of FOO propagules during the Pesta formulation process.

| *Experiment I* | |
|---|---|
| Additive (per 40 g batch) | Cfu $g^{-1}$ formulated material ($\times 10^6$) |
| No additive (standard granules) | 5 (0.3) **a** |
| Flory 72-Fe 0.1g | 15.7 (2.6) **b** |
| Flory 72-Fe 0.5 g | 28.4 (3.4) **c** |
| Flory 72-Fe 1g | 36.2 (5.7) **c** |
| *Experiment II* | |
| Additive (per 40 g batch) | Cfu per g formulated material ($\times 10^6$) |
| No additive (standard granules) | 5.7 (0.6) **a** |
| Flory 72-Fe 1 g | 64.3 (4.0) **b** |
| Flory 72-Fe 1.5 g | 63.3 (4.0) **b** |
| Flory 72-Fe 2 g | 60.5 (7.4) **b** |

Values are the means of two experiments. Means followed by the same letter are not significantly different according to Tukey's HSD test at the $\alpha = 0.05$ level of significance. Values in parentheses are SEs.

Table II. Effect of Flory 72-Fe and Flory 72-Fe+yeast extract added to the dough on the survival of FOO propagules during the Pesta formulation process.

| Additive (per 40 g batch) | Cfu $g^{-1}$ formulated material ($\times 10^6$) |
|---|---|
| No additive (standard granules) | 4.2 (0.8) **a** |
| Flory 72-Fe 1 g | 71.9 (9.2) **b** |
| Flory 72-Fe 1 g+yeast extract 2 g | 61.0 (7.6) **b** |

The repeated experiments could not be analyzed together due to heterogeneity of variances. Values presented are the means of one experiment, the second experiment gave identical statistical significances. Means followed by the same letter are not significantly different according to Tukey's HSD test at the $\alpha = 0.05$ level of significance. Values in parentheses are SEs.

does apparently not influence the storability of the formulated material over the first 3 months.

It can only be speculated what mechanisms cause the increased survival of propagules after adding Flory 72-Fe. Since pure FeEDDHA is not commercially available and the amount of EDDHA available for the experiments was only very limited, FeEDDHA was not tested as a single additive to the formulation. Generally, it can be assumed that a reaction of Flory 72-Fe with the other components of the formulation or an influence of one of the ingredients on the ability of the fungal spores to survive the drying process or to germinate again out of the dry granules is the reason for the observed effect. Conidia harvested from media containing Flory 72-Fe survived the formulation process only slightly or marginally better than the conidia from the non-amended medium ($1.1 \times 10^6$ cfu $g^{-1}$ and $8 \times 10^5$ cfu $g^{-1}$, respectively). This indicates that the conidia are not predisposed to surviving the formulation process by adding FeEDDHA such as the addition of sucrose did for *Sclerotinia sclerotiorum* (Lib.) de Bary as reported by Quimby et al. (2004), but that the observed effect occurs during formulation. Since the addition of Flory 72-Fe increases the pH of the inoculum from about 4.4 to 7.4, we tested if the observed effect could be attributed to this increase. When the pH was increased to the same level by adding NaOH, only a slight increase in propagule survival could be observed in one experiment, which could not be repeated (Table IV).

Adding the chelator EDDHA alone (Table V) did not result in any change in propagule survival which indicates that this substance is not responsible for the observed effect. Several additives are known to protect microorganisms during drying, especially sugars such as sucrose and trehalose (Elbein et al. 2003) that prevent phase

Table III. Efficacy of FOO formulated in Pesta granules without and with Flory 72-Fe on the control of *O. cumana* in a greenhouse experiment.

| | Total number of *Orobanche* shoots | Number of emerged *Orobanche* shoots |
|---|---|---|
| Control | 10.6 (1.7) **a** | 9.4 (1.7) **a** |
| Standard Pesta granules | 3.3 (2.1) **b** | 2.8 (2.1) **b** |
| Pesta granules amended with 1 g Flory 72-Fe per 40 g batch | 3.8 (0.8) **b** | 3.4 (0.7) **b** |

The repeated experiments could not be analyzed together due to heterogeneity of variances. Values presented are the means of one experiment. In the second experiment, no statistically significant differences between the standard granules and the Flory 72-Fe -amended granules were observed again. Means followed by the same letter are not significantly different according to Tukey's HSD test at the $\alpha = 0.05$ level of significance. Values in parentheses are SEs.

Table IV. Influence of an increased pH on the subsequent survival of FOO propagules in Pesta granules.

| pH of inoculum | Cfu $g^{-1}$ formulated material ($\times 10^6$) |
|---|---|
| *Experiment I* | |
| 4.1 (no additive) | 2.3 (0.2) **a** |
| 7.1 (Flory 72-Fe 1 g per 20 mL) | 10.1 (0.3) **c** |
| 7.1 (by adding NaOH 0.5 M) | 3.6 (0.1) **b** |
| *Experiment II* | |
| 4.6 (no additive) | 6.8 (0.3) **a** |
| 7.6 (Flory 72-Fe 1 g per 20 mL) | 37.9 (8.0) **b** |
| 7.7 (by adding NaOH 0.5 M) | 5.5 (0.7) **a** |

Means followed by the same letter are not significantly different according to Tukey's HSD test at the $\alpha = 0.05$ level of significance. Values in parentheses are SEs.

transitions in the membrane from the liquid-crystalline to the gel-phase during dehydration (Tan 1997). However, we do not know of any reports on the effect of micronutrients on fungal cells undergoing dehydration. In contrast, the influence of iron on fungal germination and germ tube elongation is documented (Scher & Baker 1982; Simeoni et al. 1987). However, the fact that neither $FeSO_4$ nor Fe chelated by ethylenediaminetetraacetic acid (Fetrilon, Table V) increased propagule survival could be a hint that iron is not the causal agent of the observed effects. It could also be that Fe in these forms is not available to the fungal cells or that other components had a negative impact on propagule survival. For example, EDTA was shown to have antifungal activity and is therefore used for medical or food-preserving purposes (Kubo et al. 2005; Hachem et al. 2006). Grcman et al. (2001) reported toxic effects of EDTA on soil fungi. Similarly, in our own experiments, the addition of NaEDTA to the formulation had an adverse effect on the number of viable cfu (data not shown). Negative effects of the iron itself on the survival of fungal cells cannot be excluded either, especially when $FeSO_4$ had been incorporated at the higher rate. Fe can exhibit strong toxicity to living cells, especially by the formation of reactive oxygen species (Schützendübel & Polle 2002).

It can thus be summarized that there is a pronounced positive effect on FOO propagule survival when adding the commercial fertilizer to the granular formulation

Table V. Effect of various iron compounds in different levels and the chelator EDDHA on the survival of FOO propagules during the Pesta formulation process.

| Additive (per 40 g batch) | Cfu $g^{-1}$ formulated material ($\times 10^6$) |
|---|---|
| *Experiment I* | |
| No additive (standard granules) | 3.9 (0.9) **a** |
| Fetrilon 0.1 g | 3.7 (0.7) **ab** |
| Fetrilon 1g | 2.8 (0.2) **ab** |
| $FeSO_4$ 0.03g | 3.1 (0.8) **ab** |
| $FeSO_4$ 0.3 g | 0.04 (0.01) **b** |
| *Experiment II* | |
| No additive (standard granules) | 0.3 (0.1) **a** |
| EDDHA 0.1 g | 0.3 (0) **a** |
| Flory 72-Fe 0.1 g | 1.0 (0.4) **b** |

Values are the means of two experiments. Means followed by the same letter are not significantly different according to Tukey's HSD test at the $\alpha = 0.05$ level of significance. Values in parentheses are SEs.

which significantly increases the yield of viable inoculum. However, the mechanisms behind this effect are in need of further investigation.

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