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Improving the formulation and timing of application of endophytic biocontrol and chemical agents against frosty pod rot (*Moniliophthora roreri*) in cocoa (*Theobroma cacao*)

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ABSTRACT

Frosty pod rot (FPR), caused by *Moniliophthora roreri*, reduces yields of cocoa (*Theobroma cacao*) by over 80% within a few years of disease outbreak. Both biological and chemical control approaches are being developed to supplement cultural management. Our objectives were to optimize the agent(s), their formulation and application regime for FPR control in Costa Rica. In order to economize scarce resources for field research, we opted for an iterative optimization of treatments. We aimed to (partly) replace copper hydroxide of proven efficacy with two systemic agents, the coevolved endophyte *Trichoderma ovalisporum* and the oxathiin flutolanil, and assessed four stickers for improving the agents' performance. Over 3 years, we selected effective agents and their combinations: eight of 18 treatments increased yields. Orthogonal contrasts extracted three general trends: (1) Copper should be applied in water, whereas the systemic agents were best formulated with a sticker. There was no difference between the stickers BreakThru and NP-7. (2) If the first rule was observed, a switch from systemic agent to the contact fungicide in mid season was beneficial. The optimum timing for this change requires further definition. (3) *T. ovalisporum*-containing agents controlled black pod better than flutolanil-based ones; both systemic agents were equally effective in controlling the more severe FPR. We discuss potential benefits of switching agents in the context of organic agriculture and argue that coevolved endophytes may be particularly suited for the classical biocontrol of *Moniliophthora* spp. in cocoa planted outside its South American centre of origin.

1. Introduction

The centre of diversity, and thus the assumed centre of origin, of cocoa (*Theobroma cacao* L.) is the upper Amazon basin in Peru. Interestingly, the centre of domestication is Mexico and Central America (Wood, 1985; Evans et al., 1998). Two major fungal diseases limit cocoa production in the Americas: witches' broom disease, caused by *Moniliophthora* (formerly: *Crinipellis*) *perniciosa* (Stahel) Aime and Phillips-Mora, and frosty pod rot (FPR), caused by *Moniliophthora roreri* (Cif) Evans. Witches' broom is endemic throughout the cocoa-producing areas of South America from Bolivia to Guyana, and on some Caribbean islands. FPR is a highly invasive disease, currently affecting Peru, Ecuador, Colombia, Venezuela, Panama, Costa Rica, Nicaragua, Honduras, Guatemala, Belize, and Mexico (Phillips-Mora et al., 2007). Within a few years of establishment, FPR reduced yields in Central America by over 80% (Krauss et al., 2003). *M. perniciosa* is believed to have coevolved with *T. cacao* on the eastern side of the Andes, whereas *M. roreri* probably evolved in Colombia and/or Ecuador on the Pacific (Western) side of the Andes on the forest host *Theobroma gileri* L. (Evans et al., 2003a; Phillips et al., 2003).

Numerous strategies for control of both diseases have been investigated and some are recommended. These, invariably, centre on cultural control with various supplementary control options, such as biological and chemical control (Bateman et al., 2005a), at different stages of development. Bateman et al. (2005b) evaluated modern fungicides, such as triazoles and oxathiins, in comparison with a copper standard for FPR control. Copper hydroxide at 1500 g a.i. ha^{-1} per production season was most effective in FPR

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control. However, even targeted applications of these fungicides, using a motorized mistblower configured for cocoa, become cost-effective in Costa Rica only when farm-gate prices reach \$1.25 kg^{-1}, which is rarely the case, at least for conventional cocoa. Of the systemic fungicides, the oxathiin flutolanil improved total pod production at the relatively high rate of 300 g a.i. ha^{-1}, but gave proportionately less control of FPR at the height of the season. The authors, thus, suggested testing a combination regime in which the systemic agent is applied during the early season, followed by the contact fungicide later in the season (Bateman et al., 2005b).

Biological control of *Moniliophthora* spp. can be categorized into two approaches: classical and inundative biocontrol. Inundative biocontrol resorts to antagonists native to the area where the pathogen is to be controlled. The argument in favor of inundative biocontrol is adaptation of the agent to local agroecological conditions. In order to achieve good control, these agents typically have to be applied in relatively large quantities and repeatedly throughout the season, which is expensive. This approach has been developed for the control of cocoa pod diseases in Peru with yield increases of up to 16.7% (Krauss and Soberanis, 2001, 2002), but proved less promising in Costa Rica (Krauss et al., 2003).

In contrast, the classical approach is applicable in situations where a pest has been introduced into new regions. In the absence of natural enemies, the exotic pest escalates into a problem, frequently far more serious than in the country of origin. In such cases, the strategy is to introduce a coevolved, natural enemy of the pest into the new location to re-establish the natural equilibrium (Evans, 1999; Bateman et al., 2005a). Establishment can be the bottleneck with this approach, but, if successful, the introduced agent becomes self-perpetuating. Classical biocontrol was originally developed for the control of exotic insect pests and weeds. For control of pathogens, research is in its infancy, but Evans et al. (2003b) collected numerous candidates for FPR control from *T. gileri* in Western Ecuador, most notably *Clonostachys* spp. and *Trichoderma* spp. Subsequent field screens in Costa Rica demonstrated that *Trichoderma ovalisporum* Samuels and Schroers strain TK-1 could be reisolated from cocoa pods 10 weeks after inoculation and suggested an adverse effect on sporulation by *M. roreri* (Evans et al., 2003c; Holmes et al. 2006). *T. ovalisporum* TK-1 is an endophyte, i.e. a fungus that develops asymptomatic infections within healthy plants to form a mutualistic symbiosis. Arnold et al. (2003) showed the potential of cocoa endophytes to protect seedlings from leaf infection by *Phytophthora* sp. One argument in favor of endophytic biocontrol agents (BCAs) is that these would face a much wider window of opportunity for antagonism, given that spores of *M. roreri* germinate and penetrate the pod surface soon after landing there and establish a systemic pod infection which can be latent for 2 months (Evans, 1981).

Our objectives in this study were to optimize the treatments for FPR control in Costa Rican cocoa in terms of the agent(s) used – biological and chemical, their formulation and application regime. In particular, we aimed to reduce the application of copper, while maintaining or increasing productivity.

2. Materials and methods

2.1. Control agents and adjuvants

Trichoderma ovalisporum strain TK-1 (= DIS70a, IMI390990) was isolated as an endophyte of the liana *Banisteriopsis caapi* (Spruce ex Griseb.) Morton (Malpighiaceae) from the high tropical forest in the Sucumbios Province, Ecuador, in 1999. It was closely associated with the L-type of witches' broom pathogen *M. perniciosa* (Holmes et al., 2004). This isolate colonized stems and unhardened meristems of cocoa seedlings. It also parasitized *M. roreri in vitro* and on infected cocoa pods (Holmes et al., 2005). Preliminary field screens in La Lola, Costa Rica, indicated that *T. ovalisporum* TK-1 persisted on the surface of non-sterilized cocoa pods for 10 weeks (Holmes et al., 2004, 2005). Subsequently, its endophytic establishment was also proven, at least if pods had been surface-sterilized prior to inoculation (Holmes et al., 2006). We, therefore, interpret *T. ovalisporum* as a systemic, classical biocontrol candidate.

Inoculum for laboratory assays was prepared in Petri dishes containing half-strength potato-dextrose agar (PDA) (10 g PDA and 20 g agar l^{-1}, Difco, Sparks, MD). Inoculum for field trials was prepared using the rice method as described by Krauss et al. (2002). Spores were separated from the rice using a mycoharvester MH1 (http://www.dropdata.net/mycoharvester) and the dry spores were stored at 4 °C in air-tight containers until use (max. 4 weeks). Germination of field inoculum on half-strength PDA was consistently >98% (data not shown).

Two chemical fungicides were used: copper hydroxide (Kocide; Abonos Superior S.A., Cartago, Costa Rica: distributor for Griffin Corp.), a contact fungicide, and flutolanil (Moncut 50% WP; Aventis), an oxathiin and thus systemic agent with specificity against the basidiomycetes. Both fungicides had previously shown promise in the control of FPR (Hidalgo et al., 2003; Bateman et al., 2005b).

The stickers tested here were BreakThru 100SL (BASF), a non-ionic wetter, spreader and penetrant surfactant, active ingredients: polyether–polymethylsiloxane copolymer; Citowett™ 100% (BASF), an alky aryl polyglycol ether; NP-7™ (Bayer), a nonylphenol polyglycol ether; Nu Film®-17 (DuPont), active ingredient the pine resin Pinolene® (di-1-*p*-menthene); and Pegafix (Abonos Superior, Costa Rica), active ingredient: polyethylene 11% v/v.

2.2. Effect of sticker on germination of potential biocontrol agents

Spore suspensions of *T. ovalisporum* TK-1 were adjusted to 3.7×10^7 spores ml^{-1} in water with or without a sticker, using a hemocytometer. Stickers were used at their recommended range of formulated product: BreakThru: 0.30 ml l^{-1}, Citowett: 0.25 ml l^{-1}, NP-7: 1.0 ml l^{-1}, Nu Film-17: 0.45 ml l^{-1} and Pegafix: 1.5 ml l^{-1}, at half and at double that concentration. Water served as control. Conidial suspensions were plated out onto 20% PDA and incubated at 25 °C. After 14–16 h of incubation, between 100 and 200 conidia per sample were assessed for germination under a compound microscope. A spore was considered germinated if the germtube had reached at least the length of half the spore diameter.

2.3. Field survival of T. ovalisporum with and without the sticker NP-7

This trial was installed at CATIE's La Lola field station in Costa Rica (altitude 70 m; access point: 10° 5′ 18.7″ N; 83° 23′ 20.2″W) in March 2004 and monitored for 7 weeks. The experimental area was planted with 45 different hybrid seedlings at a spacing of 3 m × 3 m in 1992. Trees height was maintained at approximately 3 m. A total of 120 healthy pods (30 per treatment) of 3–5 months of age were chosen randomly and surface sterilized by wiping them with 70% alcohol.

The following four treatments were applied to an initial 30 pods per treatment: *T. ovalisporum* at 10^6 conidia ml^{-1} suspended either in water or sticker (NP-7 at 1 ml l^{-1}), and water or sticker as controls. Agents were applied with hand-held, pressurized sprayers (Volpi & Bottoli, Piadena, Italy). Each pod received approximately 8 ml of liquid. The persistence of *T. ovalisporum* TK-1 and recolonization of surface-sterilized pods by other fungi was assessed using the pre-colonized-plate baiting technique with the highly susceptible black pod pathogen, *Phytophthora palmivora* (Butl.) Butl. strain CPhy13, as follows. Every week, starting from the day of

inoculation and continued for 7 weeks, three peel disks (diameter 12 mm) were excised from each of 10 replicate pods. The remaining 20 pods served as replacement for the ones that decayed after earlier sampling. This resulted in a factorial (2 × 2) design with 10 effective replicate pods for each sampling date. The surface layer of the discs was removed with a scalpel and air-dried (ca 3 days). The dried discs were then placed onto agar plates pre-colonized with *P. palmivora*, with the cuticula facing down so that the original pod surface was resting on the host mycelium. The development of *T. ovalisporum* on the host was observed for up to 1 week and scored as presence or absence per peel disc.

2.4. Efficacy test of biological and chemical control agents with and without sticker

2.4.1. Trial layout

A series of three field trials was carried out at La Lola with the objective to assess the efficacy of biological and chemical control agents with and without sticker in different combinations, which were optimized from year to year, thus leading to an overall unbalanced design. Table 1 gives an overview of treatments applied in each season. All trials followed the randomized complete block design. Plots (experimental unit) consisted of 8 × 8 trees, comprising a 4 × 4 tree assessment zone of trees that were both sprayed and assessed, surrounded by one row of trees that were sprayed but not assessed in order to prevent edge effects (Hidalgo et al., 2003). A perimeter row of unsprayed guard trees per plot prevented cross-contamination. Thus, treatments in neighboring plots were at least 9 m apart and separated by at least two rows of guard trees. Three to five replicate plots per treatment were marked, depending on the number of treatments to be tested in that year (Table 1). Prior to installation, all developing pods were removed from the assessed trees to pre-empt quantifying latent infections. During the trial, phytosanitary removal of diseased pods and epiphytic plants (mostly mosses) was performed according to local practices throughout the experimental area, including control plots and border rows. Less well-maintained fields surrounded the trial site.

The first trial, which was conducted from September 2003 to May 2004, was based on the hypothesis that a systemic agent is best in the early stages of pod development, when the pod surface expands rapidly; whereas, a copper-based contact fungicide is better later on. This idea emerged from the previous season's trial, where the systemic flutolanil gave a marked increase in the number of young healthy pods, but then fell back behind copper due to FPR (Bateman et al., 2005b). Therefore, we decided to investigate three individual agents and their pairwise combination in time, i.e. after 5 months, we switched from the systemic agent (the BCA *T. ovalisporum* TK-1 or the chemical flutolanil) to copper for the last two applications (Table 1). For statistical analysis, a treatment was represented either by an agent applied throughout the season or by the sequential combination of two agents in time. Thus, there were five treatment levels for agents; a second factor was the presence of absence of the sticker NP-7, resulting in a treatment arrangement of: 5 × 2 factorial + control with three replicate plots per treatment.

Based on the results from this first (2003/2004) trial, we further hypothesized the sticker NP-7 to be beneficial to the systemic agent, but not to the copper fungicide. Thus, such combinations were prioritized for the second trial (Table 1), which was conducted from April 2005 to February 2006. Our specific objective here was to optimize the date for switching from the systemic agent (biological or chemical) to copper hydroxide. Therefore, we compared a switch-over after 4 months with a switch-over after 6 months and with no switch-over at all, again with each of the three sequences constituting a replicated treatment (see Table 1 for treatment details). A second comparison was between the BCA *T. ovalisporum* TK-1 and the chemical flutolanil. Both an absolute control and the copper standard were maintained as controls, resulting in a treatment arrangement of 3 × 2 factorial + 2 controls. The lower number of treatments prioritized in 2005/2006, allowed the installation of five replicate plots per treatment (Table 1).

Table 1
Treatments [a] tested (+) in cocoa field trials La Lola, Costa Rica, over three growing seasons.

	2003/2004	2005/2006	2006/2007
Kocide in water	+	+	+
Kocide with NP-7	+	–	–
Flutolanil in water	+	–	–
Flutolanil in water for 5 months, followed by Kocide in water	+	–	–
Flutolanil with BreakThru	–	–	+
Flutolanil with BreakThru for 6 months, followed by Kocide in water	–	–	+
Flutolanil with NP-7	+	+	–
Flutolanil with NP-7 for 4 months, followed by Kocide in water	-	+	–
Flutolanil with NP-7 for 5 months, followed by Kocide with NP-7	+	–	–
Flutolanil with NP-7 for 6 months, followed by Kocide in water	–	+	+
Trichoderma ovalisporum TK-1 in water	+	–	–
Trichoderma ovalisporum TK-1 in water for 5 months, followed by Kocide in water	+	–	–
Trichoderma ovalisporum TK-1 with BreakThru	–	–	+
Trichoderma ovalisporum TK-1 with BreakThru for 6 months, followed by Kocide in water	–	–	+
Trichoderma ovalisporum TK-1 with NP-7	+	+	-
Trichoderma ovalisporum TK-1 with NP-7 for 4 months, followed by Kocide in water	-	+	-
Trichoderma ovalisporum TK-1 with NP-7 for 5 months, followed by Kocide with NP-7	+	–	–
Trichoderma ovalisporum TK-1 with NP-7 for 6 months, followed by Kocide in water	–	+	+
Untreated control	+	+	+
Number of treatments tested	11	8	8
Replicate plots	3	5	5
Total number of plots	33	40	40
Number of spray applications	7	13	10
Arrangement	5 × 2 factorial + control	3 × 2 factorial + 2 controls	3 × 2 factorial + 2 controls

[a] Application rates of agents were: Kocide (copper hydroxide), 1500 g ha^{-1}; *T. ovalisporum* TK-1: 5 × 10^{12} cfu ha^{-1}; flutolanil: 300 g ha^{-1}. Concentrations of stickers were: NP-7: 1 ml l^{-1} and BreakThru: 0.3 ml l^{-1}.

The switch-over to copper after 6 months appeared somewhat preferable during the second trial season, so this application regime was chosen for the key comparison of the third trial season, which ran from April 2006 to March 2007: the sticker NP-7 was compared with a novel adjuvant for formulating the systemic agents: BreakThru. For comparison, both systemic agents (biological and chemical), formulated with BreakThru and applied throughout the season, were also tested on their own (Table 1). These agents, formulated with NP-7, had already been evaluated for two growing seasons and were thus not tested in 2006/2007. The second comparison was again between the BCA *T. ovalisporum* TK-1 and the chemical flutolanil. The two controls of the previous trial season were maintained, thus resulting in the same treatment arrangement as the second trial season (3×2 factorial + 2 controls with five replicates; Table 1).

2.4.2. Application techniques

The final concentrations of our agents were: *T. ovalisporum* TK-1: 5×10^{12} cfu ha^{-1}, copper hydroxide: 1500 g ha^{-1}, flutolanil: 300 g ha^{-1}, and for the stickers: NP-7: 1 ml l^{-1} and BreakThru: 0.3 ml l^{-1}. All agents were pre-weighed in the laboratory and freshly suspended in the field immediately before application.

Agents were applied using a Stihl SR400 (Andreas Stihl AG & Co., Waiblingen, Germany), with a 1.0 restrictor. When calibrated with the motor operating at full-throttle and the nozzle directed upwards at 45°, the measured flow rate was approximately 550 ml min^{-1}, delivering an estimated equivalent of 190 l ha^{-1} (Bateman et al., 2005b). At this flow rate, the sprays have a volume median diameter (VMD) of approximately 50 μm with water-based formulations. No comparable information is available for the sticker-containing formulations. The VMD is mostly governed by nozzle characteristics, but can be markedly reduced by organo-silicone surfactants (Stevens, 1992), such as BreakThru.

A total of 11 operators applied control agents during the three trial seasons, but not all operators in all years. They used three different mistblowers, one dedicated to the BCA, one to copper and one to flutolanil. Each year, operators were randomized across treatments and mistblowers, which were partially confounded within treatments. In order to assess the effectiveness of this restricted randomization, spray logs were maintained as follows: for each treatment, the amount of tank mixture prepared exceeded the volume needed to treat the plots. After spraying, the excess was recorded for subsequent analysis of the volume application rates (VARs).

Applications were made in approximately monthly intervals throughout the production cycle. This resulted in seven sprays in the 2003/2004 seasons, 13 in 2005/2006 and 10 in 2006/2007 (Table 1). Occasionally, spraying had to be postponed by one or two days because of rain. Other weather conditions posed no hindrance to applications.

Samples of the harvested, fermented and dried cocoa seeds of the plots with flutolanil applied throughout the production cycle were analyzed for flutolanil residue using HPLC chromatography by the *Centro Químico de Investigación y Asistencia Técnica* (CEQUIATEC), Cartago, Costa Rica. A total of eight samples were submitted for the 2005/2006 and 2006/2007 seasons.

2.5. Data recording and statistical analysis

The germination test had a 5×3 factorial arrangement as five stickers were assayed at three concentrations each, excluding the water controls. To render experiments carried out at different times comparable, data were scaled: percentage germination was divided by the percentage germination in SDW. As this occasionally lead to values over 100%, no transformation was applied prior to two-way analysis of variance (ANOVA) on Infostat (Infostat, 2004). The Duncan test was used to separate means whenever ANOVA indicated significant effects.

Presence/absence data of *T. ovalisporum* TK-1 on excised cocoa peel disc were averaged to obtain an estimate of percentage pod colonization. The change in the percentage colonization was analyzed by Gompertz regression on SAS (SAS Institute, 1985). The Gompertz curve is given by the equation:

$$Y = A + C\exp^{(-\exp)-B(X-M)}$$

where X is the is time in (independent variable); Y is the percentage colonization of cacao pods with *Trichoderma* TK-1 (dependent variable); A is the lower asymptote, i.e. final colonization percentage for declines; C is the upper asymptote, i.e. initial colonization percentage for declines; B is a measure (unit: $week^{-1}$) of the maximum rate of colonization, which is derived by the equation: $C \times B \times e^{-1}$; and M is the point of inflexion (unit: weeks).

Meteorological data were measured on site and are available on-line (Salas, undated). VARs (dosages applied per hectare) recorded in spray logs were subjected to three-way ANOVA for treatment, sprayer and operator, on SAS. Because the factor sprayer was partly confounded in treatment, only main effects and first-order interactions were tested (Zar, 1996).

Efficacy evaluation consisted of fortnightly harvesting and counting healthy, mature pods, and quantifying and removing any diseased pod of at least 8 cm in length. Dead pods shorter than 8 cm were attributed to cherelle wilt, a physiological disorder, which affects young pods and cannot be distinguished with certainty from diseases at this stage.

Disease incidence (percentage data) was arcsine-transformed prior to analysis to normalize the error distribution. Initially, each year's data were analyzed for differences between treatments on InfoStat (InfoStat, 2004). Means of field data with significant effects were separated by the Duncan test, as this test matched the outcome of the preceding ANOVA best. When few significant effects were found for the first two trial seasons individually, the 3 years' data were subjected to a two-way ANOVA with the factors treatment (which consisted of each combination of the agent(s), adjuvant and/or switch-over) and trial season. While giving a reasonable indication of the reproducibility of effects and thereby aiding decision-making (formulating interim hypotheses and prioritizing treatments), this analysis missed some critical trends because it ignored much of the systematic design, i.e. the sequential optimization of treatments (Table 1). These trends were extracted by a number of orthogonal contrasts that reflected the various objectives over the three seasons (Table 6), also on InfoStat.

3. Results

3.1. Effect of sticker of germination of potential biocontrol agents

The two-way ANOVA indicated that NP-7 was more inhibitory ($P < 0.001$) to germination of *T. ovalisporum* TK-1 than the other stickers. Average germination in NP-7 was 93.2% of the water control; germination for the other stickers averaged 97.9%. The factor concentration was significant ($P = 0.004$) and interacted with sticker ($P = 0.002$). Whereas most stickers produced no significant change in germination at the range of concentrations tested, NP-7 lowered germination at increasing dosages (Fig. 1).

Based on these results, our stickers of choice for field trials were BreakThru, Nu Film-17 and Citowett, after Pegafix had just become unavailable in Costa Rica. However, the latter two were about to be withdrawn from the national market (Abonos Superior, pers. comm., 2004). Therefore, they were not field-tested due to the likely lack of availability. This left BreakThru and NP-7, the sticker least compatible with *T. ovalisporum* TK-1. Before investing into a

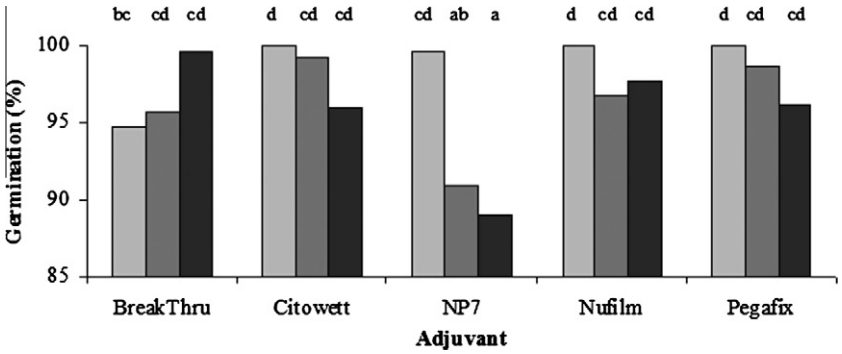

Fig. 1. Percentage germination of *Trichoderma ovalisporum* TK-1, compared with water control, of four endophytic antagonists after incubation on 20% PDA in five different stickers at different dosages: half-strength (□), the recommended dosage (▩), and twice that concentration (■). Bars carrying the same letter do not differ at $P = 0.05$ (two-way ANOVA of scaled percentages, based on germination in water, followed by Duncan test).

full-scale field trial, we opted to test NP-7 at the recommended concentration in a short-duration field survival trail on order to be able to detect any effect of adding this sticker on the establishment and survival of *T. ovalisporum* TK-1 on cocoa pods, be it positive or negative.

3.2. Field survival of T. ovalisporum with and without the sticker NP-7

The effect of NP-7 on the persistence of *T. ovalisporum* applied to surface-sterilized pods was investigated by monitoring the presence of the applied strain for 7 weeks after application. *T. ovalisporum* TK-1 or indistinguishable isolates were very rarely detected on pods to which TK-1 had not been applied (Fig. 2). The recommended dosage of NP-7 had no significant effect on any of the Gompertz parameters used to quantify the persistence of *T. ovalisporum* TK-1 on the pod surface (Table 2). Therefore, this concentration of sticker was used in the larger field trial. The application frequency was set at monthly intervals, as is common practice; this time is also in agreement with the time of inflexion (parameter *M*) of 3.25 weeks measured here.

3.3. Weather conditions

Weather conditions during this field survival experiment were typical for the time of year. Overall precipitation was normal, despite some heavy rains in May 2004. A total of 315 mm of rain was received, while 343 mm is the long-term average for the same 7 weeks. Heavier rains concentrated around the end of the fifth week, when populations of *T. ovalisporum* TK-1 had already declined substantially (data not shown).

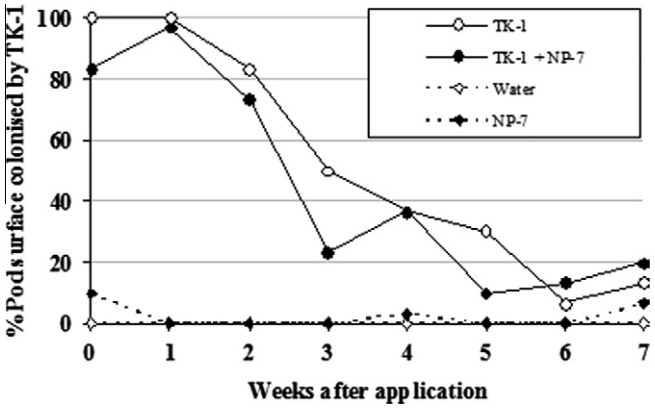

Fig. 2. Percentage surface colonization of surface-sterilized cocoa pods after inoculation with *Trichoderma ovalisporum* TK-1 (10^6 conidia ml^{-1}) suspended in water or with the sticker NP-7 at 1 ml l^{-1}obtained by averaging presence/absence data on excised cocoa peel discs.

Table 2
Changes in percentage surface colonization of cocoa pods over time by biocontrol candidate *Trichoderma ovalisporum* TK-1 that had been applied (10^6 conidia ml^{-1}) to surface-sterilized cacao pods with or without the adjuvant NP-7 (1 ml l^{-1}). Estimates are derived by Gompertz regression (95% confidence limited in parentheses), using the equation: $Y = A + C\ \exp^{(-\exp)\text{-}B(X-M)}$.

	Trichoderma ovalisporum TK-1 applied	
Parameters	With NP-7 (1 ml l^{-1})	In water only
Initial colonization (%) (Gompertz parameter *C*)	70.0 (54.7–85.0)	100.0 (50.3–100.0)
Final colonization (%) (Gompertz parameter A)	20.3 (12.3–28.3)	6.7 (0–29.0)
Time to inflexion (weeks) (Gompertz parameter M)	3.52 (2.95–4.08)	5.01 (3.97–6.06)
Maximum rate of change (% $week^{-1}$) ($C\ B\ e^{-1}$)	−68.8 (−92.2–6.0)	−19.2 (−56.4–0.7)

For temperature and humidity, all three seasons of the efficacy trials were well within the long-term average ranges for La Lola. Precipitation during the first 5 months of 2004 was 31% above the long-term average. This was driven by heavy rains in May and especially March, when more than twice the normal amount was received. Experimental periods in 2006 and 2007 were 22% drier than the long-term average. Shortfalls were relatively well-distributed, with the driest month (October) receiving 20% of normal rain; all others were above 30%. The other seasons were within 20% of the long-term average for precipitation. Distinct monthly variations cancelled each other out, e.g. in April 2005, 121% of normal rain was received, while December 2005 yielded only 19% of normal rain. Solar radiation data only became available in July 2007, but appeared typical for the area throughout the experimental period.

3.4. Application of agents in 3-year field trial

There was no difference between the different motorized mist-blowers used ($P = 0.204$); however, application rates differed significantly between the 11 persons ($P = 0.002$) who had operated the sprayers at some point during the three trials. Application rates did not differ between years ($P < 0.101$) and were adequately randomized across treatments ($P = 0.106$). An average of 165 l ha^{-1} suspension applied was within the range of the target rate, namely 190 l ha^{-1}. First-order interactions were generally due to the unbalanced design and not a biased application, as evidenced by the fact that volumes for individual operators who participated for two or more seasons did not differ between treatments or sprayers (data not shown).

Flutolanil residues in dried cocoa seeds were consistently below the detection limit of 0.05 mg kg^{-1}.

3.5. Efficacy test of biological and chemical control agents with and without sticker

FPR destroyed 83.9% of untreated pods on average, across years. Black pod (BP) was of minor importance, with an average loss of 2.3% in the controls. Initially, data for each year were analyzed individually in order to prioritize a subset of treatments for further testing and development. The only parameter with a consistently significant treatment effect during all seasons was the number of healthy pods (Table 3). While in the first season (2003/2004) the Duncan tests did not identify any individual treatment that improved yield, one treatment reduced yield significantly: flutolanil with NP-7 applied for 5 months followed by copper hydroxide with NP-7 (dotted arrow in Fig. 3). This treatment was not applied in the subsequent trial cycles, when copper hydroxide was always applied in water only, while the systemic agents were applied with a sticker (Table 1). This modification enhanced performance: Flutolanil with NP-7 applied throughout the 2005/2006 season or followed by copper hydroxide (in water) after 4 months increased yields significantly in this second year; no treatment reduced yield. In the third cycle of optimization (2005/2006), all treatments improved yield compared with the control (Fig. 3).

Disease incidences as well as the percentage of healthy pods showed no significant treatment effect in 2003/2004 or 2005/2006 when analyzed separately by season. After further optimization, in the third year, all parameters except the two related to sporulation of FPR-infected pods were significant (Fig. 4 and Table 3). All treatments now improved the number ($P < 0.001$) and the percentage ($P = 0.001$) of healthy pods harvested; no difference was found between the two stickers for the number ($P = 0.747$) or percentage ($P = 0.936$) of healthy pods, respectively (Table 6). Increased yield was driven by a reduction of FPR ($P = 0.003$, Fig. 4), again brought about by all agents tested during that season, with no difference between stickers ($P = 0.931$, Table 6). Two treatments reduced BP significantly ($P = 0.003$) in 2006/2007: *T. ovalisporum* TK-1 with BreakThru applied either throughout the season or followed by copper hydroxide after 6 months. Orthogonal contrast established that *T. ovalisporum* TK-1-containing agents controlled

Table 3
Error probability of treatment effect on field parameters in Costa Rica (one-way ANOVA of arcsine-transformed percentages or log-transformed counts for each trial season).

Treatment	2003/2004	2005/2006	2006/2007
Yield (pods per plot)	0.032	0.031	<0.001
Healthy pods (%)	0.167	0.520	0.001
Black pod (%)	0.254	0.618	0.003
Frosty pod rot (%)	0.887	0.409	0.003
FPR-infected pod reaching sporulation (%)	0.783	0.605	0.932
(number)	0.662	0.512	0.167

Table 4
Effect of trial season on average parameters of cocoa pod health in Costa Rica.

	Parameter					
	Healthy pods		Black pod	Frosty pod rot	FPR-infected pods sporulating	
Season	(Number)	(%)	(%)	(%)	(%)	(Number)
2003/2004	73.3	25.9[b]	2.7[b]	71.4[a]	30.1[b]	62.7
2005/2006	128.0	19.7[a]	2.3[b]	78.0[b]	34.2[c]	177.3
2006/2007	134.0	22.7[a,b]	0.7[a]	76.6[b]	28.7[a]	134.6
Error probability *P*	0.144	0.002	0.003	<0.001	<0.001	0.376

[a,b,c] Means in a column followed by the same letter do not differ at $P = 0.05$ (ANOVA of arcsine-transformed percentages or log-transformed counts, followed by Duncan test). Retransformed means (for all treatments) are presented.

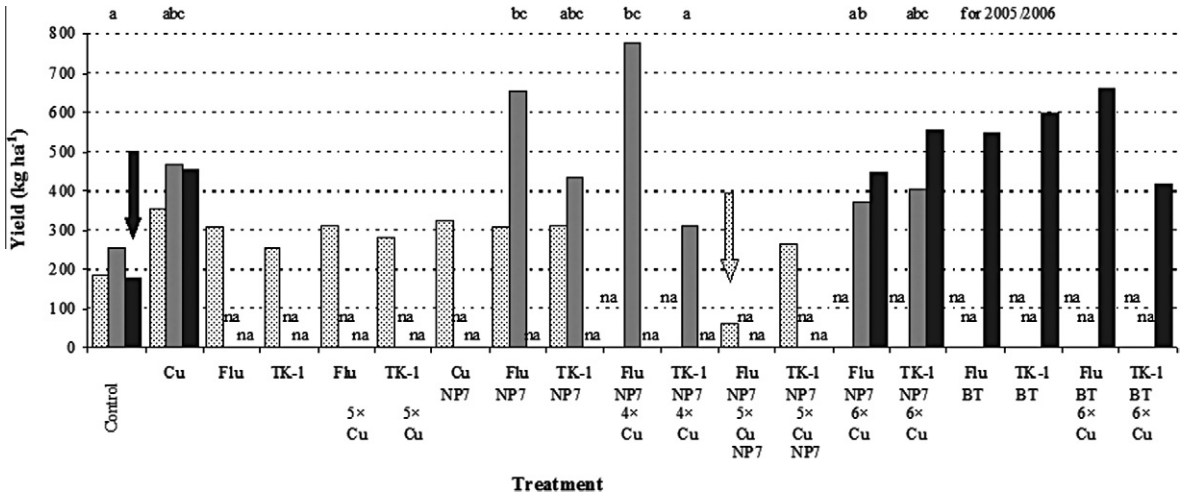

Fig. 3. Yield (extrapolated to kg ha^{-1}) in three IPM field trials in La Lola, Costa Rica, in 2003/2004 (■), 2005/2006 (▒) and 2006/2007 (■); na = not applied. Bars of treatments that differed from all the others in 2003/04 and 2006/07 are marked with an arrow in the respective color for that season. For 2005/2006, grey bars carrying the same letter do not differ significantly (ANOVA followed by Duncan test, $P < 0.05$). Treatment codes: Agents: Cu, copper hydroxide (1500 g ha^{-1}); Flu, flutolanil (300 g ha^{-1}); TK-1, *Trichoderma ovalisporum* (5×10^{12} cfu ha^{-1}); Stickers: NP-7 (1 ml l^{-1}); BT, BreakThru (0.3 ml l^{-1}); Application regime: First agent applied for four (4×), five (5), or six (6×) months, followed by the second agent for the remainder of the season.

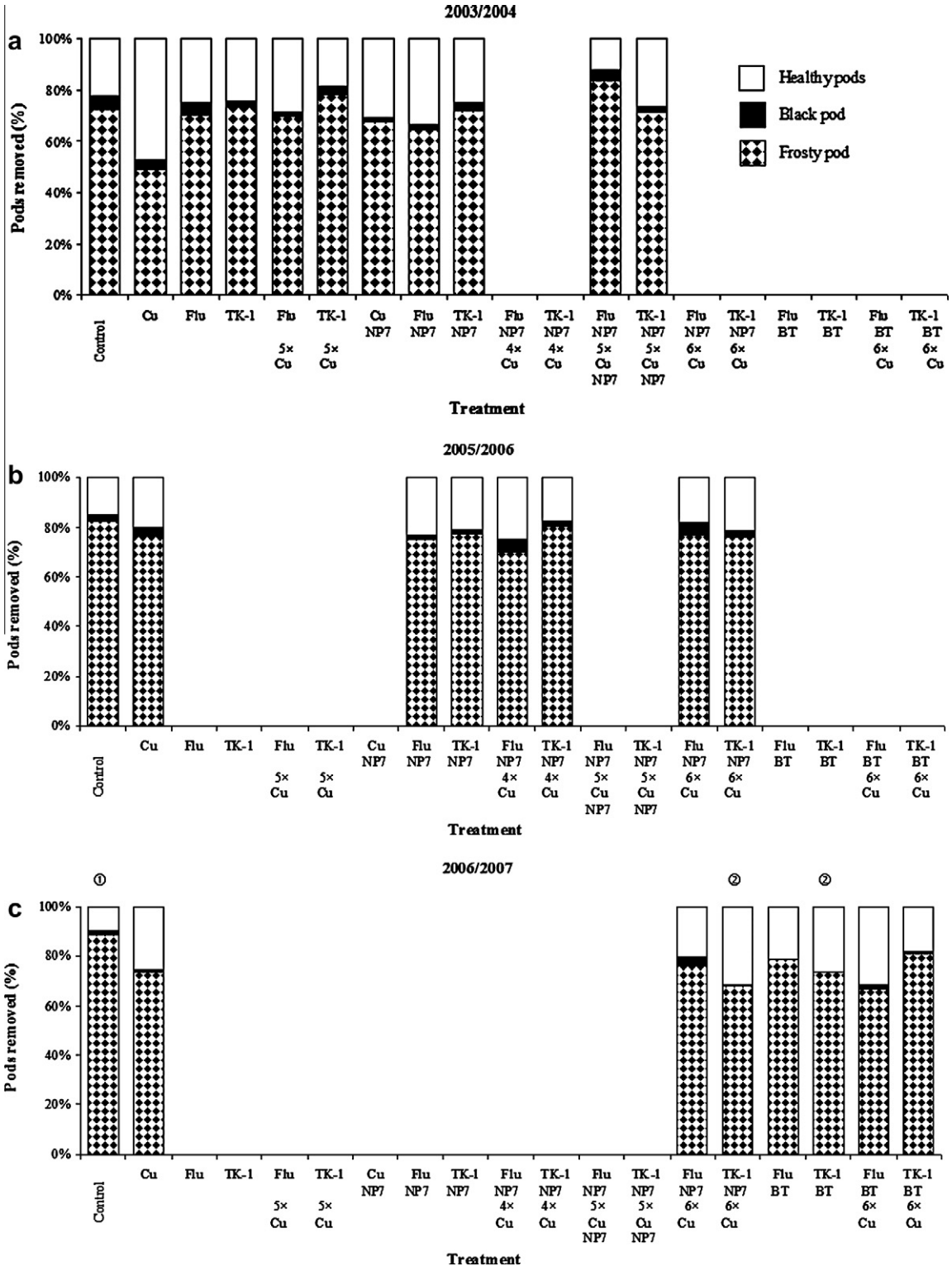

Fig. 4. Disease incidence and percentage healthy mature pods harvested in cocoa IPM field trial in La Lola, Costa Rica, over three trial seasons. Treatment codes: Agents: Cu, copper hydroxide (1500 g ha^{-1}); Flu, flutolanil (300 g ha^{-1}); TK-1, *Trichoderma ovalisporum* (5×10^{12} cfu ha^{-1}); Stickers: NP-7 (1 ml l^{-1}); BT, BreakThru (0.3 ml l^{-1}); Application regime: First agent applied for four (4×), five (5×), or six (6×) months, followed by the second agent for the remainder of the season. For 2006/2007: (1) all treatments increased the percentage health pods and decreased the incident of frosty pod rot compared with control, (2) these two treatments reduced black pod compared with the control at $P = 0.05$ (ANOVA of arcsine-transformed percentages, followed by Duncan test; untransformed means are presented).

BP better than flutolanil-based ones ($P < 0.001$), whereas both systemic agents were equally effective ($P = 0.277$) in controlling the more severe FPR.

Tables 5 and 6 summarize the main effects over the entire trial period of 3 years, analyzed as a factorial experiment for treatment and year. Although this analysis does not give detailed consideration to the treatment structure, it provides an indication of reproducible effects. The factor "year" was significant ($P \leqslant 0.003$) for percentage of healthy pods, BP and FPR incidence, as well as the percentage of FPR-infected pods that reached

Table 5
Effect of different agents and their combination[A], compared with an untreated control, on parameters of cocoa pod health averaged over 3 years.

Treatment	Healthy pods		Black pod	Frosty pod	FPR-infected pods sporulating	
	(Number)	(%)	(%)	Rot (%)	(%)	(Number)
Control	57.5^{b}	$14.4^{a,b}$	$2.4^{c,d,e}$	83.2	28.4	$102.3^{b,c,d}$
Kocide	$117.5^{c,d,e}$	31.0^{c}	$1.9^{a,b,c,d,e}$	67.1	30.4	$77.6^{a,b,c,d}$
Kocide + NP-7	$95.5^{b,c,d}$	29.9^{c}	$1.4^{a,b,c,d,e}$	68.6	35.3	$79.4^{a,b,c,d}$
Flutolanil	$91.2^{b,c,d}$	24.7^{b}	$4.6^{d,e}$	70.7	25.2	$66.1^{a,b}$
Flutolanil (5), Kocide [B]	$91.2^{b,c,d}$	28.4^{c}	$1.4^{a,b,c,d,e}$	70.2	33.2	$74.1^{a,b,c}$
Flutolanil + BreakThru	$141.3^{c,d,e}$	$21.9^{a,b,c}$	$0.2^{a,b,c}$	77.9	25.1	$125.9^{b,c,d,e}$
Flutolanil + BreakThru (6×), Kocide	$166.0^{d,e}$	31.5^{c}	$1.4^{a,b,c,d,e}$	67.1	27.1	$95.5^{b,c,d}$
Flutolanil + NP-7	$131.8^{c,d,e}$	$27.2^{b,c}$	$1.6^{a,b,c,d,e}$	71.1	33.7	$120.2^{b,c,d,e}$
Flutolanil + NP-7 (4×), Kocide	229.1^{e}	$24.6^{b,c}$	4.7^{e}	70.7	32.8	218.8^{e}
Flutolanil + NP-7 (5×), Kocide + NP-7	17.8^{a}	11.7^{a}	$4.1^{d,e}$	84.3	30.8	40.7^{a}
Flutolanil + NP-7 (6×), Kocide	$120.2^{c,d,e}$	$19.5^{a,b,c}$	$3.2^{d,e}$	77.3	34.6	$166.0^{d,e}$
Trichoderma ovalisporum TK-1	$75.9^{b,c}$	$23.8^{a,b,c}$	$2.2^{b,c,d,e}$	74.0	30.7	$72.4^{a,b,c}$
TK-1 (5×), Kocide	$83.2^{b,c,d}$	$18.5^{a,b,c}$	$2.8^{d,e}$	78.7	29.8	$100.0^{b,c,d}$
TK-1 + BreakThru	$128.8^{c,d,e}$	$26.7^{b,c}$	0.1^{a}	73.3	31.7	$109.6^{b,c,d,e}$
TK-1 + BreakThru (6×), Kocide	56.2^{b}	$18.5^{a,b,c}$	$0.1^{a,b}$	81.4	30.3	$77.6^{a,b,c,d}$
TK-1 + NP-7	$107.2^{b,c,d}$	$22.7^{a,b,c}$	$1.8^{a,b,c,d,e}$	75.5	27.7	$97.7^{b,c,d}$
TK-1 + NP-7 (4×), Kocide	$91.2^{b,c,d}$	$17.4^{a,b,c}$	$1.6^{a,b,c,d,e}$	81.0	33.1	$144.5^{b,c,d,e}$
TK-1 + NP-7 (5×), Kocide + NP-7	$79.4^{b,c}$	$25.1^{b,c}$	$2.4^{c,d,e}$	72.5	31.0	$74.1^{a,b,c}$
TK-1 + NP-7 (6×), Kocide	$141.3^{c,d,e}$	$25.6^{b,c}$	$1.0^{a,b,c,d}$	73.4	36.2	$154.9^{c,d,e}$
Error probability *P*	<0.001	0.010	0.004	0.061	0.376	0.002

[a,b,c,d,e] Means within a column followed by the same letter do not differ at $P = 0.05$ (ANOVA of arcsine-transformed percentages or log-transformed counts, followed by Duncan test). Retransformed means are presented.

[A] Application rates of agents were: Kocide (copper hydroxide), 1500 g ha^{-1}; *T. ovalisporum* TK-1: 5×10^{12} cfu ha^{-1}; flutolanil: 300 g ha^{-1}. Concentrations of stickers were: NP-7: 1 ml l^{-1} and BreakThru: 0.3 ml l^{-1}.

[B] First agent applied for four (4×), five (5×), or six (6×) months, followed by the second agent for the remainder of the season.

Table 6
Error probability of orthogonal contrast applied to field data of three trial seasons in Costa Rican cocoa.

Contrast	Healthy pods		Black Pod (%)	Frosty pod Rot (%)	FPR-infected pods sporulating	
	(Number)	(%)			(%)	(Number)
Switch of agent versus single-agent-throughout-season	0.140	0.083	0.205	0.167	0.131	0.247
Switch after 4 versus 6 months (2005/2006 only)	0.304	0.638	0.904	0.738	0.097	0.853
All with Sticker versus water only	0.969	0.542	0.079	0.473	0.795	0.309
Copper with sticker versus copper in water	<0.001	0.503	0.352	0.691	0.875	<0.001
Flutolanil with sticker versus flutolanil in water	0.320	0.245	0.650	0.247	0.639	0.015
Trichoderma ovalisporum TK-1 with sticker versus TK-1 in water	0.080	0.537	0.071	0.744	0.735	0.183
Systemic agent applied with sticker, Cu with water versus smart formulation rule ignored	<0.001	0.562	0.016	0.915	0.977	<0.001
Smart[a] switch of agent versus smart single agent	<0.001	0.534	0.284	0.485	0.081	0.002
Any copper-containing agent versus control	<0.001	0.001	0.437	0.006	0.104	0.925
Any flutolanil-containing agent versus control	<0.001	0.003	0.867	0.009	0.435	0.981
Any *Trichoderma ovalisporum* TK-1-containing agent versus control	<0.001	0.003	0.061	0.017	0.253	0.975
Any *Trichoderma ovalisporum* TK-1-containing agent versus any flutolanil	0.884	0.939	0.018	0.739	0.614	0.993
Trichoderma ovalisporum TK-1 with sticker versus Flutolanil with sticker	0.841	0.394	0.005	0.735	0.677	0.623
Trichoderma ovalisporum TK-1 with sticker versus copper in water throughout season	0.611	0.243	0.050	0.165	0.704	0.161
Flutolanil with sticker versus copper in water throughout season	0.418	0.124	0.119	0.074	0.617	0.031
NP-7 versus BreakThru (2006/2007 orthogonals only)	0.747	0.936	0.263	0.931	0.264	0.573

[a] The so-called "smart formulation" denotes application of systemic agents (*T. ovalisporum* or flutolanil) with a sticker, and of the contact fungicide (copper hydroxide) in water.

sporulation. The number of FPR-infected pods that reached the sporulation stage ($P = 0.376$) and of healthy mature pods harvested ($P = 0.144$) remained relatively stable over time (Table 4). The only trend discernible in Table 4 is a slight increase of FPR over time at the expense of BP. A highly significant ($P \leqslant 0.010$; Table 5) treatment effect was found for parameters: number and percentage of healthy pods, BP incidence, and number of FPR-infected pod reaching sporulation, but not their percentage ($P = 0.376$). FPR incidence narrowly failed to reach statistical significance ($P = 0.061$). In no case was the treatment × year interaction significant ($P \geqslant 0.210$), indicating that despite the unbalanced design, the progressive optimization of treatments under evaluation did not seriously bias treatment means or their statistical interpretation.

Yield, measured as the number of healthy pods harvested, was significantly improved, compared with the control, by eight of the 18 treatments tested: copper hydroxide, *T. ovalisporum* TK-1 plus BreakThru applied throughout the season, *T. ovalisporum* TK-1 plus NP-7 for 6 months, followed by copper hydroxide, flutolanil with BreakThru applied throughout the season or followed by copper hydroxide after 6 months, and flutolanil with NP-7, applied throughout the season or followed by copper hydroxide after either 4 or 6 months (Table 5). However, if flutolanil with NP-7 was followed by copper hydroxide with NP-7 after 5 months, yields were reduced. In contrast to absolute yield, the percentage healthy pods was lowest in 2005/2006 and highest in 2003/2004; 2006/2007 was intermediate (Table 4). Four treatments increased the percentage healthy pods: copper hydroxide alone or with NP-

7, flutolanil applied for 5 months followed by copper hydroxide, and flutolanil with BreakThru applied for 6 months followed by copper hydroxide (Table 5). The first and last of these also exhibited increased absolute yields (Fig. 3).

BP incidence was lower in 2006/2007 than in the other two seasons (Table 4). For all treatments, it remained below 5% (Table 5). Two treatments reduced BP incidence significantly below the control value: *T. ovalisporum* TK-1 applied with BreakThru either throughout the season or followed by copper hydroxide after 6 months.

With 71.4% FPR incidence was lower in 2003/2004 than in the other two seasons (average 77.3%; Table 4). The percentage of FPR-infected pods that reached sporulation was not affected by treatment ($P = 0.376$, Table 5). The total number of FPR-infected pods that reached sporulation, which is a function of both the number of FPR-infected pods and their percentage reaching sporulation, exhibited great variability. However, only one treatment significantly reduced this number: flutolanil with NP-7 applied for 5 months, followed by copper hydroxide also with this sticker. Contrary, flutolanil with NP-7 applied for 4 months, followed by copper hydroxide in water, increased the number of FPR-infected pods that reached sporulation (Table 5).

Orthogonal contrast permitted to further extraction of yield-improving disease control strategies (Table 6). The change from a systemic agent to copper hydroxide at some point in mid season did not, *per se*, have a significant effect on any parameter measured ($P \geqslant 0.083$); nor was there a difference between a switch after 4 months compared with 6 months ($P \geqslant 0.097$). The addition of an adjuvant did not result in any generalized change compared with the water formulation ($P \geqslant 0.079$). Stickers were detrimental to the efficacy of copper hydroxide, with yield significantly reduced from 121.8 to 64.2 pods per plot ($P < 0.001$; Table 6); however, the number of pods per plot reaching sporulation was reduced from 123.2 to 64.8 in the copper plus adjuvant formulation ($P < 0.001$). Similarly, the addition of an adjuvant by itself did not significantly improve the efficacy of *T. ovalisporum* TK-1 ($P \geqslant 0.071$) or flutolanil ($P \geqslant 0.245$) for any parameter; in fact, in the latter combination, it increased the number of sporulating pods from 70.1 to 127.8 per plot ($P = 0.015$). However, the application of systemic agents with a sticker, and the contact fungicide applied in water, was highly beneficial for overall yield ($P < 0.001$), increasing the number of healthy pods harvested per plot from 76.3 to 130.0, and for BP control ($P = 0.016$), decreasing the disease incidence from 2.7% to 1.6%. Thus, this application regimen was called the "smart formulation". On the other hand, smart formulation increased the number of FPR-infected pods that reached sporulation from 72.4 to 126.2 per plot ($P < 0.001$). We can rule out that the latter is mediated by an effect on the percentage of FPR-infected pods reaching sporulation ($P = 0.977$). With smart formulation, the switch-over from systemic (with adjuvant) to contact fungicide (in water) significantly ($P < 0.001$) improved pod yield further from 125.3 for single agent to 134.0 pods per plot for the sequence of agents, although the number of FPR-infected pods that reached the sporulation stage was also increased ($P = 0.002$).

Largely parallel trends were observed for pod yield, the percentage of healthy pods and FPR incidence, but most significant contrasts were observed for the former (Table 6). Agents containing either copper hydroxide, flutolanil or *T. ovalisporum* TK-1 reduced FPR ($P \leqslant 0.017$) and increased absolute ($P < 0.001$) and relative yield ($P \leqslant 0.003$) compared with the control, demonstrating the principal effectiveness of all tested agents against the dominant disease, FPR. No difference between these agents was found with respect to FPR control ($P \geqslant 0.074$), absolute ($P \geqslant 0.418$) or relative ($P \geqslant 0.124$) yield. Inoculum production, quantified via the number of pods sporulating in the field, was not reduced by any agent ($P \geqslant 0.925$). However, copper applied in water decreased the number of sporulating pods per plot from 123.1 to 77.6 compared with flutolanil with a sticker, if either fungicide was applied throughout the season.

T. ovalisporum TK-1 was superior to flutolanil in controlling BP, reducing its incidence from 2.6% to 1.5% if all formulations were considered ($P = 0.018$), and from 2.5% to 1.2% if only the sticker formulations favorable to BP control were compared ($P = 0.005$). No difference was detected between the two stickers for any parameter considered ($0.263 \leqslant P \leqslant 0.936$, Table 6).

4. Discussion

During field research with perennial crops where long-term trials are obligatory, rationing of limited resources is a pragmatic tenet. Here, this meant we had to select only the most promising treatments from each season's trial, omitting others that would have completed a neat factorial design with multi-season replication, for the next experimental cycle. Our iterative approach was supported by statistical analyses selected to detect prevalent trends and then, based on successive observations, pinpoint specific hypotheses for testing regarding the agents(s), their formulation as well as their application regime. The conclusions drawn from this research were based on orthogonal contrasts applied to data derived from three field seasons, while the initial ANOVAs were decision-making aids only, guiding us in the formulation of interim hypotheses and priorization of treatments. This was a rewarding strategy: Whereas none of the 10 treatments improved cocoa yield in the first season and one actually reduced yield compared with the control, two out of seven treatments improved yield and none reduced yield, in the second season. In the third and final trial year, all seven treatments tested improved yield significantly compared with the control (Fig. 3). The approach was also robust and not adversely affected by the need to work with different sprayers and staff over the 3-year period. Consequently, in this paper, we report two breakthroughs: This is the first of the La Lola trials (Hidalgo et al., 2003; Bateman et al., 2005b) in which the BCA achieved a significant yield improvement and also the first in which an advantageous adjuvant was identified.

Copper was again the best agent, but both *T. ovalisporum* and flutolanil increased clean cocoa yield significantly when applied with a sticker. FPR control was the main driver of percentage healthy pods. Interestingly, the number of treatments that increased absolute yield far exceeded that of treatments improving the percentage of healthy pods (Table 5). However, the percentage healthy pods was increased by two treatments that did not improve absolute yield (Table 5) and were thus deprioritized after one season: copper hydroxide with NP-7 and flutolanil followed by copper hydroxide after 5 months, both applied in water. Krauss et al. (2006a) had also observed absolute yield to be more variable than relative yield in neighboring Panama.

One key hypothesis emerging for testing in the following season was that the sticker was beneficial to the systemic agents, *T. ovalisporum* TK-1 and flutolanil, but detrimental to the contact fungicide, cooper hydroxide. At least in terms of pod yield, this was indeed confirmed (Table 6). These results contrast those of Hidalgo et al. (2003), who found an emulsifiable rape-seed adjuvant oil to enhance the efficacy of copper, but not that of *Clonostachys rosea* Schroers-based epiphytic BCAs. Hanada et al. (2009) also reported a lack of effect of an adjuvant oil on the persistence of the endophytic *Trichoderma martial* Samuels on cocoa pods in Brazil. The so-called smart formulation rule postulated here was incorporated in subsequent trials, where it almost doubled yields, but also increased the inoculum produced by *M. roreri* by 74%. Future research should aim to simultaneously reduce *M. roreri* inoculum – and thereby pathogen inoculum. In this context, epiphytic BCAs

applied inundatively to the pod surface have shown considerable promise (Hidalgo et al., 2003; Krauss et al., 2006a). Their combination with endophytes into mixed inocula merits consideration.

Whenever flutolanil was applied employing smart formulation, a significant raise in the number of healthy pods was recorded that was in the same range as the copper fungicide standard (Table 5). The BCA was somewhat more erratic, with only two treatments in the same league as copper: *T. ovalisporum* TK-1 with NP-7 for 6 months followed by copper hydroxide in water for the remainder of the season, and TK-1 with BreakThru applied throughout the season (Table 5). Nevertheless, the success of the endophyte is noteworthy: it improved healthy pod yields (absolute and relative) as effectively as flutolanil. Moreover, TK-1 performed as well as the systemic chemical in controlling FPR and even exceeded flutolanil, which targets basidiomycetes specifically, in BP control (Table 6). Further improvement of formulation may well be the key to successful development of BCAs against FPR and will thus be our research priority for up-coming trials.

For the first time, we identified beneficial adjuvants, although we were obliged to work with a sticker of sub-optimal compatibility with *T. ovalisporum* TK-1. At *in vitro* germination rates of 91% and 89% that of the water control at the recommended and double that dosage, respectively, NP-7 seemed unlikely to have a detrimental effect on the antagonist in the field when applied at the recommended dosage. This was corroborated by a short-duration field test (Fig. 2). The field survival test was not performed for BreakThru, the only viable alternative to NP-7, as BreakThru did not raise concerns in laboratory germination tests. In the main field trials, no difference between the two stickers was found to date (Table 6), but this hypothesis should be tested in at least one further field season.

Our experience of prioritized stickers being phased out highlights the importance for scientists to communicate with private sector stakeholders in order to focus adaptive research. Costa Rica is not only a major market for agrochemicals and one of the region's largest for commercial BCAs, but also acts as regional intermediary, re-exporting substantial quantities of commercial BCAs and agrochemicals to other Central American countries (Carballo et al., 2003; Alves and Lopes, 2008). Having assessed market intelligence in a timely manner allowed us to arrive at a somewhat riskier prioritization than a pure research result-based process would have led us to.

Progress was also made with respect to the hypothesized benefits of using a systemic agent in the early season, when pod expansion is most rapid, followed by the contact fungicide later on, although recommendations on the best switch-over time remain to be further refined. The effectiveness of copper in disease control is beyond doubt. However, the International Federation of Organic Agriculture Movements (IFOAM) allows this temporary exception in organic agriculture only because there are no viable alternatives at the present (IFOAM, 2002). Thus, a worthwhile research objective would be aiming for minimal copper applications. Four to five applications of Kocide at the rate we used here are within the upper limit of 8 kg ha^{-1} yr^{-1} set by IFOAM for use of copper in organic agriculture, whereas six applications would exceed them.

It is noteworthy that the benefits of the change of agents was only realized when the smart formulation was employed and vice versa. In this context, it is particularly remarkable that, on average, a smart switch-over lead to a substantial yield increase, although the most effective single treatment, copper hydroxide in water, fell into the single-agent-throughout-season group. In order to further optimize the switch-over strategy, additional systemic agents (biologicals as well as chemicals) should be tested.

Laker and Rudgard (1989) expressed concerns about a possible risk of residues of systemic compounds accumulating in cocoa beans. Our results suggest that this apprehension may be unfounded, as monthly flutolanil applications throughout the production cycle did not lead to measurable residues. Nevertheless, not applying flutolanil during the latter half of the season when the beans mature constitutes an additional safeguard.

The classical biological control approach has not widely been applied for the management of plant diseases; however, it has been used extensively and successfully for the control of insect and weed pests. This is the first report of an endophytic fungus being used as classical biocontrol agent against a plant disease under field conditions. *M. roreri* is believed to have evolved in the forests of western Colombia and/or Ecuador on *T. gileri.* The pathogen "escaped" from this geographic isolation only relatively recently, following transport of cryptically infected cocoa germplasm by man (Evans, 2003b). Our investigation was based on the hypothesis that coevolved populations of endophytes, which colonize cocoa and its near relatives in natural ecosystems, are gradually diluted out during the production of planting material. Cocoa acquires at least some of its sporulating endophyte flora through leaves via horizontal transmission from neighboring forest trees (Arnold and Herre, 2003). Thus, coevolved endophytes are gradually displaced or simply do not occur in exotic situations, far from the South American centre of origin, and the forest ecosystem in general. Any protective role that the endophytes may perform within the native range would be lost, rendering the cultivated trees more susceptible to pathogens in location to which cocoa was introduced. Inoculating planting material with coevolved endophytes could overcome this problem and thus be particularly appropriate for the control of *Moniliophthora* spp. in exotic locations. Inoculated cocoa endophytes protect seedlings from leaf infection by *Phytophthora* sp. in Panama (Arnold et al., 2003). Holmes et al. (2004, 2005) showed that *T. ovalisporum* TK-1 could enter the intact plumule of cocoa seedling and establish itself in the apical meristem and the younger tissues of the stem. It still remains to be determined, however, if cocoa endophytes can proliferate systemically and persist as permanent residents within cocoa pods, thereby conferring resistance to systemic pathogens such as *Moniliophthora* spp. Several teams are working on colonization of cocoa by fungal endophytes isolated from different tissues as well as the most conducive inoculation method (Bailey et al., 2008; De Souza et al., 2008; Mejía et al., 2008). However, the establishment of endophytes in pods requires a systematic and focused effort.

Since both *M. perniciosa* and *M. roreri*, for long periods of their life-cycles, grow asymptomatically and intercellularly within the meristematic tissues of cocoa, any endophytes which pre-colonizes these potential infection courts, could potentially prevent invasion by the pathogens through exclusion (competition), mycoparasitism, antibiosis, and induced resistance. All these mechanisms have been reported for endophytic trichodermas from cocoa (Bailey et al., 2006, 2008; Mejía et al., 2008) and could drastically enhance early disease control, at a stage where the systemic pathogen is not only relatively inaccessible, but also almost impossible to diagnose in the field. Early detection and intervention is crucial for the cost-effective control of *M. roreri*, because within 1 week of the appearance of clear symptom, the pathogen already sporulates prolifically on the pod surface (Krauss et al., 2006b). Thus, for early niche exclusion, special attention should be paid to pod entry of the endophyte into cherelles via flower cushions.

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References

Alves, S.B., Lopes, R.B. (Eds.), 2008. Controle Microbiano de Pragas na América Latina. Avanços e Desafios. Biblioteca de Ciências Agrárias Luiz de Queiroz. VOL. 14. Fundação de Estudios Agrários Luiz de Queiroz, SP, Brazil. pp. 414.

Arnold, A.E., Herre, E.A., 2003. Canopy cover and leaf age affect colonization by tropical fungal endophytes: ecological pattern and process in *Theobroma cacao* (Malvaceae). Mycologia 95, 388–398.

Arnold, A.E., Mejía, L.C., Kyllo, D., Rojas, E.I., Maynard, Z., Robbins, N., Herre, E.A., 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proc. Nat. Acad. Sci. USA 100, 15649–15654.

Bailey, B.A., Bae, H., Strem, M.D., Roberts, D.P., Thomas, S.E., Crozier, J., Samuels, G.J., Choi, I.Y., Holmes, K.A., 2006. Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four *Trichoderma* species. Planta 224, 1449–1464.

Bailey, B.A., Bae, H., Strem, M.D., Crozier, J., Thomas, S.E., Samuels, G.J., Vinyard, B.T., Holmes, K.A., 2008. Antibiosis, mycoparasitism, and colonization success for endophytic *Trichoderma* isolates with biological control potential in *Theobroma cacao*. Biol. Control 46, 24–35.

Bateman, R.P., Holmes, K.A., Krauss, U., Padi, B., 2005a. Future tactics and tools for pest management. In: Flood, J., Murphy, R. (Eds.), Cocoa Futures. CABI Commodities Press, Cali, Colombia, pp. 77–93.

Bateman, R.P., Hidalgo, E., García, J., ten Hoopen, G.M., Krauss, U., Adonijah, V., 2005b. Application of chemical and biological agents for the management of frosty pod rot (*Moniliophthora roreri*) in Costa Rican cocoa (*Theobroma cacao*). Ann. App. Biol. 147, 129–138.

Carballo, M., Carranza, C., Varela, G., 2003. Uso de Bioplaguicidas en Centro América. In: Röttger, U., Muschler, R. (Eds.), Simposio Internacional de Bioplaguicidas para Países en Desarrollo, CATIE, Turrialba, Costa Rica, pp. 144–152, available from <http://www.proamo.org/biblioteca/bioplaguicidas/SimposioMemoria.pdf>.

De Souza, J.T., Bailey, B.A., Pomella, A.W.V., Erbe, E.F., Murphy, C.A., Bae, H., Hebbar, P.K., 2008. Colonization of cocoa seedlings by *Trichoderma stromaticum*, a mycoparasite of the witches' broom pathogen, and its influence on plant growth and resistance. Biol. Control 46, 36–45.

Evans, H.C., 1981. Pod rot of cocoa caused by *Moniliophthora* (*Monilia*) *roreri*. Phytopathological Papers 24. CAB Commonwealth Mycological Institute, Kew, UK.

Evans, H.C., 1999. Classical biological control. In: Krauss, U., Hebbar, P. (Eds.), Research Methodology in Biocontrol of Plant Diseases with Special Reference to Fungal Diseases of Cocoa. CABI, CATIE, USDA, pp. 29–37.

Evans, H.C., Krauss, U., Ríos, R., Zecevich, T., Arévalo, E., 1998. Cocoa in Peru. Cocoa Growers' Bull. 51, 7–22.

Evans, H.C., Holmes, K.A., Reid, A.P., 2003a. Phylogeny of the frosty pod rot pathogen of cocoa. Plant Path. 52, 476–485.

Evans, H.C., Holmes, K.A., Thomas, S.E., 2003b. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. Mycol. Prog. 2, 149–160.

Evans, H.C., Holmes, K.A., Thomas, S.E., 2003c. Crowd control: Natural enemy biodiversity in a coevolved forest host-pathosystem and its potential as a source of novel biocontrol agents for frosty pod rot of cocoa. In: Akrofi, A.Y., Ackonor, J.B., Ollennu, L.A.A. (Eds.), Proc. INCOPED 4th Intern. Seminar on Cocoa Pest and Diseases, Accra, Ghana, 19–21 October 2003. Ghana Cocoa Board, Accra, Ghana, pp. 126–135.

Hanada, R.E., Pomella, A.W.V., Soberanis, W., Loguercio, L.L., Pereira, J.O., 2009. Biocontrol potential of *Trichoderma martiale* against the black-pod disease (*Phytophthora palmivora*) of cacao. Biol. Control 50, 143–149.

Hidalgo, E., Bateman, R.P., Krauss, U., ten Hoopen, G.M., Martínez, A., 2003. A field investigation into delivery systems for agents to control *Moniliophthora roreri*. Eur. J. Plant Path. 109, 953–961.

Holmes, K.A., Schroers, H.-J., Thomas, S.E., Evans, H.C., Samuels, G.J., 2004. Taxonomy and biocontrol potential of a new species of *Trichoderma* from the Amazon basin of South America. Mycol. Prog. 3, 199–210.

Holmes, K.A., Krauss, U., Samuels, G., Thomas, S.E., Bateman, R.P., Hidalgo, E., García, J., Schroers, H.-J., Brown, N.A., Arroyo, C., Evans, H.C., 2005. *Trichoderma ovalisporum*, a novel biocontrol agent of frosty pod rot (*Moniliophthora roreri*) of cocoa (*Theobroma cacao*): from discovery to field. Invited keynote at the 1st International Conference on Plant-Microbe Interactions: Endophytes and Biocontrol Agents 18–22 April, 2005, Saariselkä, Lapland, Finland.

Holmes, K.A., Krauss, U., Samuels, G., Bateman, R.P., Thomas, S.E., Crozier, J., Hidalgo, E., García, J., Arroyo, C., Evans, H.C., 2006. *Trichoderma ovalisporum*: a potential biocontrol agent of frosty pod rot (*Moniliophthora roreri*). In: Proceeding of the 15th International Cocoa Research Conference, vol. II, San José, Costa Rica, 9–14 Oct., 2006, pp. 1001–1006.

Infostat, 2004. Infostat, versión 2004. Grupo Infostat, FCA, Universidad Nacional de Córdoba, Argentina.

International Federation of Organic Agriculture Movements (IFOAM), 2002. Substance List, available from <http://www.ifoam.org/about_ifoam/standards/OGS_Revision/20071013_Substance_Lists.pdf> (accessed 15 Oct, 2007).

Krauss, U., Soberanis, W., 2001. Biocontrol of cocoa pod diseases with mycoparasite mixtures. Biol. Control 22, 149–158.

Krauss, U., Soberanis, W., 2002. Effect of fertilization and biocontrol application frequency on cocoa pod diseases. Biol. Control 24, 82–89.

Krauss, U., Martínez, A., Hidalgo, E., ten Hoopen, G.M., Arroyo, C., 2002. Two-step liquid/solid state mass production of *Clonostachys rosea*. Mycol. Res. 106, 1449–1454.

Krauss, U., ten Hoopen, G.M., Hidalgo, E., Martínez, A., Arroyo, C., García, J., Portuguéz, A., Sánchez, V., 2003. Manejo integrado de la moniliasis (*Moniliophthora roreri*) del cacao (*Theobroma cacao*) en Talamanca, Costa Rica. Agroforestería en las Américas 10, 52–58.

Krauss, U., ten Hoopen, G.M., Hidalgo, E., Martínez, A., Stirrup, T., Arroyo, C., García, J., Palacios, M., 2006a. The effect of cane molasses amendment on biocontrol of frosty pod rot (*Moniliophthora roreri*) and black pod (*Phytophthora* spp.) of cocoa (*Theobroma cacao*) in Panama. Biol. Control 39, 232–239.

Krauss, U., Steuten, C., Adonijah, V., Holmes, K., 2006b. Early detection of frosty pod rot as key to cost-effective control. In: Akrofi, A.Y., Baah, F. (Eds.), Proceeding of 5th INCOPED Seminar, San José, Costa Rica, pp. 8–13.

Laker, H.A., Rudgard, S.A., 1989. A review of the research on chemical control of witches' broom disease of cocoa. Cocoa Growers' Bull. 42, 12–24.

Mejía, L.C., Rojas, E.I., Maynard, Z., Van Bael, S., Arnold, A.E., Hebbar, P., Samuels, G.J., Robbins, N., Herre, E.A., 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biol. Control 46, 4–14.

Phillips, W., Krauss, U., Evans, H.C., Wilkinson, M., 2003. Genetic diversity of the cocoa pathogen *Moniliophthora roreri* (Cif.) Evans et al. in tropical America. In: Proceeding of the 14th International Cocoa Res. Conference, Accra, Ghana, 12–18 October 2003, vol. II. Lagos, Nigeria: Cocoa Producers' Alliance, 2005, pp. 613–618.

Phillips-Mora, W., Ortiz, C.F., Aime, M.C., 2007. Fifty years of frosty pod rot in Central America: Chronology of its spread and impact from Panama to Mexico. In: Proceedings of the 15th International Cocoa Research Conference, vol. I, pp. 1039–1047.

Salas, A., undated. Datos meteorológicos La Lola, available on-line up to 2008, available from <http://tiny.cc/th4Xi>.

SAS Institute, 1985. SAS User Guide: Statistics, SAS Institute, Cary, NC, USA.

Stevens, P.J.G., 1992. Organosilicone surfactants as adjuvants for agrochemicals. Paper presented at the 3rd International Symposium on Adjuvants for Agrochemicals, Cambridge, UK, 3–7 August, 1992.

Wood, G.A.R., 1985. History and development. In: Wood, G.A.R., Lass, R.A. (Eds.), Cocoa. Longman Scientific & Technical, Harlow, UK, pp. 1–10.

Zar, J.H., 1996. Biostatistical Analysis, third ed. Prentice-Hall, Englewood Cliff, NJ, USA.