PennState College of Agricultural Sciences

THE PENN STATE LINES

Final Report for the SCRI Project 2012 – 2017
Addressing Management Gaps with Sustainable Disease and Pest Tactics for Mushroom Production

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Fungal Pathogens

Cropping experiments were used to evaluate bio-pesticides for efficacy in controlling *Trichoderma aggressivum aggressivum, (Ta2) (Green Mold)* and *Lecanicillium fungicola* (Dry Bubble) two fungal pathogens of *Agaricus bisporus*, white and brown button mushroom. *A. bisporus* was inoculated with spore suspensions of *L. fungicola* several days after casing. The trials had combinations of un-inoculated and inoculated-untreated controls compared to treatments of registered chemicals. Different rates of application and combinations of bio-fungicides were tested including: Sesame Oil (Organocide®), *Coniothyrium minitans* (Contans WG®), *Gliocladium catenulatum* (Prestop®), *Streptomyces griseoviridis* (Mycostop®), and Saponins of *Chenopodium quinoa* (Heads-Up Plant Protectant®). Only *Streptomyces griseoviridis* applied after casing demonstrated a reduction in the incidence of disease (Table 1).

Table 1. Total yield and dry bubble incidence as measured by spotting or bubble symptoms on 2nd break. Data within a column followed by the same letter are not significantly different (P<0.05).

Treatment	Total Yield Kg/m2		Disease Incidence	
Control Uninoculated	20.36	bcd	0.00	c
Control Inoculated	19.83	cd	15.5	a
Thiabendazole/Chlorothalonil Inoculated	21.99	a	1.0	c
Gliocladium catenulatum Inoculated	21.84	ab	15.0	a
Streptomyces griseoviridis Inoculated	19.07	d	5.3	bc
Coniothyrium minitans Inoculated	21.11	abc	13.0	a
Sesame Oil Inoculated	20.34	cd	13.3	a
Saponins of *Chenopodium quinoa* Inoculated	20.00	cd	10.0	a

The most efficient method and timing of application for use of these chemicals against *Ta2* was determined by comparing fresh mushroom yields and incidence of *Ta2* growth on the casing surface. Our results suggest that most bio-fungicides tested against *Ta2* were not effective against either pathogen. However, our preliminary results did suggest that synthetic spawn formulations and one bio-fungicide, *Gliocladium catenulatum,* may be effective in reducing the incidence and severity of *Ta2* green mold disease (Table 2).

Table 2 Total yield and green mold incidence as measured by percent of surface area infected at the end of the crop. Data within a column followed by the same letter are not significantly different (P<0.05).

Treatment	Total Yield Kg/m2		Disease Incidence	
Control Uninoculated	29.11	a	0.0	b
Control Inoculated	26.93	a	24.8	a
Thiabendazole/Chlorothalonil Inoculated	27.28	a	1.0	b
Streptomyces griseoviridis day 9 Inoculated	28.45	a	25.3	a
2X *Streptomyces griseoviridis* day 9 Inoculated	26.67	a	9.5	b
Streptomyces griseoviridis day 7, 9,15 Inoculated	28.45	a	3.0	b

Trichoderma aggressivum volatile compounds

Ta2 persists despite advanced composting technologies. Genetic approaches to detect *Ta2* in bulk substrate are inadequate and tunnel systems limit regular sampling. Monitoring volatile compounds (VC) of phase III (P3) compost may provide an early detection method of *Ta2*. Volatiles were analyzed using Agilent Technologies GC-MS method set to "scavenge" mode with a slow ramp speed over 36 minutes to detect identifier VCs. Our results suggest similarities between treatments require targeting of 150-300g/mol compounds using selective-ion monitoring (SIM) (Table 3). Differentiation between *A. bisporus* and *Ta2* VCs *were* detected as early as spawn run day 6.

Treatment	RT (minutes)	% Area	NIST Library Compound Name	% Match
Compost	14.5405	0.5729	Decanal	91
A.bisporus	14.5405	0.4418	Decanal	91
Ta2	14.5405	0.4548	Decanal	90
Compost	11.5177	0.1435	D-Limonene	95
A.bisporus	11.5177	0.3805	D-Limonene	98
Ta2	11.5176	0.2584	D-Limonene	98
Compost	19.0029	0.3519	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	94
A.bisporus	19.01	0.6842	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	93
Ta2	19.0173	0.3437	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	92
Compost	13.0363	2.9048	Nonanal	91
A.bisporus	13.036	4.6515	Nonanal	91
Ta2	13.0433	2.2458	Nonanal	91
A.bisporus	9.9631	0.2741	Bicyclo[4.2.0]octa-1,3,5-triene	93
Ta2	9.9704	0.2478	Bicyclo[4.2.0]octa-1,3,5-triene	87
A.bisporus	16.7465	0.1957	.alpha.-Copaene	78
Ta2	16.7466	0.1269	Copaene	93
A.bisporus	11.0951	0.7077	3-Octanone	91
Ta2	11.1022	0.8044	3-Octanone	91
Ta2	29.4968	1.256	1,10b(2H)-Dihydropyrano[3,4,5-jk]fluorene	90
Ta2	18.0361	0.6084	1,3,5,7-Cyclooctatetraene	76
Ta2	16.0734	0.1603	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	98
Ta2	24.1891	0.9807	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester	93
Ta2	9.9777	0.1424	Phthalic acid, di(2-propylpentyl) ester	93
Ta2	9.6409	0.3204	n-Tridecan-1-ol	97

Table 3 Subset of GC-MS results acquired from NIST library showing unique and shared VCs across treatments.

Flies

Colonies of phorid and sciarid flies have been established and maintained for both semiochemical and pesticide screening. Establishing colonies of phorid flies was a major

accomplishment as this has not been done before. Previous research on phorids relied on random numbers of eggs, larvae and flies collected from commercial farms, making the results somewhat speculative and inconsistent.

We reported that *Beauveria bassiana*, a fungal parasite of insects, was found to be ineffective in controlling larval stages of sciarid and phorid flies. However, when used as a premise spray, Botanigard® (commercial *B. bassiana* insecticide) is effective at killing adults of both species, when flies are exposed to the sprayed surface soon after application. However, while the speed of kill was found to be significantly different compared to the control population for both fly species, it was not sufficiently fast (8 days and 11 days to 100% mortality for sciarid and phorid flies respectively), to recommend its use to mushroom growers. Since the lack of efficacy of *B. bassiana* had been established by the end of 2016, a range of alternative biopesticide products, which are registered for use on other crops, were obtained and evaluated for efficacy.

The following products were evaluated using direct spray application to egg, larval and pupal stages of sciarid and phorid flies: Entrust®, Dow AgroSciences, PFR-97™, Certis, Requiem, Bayer Crop Sciences, Grandevo ® and Venerate®, Marrone Bio Innovations, BioCeres, BioSafe Systems and AzaGuard ®, BioSafe Systems. Of these, Entrust, PFR-97 and Grandevo, showed promising results and were progressed to a larger bioassay system in compost cups. These larger scale trials are ongoing and additional funding has now been obtained from the USDA IR-4 program to determine dose rates and use strategies for these three promising products. If these trials are successful, label extensions for use of these products in mushroom crops could be obtained.

The females of the mushroom sciarid, *Lycoriella ingenua* (Dufour, 1839) (Diptera: Sciaridae), are one of the most severe pests of the cultivated white button mushroom, *Agaricus bisporus*). We have shown that the flies are attracted to the mushroom compost that mushrooms are grown on and not to the mushrooms themselves.

We have identified a small set of fungal species, present during mushroom cultivation, that are attractive to female sciarid mushroom flies, i.e., the fungus gnat species, *Lycoriella ingenua.* Some of these were found on the bodies of pregnant females and may explain how infestations get started in mushroom houses. Other fungal species demonstrating different degrees of attractiveness to females are those commonly found in mushroom compost. We found that some fungal species that were not attractive to females were very strong oviposition stimulants to females, therefore we hypothesize that the volatiles from some fungi attract the females to parts of the compost, and other fungi then stimulate the females to lay their eggs there. We found that larvae can develop to adulthood by feeding on the mycelia of only three different fungal species, and for all of the other species, the larvae died before making it past the first larval stage. We have also isolated an active female-emitted sciarid mushroom fly sex pheromone component, a sesquiterpene alcohol that is a type of germacradienol, that is highly active in attracting males. This work proves that a compound identified as a sex pheromone in 1980, by a different research group, was an erroneous and misleading identification that has hampered research into finding a true and highly active sex pheromone such as the one we have isolated.

We are currently attempting to characterize the precise stereochemical structure of this very difficult and first-ever discovered form of germacradienol, and have formed collaborative research efforts with top-notch sesquiterpene chemists from Germany, England, the U. S., and

Costa Rica. Additional manuscripts are being prepared on: a) the use of Botanigard for phorid control and b) methods for optimization and maintenance of phorid colonies.

We also showed that females are attracted to the parasitic green mold, *Trichoderma aggressivum*. We have attempted to begin to identify what is in the mushroom compost that attracts female *L. ingenua*. We isolated several species of fungi from adult males and females, third instar larvae, and the mushroom compost itself. We then analyzed the attraction of females to these substrates using a static-flow two choice olfactometer (**Fig. 1**), as well as their oviposition tendencies in another type of assay in glass petri dishes under choice and no-choice conditions. We also assessed the survival of larvae to adulthood when 1st instar larvae were placed on each of the isolated fungal species. We found that female flies were attracted most to the mycoparasitic green mold, *T. aggressivum*, to *Penicilium citrinum* isolated from adult female bodies, and to *Scatylidium thermophilium* isolated from the mushroom compost (**Fig. 2**). Pregnant female flies laid the most eggs on *T. aggressivum*, *Aspergillus flavus* isolated from third instar larval feces, *Aspergillus fumigatus* isolated from adult male bodies, and on *P. citrinum* (**Fig. 3**). This egg-laying trend remained consistent under no-choice conditions as females aged (**Fig. 4**). First instar larvae developed to adulthood only on *S. thermophilium* and *Chaetomium* sp. isolated from mushroom compost, and on *P. citrinum* from female bodies (**Fig. 5**).

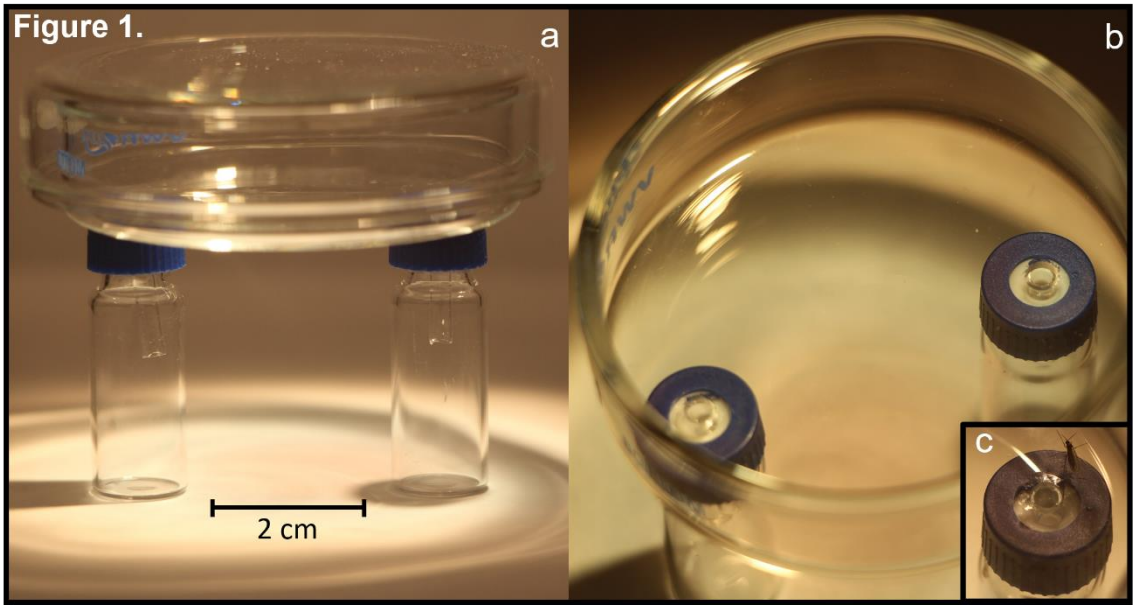

Figure 1. Two-choice, static-flow olfactometer. (a) The olfactometer consists of a 5-cm-diameter glass Petri dish release arena attached to two 2-ml glass vial pitfall traps spaced 3.5-cm apart through two 3-mm-diameter holes. A 4-mm-diameter, 1.5-cm-long glass tube extends into each of the two 2-ml glass vial pitfall trap lids. These glass tubes are affixed into the lids of the 2-ml glass vial pitfall traps. (b) The tips of the tubes extend out of the 2-ml glass vial pitfall traps and are positioned directly under 3-mm diameter holes drilled into the release arena floor. Because the tubes are 1-mm diameter larger than the drilled holes, the release arena is able to be set directly over these holes such that they lay flush with the release arena floor. This is critical in facilitating female *Lycoriella ingenua* entrance into the pitfall traps. (c) A single female fly is shown here resting next to the glass tube leading to a 2-ml glass vial pitfall trap.

Figure 2.

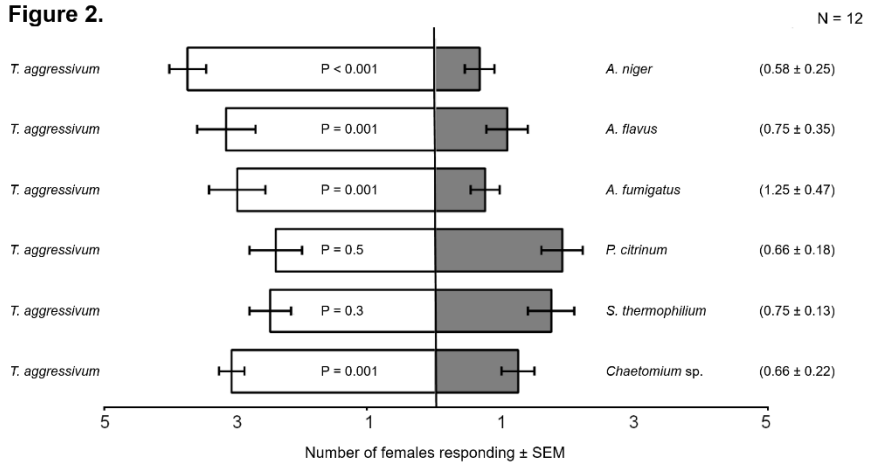

Figure 2. Mean number (± SEM) of two-day-old gravid female *Lycoriella ingenua* flies attracted to various pure fungal cultures grown on potato dextrose agar in two-choice, static-flow olfactometer assays. Each horizontal bar is the mean of responses of 5 females to each treatment over 12 replicates (N=12). The mean number of non-responders (± SEM) for each combination is included in parentheses to the right. Sixty individual flies were thus tested for each choice between different fungal cultures. All data were normally distributed and female choices for each pair of cultures were analyzed via the Students *t*-test (two-tailed, df = 11). All non-responders were excluded from the analysis.

Figure 3.

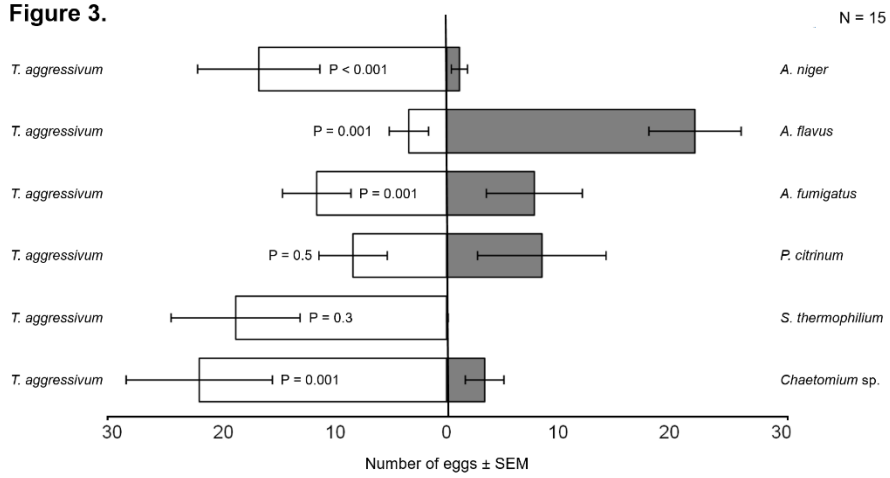

Figure 3. Mean number (± SEM) of eggs laid by two-day old gravid female *Lycoriella ingenua* flies on various pure fungal cultures grown on potato dextrose agar in two-choice oviposition assays. Each horizontal bar shows the mean number of eggs laid on the two choices of fungi by 15 two-day-old gravid female flies, tested in groups of three in three individual chambers with 5 different cohorts of females being tested over 5 different nights. Thus, a total of 15 females were tested for each pair of fungal cultures, with all pairs of cultures tested simultaneously on the same nights. All data were non-normally distributed and differences between the mean number of eggs deposited on each pair of fungal cultures were analyzed via the Mann-Whitney test (two-tailed, df = 14).

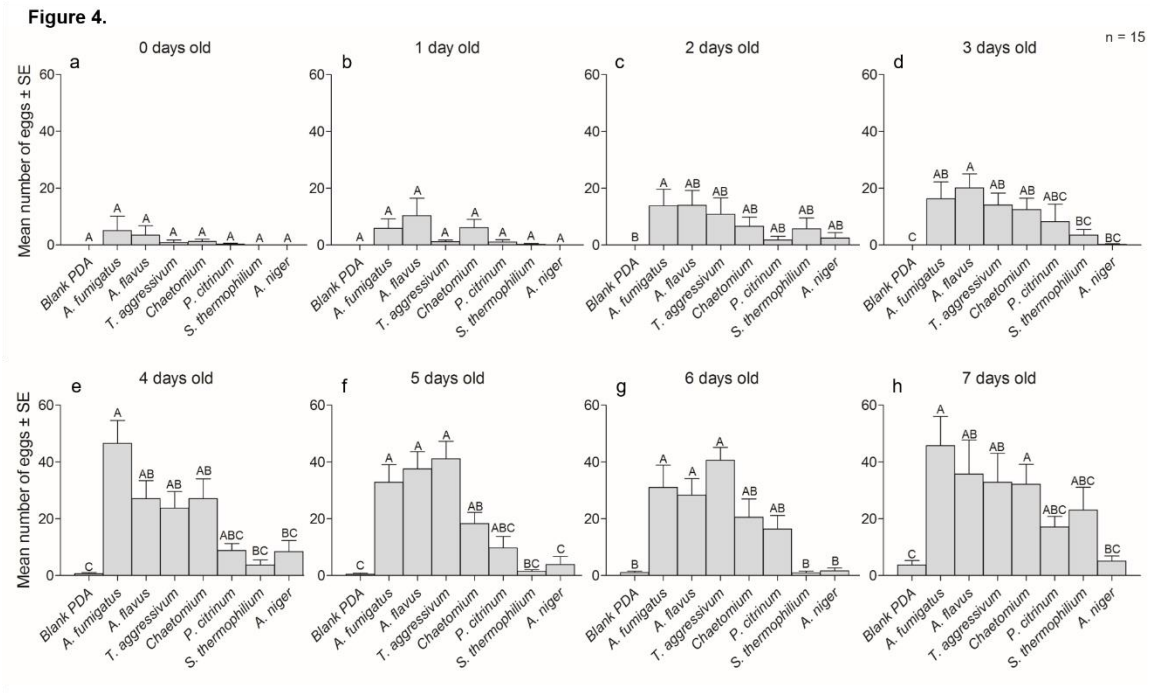

Figure 4. Mean number (± SEM) of eggs laid by 0- (day of emergence) to-7 day-old female *Lycoriella ingenua* flies on various pure fungal cultures grown on potato dextrose agar in no-choice oviposition assays. Each panel shows the mean number of eggs laid by 15 different female flies of that age on different fungal cultures. The 15 flies were tested in groups of three in three individual chambers, with 5 different cohorts of females being tested over 5 different nights. Thus, a total of 15 flies of a particular age were tested for their tendency to lay eggs on each of the 8 fungal cultures under no-choice conditions, resulting in a total of 120 females (15 flies x 8 fungal cultures) of each age that were tested for oviposition on all fungal cultures. There were 8 age groups tested (panels a-h), and thus 840 different female flies were used in this experiment. All data were non-normally distributed and differences among mean egg numbers within each age group were first analyzed via the Kruskall-Wallis test, then differences between mean egg numbers for each age group were compared via the Dunn's Multiple Comparisons Test. No comparisons were made between mean numbers of eggs in different age groups. Different letters above histograms within the same age group indicate a significant difference (df =14; $P < 0.05$).

Figure 5.

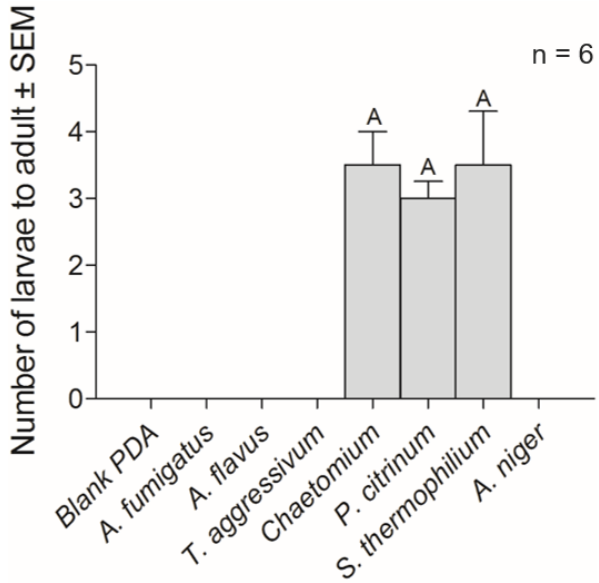

Figure 5. Mean survival (± SEM) of *Lycoriella ingenua* larvae to adults on various pure fungal cultures grown on potato dextrose agar. Each histogram is the mean survival of 5 newly emerged first instar larvae, replicated from 6 different cohorts of larvae. Thus, a total of 30 larvae were tested for each of the 7 single fungal cultures and a blank PDA control. All data were non-normally distributed and differences among mean survival on different fungal cultures were first analyzed via the Kruskall-Wallis test, then differences between survival on different fungal cultures were compared via the Dunn's Multiple Comparisons Test. Different letters above bars indicate a significant difference (df =5; $P < 0.05$).

We have made progress in identifying the sex pheromone of *Megaselia halterata*, which differs from the structure identified in 1983 by Ray Baker and colleagues that was never shown to be behaviorally active. In anticipating the use of the sex pheromone that we are nearing the identification of for Lycoriella ingenua, we examined whether delayed mating of females caused by mating disruption of this species could reduce fecundity and fertility. We performed extensive experiments delaying the mating of females by from one to seven days. We found that there was no negative effect on female fecundity or fertility when mating was delayed by up to five days. We have successfully extracted pheromone from wild-caught *M. halterata* females and shown, using coupled gas chromatograph/electroantennogram recordings (GC/EAG), that there are several EAG-active peaks using male antennae but not from female antennae. We have performed coupled GC-Mass Spectrometer (GC/MS) analyses of these peaks and found two peaks that do not conform to the sex pheromone of this species previously identified by Ray Baker and colleagues (1983). We are pursuing obtaining synthetic samples of this prior-identified compound plus chemically characterizing the other two EAG-active peaks.

Utilizing our supply of phorid flies, we have conducted a series of replicated bioassays to determine the potential efficacy of *Beauveria bassiana* (specifically Botanigard®) for control of phorids. Results obtained are similar to those previously reported for sciarid flies, and it appears that Botanigard® is not effective as a compost drench for phorid fly control. However, when used as a premise spray, Botanigard is effective at killing adults of both species, when flies are exposed to the sprayed surface soon after application. However, while the speed of kill was found to be significantly different to the control population for both fly species, it was not

sufficiently fast enough (8 days and 11 days to 100% mortality for sciarid and phorid flies respectively), to recommend its use to mushroom growers. Since the lack of efficacy of *B. bassiana* had been established by the end of 2016, a range of alternative biopesticide products, which are registered for use on other crops, were obtained and evaluated for efficacy.

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