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Indirect Bacterial Effect Enhanced Less Recovery of Neonicotinoids by Improved Activities of White-Rot Fungus *Phlebia brevispora*

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Bacterial strains that improve mycelial morphology and growth of white-rot fungi in liquid medium could enhance the impact of white-rot fungi towards lesser recovery of neonicotinoids when cocultured. This was demonstrated by the recovery of clothianidin and acetamiprid from cocultures of the white-rot fungus *Phlebia brevispora* strains with two mycelial-growth-promoting bacteria, *Enterobacter* sp. TN3W-14 and *Pseudomonas* sp. TN3W-8. Clothianidin recovery from cocultures of white-rot fungi and bacteria was over 40% lower than that from axenic microbial cultures and mixed-bacterial cultures. About 20% less acetamiprid was equally recovered from both TMIC33929+TN3W-14 cocultures and mixed-bacterial cultures than from axenic fungal and bacterial cultures.

Keywords: Bioremediation, fungal remediation enhancement, insecticides, growth-promoting bacteria, white-rot fungi

Neonicotinoids, which are the most widely used insecticides in the world, affect the central nervous system of insects, resulting in paralysis and death [1]. These compounds invariably cause less toxicity to birds and mammals than insects; however, they have been implicated in the death of beneficial insects such as honey bees [2]. Because some neonicotinoids equally degrade slowly and can remain in the environment for months or even years after application [1], there is a need for more proactive degradation and detoxification measures.

Fungi and bacteria have been shown to possess the ability to convert pesticides into simpler, non-toxic compounds. Moreover, white-rot fungi, which grow naturally on wood, are characterized by their ability to decompose lignin and other organic substances [3]. Recent research has focused on developing microbial consortia for complete degradation of such pollutants. This is because microbial consortia consisting of various bacteria and fungi have been found to have superior biodegradation performance than single microbial strains [4].

Our study [5] revealed that two bacterial strains, TN3W-8 and TN3W-14, which showed high homology with *Pseudomonas* and *Enterobacter*, respectively, were able to improve mycelia growth of the white-rot fungi *Phlebia brevispora* in liquid medium via dissociation of mycelial gathering. The white-rot fungus *P. brevispora*, which is one of the most studied species, has the ability to degrade organohalogen compounds such as polychlorinated biphenyls, polychlorinated dioxins, and organohalogen pesticides [6–8]. With the hypothesis that this growth enhancement would translate to improved bioremediation traits in white-rot fungus, the present study was conducted to evaluate the impact of *P. brevispora* with or without coculturing with growth-promoting bacteria on recovery of two neonicotinoid insecticides, clothianidin and acetamiprid.

The white-rot fungi *P. brevispora* strains TN3F and TMIC33929, and its growth-promoting bacteria *Enterobacter* sp. TN3W-14 or *Pseudomonas* sp. TN3W-8 were incubated on PDB or PDA medium (Difco, France) according to our previous study [5].

Growth media were prepared by adding two 2-cm-diameter mycelial disks to fungal cultures in autoclaved 100-ml Erlenmeyer flasks containing Kirk's low nitrogen medium (LNM) [9] at different culture formulations. Fungal axenic cultures (TMIC33929 or TN3F) contained 10 m1 LNM, while bacterial axenic cultures (TN3W-8 or

TN3W-14) and fungal-single bacterial (TMIC33929+TN3W-8; TMIC33929+TN3W-14 or TN3F+TN3W-8; TN3F+TN3W-14) cocultures contained 8 ml LNM and 2 ml of corresponding preincubated bacterial cells, with an initial cell population of approximately 10^6 cells ml^{-1}. For fungal-mixed bacterial cocultures (TMIC33929+TN3W-8+TN3W-14 or TN3F+TN3W-8+TN3W-14) and mixed-bacterial cultures (TN3W-8+TN3W-14), 1 ml each of the preincubated bacterial cultures was added to 8 ml of LNM. PDB medium from 0.0 ml to 2.0 ml was added to fungal cultures, in place of bacterial cells, to evaluate its effect on fungal activity. Cultures were then pre-incubated for 5 days on a rotary shaker at 120 rpm, after which 200 µl of 10 mM substrate (final conc. 0.05 mM) in *N*,*N*-dimethylformamide was added. The headspace of each flask was subsequently aseptically flushed with oxygen for 5 s at inoculation, sealed with a glass stopper and sealing tape, and incubated in the dark on a rotary shaker at 25°C for 15 days. For this experiment, abiotic cultures served as a control

At 15 days after sampling, triplicate samples of each culture were homogenized with 10 ml of acetonitrile after addition of internal standard (coumarine, final conc. 0.01 mM). To quantify the amount of substrate recovery, the resulting supernatants were analyzed by high performance liquid chromatography (HPLC). HPLC was performed on a Shimadzu LC-10AD with a SPD-20A UV/VIS (254 nm) detector and a STR ODS-II 250 × 4.6 mm column. T-tests were used to determine the level of significance at a *p*-value of 0.01 for clothianidin and 0.05 for acetamiprid. To determine the stability of the reactants, half-lives and rate constants of the first order reaction were calculated using the first order degradation rate model [10] as described below:

$$X_t /2 = X_0 e^{-kt_{1/2}},$$

where X_t was the concentration of neonicotinoid insecticides (µM) at time t (days), k was a first order degradation constant (d^{-1}) and $t_{1/2}$ was the half-life. By applying non-linear regression to the experimental data, $k = 0.693/t_{1/2}$ was obtained.

To determine metabolites, the 10-ml contents from each flask were adjusted to pH 2.5 then transferred to separating funnels and extracted twice with an equal volume of ethyl acetate. The ethyl acetate phase was pooled and dried by a rotary evaporator and nitrogen gas. The dried samples were subsequently dissolved in methanol (600 µl) for HPLC analysis.

In the present study, axenic fungal and bacterial cultures gave high recovery of both clothianidin and acetamiprid. For clothianidin (Table 1), cultures of fungal strains TN3F and TMIC33929 recovered 68.9% and 87.7% of substrate at estimated half-lives of 29.7 days (k = 0.023) and 37.8 days (k = 0.018) respectively, which were significantly higher ($p > 0.01$) than the recovery from the coculture samples, while cultures of bacterial strains TN3W-14 and TN3W-8 recovered almost all substrate added, similar to the abiotic control. Fungal cocultures with each of the bacterial strains recovered about 30% of clothianidin after 15 days of incubation. TMIC33929+TN3W-14 and TMIC33929+TN3W-8 cocultures recovered 22.9% and 24.2% of the substrate at an estimated half-life of 10 days (k = 0.070 and 0.066) respectively, while TN3F+TN3W-14 and TN3F+TN3W-8 cocultures recovered 31.3% and 26.4% of the clothianidin at estimated half-lives of 13.5 days (k = 0.051) and 11.4 days (k = 0.061) respectively. Substrate recovery from mixed-bacterial cultures was also about 50% higher than that from fungal-bacterial cocultures. The half-life of clothianidin has been noted to range from 148–1155 days [11], although half-life and behavior of clothianidin are governed by a variety of complex dynamic physical, chemical, and

Table 1. Clothianidin recovery R (%), half-life $t_{1/2}$ (d) and rate constant k (d^{-1}) for fungal axenic cultures and cocultures with bacterial strains.

	Fungal and bacterial combinations										
Fungi	TN3F	TN3F	TN3F	TN3F	TMIC33929	TMIC33939	TMIC3393	TMIC33929			
Bacteria		TN3W-8	TN3W-14	TN3W-8 TN3W-14		TN3W-8	TN3W-14	TN3W-8 TN3W-14	TN3W-8	TN3W-14	TN3W-8 TN3W-14
R (%)	68.9	26.4*	31.3*	17.2*	87.7	24.2*	22.9*	15.1*	109.4	96.7	76.8
	±2.4	±3.5	±2.1	±4.9	±11.6	±1.8	±1.1	±1.8	±15.4	±4.3	±1.8
$t_{1/2}$(d)	29.7	11.4	13.5	7.4	37.8	10.4	9.9	6.5	47.1	41.7	33.1
k (d^{-1})	0.023	0.061	0.051	0.094	0.018	0.066	0.070	0.107	0.015	0.017	0.021

Recovery values are means ± standard deviations of triplicate samples.

*Asterisks represent coculture recovery values, which are significantly different from corresponding fungal axenic cultures at $p > 0.01$. Recovery from abiotic control was 99.8 ± 5.9.

Table 2. Acetamiprid recovery R (%), half-life $t_{1/2}$ (d) and rate constant k(d^{-1}) for axenic cultures of *Phlebia brevispora* TMIC33929, cultures of bacterial strains TN3W-14 and TN3W-8 and fungal-bacterial cocultures.

	Fungal and bacterial combinations						
Fungi	TMIC33929	TMIC33939	TMIC3393	TMIC33929			
Bacteria		+TN3W-8	+TN3W-14	+TN3W-8 +TN3W-14	TN3W-8	TN3W-14	TN3W-8 +TN3W-14
R (%)	81.59	76.88	63.37*	64.63*	103.90	78.64	63.35
	±2.6	±12.3	±2.5	±3.6	±1.8	±4.7	±8.9
$t_{1/2}$(d)	35.140	33.110	27.290	27.836	44.749	33.870	27.286
k (d^{-1})	0.020	0.021	0.025	0.025	0.015	0.020	0.025

Recovery values are means ± standard deviations of triplicate samples.

*Asterisks represent coculture recovery values, which are significantly different from corresponding fungal axenic cultures at $p > 0.05$. Recovery from abiotic control was 101.1 ± 0.3.

biological processes [12]. Few studies have however achieved shorter half-lives in non-soil conditions [13, 14], especially with enriched microbial cultures. In the present study, shorter half-lives of clothianidin in fungal-bacterial cocultures along with clear transformation of the substrate were observed, representing strong points of this less recovery strategy. Similarly for acetamiprid (Table 2), coculture of TMIC33929+TN3W-14 recovered 63.3% of the substrate at an estimated half-life of 27.3 days (k = 0.025), which was significantly lower ($p > 0.05$) than the 81.5% recovery observed for TMIC33929 cultures at an estimated half-life of 35.1 days (k = 0.020). Acetamiprid has generally been noted to degrade more slowly in water than in soils [15, 16].

To avoid the concerns about the effect of PDB addition on fungal activity, PDB medium without bacterial inoculation was added to the LNM with *P. brevispora*. The results showed that the addition of little or no PDB gave relatively the same quantity of insecticide recovery as axenic fungal cultures, while the addition of higher levels of PDB tended to increase substrate recovery (data not shown). Few studies have shown that bacterial strains with little or no degradative activity for an insecticide could enhance fungal reduction of the same insecticide. Nevertheless, the present study revealed a remarkable increase in the mycelial growth of coculture samples, along with less recovery of neonicotinoids by improved basidiomycetes' activities.

Possible metabolic products from fungal and bacterial treated cultures of acetamiprid were found in ethyl acetate extracts by HPLC analysis (Fig. 1). A peak corresponding to the compound, I, appeared on the axenic TMIC33929 chromatogram at a retention time of 2.74 min, while another peak, II, appeared on chromatograms of TN3W-14+TN3W-8 coculture at a retention time of 3.41 min. Furthermore, detection of compound III at 4.36 min from fungal-bacterial cocultures alone suggests cooperative degradation by the fungus and the bacterium. Although further detailed study should be needed to identify these compounds, these detected compounds did not appear on chromatograms of biotic control samples.

This paper reported the indirect use of bacterial strains (of low insecticide metabolism) in achieving enhanced fungal activity for lesser insecticide recovery, by establishing a

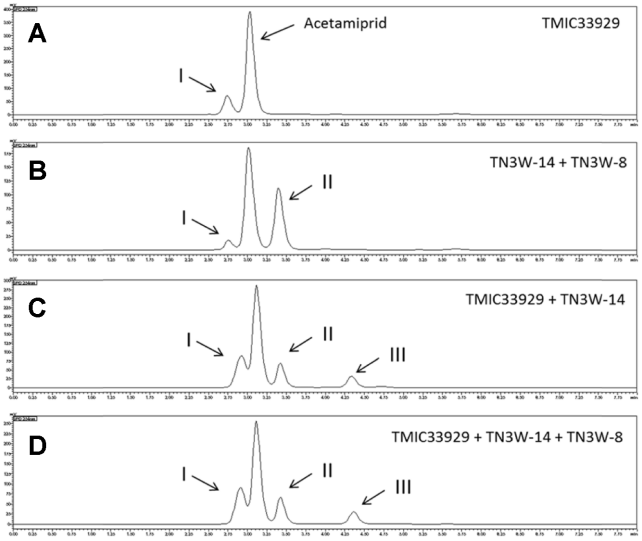

Fig. 1. Possible metabolic compounds during acetamiprid recovery in Kirk low nitrogen medium.

Products in acetamiprid culture with fungal strain TMIC33929 (**A**), mixed bacterial coculture of strains TN3W-14 and TN3W-8 (**B**), fungal-bacterial coculture of fungal strain TMIC33929 with bacterial strain TN3W-14 (**C**), and fungal-mixed bacterial coculture of fungal strain TMIC33929 with bacterial strains TN3W-14 and TN3W-8 (**D**).

mycelial morphological and growth-promoting relationship between basidiomycetes and bacteria in liquid medium.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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