



Toxicity of delta endotoxins from native *Bacillus thuringiensis* isolates against the larger grain borer *Prostephanus truncatus* (Horn) under different maize-based toxin delivery forms

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ABSTRACT

Objectives: To evaluate the toxicity of delta endotoxins from native *Bacillus thuringiensis* isolates against adult *Prostephanus truncatus* and determine whether maize-based toxin delivery form and toxin solubility influence mortality under laboratory conditions.

Methodology and Results: Adult *P. truncatus* were exposed separately to soluble toxin fractions or insoluble spore–crystal preparations from eight *B. thuringiensis* isolates delivered on whole maize grains, coarsely ground maize particles or maize flour. Re-entry of the surviving mortality worksheet at the ten-assessment structure documented in the thesis appendix gave 539 observations. Three-way ANOVA detected effects of isolate ($F_{8,485} = 4.268$, $p < 0.001$) and maize form ($F_{2,485} = 3.676$, $p = 0.026$), but not solubility ($F_{1,485} = 0.249$, $p = 0.618$); all interactions were non-significant.

Conclusions and application of findings: Toxicity was isolate-dependent, with KG 20 producing the highest pooled mortality. Dunnett-adjusted comparisons showed greater mortality than the untreated control for KG 12, KG 20, KG 411, TEN, KUR and KM 31, whereas KM 24 and ISR were not significant after multiplicity adjustment. Maize form affected mortality, but the absence of a solubility effect indicates that a less processed spore–crystal formulation may be a practical development route. The findings support confirmatory dose–response, persistence, food-safety and whole-grain storage trials.

Keywords: *Bacillus thuringiensis*; delta endotoxins; *Prostephanus truncatus*; stored maize; biological control

INTRODUCTION

Post-harvest losses caused by insect pests remain a major constraint to food security in sub-Saharan Africa. The larger grain borer, *Prostephanus truncatus* (Horn), is a destructive pest of stored maize. Its feeding damages kernels and produces frass, increasing quantitative and quality losses (Muatinte *et al.*, 2019; Panagiotakis *et al.*, 2023). Reliance on synthetic insecticides also raises concerns about resistance, residues, environmental contamination and human exposure (Pathak *et al.*, 2022). *Bacillus thuringiensis* (Bt) is used as a microbial insecticide because its pesticidal proteins can act selectively after ingestion. Toxicity

nevertheless varies among Bt strains and insects because toxin composition, solubilization, proteolytic activation and midgut receptor interactions differ (Jurat-Fuentes *et al.*, 2021; Endo, 2022; Aswathi *et al.*, 2024). Formulation can also affect stability, adherence and ingestion of Bt products (de Oliveira *et al.*, 2021). This study evaluated delta endotoxins from five archived Kenyan Bt isolates and three reference varieties against adult *P. truncatus*. It tested whether Bt isolate, maize-based delivery form and toxin solubility affected mortality, with the aim of identifying candidates for further development for stored-maize protection.

MATERIALS AND METHODS

The activity of *Bacillus thuringiensis* (Bt) delta endotoxins was determined following confirmation of isolate identity and subsequent purification and extraction of the toxins using the procedures described below.

***Bacillus thuringiensis* isolates:** Eight *Bacillus thuringiensis* preparations were evaluated: Kenyan isolates KG 411, KG 12-0 and KG 20 from Gazi in coastal Kenya, and KM 31 and KM 24 from Machakos in eastern Kenya, together with *B. thuringiensis* var. tenebrionis, var. israelensis and var. kurstaki used as reference comparators. The cultures were obtained from the Centre for Biotechnology and Bioinformatics, University of Nairobi, where they had been maintained at -20°C for approximately three years before this study.

Culture and toxin preparation: The Bt isolates were cultured following Wang'ondut *et al.* (2003), with study-specific modifications. Cultures were grown in nutrient broth at $28 \pm 2^{\circ}\text{C}$ and 200 rpm until approximately 90% cell lysis after 72 h. Spores and endotoxins were harvested by centrifugation at 5,000 rpm and 5°C for 15 min, washed with phosphate buffer (0.1 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 0.1 M NaCl, pH 6.0), and prepared for the assays described below.

Microscopy: Purity of crystal preparations was assessed using Gram staining (Poinar, 2012). Gram-positive cells appeared dark violet with visible refractile spores, while Gram-negative cells appeared pink following counterstaining.

Protein Estimation and Solubilization of Bt Toxins : Protein concentration of each Bt isolate was estimated using bovine serum albumin (BSA) standards spanning 0–2.0 mg mL^{-1} . Absorbance was measured at 280 nm, and the original standard readings were fitted by ordinary least-squares linear regression. Stock concentrations were back-calculated from the fitted equation before preparation of the working bioassay suspensions. The archived enzymatic-activity readings at 750 nm were not used for protein quantification. Purified Bt crystals (0.5 mg) were solubilized in 50 mM Na_2CO_3 –HCl buffer (pH 9.5) containing 10 mM dithiothreitol (DTT). The mixture was incubated in a water bath at 37°C for 14 h following a modification of the protocol by Haider *et al.* (1986). Insoluble material was removed by centrifugation at $4,000 \times g$ for 10 min at 4°C . The insoluble protoxin fraction was washed in phosphate-buffered saline (PBS) by centrifugation to remove residual Na_2CO_3 , HCl and DTT.

Protein estimation was carried out for both soluble and insoluble protoxin fractions as described above, and samples were stored at -20°C .

Protease Treatment of Bt Protoxins :

Solubilized protoxin fractions were dialysed in 0.1 M Tris-HCl buffer (pH 8.0) at 4°C for 24 h, while insoluble protoxin fractions were washed three times in the same buffer by centrifugation at $4,000 \times g$ for 10 min at 4°C . Samples were treated with bovine pancreatic trypsin and α -chymotrypsin (Serva, Heidelberg, Germany), as well as partially purified trypsin-like protease from *Glossina* spp. midgut. Crude gut homogenate was prepared from adult *P. truncatus* by dissecting the guts and homogenising them for 1 min in ice-cold 0.1 M Tris-HCl (pH 8.0). After centrifugation at $12,000 \times g$ for 10 min at 4°C , trypsin activity in the supernatant was measured using α -N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Wamunyokoli, 1992). Protoxin was also treated separately with bovine pancreatic trypsin, α -chymotrypsin, or partially purified trypsin-like protease from *Glossina* midgut; enzyme–protoxin mixtures were incubated at 37°C for 60 min and then held at 4°C .

Screening of *Bacillus thuringiensis* Toxicity against *Prostephanus truncatus*: Adult *P. truncatus* (at least 30 days old) were obtained from KARI-NARL (now KALRO). The laboratory colony was maintained on autoclaved maize grains at $27 \pm 2^{\circ}\text{C}$ and $65 \pm 5\%$ relative humidity. Unsexed adults were

used. Soluble toxin fractions and insoluble spore–crystal preparations were evaluated separately for each isolate; toxins from different isolates were not mixed. For each treatment, 2 mL of preparation was mixed with 10 g of whole maize grain (MG), coarsely ground maize particles (MP), or finely ground maize flour (MF). Treated diets were air-dried for 24 h at 30°C . Ten adults were placed in each Petri dish, with three replicate dishes per treatment. Mortality was recorded over 21 days, and the experiment was conducted three times. Reported descriptive means are numbers of dead adults on a 0–10 scale for the experimental unit, not totals out of 30 adults.

Data Analysis: The analysis was carried out by having dead-adult counts were entered from the ten numbered assessment blocks corresponding to the $N = 10$ cell structure using SPSS version 21 and in Python 3.12 using SciPy 1.17. Available replicate-dish entries were averaged within each assessment $\times$ isolate $\times$ maize form $\times$ solubility cell. One KUR $\times$ maize-particle $\times$ insoluble assessment was missing, giving $N = 539$ and error $df = 485$. Mortality was analysed by three-way fixed-effects ANOVA using sum-to-zero contrasts. Partial η^2 was calculated for each effect. Unadjusted least significant difference (LSD) comparisons were used to derive compact-letter groupings, while two-sided Dunnett simultaneous tests compared each Bt isolate with the untreated control. Statistical significance was assessed at $\alpha = 0.05$.

RESULTS

Growth Characteristics and Microscopy of *Bacillus thuringiensis*: Growth and microscopic characteristics confirmed all isolates as *B. thuringiensis*. Reanalysis of the original BSA standards showed a strong positive linear relationship between absorbance and protein concentration ($r = 0.9637$; $R^2 = 0.9286$; $p < 0.001$). All *B. thuringiensis* isolates formed pale cream

colonies on nutrient agar (Plate 1a). Microscopic examination revealed actively multiplying vegetative cells and the presence of spores in sporulating cultures (Plate 1b). Gram staining showed that the isolates were Gram-positive rods, occurring singly or in chains, with refractile endospores visible either within vegetative cells or as free spores.

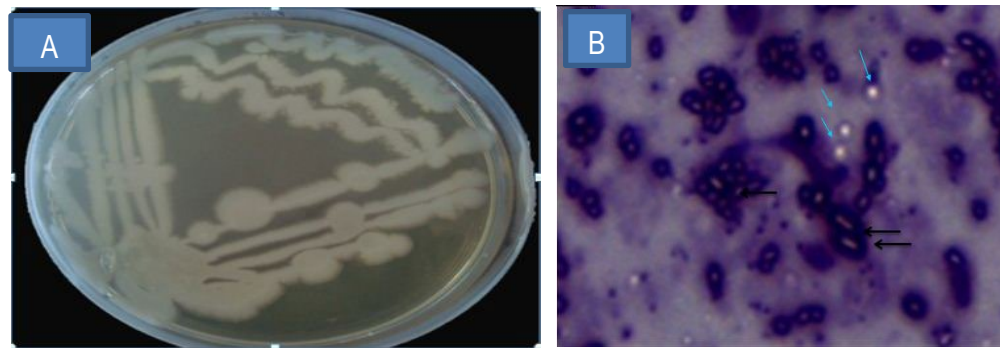


Plate 1. (a) Colony morphology of *Bacillus thuringiensis* on nutrient agar; (b) Gram-stained cells showing vegetative cells and refractile spores ($\times 1000$). Black arrows indicate intracellular spores; blue arrows indicate free spores.

Protein Estimation : Protein concentrations of Bt delta endotoxins were estimated from the author-supplied BSA standard series (Fig. 1). The fitted equation was absorbance = $0.2720 \times$ concentration + 3.0751 ($r = 0.9637$; $R^2 =$

0.9286; $p < 0.001$). Back-calculated stock estimates ranged from 0.389 mg mL^{-1} for KG 12-0 to 1.492 mg mL^{-1} for KUR (Supplementary Table S4).

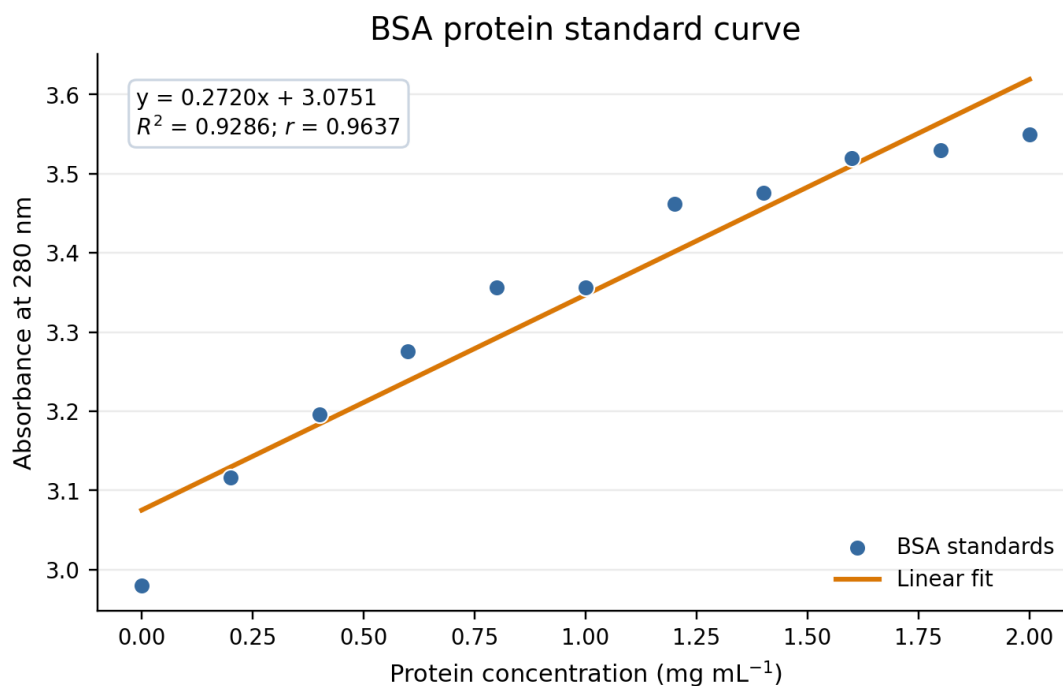


Figure 1: Original bovine serum albumin standard curve used to estimate Bt protein concentrations. Points are the supplied standards; the line is the ordinary least-squares fit ($n = 11$).

Mortality of *P. truncatus* differed among *B. thuringiensis* isolates ($F_{8,485} = 4.268$, $p < 0.001$, partial $\eta^2 = 0.066$) and maize delivery forms ($F_{2,485} = 3.676$, $p = 0.026$, partial $\eta^2 = 0.015$), but not between soluble and insoluble preparations ($F_{1,485} = 0.249$, $p = 0.618$). No interaction was significant (Table 1).

Table 1: Effects of *Bacillus thuringiensis* isolate, maize form and toxin solubility on mortality of *Prostephanus truncatus*

Source of variation	df	F-value	p-value	Partial η^2
Bt isolate	8	4.268	< 0.001*	0.066
Maize form	2	3.676	0.026*	0.015
Solubility	1	0.249	0.618 ns	0.001
Bt $\times$ maize form	16	1.436	0.120 ns	0.045
Bt $\times$ solubility	8	0.991	0.442 ns	0.016
Maize $\times$ solubility	2	1.250	0.288 ns	0.005
Bt $\times$ maize $\times$ solubility	16	0.955	0.505 ns	0.031

Significant at $p \leq 0.05$; ns = not significant. Three-way fixed-effects ANOVA; N = 539, error df = 485. Partial η^2 is reported as the effect-size estimate.

Pooled estimated marginal mortality was highest for KG 20 (3.519 adults per dish). LSD comparisons showed that KG 20 exceeded KM 24 ($p = 0.006$), ISR ($p = 0.004$) and TEN ($p = 0.037$); no other Bt-to-Bt LSD comparison was significant. Dunnett-adjusted comparisons with the untreated control were significant for

KG 12 ($p = 0.001$), KG 20 ($p < 0.001$), KG 411 ($p < 0.001$), TEN ($p = 0.009$), KUR ($p = 0.003$) and KM 31 ($p = 0.004$), but not KM 24 ($p = 0.059$) or ISR ($p = 0.085$). Compact-letter groups were KG 20 (a); KG 411, KG 12, KUR and KM 31 (ab); TEN, KM 24 and ISR (b); and control (c) (Fig. 4; Supplementary Table S3).

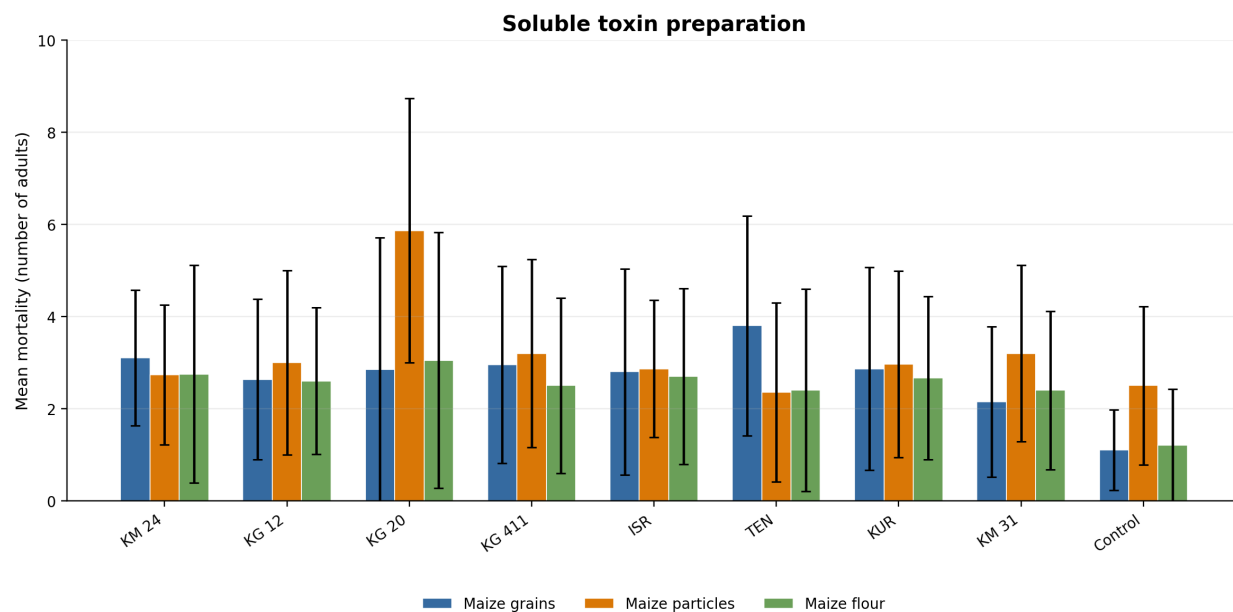


Figure 2: Mean mortality ($\pm$ SD) of *Prostephanus truncatus* exposed to soluble *Bacillus thuringiensis* preparations delivered on maize grains (MG), maize particles (MP) or maize flour (MF). Values are assessment-level mean numbers of dead adults per dish on a 0–10 scale (n = 10 assessments per cell).

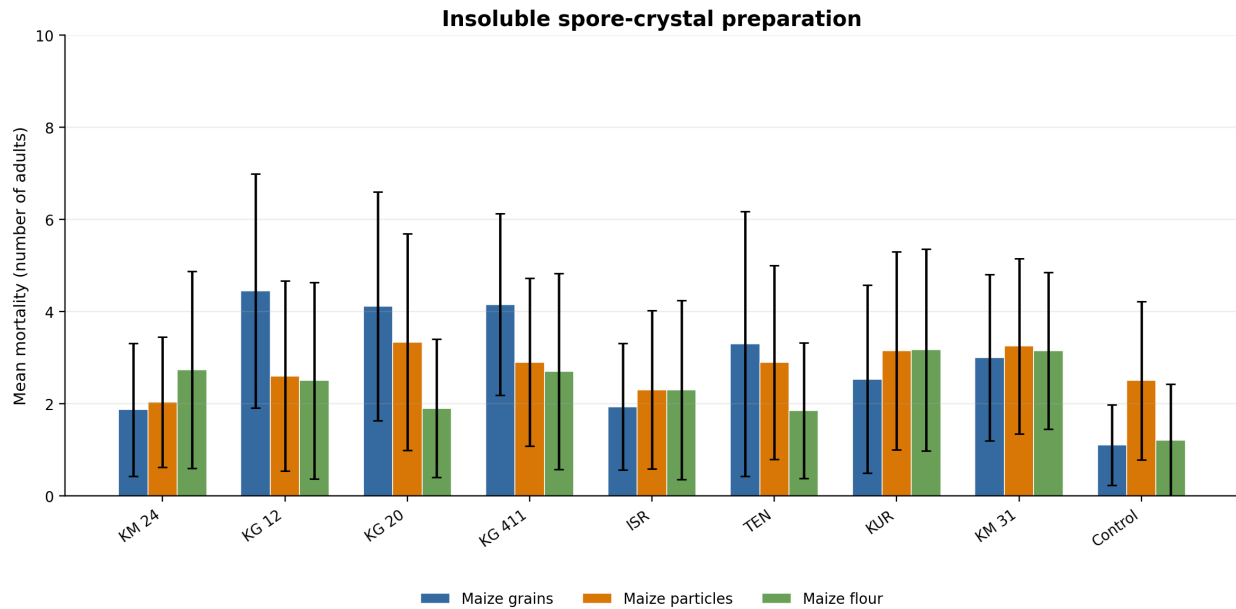
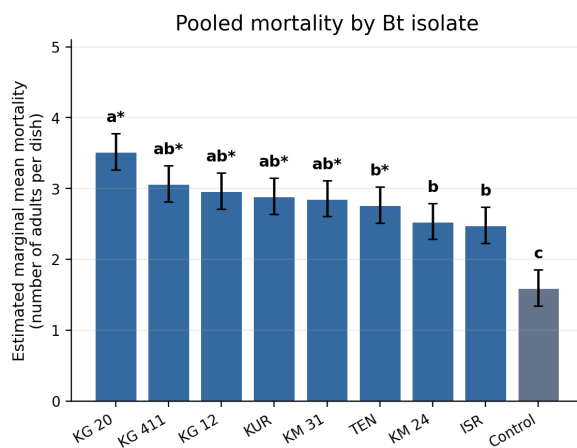


Figure 3: Mean mortality ($\pm$ SD) of *Prostephanus truncatus* exposed to insoluble *Bacillus thuringiensis* spore–crystal preparations delivered on maize grains (MG), maize particles (MP) or maize flour (MF). Values are assessment-level mean numbers of dead adults per dish on a 0–10 scale; $n = 10$ assessments per cell except KUR–MP ($n = 9$).



Bars show estimated marginal means $\pm$ SE. Shared letters indicate no LSD difference at $p \leq 0.05$; * indicates a significant Dunnett comparison with the untreated control.

Figure 4: Estimated marginal mean mortality ($\pm$ SE) pooled across maize form and solubility. Isolates sharing a letter are not different by unadjusted LSD at $p \leq 0.05$. An asterisk marks a significant two-sided Dunnett comparison with the untreated control after simultaneous adjustment.

DISCUSSION

The isolate main effect confirms that Bt activity against *P. truncatus* was strain-dependent. KG 20 had the highest pooled estimated marginal mortality, while KG 12, KG 20, KG 411, TEN, KUR and KM 31 each differed from the untreated control after Dunnett adjustment. KM 24 and ISR did not

meet the adjusted 0.05 threshold. Strain-dependent activity is biologically plausible because pesticidal-protein composition, activation and receptor binding determine potency (Jurat-Fuentes *et al.*, 2021; Endo, 2022; Aswathi *et al.*, 2024). KG 20 is therefore the clearest lead from the pooled raw-data

reanalysis, with KG 12, KG 411, KUR and KM 31 sharing an overlapping intermediate LSD group. These isolates are research leads rather than finished products. Performance should be confirmed through concentration–response assays, appropriate positive controls, independent culture batches and time-to-mortality analysis; characterization of pesticidal-protein profiles would help explain the isolate effect. Maize was both food and toxin carrier, so substrate structure could alter contact, adherence and ingestion. The significant maize-form main effect indicates that the delivery matrix mattered. However, treatment means were heterogeneous: particles did not produce the highest mortality for every isolate and solubility combination. The result should therefore be interpreted as evidence that formulation and substrate require joint optimization, rather than as a universal particle-size effect. Whole grain is the relevant storage commodity for most farmers. Grinding before storage would increase exposed surface area and may worsen handling and contamination risks; this study does not recommend that farmers store maize as particles. The practical route is to develop a formulation that adheres to intact kernels and remains available during *P. truncatus* feeding. Candidate formulations should be evaluated for persistence, grain quality, mould and mycotoxin risk, non-target effects and acceptability before recommendation.

CONCLUSION AND APPLICATION OF RESULTS

Raw-data reanalysis identified KG 20 as the strongest pooled *Bacillus thuringiensis* candidate against *Prostephanus truncatus*; KG 12, KG 411, TEN, KUR and KM 31 also produced greater mortality than the untreated control after Dunnett adjustment. Maize delivery form influenced mortality, whereas

Solubility had no detectable main effect ($p = 0.618$), and none of the tested interactions was significant at $\alpha = 0.05$. The additional solubilization step therefore did not measurably improve mortality under these assay conditions. A simpler spore–crystal formulation could reduce processing requirements, but that remains a product-development hypothesis requiring dose standardization, shelf-life testing and validation on whole grain (de Oliveira *et al.*, 2021). Direct efficacy comparisons with entomopathogenic fungi or contact insecticides are inappropriate because exposure routes, doses, endpoints and mechanisms differ. Bt preparations should be compared with Bt-based benchmarks under matched bioassay conditions. Recent work nevertheless confirms the continuing need for safer, effective *P. truncatus* management in stored maize (Muatinte *et al.*, 2019; Panagiotakis *et al.*, 2023; Mlambo *et al.*, 2026). Together, the isolate, solubility and delivery-form results identify KG 20 as the primary candidate for further formulation work and support confirmatory evaluation of KG 12, KG 411, KUR and KM 31. Whole-grain performance should be strengthened through improved adherence and persistence while retaining the low-processing advantage suggested by comparable soluble and insoluble preparations.

toxin solubility did not. Further work should standardize dose, characterize pesticidal proteins, test grain-adherent formulations on intact maize, and assess persistence, food safety, mould and mycotoxin risk under realistic storage conditions before any farmer recommendation or product claim.

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