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Comparative Analysis of Determination Methods of Glyphosate Degradation by *Trichoderma asperellum* Strain JK-28: A Multivariate Statistical Approach

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Authors' contributions

This work was carried out in collaboration between both authors. Author AA proposed the research, designed the protocol and conducted the experiments while author ME conducted the statistical analyses and drafted the first manuscript. Both authors prepared the final manuscript for submission.

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ABSTRACT

Aims: This study aimed at demonstrating the ability of a mold species, isolated from a rice farm agricultural soil, chronically treated with glyphosate herbicide, to degrade glyphosate and to compare different evaluation methods of biodegradation.
Study Design: A completely randomized design of a factorial experiment was used.
Place and Duration of Study: The study was conducted at the Department of Microbiology, University of Calabar, Nigeria between February and April.
Methodology: Molds were isolated from nine rice agricultural soil samples using potato dextrose agar. The molds were screened for glyphosate degradation using different media to determine their requirement for supplementation or not at all. A completely randomized design of a factorial experiment involving 3 factors at 3 levels each, with three replications, was adopted for glyphosate degradation studies. Degradation was monitored by dehydrogenase activity, carbon dioxide evolution, pH changes, fungal dry weight and residual glyphosate determination. A comparative evaluation of the different methods was made using multivariate statistics.

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Results: The mold, identified as *Trichoderma asperellum* strain JK-28, was incapable of using glyphosate as sole sources of phosphorus and nitrogen and so required nutrient supplementation. Largest significant correlation between dependent variables (P < .001; r = - 779) occurred between dehydrogenase activity and fungal dry weight while the lowest was between residual glyphosate and fungal dry weight. Residual glyphosate and pH had negative correlations with other dependent variables. Principal component analysis extracted only one component with a cumulative variance of 70%. Multivariate analysis of variance (three-way MANOVA) of data, interpreted from the Pillai's Trace test, was significant at P <.001 for the main, two- and three-way interactions.
Conclusion: *Trichoderma asperellum* strain JK-28 could degrade glyphosate using it as sole carbon source but with nutrient supplementation. Residual glyphosate determination is recommended as most reliable method for evaluating organic pollutant biodegradation.

Keywords: Trichoderma asperellum strain JK-28; glyphosate; degradation; correlation analysis; principal component analysis; three-way MANOVA.

1. INTRODUCTION

Glyphosate is a broad spectrum, non-selective herbicide used in the control and/or killing of grasses, herbaceous plants, including deep rooted perennial weeds, brush, some broad-leaf trees and some shrubs [1]. It can be used in no-till agriculture, to prepare fields before planting, during crop development and after crop harvest. Its mode of action is the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase, resulting in the depletion of essential aromatic amino acids needed for plant survival [2]. On application, glyphosate remains unchanged in the soil for varying lengths of time, as a result of its adsorption on clay particles and organic matter present in the soil [3]. There is increasing report of glyphosate seeping into and contaminating groundwater, even polluting it, at high concentrations. Attendant health and environmental effects of the herbicide contamination which therefore necessitates decontamination (remediation) are extensively discussed in Cox [4].

A recommended technique for environmental (soil or groundwater) remediation is the use of microorganisms. This is because the removal of glyphosate from the environment is usually by microbiological processes as chemical processes of degradation are ineffective because of the presence of highly stable carbon-phosphorus bonds present in the compound [5]. Studies of glyphosate-degrading bacteria have involved isolation of pure bacterial and fungal strains with novel degradation capabilities for potential uses in biotechnological industry and bioremediation of polluted soils and water [6]. Reports also show that glyphosate inhibit microbial activities in the soil in its attempt at killing weeds thus compromising soil fertility [7]. However, microorganisms significantly degrade glyphosate by breaking the carbon-phosphorus (C-P) bonds and releasing sarcosine as the major degradation product. The ability of microorganisms to utilize organophosphorus compounds as sole phosphorus source is well documented [6,8]. The first evidence of the biological cleavage of the C-P bond was obtained with *E. coli* as the bacterium utilized methyl-phosphonic or ethyl-phosphonic acids as sole sources of phosphorus. It is possible that after depletion of easily assimilated phosphorus sources in the environment, only the phosphites and phosphonates are left for microorganisms as sources of phosphorus.

In the present study, we demonstrate the ability of a rarely encountered mold, isolated from a rice field, to degrade glyphosate present in the frequently used herbicide 'Round-up' and compared, using multivariate statistics, the reliability of five major evaluation methods often employed in organic biodegradation measurements in laboratory and field experiments .

2. METHODOLOGY

2.1 Sample Collection and Selection of Glyphosate-degrading Molds Using Different Media

Nine rice agricultural soil samples, collected from three rice farms at Obubra Local Government Area of Cross River State, Nigeria, were plated onto potato dextrose agar by the spread plate technique and molds isolated and purified by repeated sub-culturing [8]. Pure cultures of molds were further inoculated into minimal media of dissimilar compositions to identify their growth requirement, if at all. Its composition varied

according to nutrient tested since glyphosate is reported to serve as sole sources of carbon, nitrogen and phosphorus [6,9]. All minimal media were supplemented with 1 mL of trace mineral solution containing (g/L): $ZnSO_4.7H_2O$ 0.005; $MnSO_4.4H_2O$ 0.005; H_3BO_3 0.005; $CuSO_4.5H_2O$ 0.005; $CoCl_2.6H_2O$ 0.005; $MoNa_2O_4.2H_2O$ 0.005 and $NiCl_2.6H_2O$ 0.005 [10]. The minimal medium, supplemented with 5000 µg/L glyphosate-based herbicide 'Round-up' was used throughout the study. Duplicate plates were prepared and incubated at room temperature for 14 days. Only the mold culture with the fastest and densest growth on any of the media was used for further studies.

Medium 1: Minimal medium containing 5,000 µg/L glyphosate as sole sources of carbon, nitrogen and phosphorus and supplemented with 1 mL of trace mineral solution with pH adjusted to 5.8 and solidified with 1.5% agar-agar, was dispensed in 49 mL volumes into 250-mL Erlenmeyer flasks and sterilized by autoclaving at 121ºC for 15 min.

Medium 2: Minimal medium containing 5,000 µg/L of glyphosate and supplemented with 1% (w/v) ammonium chloride (NH_4Cl), 1% (w/v) dipotassium hydrogen phosphate (K_2HPO_4), 1% (w/v) potassium orthophosphate (KH_2PO_4) and 1 mL of trace mineral solution with pH adjusted to 5.8, and solidified with 1.5% agar-agar, was dispensed in 49 mL volumes into 250 mL Erlenmeyer flasks and sterilized by autoclaving at 121ºC for 15 min. Here, glyphosate only served as sole carbon source with nitrogen and phosphorus sources boosted by ammonium and phosphate supplementations respectively.

Medium 3: Minimal medium containing 5,000 µg/L of glyphosate and supplemented with 1% (w/v) dipotassium hydrogen phosphate (K_2HPO_4), 1% (w/v) potassium orthophosphate (KH_2PO_4) and supplemented with 1 mL of trace mineral solution with pH adjusted to 5.8, and solidified with 1.5% agar-agar, was dispensed in 49 mL volumes into 250 mL Erlenmeyer flasks and sterilized by autoclaving at 121ºC for 15 min. The glyphosate served as sole source of carbon and nitrogen but not of phosphorus as it received a boost from phosphate supplementation.

Medium 4: Minimal medium containing 5,000 µg/L of glyphosate and supplemented with 1% (w/v) ammonium chloride (NH_4Cl) and 1 mL of trace mineral solution with pH adjusted to 5.8, and solidified with 1.5% agar-agar, was dispensed in 49 mL volumes into 250 mL Erlenmeyer flask and sterilized by autoclaving at 121ºC for 15 min. The glyphosate served as sole sources of carbon and phosphorus but not of nitrogen as it received a boost from ammonium chloride supplementation.

2.2 Morphological and Molecular Identification of Glyphosate-degrading Mold

The mold species with the densest growth on the glyphosate medium was identified morphologically using surface and reverse colouration, spore and mycelium patterns, microscopy for conidia and conidiophores after staining with lacto-phenol cotton blue and molecular characterization by ITS sequencing using universal ITS primers (ITS1-F and ITS4) for quantitative PCR and heterogeneity analysis as described in Manter and Vivanco [11].

2.3 Glyphosate Degradation Studies with Strain JK-28

Minimal medium 2 was selected for degradation studies by the mold and was dispensed in 50 mL volumes into 250 mL Erlenmeyer flasks in triplicates. Flasks and contents were sterilized by autoclaving at 121ºC for 15 min. Upon cooling, degradation study flasks were inoculated with an earlier prepared spore suspension of strain JK-28 at 2% (v/v) spore concentration of10^8 spores/mL. Briefly, spore suspension was prepared by allowing the mold to grow for 72 h on potato dextrose agar (PDA) slants in Roux bottles for 96 h. Spores were harvested by addition of 5 mL sterile phosphate buffer to mold growth, followed by gentle up and down, side to side swirling of the bottle. Dislodged spores were transferred to a sterile receptacle and dilutions prepared using phosphate buffer.

2.3.1 Preparation of glyphosate standard curve

A stock solution of analytical grade glyphosate containing 1000 µg/mL was prepared by dissolving 0.1 g of glyphosate in 20 mL distilled water and then making up to the 100 mL mark in a volumetric flask. Additionally, 1000 µg/mL of ammoniacal copper nitrate [$Cu(NO_3)_2$] solution was prepared by dissolving 0.1 g of the compound in 20 mL distilled water and diluting to 100 mL with ammonia solution. Finally, a 1% solution of carbon disulphide (CS_2) was prepared by mixing 0.5 mL CS_2 in chloroform and diluting to 50 mL mark with chloroform [12]. An aliquot of variable concentrations of glyphosate solution (≤

20 µg/mL) were added to a series of 100 mL separating funnels. To each flask was added 5 mL of 1% v/v CS_2 solution and the mixture shaken until formation of dithiocarbamic acid. Afterwards, 1 mL of ammoniacal solution of Cu (II) (100 µg/mL) was added to the mixture for complexation with dithiocarbamic acid. The mixture was again shaken vigorously for 3 min and left for separation of two phases. The yellow-coloured chloroform layer containing the complex was separated in a 10 mL flask and diluted with ethanol. The absorbance of the complex was read off at 435 nm wavelength using a UV-Vis Spectrophotometer.

2.3.2 Experimental design and degradation studies

Independent variables considered in the degradation studies were glyphosate concentration, nature of ammonium-nitrogen and fermentation time. Three levels of the herbicide 'Round-up' concentrations used included 5,000, 7,500 and 10,000 µg/L which corresponded to 2050, 3075 and 4100 µg/mL of glyphosate respectively; the three ammonium- or amino-nitrogen sources included ammonium chloride, urea and L-asparagine at 1% (w/v); and three levels of time investigated included 10, 20 and 30 days. The experiment was set up in 250 mL Erlenmeyer flasks provided with outlets to trap CO_2. Degradation flasks were incubated at room temperature (28 ± 2ºC) for 30 days on a rotary shaker agitating at 130 rpm. At 10-day intervals, 10 mL of flask contents were withdrawn for determination of dehydrogenase activity, fungal dry weight, residual glyphosate, amount of carbon dioxide evolved and pH.

2.3.2.1 Measurement of dehydrogenase activity

Dehydrogenase activity was determined as described by Casida et al. [13]. The activity was based on the reduction of the water-soluble 2, 3, 5 triphenyltetrazolium chloride (TTC) substrate to the reddish-colored water-insoluble formazan products whose intensities were measured spectrophotometrically at a wavelength of 485 nm with methanol as blank [14]. The results of the periodic samples were compared with triphenyl formazan standards.

2.3.2.2 Determination of fungal biomass by dry weight

The mold strain JK-28 was grown on potato dextrose agar belly-slant in Roux bottle for 168 h at room temperature (28 ± 2ºC) to obtain spores. Fungal spores were harvested and suspension prepared in sterile phosphate buffer solution containing 0.05% Tween 80 [15]. The stock suspension was diluted through a series of ten triplicate ten-fold dilutions. Optical density (OD_{600}) of each dilution was determined by means of a spectrophotometer. One milliliter (1 mL) of each spore dilution was also plated onto potato dextrose agar (PDA) and plates incubated at room temperature for 72 h. Counts were taken by means of a Neubauer counting chamber and reported as spore forming units per milliliter (sfu/mL). The result was used to prepare a standard curve to establish a relationship between OD_{600} and spore count. Mycelium from each triplicate plate was separated by melting agar, diluting and filtering through Whatman No.1 filter paper. The mycelial pellet was repeatedly washed with distilled water and dried at 70ºC overnight. Dry weight of the fungus was calculated by subtracting the weight of filter paper from the combined weight of filter paper and mycelium using the equation;

$$MDW = wf - wo \qquad Eqn.1$$

where, MDW is the mean fungal dry weight (g), w_f is combined weight of filter paper and fungal mycelium (g) and w_0 is weight of filter paper alone (g).

Once again, a standard curve was prepared by regressing mean dry weight on absorbance at a wavelength of 600 nm. Fungal biomass was determined by dry weight using the regression equation of the standard curve;

$$y = 1.6144x - 1.7393 \qquad Eqn.2$$

2.3.2.3 Determination of residual glyphosate

Residual glyphosate was determined by the spectrophotometric method described in Jan et al. [12], using sterile filtrate obtained from a two-stage filtration of flask contents using first Whatman No.1 and next 0.22 µM membrane filter (Millipore). The procedure is as described in section 2.4.2. The mean absorbance reading was inserted into the regression equation obtained from residual glyphosate calibration curve*;*

$$y = 0.0524x - 0.0654 \qquad Eqn.3$$

2.3.2.4 Measurement of glyphosate degradation by carbon dioxide evolution

Amount of carbon dioxide evolved during glyphosate degradation was determined using the titrimetric method described in Ekpenyong and Antai [16] and Asitok et al. [17] using the equation

$$C - CO2\,(mg) = (B - V)NE \qquad Eqn.4$$

where *C-CO_2* is the amount of carbon (IV) oxide evolved; *B*, the volume (milliliters) of acid used to titrate the alkali in carbon (IV) oxide collectors to end point in the control; *V*, the volume (milliliters) of acid used to titrate the alkali in the carbon (IV) oxide collectors to end point in the treatments; *N*, normality of acid (HCl); *E*, equivalent weight; if data are expressed as carbon (IV) oxide, *E* = 22, but if expressed as carbon, then *E* = 6.

2.3.2.5 pH determination

pH measurements of all test samples were made using Orion 710A pH Meter equipped with glass electrode after calibration with appropriate buffers.

2.4 Statistical Analyses

Correlation analysis (CA) among dehydrogenase activity (DHA), amount of carbon dioxide evolved (CDE), fungal dry weight (FDW), residual glyphosate (RGP) and pH, principal component analysis (PCA) and multivariate analysis of variance (MANOVA) of data, were all conducted using SPSS ver. 20 (IBM, USA).

3. RESULTS AND DISCUSSION

Medium 2 was selected for glyphosate degradation studies because residual glyphosate was least in it suggesting that the mold could only use glyphosate as sole source of carbon and not as sole sources of nitrogen and phosphorus. A total of five molds demonstrated abilities to degrade the glyphosate-based herbicide (Round-up) within the study time. One particular strain, JK-28, was outstanding and was employed in further degradation studies. The white mycelial mold possessed velutinous colonial growth. Conidia were green and started off from the center to spread en masse with the reverse of the plate remaining un-coloured or white [18]. Microscopy revealed globose conidia with terminal chlamydospores and flask-shaped phialides. Molecular sequencing using universal ITS primers (ITS1-F and ITS4), NCBI BLAST and phylogenetic analysis using MEGA 4.0 revealed that the mold shared 100% sequence homology with *Trichoderma asperellum* GDFS1009 (Accession Number: KF367522) earlier isolated by Wu et al. [18] and studied for its biocontrol efficacy. The mold was therefore identified as *Trichoderma asperellum* strain JK-28, maintained in sterile soil and deposited with University of Calabar Collection of Microorganisms (UCCM).

Fig. 1 is the calibration curve for the determination of residual glyphosate concentration. The regression model equation, Eqn. 2, was significant at $P < .001$, with an adjusted R^2 of 0.9925 suggesting that 99.25% of the variations in the outcome variable was due to the independent variable and so could be used to determine residual glyphosate concentrations from test samples. Fig. 2 presents the standard curve used for the determination of fungal dry weight from spore concentration. The model equation, Eqn. 2 was significant at $P < 0.001$, with an adjusted R^2 of 0.9682 suggesting that the model equation could explain 96.82% of the variations about the data. The model equation was therefore used to determine fungal dry weight during glyphosate degradation studies.

Multivariate statistics are a robust statistical tool with potential to identify, classify, quantify and interpret interrelationships that exist between and among experimental variables [19,20]. In this study, we adopted correlation analysis (CA), three-way multivariate analysis of variance (MANOVA) and principal component analysis (PCA) to explain the nature of relationships that existed between and among the dependent variables (DVs) and the main and interactive effects of independent variables (IVs) that controlled the values of the DVs.

In multivariate statistics, CA is first conducted to establish and weigh the interrelationships that exist among the DVs. Even though each pair of correlates is expected to significantly relate one to another, the correlation coefficient, *r*, is expected to lie between the weak and moderately strong values say between 0.15 and 0.799. Correlation coefficients from 0.8 and above are described as very strong correlations and interpreted as over-correlation which violates the multicolinearity assumption of a multivariate statistical analysis. In Table 1, all the correlations were very significant ($P = .01$), but the most significant correlations were those between DVs other than residual glyphosate (RGP) and

dehydrogenase activity (DHA). Coefficients of these correlates were: DHA/fungal dry weight (FDW), r = .779, DHA/CO_2 evolved (CDE), r = .767 and DHA/pH, r = -.751.The relationship between DHA and FDW was a strong positive correlation suggesting that FDW increased as dehydrogenase activity increased. Since biological oxidations of organic compounds are mostly, if not entirely, dehydrogenase-mediated reactions [21], DHA, a measure of respiratory metabolism, correlated strongly and positively with microbial biomass-FDW [22]. As microbial oxidations progress, acidic degradation intermediates accumulate in systems causing decreases in pH which relate negatively but strongly with DHA and with FDW [23].

One of the major end products of complete respiratory metabolism is carbon dioxide (CO_2) which volume indicates the amount of mineralized carbon from the organic compound. The higher the respiratory metabolism, the more the amount of CO_2 evolved, and if properly trapped, then quantified. Expectations were that there will be a strong positive relationship between DHA and amount of CO_2 evolved, which was the case in this study. Assessment of glyphosate degradation using carbon dioxide evolution method had earlier been reported by Principal component analysis (PCA) is a mathematical dimension-reduction procedure that transforms a large number of weakly to moderately correlated variables into a smaller number of uncorrelated variables called principal components. A correlation matrix that shows only a few correlations above .30 suggests discontinuation of the analysis [24]. Our correlation analysis results showed that all the correlations were above .30 suggesting that PCA could be conducted on the data. Our results

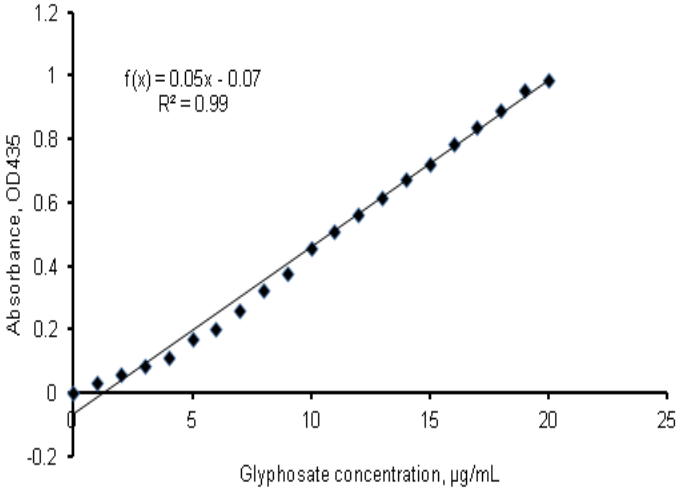

Fig. 1. Calibration curve for the determination of residual glyphosate

Table 1. Pearson's bivariate correlation analysis of dependent variables of glyphosate degradation by *Trichoderma asperellum* strain JK-28

		DHA	CO_2	FDW	pH	RGP
DHA	Correlation	1				
	Significance					
CO_2	Correlation	.767**	1			
	Significance	.000				
FDW	Correlation	.779**	.551**	1		
	Significance	.000	.000			
pH	Correlation	-.751**	-.550**	-.676**	1	
	Significance	.000	.000	.000		
RGP	Correlation	-.593**	-.691**	-.424**	.430**	1
	Significance	.000	.000	.000	.000	

***. Correlation is significant at the 0.01 level (2-tailed); DHA – Dehydrogenase activity; CO_2 – Amount of carbon dioxide evolved; FDW – Fungal dry weight; RGP – Residual glyphosate*

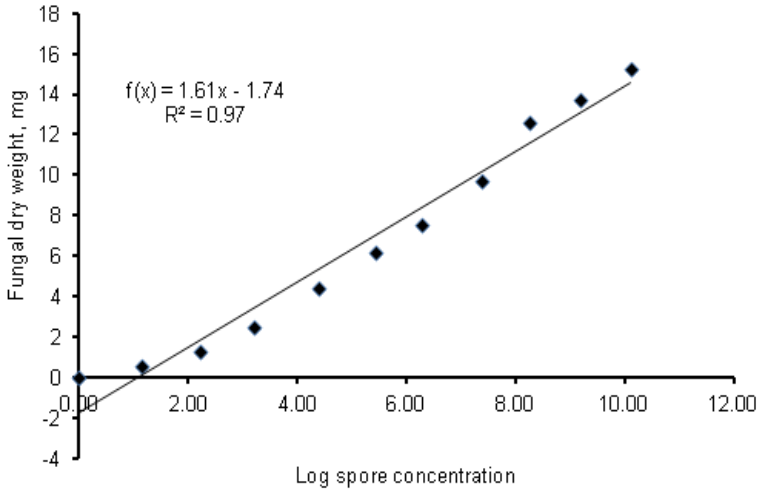

Fig. 2. Calibration curve for the determination of fungal dry weight

revealed a Kaiser-Meyer-Olkin measure of sampling adequacy (KMO) of .812 and a significant (P < .0005) Bartlett's test of Sphericity suggesting that the null hypothesis which assumed an identity matrix in the data should be rejected and a PCA accordingly performed.

Only one principal component, with initial eigenvalues ≥1.0, was extracted suggesting that the PCA constructed one principal component from the survey items based on eigenvalues. An eigenvalue is a ratio of the shared variance to the unique variance accounted for in the construct of interest by each factor obtained from the extraction by principal component(s). Eigenvalues of 1.0 or greater are an arbitrary criterion accepted to help decide if a factor should be further interpreted or not. Table 2 presents the extracted components and their eigenvalues that guided the extraction. Extraction of one component with a cumulative explained variance of 70.07% suggested that one factor solution was sufficient to study the degradation of glyphosate by the mold. Put another way, any one of the dependent variables would have sufficed for the evaluation of glyphosate degradation. The only difference would be in the sensitivity of the variable which could be given by their R^2 values. Fig. 3 presents the number of extracted components as a Scree plot to show the elbow (break) point of the plot confirming the only one extracted principal component.

A three-way multivariate analysis of variance (3-way MANOVA) was conducted on data to evaluate the main effects of glyphosate concentration, nitrogen source and duration of exposure on the five moderately correlated dependent variables (DVs). The assumption of homogeneity test for equality of error variance was met, as presented in Table 3. The Table shows that Levene's test of equality of error variance was not significant for any of the five DVs and this permitted the interpretation of the multivariate test results.

Table 4 presents the four different multivariate tests conducted to explain the main, two-way and three-way interactive effects of the IVs on the combined DVs. The Table shows that all four multivariate tests were significant (P < .001) for both main and interactive effects. Because the Box M test of homogeneity of equality of covariance was violated, we report and interpret the Pillai's Trace which is the most robust of the four multivariate tests and is able to maneuver through the violated assumption instead of the frequently interpreted Wilks' Lambda test. The Table shows that there was a statistically significant interactive effect between glyphosate concentration and nitrogen source on the combined DVs: GlyphoConc*NitroSour, F (20, 212) = 59.39, P < .001; Pillai's Trace = 3.39, partial η^2 = .849.

The interactive effect between glyphosate concentration and duration and that between nitrogen source and duration on the combined DVs were also significant: GlyphoConc*Duration, F (20, 212) = 20.31, P < .001; Pillai's Trace = 2.63, partial η^2 = .657 and NitroSour*Duration, F (20, 212) = 13.52, P < .001; Pillai's Trace = 2.24,

Table 2. Total explainable variance of a principal component analysis showing one extracted component

Component	Total variance explained					
	Initial eigenvalues			Extraction sums of squared loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	3.504	70.074	70.074	3.504	70.074	70.074
2	.737	14.734	84.809			
3	.327	6.544	91.353			
4	.297	5.940	97.293			
5	.135	2.707	100.000			

Extraction Method: Principal Component Analysis

Table 3. Levene's test of equality of error variances[a] of dependent variables

	F	df1	df2	Sig.
Dehydrogenase activity	1.092	26	54	.382
Amount of carbon dioxide evolved	.469	26	54	.981
Mean fungal dry weight	1.063	26	54	.413
pH	1.113	26	54	.361
Residual glyphosate concentration	1.030	26	54	.449

Tests the null hypothesis that the error variance of the dependent variable is equal across groups
*a. Design: Intercept + GlyphoConc + NitroSour + Duration + GlyphoConc * NitroSour + GlyphoConc * Duration + NitroSour * Duration + GlyphoConc * NitroSour * Duration*

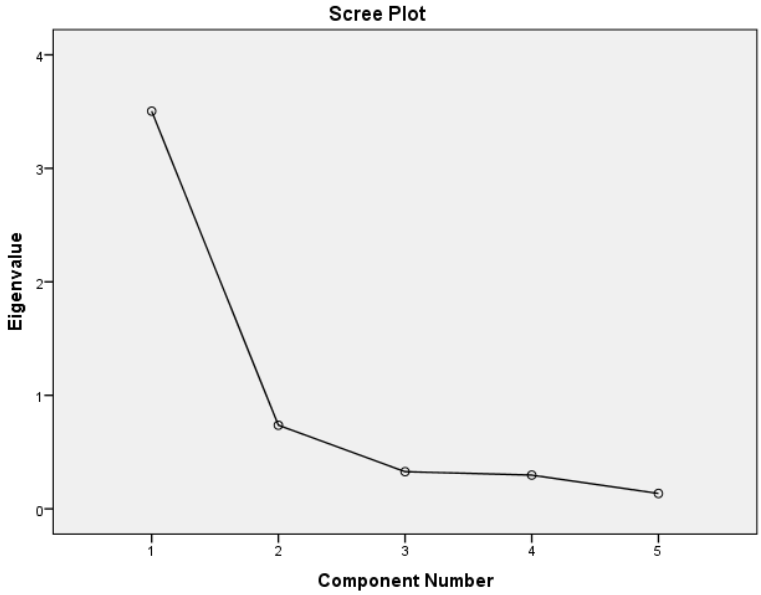

Fig. 3. Scree plot of a principal component analysis showing one extracted component

partial η^2 = .561 respectively. Finally, the interactive effect of the three IVs on the combined DVs was also significant: GlyphoConc*NitroSour*Duration, F (40, 270) = 13.39, P = .001; Pillai's Trace = 3.32, partial η^2 = .665. These results indicate that biodegradation of glyphosate by *Trichoderma asperellum* strain JK-28 was affected significantly by glyphosate concentration which was, in itself, influenced by the nature of nitrogen source in the system and the duration of exposure to the fungus.

Since the Partial Eta Squared (η^2) value has the same connotation as R^2 in regression statistics, these results indicate that the interaction between glyphosate concentration and the nitrogen sources could explain about 85% of the variations that were observed about glyphosate degradation using the combined dependent variables. Other two-way interactions yielded lower explainable values but the three-way interaction was responsible for 66.5% of the variations observed in the five dependent variables in combination during the herbicide degradation.

The main effects of the IVs were even more influential on the extent of glyphosate degradation. The Table shows that glyphosate concentration, on its own, was responsible for 99.5% of the variations observed in the response variables. Concentration of an organic substrate could influence the nature of metabolism that prevails in the organism at any given point in time. A typical example is the glucose (Crab-tree) effect in *Saccharomyces cerevisiae* and other yeasts. Nature of nitrogen source and duration of degradation studies were able to explain 98.5% and 95% respectively, of the variations about the combined DVs.

All the tests of between-subjects effects were significant, P = .000 as shown in Table 5. The

Table 4. Multivariate test[a] results of a three-way multivariate analysis of variance

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial eta squared
Intercept	Pillai's Trace	1.000	199877.438[b]	5.000	50.000	.000	1.000
	Wilks' Lambda	.000	199877.438[b]	5.000	50.000	.000	1.000
	Hotelling's Trace	19987.744	199877.438[b]	5.000	50.000	.000	1.000
	Roy's Largest Root	19987.744	199877.438[b]	5.000	50.000	.000	1.000
GPC	Pillai's Trace	1.990	1981.379	10.000	102.000	.000	.995
	Wilks' Lambda	.000	3903.205[b]	10.000	100.000	.000	.997
	Hotelling's Trace	1566.548	7676.084	10.000	98.000	.000	.999
	Roy's Largest Root	1462.946	14922.046[c]	5.000	51.000	.000	.999
NS	Pillai's Trace	1.970	665.382	10.000	102.000	.000	.985
	Wilks' Lambda	.000	1343.095[b]	10.000	100.000	.000	.993
	Hotelling's Trace	550.852	2699.173	10.000	98.000	.000	.996
	Roy's Largest Root	516.471	5268.000[c]	5.000	51.000	.000	.998
D	Pillai's Trace	1.900	194.705	10.000	102.000	.000	.950
	Wilks' Lambda	.000	1319.279[b]	10.000	100.000	.000	.992
	Hotelling's Trace	1757.179	8610.175	10.000	98.000	.000	.999
	Roy's Largest Root	1748.076	17830.378[c]	5.000	51.000	.000	.999
GPC*NS	Pillai's Trace	3.394	59.391	20.000	212.000	.000	.849
	Wilks' Lambda	.000	397.678	20.000	166.781	.000	.960
	Hotelling's Trace	543.140	1317.115	20.000	194.000	.000	.993
	Roy's Largest Root	493.207	5227.998[c]	5.000	53.000	.000	.998
GPC*D	Pillai's Trace	2.628	20.312	20.000	212.000	.000	.657
	Wilks' Lambda	.000	173.263	20.000	166.781	.000	.922
	Hotelling's Trace	310.181	752.189	20.000	194.000	.000	.987
	Roy's Largest Root	265.534	2814.663[c]	5.000	53.000	.000	.996
NS*D	Pillai's Trace	2.242	13.524	20.000	212.000	.000	.561
	Wilks' Lambda	.004	35.945	20.000	166.781	.000	.750
	Hotelling's Trace	38.622	93.659	20.000	194.000	.000	.906
	Roy's Largest Root	35.225	373.385[c]	5.000	53.000	.000	.972
GPC* NS*D	Pillai's Trace	3.325	13.394	40.000	270.000	.000	.665
	Wilks' Lambda	.000	30.203	40.000	220.739	.000	.804
	Hotelling's Trace	46.841	56.678	40.000	242.000	.000	.904
	Roy's Largest Root	33.932	229.042[c]	8.000	54.000	.000	.971

*a. Design: Intercept + GPC + NS + Duration + GPC*NS + GPC*D + NS*D + GPC*NS*D, b. Exact statistic*
c. The statistic is an upper bound on F that yields a lower bound on the significance level.
GPC-Glyphosate concentration; NS-Nitrogen source; D-Duration

Table 5. Extract of Tests of between-subjects effects of MANOVA showing only the categorical variable

Source		Type III sum of squares	df	Mean square	F	Sig.	Partial eta squared
GPC	DHA	1762.969	2	881.484	1186.715	.000	.978
	CDE	96533.759	2	48266.879	424.270	.000	.940
	FDW	44.826	2	22.413	1171.798	.000	.977
	pH	7.288	2	3.644	181.763	.000	.871
	RGP	14279775.343	2	7139887.672	37405.997	.000	.999
NS	DHA	2053.050	2	1026.525	1381.978	.000	.981
	CDE	12570.112	2	6285.056	55.246	.000	.672
	FDW	31.188	2	15.594	815.291	.000	.968
	pH	2.491	2	1.245	62.120	.000	.697
	RGP	5189005.907	2	2594502.954	13592.647	.000	.998
D	DHA	7600.614	2	3800.307	5116.234	.000	.995
	CDE	84456.160	2	42228.080	371.188	.000	.932
	FDW	80.060	2	40.030	2092.840	.000	.987
	pH	50.225	2	25.113	1252.696	.000	.979
	RGP	16559268.199	2	8279634.100	43377.149	.000	.999
GPC*NS	DHA	127.322	4	31.831	42.852	.000	.760
	CDE	8958.886	4	2239.721	19.687	.000	.593
	FDW	39.181	4	9.795	512.114	.000	.974
	pH	7.335	4	1.834	91.479	.000	.871
	RGP	4875507.462	4	1218876.865	6385.717	.000	.998
GPC*D	DHA	731.769	4	182.942	246.289	.000	.948
	CDE	5089.849	4	1272.462	11.185	.000	.453
	FDW	27.316	4	6.829	357.035	.000	.964
	pH	4.860	4	1.215	60.603	.000	.818
	RGP	2721038.030	4	680259.508	3563.892	.000	.996
NS*D	DHA	261.313	4	65.328	87.949	.000	.867
	CDE	3154.573	4	788.643	6.932	.000	.339
	FDW	21.870	4	5.468	285.856	.000	.955
	pH	8.946	4	2.237	111.564	.000	.892
	RGP	29672.060	4	7418.015	38.863	.000	.742
GPC*NS*D	DHA	99.206	8	12.401	16.695	.000	.712
	CDE	9734.572	8	1216.821	10.696	.000	.613
	FDW	4.880	8	.610	31.893	.000	.825
	pH	11.011	8	1.376	68.658	.000	.910
	RGP	261630.197	8	32703.775	171.336	.000	.962

GPC-Glyphosate concentration; NS-Nitrogen source; D-Duration; DHA-Dehydrogenase activity; CDE-Carbon dioxide evolved; FDW-Fungal dry weight; RGP-residual glyphosate

Partial Eta Squared is interpreted in much the same way as R^2 in regression analysis. In this study, the η^2 values for the main effects of glyphosate concentration, nature of nitrogen source and duration of exposure to degradation, ranged between 67.2% and 99.9% while those for the interaction effects ranged from 33.9% to 99.8%. These results suggest that the interaction effects of the independent variables were responsible for the bulk of the variations in the outcome or dependent variables but that the main effects made more significant contributions to glyphosate degradation.

A comparison of the reliability of dependent variables to measure biodegradation using partial Eta Squared η^2 values (Table 5) revealed that residual glyphosate was the most reliable. The variable ranged from 99.8% to 99.9% in its ability to explain data variations brought about by the main effects and 74.2% to 99.8% for data variations by interaction effects. Fungal dry weight measurement was second while the least reliable measurement came from carbon dioxide evolution. This observation about carbon dioxide evolution method is plausible since its measurements rely on the amount of carbon extracted from the original organic compound. The more carbon converted to biomass, the less will be evolved as carbon dioxide.

We employed both the Bonferoni's post hoc multiple comparison test and the Turkey HSD test to separate significant means. Results (not shown) showed that mean differences of all pairwise comparisons for the five dependent variables against glyphosate concentration and duration as independent variables were significant (P = .000) using both Bonferroni and Turkey HSD. However, the two post hoc tests returned dissimilar results when nitrogen sources served as the independent variable, if carbon dioxide evolution and pH fluctuations served as measures of degradation. Turkey HSD showed that when amount of carbon dioxide evolved served as the measure of glyphosate degradation, the mean difference between the use of L-asparagine and ammonium chloride as nitrogen source was not significant (P = .793 > .05) but were significantly different between either of the pair with urea. However, the corresponding Bonferroni test reported a non-significant P value of 1.000 suggesting that the later test was more discriminating and therefore less prone to error than the former. Additionally, when pH served as the measure of glyphosate degradation, the mean difference between the use of L-asparagine and urea as nitrogen sources was not significant (P = .377 > .05) but was significantly different between either of the pair with ammonium chloride. Bonferroni test also reported a non-significant P value of .552 suggesting once again that the later test was more discriminating than the former.

4. CONCLUSION

The mold *Trichoderma asperellum* strain JK-28, isolated from rice farm soil frequently treated with glyphosate herbicide, demonstrated sublime potential for glyphosate degradation. Pure culture of the mold could use the herbicide as sole source of carbon but not as sole sources of nitrogen and phosphorus and therefore required supplementations. Correlation analysis revealed that the five dependent variables selected as measures of glyphosate degradation were moderately correlated with each other. Principal component analysis returned a one-factor solution suggesting that any one of the five variables would be appropriate to monitor degradation of the organic compound. The main, two-way and three-way interaction effects from MANOVA were best explained by residual glyphosate which, in most cases, could explain ≥ 95% of the variations about the data. The variable is therefore recommended as reliable in degradation studies. A comparative post hoc multiple comparison using both Turkey HSD and Bonferroni tests revealed that Bonferroni tests were more discriminating and therefore less prone to returning erroneous results for significant mean separations.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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