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Jake OO (2002). Pharmaceutical Interactions between *Striga hermonthica* (Del.) Benth. and fluorescent rhizosphere bacteria Of *Zea mays*, L. and *Sorghum bicolor* L. Moench for Striga suicidal germination In *Vigna unguiculata*. PhD dissertation, Tehran University, Iran.

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Full Length Research Paper

Response of cucurbitacin B concentration in Nemafric-BL phytonematicide to increasing storage period

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Nemafric-BL phytonematicide, produced from fermented fruit of wild watermelon (*Cucumis africanus*), had been consistent in suppressing root-knot (*Meloidogyne* species) nematode population densities. However, due to the biological nature of Nemafric-BL phytonematicide, quality could be compromised during increasing storage period. A study was, therefore, conducted to determine the response of cucurbitacin B concentration in Nemafric-BL phytonematicide over a six-month storage period. The product, in 50 ml hermetically-sealed plastic containers, was stored in a dark room at room temperature (±25°C), with 10 samples being analysed monthly for cucurbitacin B concentrations using Shimadzu High Performance Liquid Chromatography (HPLC). Cucurbitacin concentrations over increasing storage period exhibited density-dependent growth patterns, which were characterised by increases in cucurbitacin B during the initial period of storage and followed by gradual decreases. Relative to the initial storage time (T_0), at the end of the storage period, cucurbitacin B concentration was still more than three-hundred times that at T_0, suggesting that the product was still suitable for use in managing nematode numbers.

Key words: *Cucumis africanus*, effective microorganisms, product quality, phytonematicide, shelf-life.

INTRODUCTION

Storage period of fermented products plays an important role in product quality (Rogers, 2010). Product quality (Q) is a function of its performance (P) and expectation (E), conceptualised as Q = P/E (Besterfield et al., 2003). Nemafric-BL phytonematicide had been consistent in suppressing nematode numbers in various crops (Mashela et al., 2015), with occasional incidents of stimulated plant growth (Mashela et al., 2015). The product is produced from fermented fruit of wild watermelon (*Cucumis africanus*) (Mashela et al., 2011). The product comprises cucurbitacin B ($C_{32}H_{46}O_8$) active ingredient (Chen et al., 2005), which is both stable and insoluble in water (Jeffery, 1978).

Recently, Shadung et al. (2015) observed that when *C. africanus* fruit were dried at 52°C and stored over six months prior to manufacturing the product, the

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concentration of cucurbitacin B in phyto-inventories increased quadratically. The behaviour of cucurbitacin B in the stimulation phase of the density-dependent growth (DDG) patterns was explained on the basis of the thermo-stable enzyme-driven precursors (Chen et al., 2014), whereas in the inhibition phase the decreases were explained in terms of auto-oxidation. These behavioural-concentration changes, can invariably affect product quality. The objective of this study was, to determine the response of cucurbitacin B concentration in Nemafric-BL phytonematicide over a six-month storage period.

MATERIALS AND METHODS

Study field and cultural practices

Hardened-off *C. africanus* seedlings were raised under irrigated-field conditions at the Green Technologies Research Centre (GTRC), University of Limpopo, South Africa (23°53'10"S, 29°44'15"E) in summer (October – December) 2014 and repeated in 2015. Seeds were prepared as described previously (Maila et al., 2016) and sown in seedling trays containing Hygromix-T (Hygrotech, Pretoria North, South Africa) growing medium. At two-leaf stage, seedlings were hardened-off for 5 days, selected for uniformity and at 4 weeks were transplated under field conditions. Plot size was 1 m x 1 m, each containing four equidistant-transplanted seedlings. Three days after transplanting, 3 g 2:3:2 (22) NPK fertiliser mixture/plant provided a total of 186 mg N, 126 mg K and 156 mg P per ml water, whereas 2 g 2:1:2 (43) fertiliser mixture provided 0.35 mg N, 0.32 mg K and 0.32 mg P, 0.9 mg Mg, 0.75 mg Fe, 0.075 mg Cu, 0.35 mg Zn, 1.0 mg B, 3.0 mg Mn and 0.07 mg Mo per ml water. Plants were irrigated weekly using overhead sprinklers to provide at least 20 mm water.

Experimental units

Fruit of *C. africanus* were harvested at 110 days after transplanting, cut into pieces and dried at 52°C for 72 h in an air-forced oven. Dried material was ground in a Wiley mill to pass through a 1 mm screen (Mashela, 2002). Approximately 40 g crude extract of *C. africanus* fruit, was placed in 20-l-plastic containers and 300 ml molasses, 100 g brown sugar, 300 ml effective microorganisms (EM) and 16 litre chlorine-free tapwater added and hermetically sealed (Nzanza and Mashela, 2012). The mixtures were fermented for 14 days at room temperature until pH declined to 3.7. Gases were allowed to escape from the container through a 5 mm-diameter tube with hermetically-glued end to a hole on the lid of the 20 L container, with an outlet end dangling in chlorine-free tapwater container in a litre bottle. After fermentation, 200 ml were pipetted into 300 ml plastic containers, which were hermetically sealed. Treatments, *viz*., 0, 1, 2, 3, 4 and 5 month storage time, were arranged in rando-mised complete block design, with five replications. Samples were stored in a dark room at room temperature (±25°C), with the initial storage period (T_0) being the control.

Data collection

Prior to storage (T_0) and then monthly, 1 ml subsamples were collected from 10 containers and centrifuged at 4500 rpm for 10 min before filtering through 0.22 µm-pore filter (Miller, Sigma).

Concentration of cucurbitacin B was quantified using the isocratic elution Shimadzu HPLC Prominence with CTO-20A diode array detector, with a wide pore reverse phase C18 (25 cm × 4.0 mm, 5 µm) discovery column (Sigma-Aldrich). A 2:3 (v/v) methanol and deionised water mobile phase at a flow rate of 1.0 ml/min in an oven at 35°C, was monitored at the 230 nm wavelength for 43 min. Standards (1 µg each) were dissolved in 1 ml methanol and then diluted to form 0.02, 0.04, 0.06, 0.08 and 1.0 µg/ml methanol dilutions. The retention times and peak areas of cucurbitacin B in subsamples was compared with those of pure (≈ 98%) cucurbitacin B standard (Wuhan ChemFaces Biochemical Co. Ltd., Wuhan: China).

Data analysis

Cucurbitacin B data were subjected to analysis of variance procedure using SAS software (SAS Institute Inc., 2008). When treatment effects were significant at the probability level of 5%, the degrees of freedom and their associated sum of squares were partitioned to determine the percentage contribution of sources of variation to the total treatment variation (TTV) in the observed variable (Steyn et al., 2003). Mean separation was accomplished using Waller-Duncan multiple range test. Variables with significant ($P \leq 0.05$) treatment means were further subjected to lines of the best fit using cucurbitacin responses (y-axis) versus increasing storage time (x-axis). The variables were modelled using the regression curve estimations in a quadratic equation: $Y = b_2x^2 + b_1x + a$, where Y is the concentration of cucurbitacin B and x from the $x = -b_1/2b_2$ relation is the optimum storage time. Unless otherwise stated, only treatment means significant at the probability level of 5 % were discussed.

RESULTS AND DISCUSSION

The interaction between the 2014 and 2015 growing seasons was not significant ($P > 0.05$) and therefore, data were polled (n = 60) and re-analysed. Storage period had highly significant ($P \leq 0.01$) effects on concentration of cucurbitacin B Nemafric-BL phytonematicide. Treatments contributed 68% in TTV of cucurbitacin B. Relative to T_0, storage increased concentration of cucurbitacin B Nemafric-BL phytonematicide (Table 1). Cucurbitacin B increased by between 599 and 1182% in the first three months and thereafter the rate of increase declined to 348% by the fifth month in Nemafric-BL phytonematicide. Concentration of cucurbitacin B versus storage period exhibited quadratic relationship (Figure 1). The model explained the observed relationship for cucurbitacin B by 93%. Using $x = -b_1/2b_2$ relation, concentration of cucurbitacin B in Nemafric-BL phytonematicide was optimised at 2.71 months (Table 2).

The quality of phytonematicides is dependent upon the concentration of active ingredient, which is directly associated with their performance. Active ingredients in phytonematicides, being secondary metabolites, are in a state of continuous change (Luckner, 1984) due to microbial degradation and/or auto-oxidation (Gunatilaka, 2006). Loss of product quality in commodities is a global concern (Straus, 2002; Drew and Myers, 1997), which

Table 1. Responses of cucurbitacin B concentration in stored Nemafric-BL phytonematicide (n = 60).

Storage period (month)	Nemafric-BL phytonematicide	
	Y-value[y]	Relative impact (%)[z]
0	1.233[b]±0.008	–
1	8.619[b]±0.014	599
2	14.535[a]±0.027	1079
3	15.807[a]±0.017	1182
4	9.446[ab]±0.016	666
5	6.53[ab]±0.042	348

[y]Column means followed by the same letter were not different at P ≥ 0.05.[z]Relative impact (%) = [(Treatment/Control) - 1] × 100.

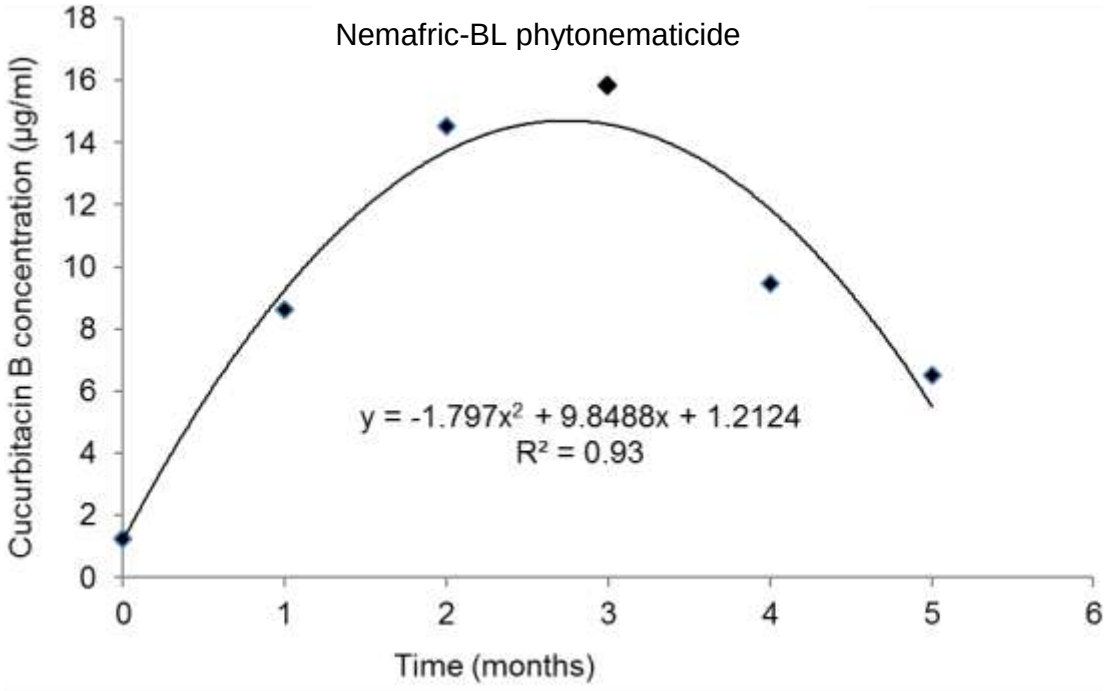

Figure 1. Quadratic curve of cucurbitacin B concentration in stored Nemafric-BL phytonematicide (n = 60).

had since necessitated the development of regulatory standards, collectively referred to as shelf-life (WHO, 2002). By definition, shelf-life is the length of time a product may be stored without becoming unsuitable for use (American Heritage® Dictionary of the English Language). In our study, the active ingredient in Nemafric-BL phytonematicide over increasing storage period exhibited strong DDG patterns, characterised by quadratic relations (Mashela et al., 2015). In a certain biofertiliser produced from EM-fermented plant materials, the chemical, physical and microbial characteristics exhibited DDG patterns over increasing storage period (Ngampimol and Kunathigan, 2008).

Gradual stimulation followed by gradual inhibition in concentration of cucurbitacin B over increasing storage period of Nemafric-BL phytonematicide within DDG patterns could be attributed to a series of both extrinsic and intrinsic factors. EM bioactivities are depended upon the availability of energy and carbon from the substrates; (Higa, 1991; Higa and Wididana, 1991). EM is widely used in fermenting plant materials to produce biofertilisers, biopesticides, phytonematicides and feeds (Pelinganga and Mashela, 2012; Pelinganga et al., 2012; Ngampimol and Kunathigan, 2008). Commercially available South African EM comprise photosynthetic bacteria, lactic acid bacteria, yeast, actinomycetes and fermenting fungi (Higa, 1991; Higa and Wididana, 1991; Higa and Parr, 1994). Upon depletion of readily available energy sources

Table 2. Quadratic relationship, coefficient of determination and computed storage time for Nemafric-BL phytonematicide (n = 60).

Plant variables	Quadratic relationship	R^2	x^z	P ≤
Cucurbitacin B	$Y = -1.797x^2 + 9.8488x + 1.2124$	0.93	2.74	0.01
	Optimum storage period (months)		2.74	

^{z}Calculated optimum storage time (x) = $-b_1/2b_2$, where for cucurbitacin B $b_1 = 9.8488$ and $b_2 = -1.797$.

(sugar + molasses), EM degrade the plant crude extracts, thereby releasing active ingredients into solution (Margarita and Dengel, 2003). However various forces come into play as the concentration of the active ingredients increases. For example, during the early stages, EM increased B in solution of Nemafric-BL phytonematicide, through degradation of fruit crude extracts as sources of readily available energy and carbon. However, with progression time, once fruit crude extracts are depleted, EM starts to attack cucurbitacin B for the same sources, thereby reducing the concentration of cucurbitacin B. Maatooq et al. (1995) noted that the observed decrease in cucurbitacin E-glycoside concentration of bitter Hawkesbury watermelon (*Citrullus vulgaris*) was primarily ascribed to microbial activities. Additionally, auto-oxidation contributed to the reduction of active ingredients in most bioactive chemical compounds (Allen, 2013).

Reductions in cucurbitacins were also observed at low pH in bitter *C. vulgaris* solution extracts stored at varying temperatures over time (Martin et al., 2002). EM-produced products like the Nemafric-BL phytonematicide are naturally acidic, with fermentation directly reducing pH of the products (Merlin et al., 2013; Rizk et al., 2007). Miller and Blackwell (1986) reported that a persistent drop in pH could result in enzyme inactivation, which would eventually stop the fermentation process. However, pH did not appear to have played a role in the DDG patterns of cucurbitacin concentrations. At the end of the fermentation process, pH of the mixture was still approximately 3.7 (Mashela et al., 2015). One should appreciate that in addition to sources of energy and carbon for immobilisation in EM, the concentration of active chemical compounds from plant materials using the fermentation process could be affected by pH, temperature, light, presence plus form of precursors and the substrate components (Kumara and Rawal, 2008; Zain et al., 2009; Bhattacharyya and Jha, 2011; Gautam et al., 2011; Jain and Pundir, 2011; Sudarkodi et al., 2012).

Conclusion

In Nemafric-BL phytonematicide the concentration of the active chemical ingredient was optimised at 2.74 months, which is equivalent to ca. 71 days. This duration should not be viewed as being equivalent to shelf-life, because six months after T_0, the concentration of cucurbitacin B was still more than three-hundred times to that at T_0. The latter suggested that the product was still suitable for use as a phytonematicide.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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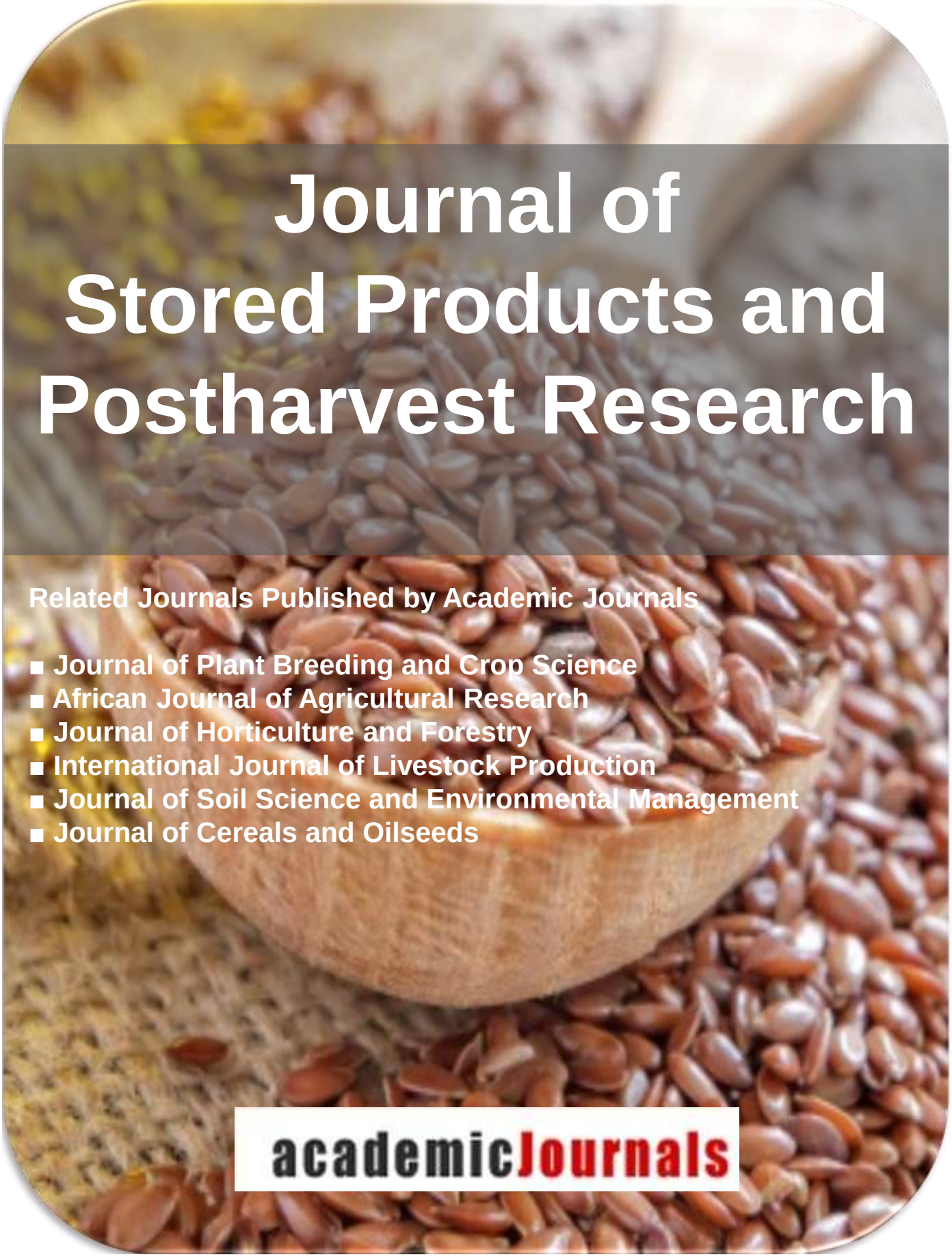

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