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RESEARCH ARTICLE

ASSESSMENT OF CYTOTOXICITY AND GENOTOXICITY OF ENTOMOPATHOGENIC FUNGI *BEUVERIA BASSIANA* USING *ALLIUM CEPA*AS A MODEL

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ABSTRACT

Application of microbial pesticides offers great potential for crop protection due to the rising costs of conventional pesticides and rising instances of pesticide toxicity. Entomopathogenic fungi are among the insect-killing fungi that hold great promise for lowering pest populations in agriculture and forestry. The wide range of entomopathogenic fungal biological control agents, which allows one isolate to cover several insect species, is one of its benefits for pest management. Due to its capacity to produce a variety of chemically diverse secondary metabolites, including Beauvericin, Beauveria bassiana, also known as "Sugar Icing Fungus," is a superior entomopathogenic fungus. However, secondary metabolite beauvericins contribute to the contamination of eggs, milk, meat, and plant products, allowing apoptosis and mitochondrial impairment.Concerns about its use should be investigated since harmful Beauveria species metabolites may infiltrate the plants. Beauveria bassiana's potential for cytotoxicity and genotoxicity was assessed using the Allium cepa test. In this work, the chromosomal aberration in Allium cepa was explicitly counted, quantified, and the dose and time response correlations between the B.bassiana crude extract and chromosomal aberrations were examined. The method described above was used to determine B.bassiana's potential for cytotoxicity and genotoxicity. In this experiment, 10 onion bulbs were suspended in distilled water as control and 10%, 30%, and 50% concentrations of Entomopathogenic fungal (EPF) culture and crude extract for 48 hours. The mitotic index, micronucleus in interphase, and chromosomal aberrations in mitotic phases were determined by looking at and counting at least 100 cells on each slide. Nine slides were grouped for each treatment group after the experiment was duplicated three times, with three roots for each replication. Mitotic index and frequency of chromosomal aberration based on the number of aberrant cells per total cells scored at each concentration of each sample were calculated and data obtained from the mitotic index calculation were analyzed using Analysis of Variance Technique (ANOVA) at significant level of $p < 0.05$ using SPSS Program Version 17. Duncan's multiple range test was performed to determine the significant differences between treatments ($p < 0.05$).It was discovered that the effects of Beauveria bassiana crude extract on Allium cepa's mitotic index and chromosomal aberrations were dose-dependent, thus B.bassiana crude extract exhibits cytotoxic and genotoxic effects at various doses and treatment intervals. This finding can serve as the foundation for further investigation into the ideal field application method and optimum B.bassiana concentration for entomopathogenic fungus.

INTRODUCTION

The increasing cost of chemical pesticides along with increasing incidences of pesticide toxicity, application of microbial pesticides holds a good promise of crop protection (1). Entomopathogenic fungi, according to Mascarin and Jaronski (2), have high potential in reducing pest populations in agriculture.

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These insect-killing fungi have high potential in reducing pest populations in agriculture and forests and even as medical vector control (3). One of the advantages of using a fungal biological control agent in pest management is its broad spectrum, whereby one isolate can cover several species of insects including lepidopteran, orthopteran, thysanopteran, heteropteran, dipteran, and coleopteran insects, compared to the narrow range of other microbial control agents, such as Bacillus thuringiensis and insect-pathogenic virus and nematodes (4,5). Secondly, entomopathogenic fungi can be easily spread and transmitted within insect populations or over different populations in favorable environmental conditions (3). Beauveria bassiana is a typical soil dwelling fungus and

has globally been used for almost a century as an eco-friendly alternative for the control of leaf and root feeding insects (1). On a recent research noted by Zimmermann (6), there are various tri-partite interactions between plant, pest insect and B. bassiana fungi demonstrated, these interactions are: plants may affect the infection by the B. bassiana fungi; plants may affect the persistence of the B. bassiana fungi and; B. bassiana can persist as an endophyte within plants. The fact that toxic metabolites of Beauveria spp. may enter the plants raises concern though such repots validating the hazardous effects of its toxins on environmental health are available (1). Beauvericin, bassianolide, bassianin, tenellin and cyclosporin A are the key secondary metabolites produced by B. bassiana (7,8). Investigations on beauvericin have demonstrated that this metabolite has insecticidal, antibiotic, cytotoxic, and ionophoric properties (1). The utilization of these fungi for crop protection advances the question of possible side-effects on non-target soil inhabiting organisms. Therefore, assessing the genotoxic potential of B.bassiana is of utmost importance. In this study, Allium cepa will be used as a model to assess the genotoxic potential of B.bassiana. The test has been used by many researchers mainly as a bio indicator of environmental pollution (9, 10) because this test uses a model that is adequately sensitive to detect innumerous substances that cause chromosomal alterations (11). This study specifically enumerated the chromosomal aberration in Allium cepa, quantify the chromosomal aberration in Allium cepa, and investigate the dose and time response relationships between the fungi and chromosomal changes. The aforementioned was recorded as the means to assess the cytogenotoxic potential of B.bassiana.

METHODOLOGY

This study was conducted in Nueva Vizcaya State University, Research, Extension and Training, MECO-TECO Laboratory February 22-26, 2021.

- ***Preparation of Entomopathogenic fungi (EPF) culture and crude extract:*** Entomopathogenic fungi (EPF) were obtained from NVSU, MECO-TECO laboratory. Cultured EPF were then inoculated in potato dextrose broth (PDB) and incubated for 15days at room temperature in a dark place. Fermented EPF broth were filtered using nylon membrane filter in Rocker glass filtration set and Rocker300 oil free vacuum pump to remove the fungal spores in the broth.

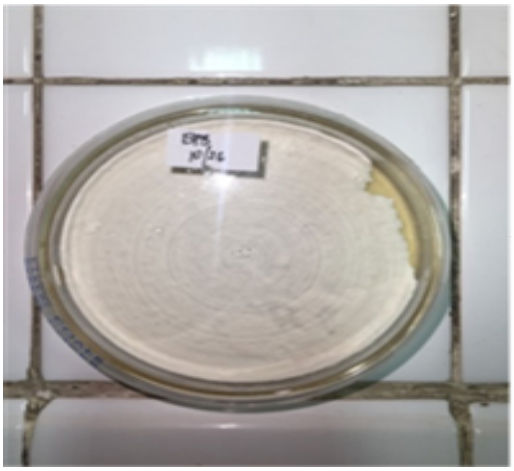

Figure 1: Entomopathogenic fungi

Figure 2. Crude extract

Figure 3. Filter process

- ***Preparation of Plant material and treatment:*** Small bulbs of Allium cepa about 1.5–2.0 cm were purchased in a local supermarket. The outer coverings of the bulbs and the dry bottom plate were scraped carefully not allowing the root primordial to be damaged (12). For the next set-up, all bulbs were suspended initially on a small plastic cup containing distilled water (pH 7.3)for 3 days (13), so rootlets can emerge. After this period, the base of each bulbs were suspended on 10%, 30%, 50% concentrations of crude extract (CE) and distilled water as control (14)in a plastic cup.

  This was kept in the dark for 24 and 48 hours and the solutions were changed daily. Although the Allium cepa test and the rooting process can be performed in either dark or light place (13), the set-up was kept in the dark given that B. bassiana spores extract is photosensitive in accordance with Edgington et. al. (15) report where hastyinactivation of B. bassiana spores happened following exposure to UV light or natural sunlight.At the end of the exposure period, the roots with the best growth (16) were obtained with the used of sharp scalpel to cut the roots from the bulb's base.

Figure 4: the bulb suspended on the crude extract

Figure 5: the bulb suspended on the crude extract covered to have a dark surrounding for growing its roots

- ***Maceration of the root tips and preparation for microscopy:*** After exposure, the root tips were cut and washed with distilled water and then placed in a glass slides. Root tips were hydrolyzed with 50µl of 1N HCl at 60–70 °C for 5 min (17). After hydrolysis, the roots were washed.
  Then, about 1–2 mm of the root tips were cut and placed on the slide. A small drop of Safranin O was dropped on the root tip and left for 2 min. The root tip was then mashed with metal rod and another small drop of Safranin O was added and left for another 2 min. The cover slip was carefully lowered on to avoid air bubbles and the sides of the slides were sealed with clear fingernail polish, ready for microscopy.

- ***Observation of Specimens:*** The slides were observed under the light microscope at 400× magnification. The mitotic index, micronucleus in interphase and chromosome aberrations in mitotic phases was identified by the examination and counting of minimum of 100 cells per slide. The experiment was replicated three times with three roots for each replicate, thus, nine slides were organized for each treatment group. Mitotic index and frequency of chromosomal aberration based on the number of aberrant cells per total cells scored at each concentration of each sample were calculated using the following formula (18) adopted the formula of FiskesjÖ (19):

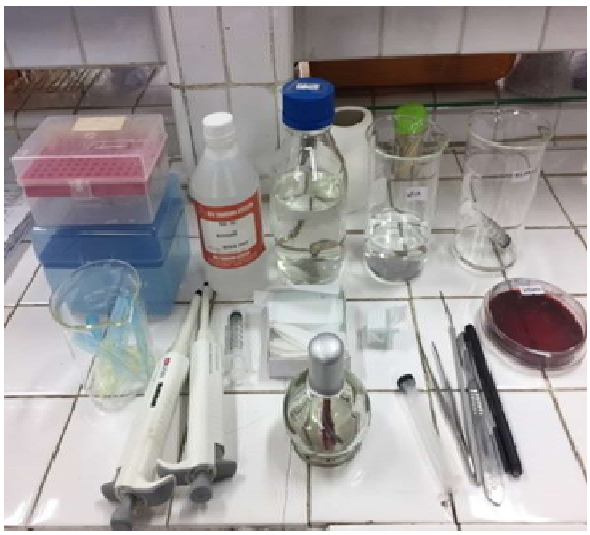

Figure 6. the materials for the Maceration Process

$$Mitotic\ Index = \frac{Number\ of\ dividing\ cells}{Total\ number\ of\ cells} \times 100 \quad (2)$$

$$\%\ Aberrant\ cells = \frac{Number\ of\ aberrant\ cells}{Total\ number\ of\ cells} \times 100 \quad (3)$$

$$\%root\ growth\ of\ control = \frac{overall\ mean\ root\ length\ of\ test\ solution}{overall\ mean\ root\ length\ of\ control} \times 100 \quad (4)$$

- ***Statistical Data Analysis:*** Data obtained from the mitotic index calculation were analysed using Analysis of Variance Technique (ANOVA) at significant level of $p < 0.05$ using SPSS Program Version 17. Duncan's multiple range test was performed to determine the significant differences between treatments ($p < 0.05$).

RESULTS AND DISCUSSION

The mitodepressive effect of the B.bassiana crude extract was obvious; moreover, a decrease of MI value was associated with the increase of crude extract concentration. The MI value recorded for control was maximum (34.8%) which significantly decreased in all crude extract (CE) treatment when compared to control (Table 2).

Table 1. Mitotic index and over all abnormality percentage obtained in different concentrations of CE for 24 and 48 h treatment time

	Conc.	Mitotic index	Abnormality percentage
24h	C	34.8%	0.0%
	10	22.7%	8.3%
	30	13.5%	24.6%
	50	9.85%	36.8%
48h	C	29.2%	0.0%
	10	19.9%	21.4%
	30	14.6%	47.9%
	50	9.49%	50.9%

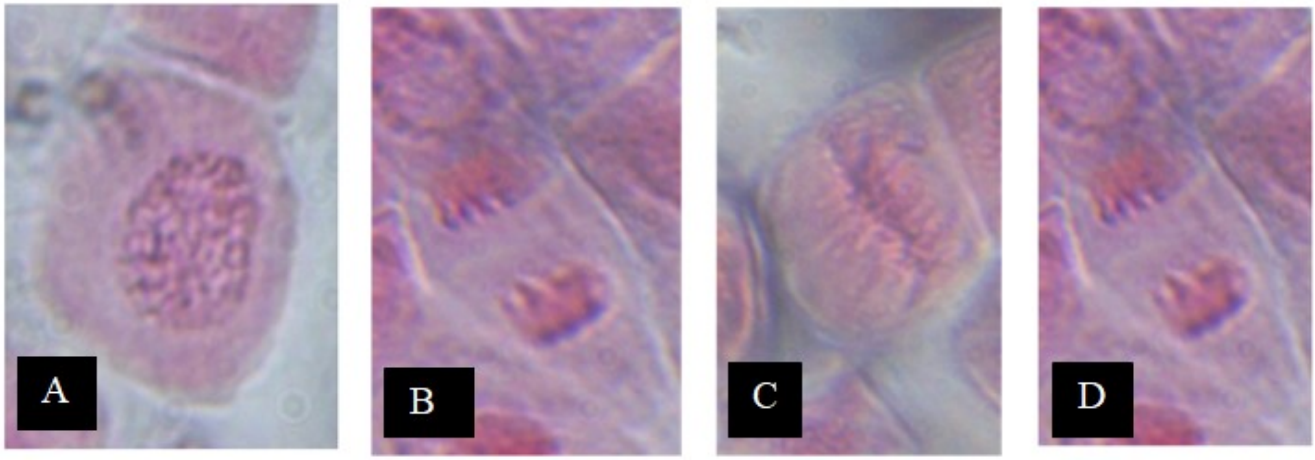

Figure 7: Mitosis stages in root tip – A. prophase; Figure 8: B. metaphase; Figure 9: C. anaphase; Figure 10: D. telophase

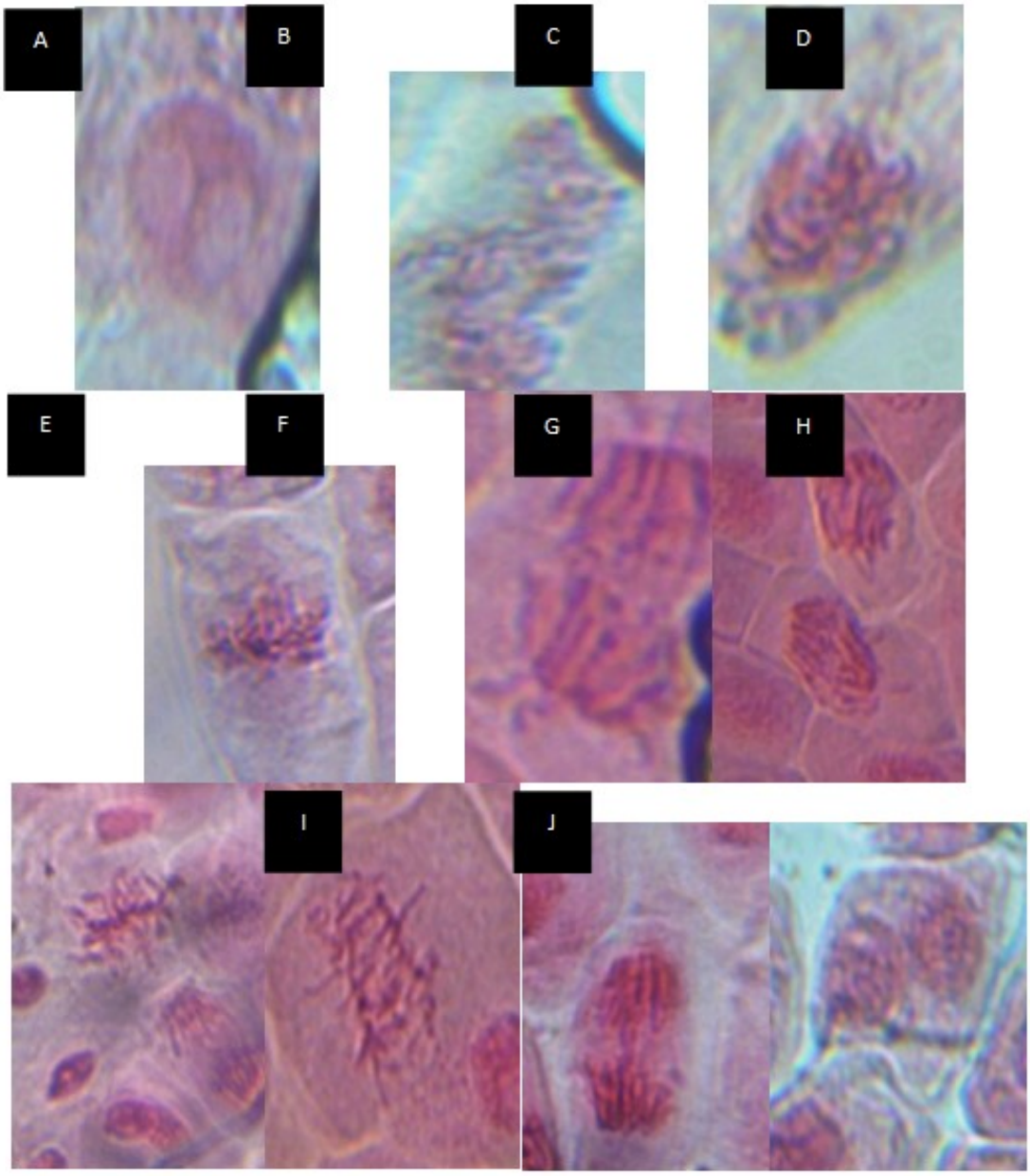

Figure 11: Different chromosomal aberrations induced by *B.bassiana* in root tip of *Allium cepa* – A; Figure 12: nuclear lesion; Figure 13: B. nuclear erosion; Figure 14: C. fault polarization in metaphase; Figure 15: D. fragmentation in metaphase; Figure 16: E. vagrant chromosome in anaphase; Figure 17: F. precocious movement of chromatids in anaphase; Figure 18: G. disturbed anaphase with lagging chromosome; Figure 19: H. c-metaphase; Figure 20: I. bridge in anaphase; Figure 21: J. Binuclei

Table 2. Relative abnormality rate (%) obtained in different concentrations of CE for 24 and 48 h treatment time

Time	Concentration	Lesion	Erosion	Fault Polarization	Fragmentation	vagrant	Precocious movement	laggard	c-methaphase	bridge	binuclei
24h	10	2.2	0.0	0.0	0.0	2.2	0.0	1.3	1.4	0.0	1.2
	30	5.5	1.7	3.6	2.8	2.7	2.8	1.9	2.3	0.0	1.8
	50	2.6	2.7	6.9	6.2	2.8	2.9	5.2	4.3	0.9	2.3
48h	10	2.7	2.1	8.1	6.5	0.0	0.0	0.0	2.0	0.0	0.0
	30	2.5	2.0	10.2	10.9	4.7	4.5	3.2	3.4	0.0	3.5
	50	3.9	3.0	13.2	11.3	3.8	8.6	6.2	4.0	1.7	5.2

CE significantly increased the frequency of abnormal cells in all concentrations and treatment times when compared to control. This increase was dose dependent as for minimum concentration of CE (10%) the recorded abnormality percentage was 8.3% and 21.4% which increased to 36.8% and 50.9% for maximum CE concentration (50%) in the 24 h and 48 h treatments respectively as shown in table 1. This reduction in mitotic activity may be due to blocking of mitotic cell cycle during interphase (20), inhibition of nuclear proteins synthesis essential for normal mitotic sequence (21), suppression of DNA synthesis (22) or change in the relative duration of the mitotic stages (23).

Different types of chromosomal abnormalities (CAs) were recorded. Nuclear lesion, nuclear erosion, fault polarization in metaphase, fragmentation in metaphase, vagrant chromosome in anaphase, precocious movement of chromatids in anaphase, disturbed anaphase with lagging chromosome, c-metaphase, bridge in anaphase and binuclei as shown in figure2. The CA frequency was increased significantly and dose dependently. While no CA was recorded in the chromosomes of Allium meristematic root cells of control bulbs thus exposure to different concentration of CE induced CA in root tips of Allium. Therefore, suggests the genotoxic effect of active substance in CE. Among the various CA lesions, fault polarization, fragmentation and c-metaphase were the most common abnormalities recorded. In addition to the mitodepressive effect, a number of chromosomal abnormalities covering all stages of mitosis were recorded after treatment with CE. The total percentage of these abnormalities increased gradually with the increase of concentrations and the period of treatments. The main effect of CE was found on metaphase and ana-telophase stages. At these stages chromosome fault polarization, fragmentation, vagrant chromosome, precocious movement of chromatids, disturbed anaphase with lagging chromosome, c-metaphase and bridge in anaphase were the most common abnormalities observed. In addition, degradation of cell wall and cytoplasm were also observed in 50% concentration at 48h time treatment.

CONCLUSION

The present study proved that the entomopathogenic fungi *B.bassiana*, decreased mitotic index in *A. cepa*, and increased chromosomal abnormalities, which suggest its cytogenotoxic potential. This study signified that *B.bassiana* crude extract has cytotoxic and genotoxic effects when tested at different concentrations and time of treatment. This result can be a basis of further research regarding the proper form of field application and appropriate concentration of B.bassiana as entomopathogenic fungi.

CONFLICT OF INTEREST STATEMENT: There is no conflict of interest in this study.

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GLOSSARY OF ABBREVIATIONS

- *A.cepa* – Allium Cepa
- ANOVA - Analysis of Variance
- *B.bassiana*- Beuveria Bassiana
- CAs- chromosomal abnormalities
- CE- crude extract
- DNA- Deoxyribonucleic acid
- EPF- Entomopathogenic fungi
- HCl- hydrochloric
- MECO-TECO- Manila Economic And Cultural Office-Taipei Economic And Cultural Office
- MI- Mitotic Index
- NVSU- Nueva Viscaya State University
- PDB- potato dextrose broth
- SPSS- **Statistical Package for the Social Sciences**
- UV –Ultra Violet

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