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Research Article

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Efficacy of Neem Seed Extract in the Control of *Fusarium Oxysporum* of Ginger (*Zingiber Officinale*) Explants in Tissue Culture and Micropropagation

Kahya, S. S[1], Pandukur, S. G[2]* and Onyenobi, F.I[3]

[1]*National Root Crops Research Institute Umudike, Potato Programme Kuru, P.M.B. 04 Vom, Jos, Plateau State Nigeria*

[2] *University of Jos, Department of Science Laboratory, Faculty of Natural Sciences, P.M.B 2084, Jos Plateau State, Nigeria*

[3]*Abia State University Uturu P.M.B.2000, Uturu, Abia State. Nigeria*

***Corresponding Author**
Pandukur, S. G, University of Jos, Department of Science Laboratory, Faculty of Natural Sciences, P.M.B 2084, Jos Plateau State, Nigeria

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Abstract

In spite the most stringent use of sterile technique during plants micro propagation, the nutrient in which the plants tissue is cultivated, is a good source of medium for microbial growth. These microbes compete adversely with the tissue culture material for nutrients. The contaminated plants culture remains a persistent problem that results to losses ranging from small number of cultures to the catastrophic lost of the whole batches of culture media and tissue cultures. The study shows the number of ginger explants initiated revealed that 62.5% were contaminated with Fusarium oxysporum which signified that these isolates constitute the major contaminants in the micropropagation of ginger which originated from the field or green house, 16.7% were contaminated with other organisms, while 20.8% uncontaminated culture tubes. The neem seed extract of water and ethanol extract at concentration of 0.5, 1.0, 1.5, 2.0 and 2.5mg/ml were compared against Benlate; a commercially available fungicide for their antifungal activities against mycelium growth of Fusarium oxysporum. The results shows that the concentrations at 2.5mg/ml of both Benlate and ethanol neem seeds extracts were highly effective in inhibiting the mycelium growth of F. oxysporum of 56.0mm and 55.6mm respectively. This is significantly ($P<0.05$) higher than the water neem seed extracts across the concentration levels. The concentrations at 2.0mg/ml of Benlate and ethanol neem seeds extracts were significantly effective for inhibiting F. oxysporum than their corresponding water extracts with the highest inhibition of 28.0mm at 2.5mg/ml. The result thus, indicates ethanol neem seeds extracts at 2.5mg/ml could be used to inhibit mycelium growth of Fusarium oxysporum to substitute Benlate. The use of antibiotics to control latent bacterial contamination in tissue culture techniques has disadvantage, they are usually heat-labile and often phototoxic and only effective against bacteria not fungi. The addition of antibiotics to tissue culture medium causes reduction in vigour, chlorosis in propagated plantlets and capable of altering the behavior of culture medium. Therefore, the use of ethanol neem seed extracts also has an added advantage of been an organic material in the medium and effective with the occurring benefit of non toxicity to the growth of in- vitro cultures.

Keywords: Neem, Fusarium, Ginger, Contamination, Micro Propagation

1. Introduction

Plant tissue culture techniques provide an alternative way of plant propagation and function as an important tool for crop improvement programmes. These biological processes can be observed and manipulated by the germination of seeds and the propagation of whole plants, plant organs, plant tissues and plant cells in vitro, which is to be maintained on various types of culture media at substantial condition free of microbial contamination [1,2].

Microbial contamination, which refers to the growth of any unwanted microorganisms, (bacteria and fungi) in a plant tissue culture, is the most important cause of losses. However, contamination has been reported as constant problem, which can compromise development of all in vitro techniques [3]. Microbial contamination in plants tissue culture techniques was reported and include *Pseudomonas flourescens, Escherichia coli, Proteus sp, Micrococcus spp, Streptococcus pneumoniae, Staphylococcus aureus, Bacillus cereus, B. subtilis, Corynebacterium sp and Erwinia sp, Alterneria tenius, Aspergillus niger, Aspergillus fumigatus, Cladosporium sp, Saccharomyces sp, Fusarium oxysporum, Rizopus nigricans* and *Fusarium culmorum* [4]. During large-scale micropropagation of some plants, certain types of slow

growing microbial contaminants persist for many generations without being noticed and cause reduction in vigour or chlorosis in propagated plantlets, even after initial surface sterilization of explants [5]. The management of microbial contaminants in tissue culture depends mainly on the use of synthetic antibiotics. These have been extensively tested for their ability to inhibit or prevent the growth of bacteria in plant culture as reported [6]. Combinations of antibiotics added to tissue culture medium reduced plant growth and induced chlorosis at higher concentrations. However, the use of antibiotics has certain limitations; they are expensive, and only effective against bacteria not fungi. The range of efficacy against the types of bacteria is often narrow, they are usually heat-labile, and they are often phototoxic or otherwise capable of altering the behavior of cultured plants tissues [7].

Ginger is affected by various fungal, bacterial, viral and mycoplasmal diseases of these are soft rot cause by *phytium aphanidermatum,* yellow of root cause by *Fusarium oxysporum,* bacterial wilt caused by *Pseudomonas solanacearum* and leaf spot caused by *Phyllosticta zingiberi* result in considerable crop losses [8]. Study showed rhizomes designated for planting material were invariably infected by *Fusarium oxysporum* [9]. Most of the fungal and bacterial diseases are eliminated during surface sterilization and culture, viruses and viroids survive through successive multiplication if the mother plant is infected [10].

The nutrient media in which the plant tissue is cultivated is a good source of nutrient for microbial growth. These microbes compete adversely with plant tissue culture for nutrient. The presence of microbes in plant cultures increased the culture mortality, latent infections can result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting [11]. However, study revealed ginger contamination was a problem and in¬corporation of an antibiotic Tetracycline and biocide PPM into culture medium helped to check contamination [12]. In spite of the strictest use of sterile techniques by the trained artisan, the contamination of plant culture vestiges a constant problem that can result in dead ranging from small numbers of cultures to the loss of the entire batches of culture medium and tissue cultures. The plant tissue cultures may be sterile when initiated but microorganisms can often contaminate cultures at any point during subsequent tissue culture manipulations.

Therefore, there is need to investigate the possible use of plant products (neem seeds extract) to control the most pathogenic fungal contamination of ginger (in vitro) in micro propagation techniques, during culture period for substantial normal development of plants, plant tissues with the occurring benefit of non toxicity, availability and cheap.

2. Materials and Methods

2.1 Collection and Preparation of Plant Extracts

Neem (*Azadirachta indica*) seeds were collected from neem trees within Kaltungo, Gombe State. The seeds were air dried and dehulled to remove the seed coat. The seed were surface sterilized with 0.75% of Sodium hypochlorite, washed and rinsed several times with sterile water and oven dried at 400C for 3 hours. 10g of neem seeds were weighed and ground in a mortar to a fine powder. The powder was weighed 0.05, 0.1, 0.15, 0.2, 0.25g transferred into a sterile conical flask and 100mls of sterile distilled water, 95% ethanol were added to the flask respectively and covered with aluminum foil. These were allowed to stand on the Laboratory bench for 24 hours. After which the suspension were filtered through a 0.45µm membrane filter into a sterile volumetric flask, and concentrations of 0.5, 1.0, 1.5, 2.0, 2.5mg/ml were prepared. The filtrates were stored in a refrigerator at 4°C until required as reported [13].

2.2 Sterilization and Incubation of Plant Cultures

100 Rhizomes of *Z. officinale (UG 1)* were collected from plants grown in the Green house at National Root Crops Research Institute Umudike and the study was conducted in Tissue Culture Laboratory at National Root Crops Research Institute Umudike. Rhizomes were first washed several times under running tap water for 30 minute to eliminate the soil particle before the roots and outer scales were removed. The clean rhizomes pieces were spread on a cleaned polythene sheet and covered to provide humidity until the buds sprouted. The buds (explants) were excised from the rhizomes and washed with sterile distilled water several times. These were surfaced sterilized by immersion into a 0.75% NaOCl (Sodium hypochloride) solution for 20 min after sterilizing with 70% ethanol for 15 seconds; these were rinsed in 4 successive changes of sterile distilled water. The explants were excised with the help of sharp blade and collected in a sterilized Petri dish for inoculation. The aseptic explants were then cultured on Murashige and Skoog medium, 3% sucrose, 0.5% agar and different concentrations of BAP (Benzylaminopurin). PH of the medium was adjusted to 5.8 before autoclaving and labeled [14]. All cultures tubes were incubated at 26 ± 2°C under 16 hours photo-period. Emergent fungal contaminants were observed on the cultures tubes within seven days of initiation. The fungi were inoculated on Potato Dextrose Agar amended with streptomycin (PDAS). Pure isolates was obtained from repeated sub-culture and stored on agar slant at 4^0C [15].

2.3 Characterization and Identification of Isolates

Fusarium oxysporum were isolated and identified using cultural character and morphology by comparison with standards [16]. The Macroscopic and microscopic identification shows mycelia extensive and cottony in structure with some tinge of pink colour. Conidiophores slender and single, bearing a whorl of phialides, grouped into sporodochia, conidia hyaline; macro conidia sever-celled slightly curve at the pointed ends, typically canoe-shaped; micro conidia 1-celled, slightly curved were observed [17].

2.4 Antifungal Activity of Neem Seed Extracts

Filtrates of varying concentrations of ethanol neem seeds and water extract were transferred into separate flasks containing 100

ml of autoclaved potato dextrose agar and streptomycin (PDAS). The solution in the flask was gently swirled and poured into sterile Petri dish. The plates were allowed to remain on laboratory bench for 24 hours. 5mm in diameter of 7-days old inoculums of test *Fusarium oxysporum* isolates was applied at the center of petri dish with a sterile swap sticks. *F. oxysporum* growth and Inhibition records in millimeter were taken at 24 hours' intervals for five weeks beginning at 24 hours after inoculation [18]. The diameter of growing mycelium was measured with millimeter scale [19]. The final concentrations of extracts per plate in descending order were 2.5, 2.0, 1.5, 1.0 and 0.5mg/ml. Each treatment was replicated three times and the culture plates were arranged in a Complete Randomized Design (CRD).

3. Statistical Analysis

The Experiments was conducted in Complete Randomized Design with three replicates. Data generated from the Experiment were subjected to simple descriptive statistics and the analysis of variance (ANOVA). Mean differences between treatments or concentration levels of the extracts were separated by Fisher's least significant difference (LSD) at 5% probability level. The ANOVA was performed with SAS ANOVA GLM procedure [20].

4. Results

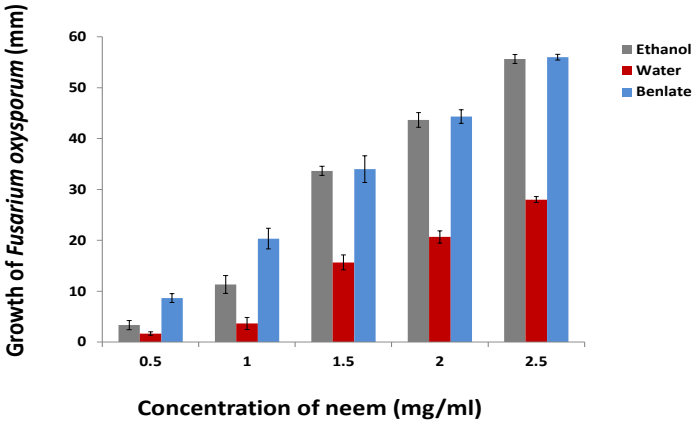

Figure 1: Effect of Neem seed extracts on the growth of F. oxysporum on PDAS agar

5. Results and Discussion

Study indicate the number of ginger explants initiated revealed that 62.5% were contaminated with *Fusarium oxysporum* which signified that these isolates constitute the major contaminants in micro propagation of ginger from the green house, 16.7% were contaminated with other organisms, while 20.8% uncontaminated culture tubes. However, the growth in diameter-recorded of Benlate at 2.5mg/ml had the highest inhibition of 56.0mm and ethanol Neem seeds extract at 2.5mg/ml had 55.6mm (Fig.1). These indicate that ethanol neem seed extracts can be used to substitute synthetic antibiotics and fungicides during tissue culture techniques. These antifungal activities of ethanol neem seed extract on *F. oxysporum* (mycelia) increases with concentration of the extracts. The results showed that neem seed extract was more effective and has a strong fungicidal activity against water neem seed extract as compared to Benlate. The water neem seeds extracts at 2.5mg/ml showed less inhibition of isolates tested which indicated less active ingredient of neem seed in water extracts. The active ingredients of neem seeds are slightly soluble in water, but freely soluble in organic solvent such as hydrocarbons, alcohols, ketones, as reported by Jacobson [21]. In contrast, distorted growth pattern was not recorded for all the plate. Statistical analysis showed that the inhibition in the test were significant from the growth pattern in Benlate and ethanol treatments. The symptom of inhibition observed on mycelium growth was the wrinkled of mycelium which was caused by plasmolysis that occurred particularly on young mycelia. The bioactive compounds of fungicides and extracts commonly cause plasmolysis on *Fusarium oxysporum.* This was due to the outer cell osmotic pressure higher than inner cell. Therefore, water run out of cell and cell then becomes dry and wrinkled [22]. The use of neem seed extracts in the control of *Fusarium oxysporum* contaminant in Tissue culture and micro propagation has gain importance, as antifungal activities of plants product. Several workers [23] now recognize antifungal activity of neem seeds and leaves. The present study showed that neem seed extract has high antifungal properties on *Fusarium oxysporum* isolates.

Conclusion

Neem seed ethanol extracts was effective at 2.5mg/ml but it was not completely inhibited as Benlate. However, spores formed on agar plate remained viable. The neem extract with the occurring benefit of non-toxicity availability to in vitro and cheapest of neem, it's advisable to carry out another evaluation of higher concentrations possibly 4.0mg/ml, 8.0mg/ml and 16.0mg/ml for complete inhibition [24-27]. To find out the concentration that will be compared effective on the control of *F. oxysporium* contamination, as indicated with Benlate 2.5mg/ml, further investigation can be carried out by directly incorporating the natural product as well as the Benlate directly into the culture media containing the explants in the process of micropropagation of ginger using tissue culture technique.

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