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In Vitro Evaluation of the Antifungal and Biocontrol Potential of Fish Amino Acid Against *Phomopsis azadirachtae*

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ABSTRACT

The current paper has experimented the antifungal activity of fish amino acid (FAA) in vitro in the absence of oxygen on the phytopathogenic fungus Phomopsis azadirachtae by fermenting the fish waste and jaggery (1:1 mixture) using fermentation. Seven days of testing in three different concentrations (10, 20 and 50) in the presence of the antifungal and an untreated control were used. The radial growth of fungi at a certain time was measured through three experimental replicates. The results indicated that FAA had an inhibitory effect on the test pathogen based on concentration. The 10 per cent treatment of FAA was somewhat inhibited and it showed averages of 1.93 cm of fungal growth on Day 7 which translates to about 62.58 per cent, as determined by colony diameter, when compared to the control. Interestingly, 20 and 50 per cent FAA totally inhibited the growth of fungi that could be observed on Day 3 to Day 7. The lowest effective concentration of the tested concentrations was found to be 20 percent and no further increase in respect to inhibition was achieved in 50 per cent. These results point to the opportunities of fish amino acid being a low-cost, naturally sourced antifungal formulation and indicate the usefulness of waste generated during fish-processing as a resource in the creation of sustainable agricultural inputs. Further biochemical characterization, antifungal mechanism of action, phytotoxicity study and field trial are required to establish its viability in application of plant diseases in an environmentally friendly manner.

Keywords: *Fish amino acid; Phomopsis azadirachtae; Antifungal activity; Neem; Biocontrol; Sustainable agriculture.*

Introduction

Limonoids (azadirachtin, nimbolide, nimbin, nimbidin, gedunin and salannin), flavonoids, phenolic compounds, tannins, terpenoids and other secondary metabolites are the main bioactive constituents of neem that have been reported. They differ in their biological actions with various components of the plant, solvent used in the extraction, concentration and preparation. Recent reviews also emphasize that neem has a lot of drug potential although much has been at preclinical levels; its effectiveness in the treatment of human beings must be further proved by well-conducted clinical trials [1]. The majority of the latest papers published in 2021-2026 have gone forward and conducted a study on the phytochemical composition, pharmacological activities, and medicinal potential of neem (*Azadirachta indica* A. Juss.). The proximate composition, bioactive constituents and antimicrobial properties of neem are reviewed as [2] point out the role of triterpenes, flavonoids and phenolic compounds in the antibacterial, antioxidant and anti-inflammatory effects of neem, and the immunomodulatory and anticancer effects of neem leaf glycoproteins. In a similar study, [3] explored the ethnopharmacological relevance, phytochemical diversity as well as pharmacological mechanism of neem, establishing over 140 reported bioactive compounds, comprising limonoids, flavonoids and phenolic acids, which have been reported to mediate the control of inflammation, oxidative stress, cancer-cell regulation and neuroprotective pathways; but noted that lack of clinical evidence, standardization of extracts and bioavailability. [4] have highlighted the importance of neem in the traditional Indian medicine particularly Ayurveda, Siddha, and Unani and have discussed the medicinal applications of different parts of the plant as leaves, bark, flowers, fruits and seeds, and reported the antimicrobial, antiviral, antifungal, anti-inflammatory, anti-plasmodial, anti-pyretic, anti-diabetic and anti-ulcer activity. [5] summarized the phytochemical and pharmacological diversity of neem, revealing several bioactive compounds, such as salannin, quercetin, nimbolinin, nimbidin, nimbidol and azadirachtin and discussed their possible antibacterial, antifungal, antiviral, anti-inflammatory, anti-diabetic, anthelmintic, hepatoprotective, immunomodulatory and wound-healing activities. The reviewed articles on the traditional and more modern medicinal uses of neem by [6], reported that it has the potential to be antioxidant, antidiabetic, anti-inflammatory, hepatoprotective, neuroprotective, anticancer, antimalarial and wound-healing properties and was applied to the treatment of certain dermatological disorders, including acne, eczema, dermatitis and microbial skin infections. [7] tested neem-based limonoids, namely, nimbolide, gedunin, azadirachtin and epoxyazadiradione, as possible anticancer agents and reported results on their effects on cancer-cell proliferation, apoptosis and their molecular signaling pathways in models. [8]

critically reviewed the phytochemistry, pharmacology and toxicology of neem with diabetes mellitus, evaluated the evidence of blood glucose regulation, lipid metabolism, oxidative stress, carbohydrate-digesting activities, glucose tolerance and glucose uptake and suggested that neem compounds could be utilized as antidiabetic medications but they needed to be tested further and their safety determined through systematic assessment. All these studies together lead to the argument that neem is a phytochemically endowed medicinal plant with a broad array of pharmacological potential although further studies on standardized extracts, characterization of the different compounds found, mechanistic validation, toxicological studies and clinical studies are required to determine the efficacy and safety of neem in therapy. Neem is an economically, medicinally and ecologically important tree species that is increasingly affected by die-back disease in several regions of India. The disease has been associated primarily with *Phomopsis azadirachtae* (syn. *Diaporthe azadirachtae*) and is characterized by progressive drying of terminal shoots, branches and twigs, followed by branch death and, in severe cases, deterioration of the entire tree. [7] reviewed the emerging threat of neem die-back caused by *P. azadirachtae*, discussing its symptoms, potential spread through airborne spores and management approaches, including removal of infected branches, maintenance of tree health and fungicide applications. [9] investigated the morphological and molecular identification of *P. azadirachtae* isolates collected from different regions of Tamil Nadu, India. Fifteen fungal isolates exhibited variations in colony colour, radial growth, colony margins and mycelial characteristics, while species-specific PCR confirmed their identity as *P. azadirachtae*. In another study, [10] evaluated antagonistic bacteria against the pathogen and reported that *Bacillus amyloliquefaciens*, *B. cereus*, *B. subtilis*, *Erwinia* sp. and *Pseudomonas aeruginosa* exhibited antifungal activity; ethyl acetate extracts of their culture filtrates completely inhibited pathogen growth at 20 ppm under the reported in vitro conditions. Renuka et al. (2026) and these recent investigations collectively emphasie the importance of pathogen detection and environmentally compatible management strategies. [11] investigated the occurrence and management of neem die-back in Telangana by characterizing seven pathogen isolates collected from different districts using cultural, morphological and ITS-based molecular approaches. The authors also evaluated 15 endophytic fungi and eight endophytic bacteria against the pathogen and tested six systemic fungicides, reporting significant inhibition of fungal growth by carbendazim and thiophanate-methyl. [12] the biocontrol potential of rhizobacteria isolated from the medicinal plant *Andrographis paniculata*. Three selected bacterial isolates showed considerable inhibition of *P. azadirachtae* in dual-culture and culture-filtrate assays, indicating their potential for further development as biological control agents. [13] examined the antifungal activity of fermented papaya (*Carica papaya*) and banana (*Musa paradisiaca*) fruit juices against the neem die-back pathogen using GC–MS profiling. Their results indicated concentration-dependent inhibition of fungal growth, with 50% fermented fruit juice treatments producing complete inhibition under the reported experimental conditions. [14] assessed neem die-back damage and recovery among trees on the Palamuru University campus, Mahabubnagar, Telangana, and reported observations following the application of formulations containing neem bark extract, garlic oil and neem seed oil.

In a related taxonomic investigation, [15] described *Cytospora azadirachtae* as a new fungal species associated with stem cankers and die-back symptoms in *A. indica* from southern India, based on morphological observations and multilocus phylogenetic analyses involving ITS, TEF1-α and TUB2 sequences. This finding indicates that neem die-back symptoms may be associated with more than one fungal pathogen and highlights the importance of accurate molecular identification before developing disease-management strategies. [16-17] also reported neem die-back disease in *Current Science*, describing the progressive drying of branches from their terminal regions and the potential for severe disease development. Earlier, [18] developed a species-specific PCR-based assay for rapid and sensitive detection of *P. azadirachtae* in naturally and artificially infected neem seeds and twigs, providing an important molecular diagnostic approach for pathogen identification. The original pathogen description and disease association were established by [18-19] respectively, recent studies demonstrate progress in the identification, diagnosis and management of neem die-back through conventional fungicides, endophytic microorganisms, antagonistic bacteria and plant-derived antifungal preparations.

Fish amino acid as a potential antifungal and biocontrol input in sustainable plant disease management

The products of fish origin are increasingly acquiring a place in sustainable farming as it offers opportunities to convert fish-processing by-products into useful agricultural products. In the years 2000-2013, research in the field of plant bio stimulants witnessed the emergence of an importance of amino acids, protein-based products and organic additions to boost plant growth, nutrient-use efficiency and stress resistance. [21] have provided a summary of agricultural applications of bio stimulants of plant origin i.e., amino acids, protein hydrolysates and described how it could lead to improvement in plant root growth and nutrient uptake and environmental tolerance of stress. [22], described what plant bio stimulants are and how to categorize such products and control them later on with the effects of such products perceived as their physiological value to plants rather than necessarily the nutrient content. Protein hydrolysates and [23], conducted a review of horticultural bio stimulants as such and provided evidence that these products, which are mostly soluble peptides and amino acids of plant or animal proteins, can enhance crop growth, nutrient uptake, productivity, and application tolerance to abiotic stresses. In the case of fish by-products, [24], specifically addressed the utilization of fish-processing by-products as plant bio-stimulants and suggested fish protein hydrolysates as a potential source of peptides and amino acids, and other bioactive compounds to harness and apply in sustainable crop production. They also talked about the possibilities of fish-based materials to enable circular bioeconomy strategies and minimize the environmental impact of fish-processing waste. [25] performed an experiment with a fish protein hydrolysate as tomato (*Solanum lycopersicum*) organic bio stimulant with the hypothesis that the compound would significantly increase the fruit production of the control with no tomato roots, and only the highest concentration would have any harmful impact on the root development. The study established that the application of the compound could significantly increase the fruit production of the control without tomato roots, and only the largest concentration of the compound had any adverse effect on the development of roots.

The article by [26] has conducted a general survey on amino acid-based bio-stimulants and Protein hydrolysates background and provided commentary on their components in plant metabolism, nutrient absorption, plant growth regulation, and the development of Environmental stressors. [27] explored the effect of fish fertilizer amino acid manufactured by fermenting mackerel waste using molasses in a hydroponic system using *Brassica rapa* L. cv. Improved height of the plant and width of the leaf of the plants treated by FAA as compared to the untreated control. [28] extensively discussed fish-viscera hydrolysates in the framework of a circular bioeconomy and as possible contributors to the nutritional aspects, growth and sustainable use of waste in crops. [29] studied how bioactive peptides are produced by the hydrolysis of fish protein, antioxidant, and antimicrobial effects of fish protein hydrolysates with a focus on the enzymatic method of hydrolysis and signaled antibacterial and antioxidant effect. The researchers also discussed how encapsulation of bioactive peptides is enhanced by using nanoliposomes as one of the mechanisms of improving its stability and functionality. Significance of amino acid make up and formation of the formula in identifying the biological reaction of the treated vegetation was additionally highlighted in the study [30]. The foliar application of the stand-alone amino acids as the plant bio stimulants were reviewed and discussed by [31], and their potential to affect the growth, metabolism, stress response and defense-related physiological processes in plants. [32] compared the different concentrations of fermented sardine residue-prepared fish amino acid mixtures with vermicompost to stimulate the growth and yield of okra (*Abelmoschus esculentus*) and further showed the potential of using fermented FAA in agriculture. In a previous study, the waste of sardine processing was converted to fish protein hydrolysate by autolytic enzyme hydrolysis and it was observed that it could assist growth and productivity parameters of tomato and sorghum and hence promising in applying fish-processing residues as bio stimulants to agriculture. [33] studied the bioactive and techno-functional properties of fish protein hydrolysates and the process of their production, its utilization and the issues that it might present. A review article of production, functionality, bioactivity and applications of fish protein hydrolysates and peptides by [34] found that enzymatic hydrolysis and microbial fermentation and other types of processing were used to obtain peptide-rich products with antioxidant, antihypertensive and anti-inflammatory efficacies. [35] evaluated extraction of fish-waste as the organic fertilizer to cultivate crops and increase their yields, including fish-amino acid production, nutrient levels, fermentation and crop production. The revision of foliar amino acid application as an abiotic stress mitigation strategy was conducted by [36] who elaborated on the mechanisms, which included osmotic regulation, antioxidant defense, photosynthetic protection, phytohormone signaling and ion homeostasis. Moreover, antimicrobial peptides are of particular interest due to their application in fish milk and fish feed supplements that result in inhibition of bacteria, fungi, viruses and parasites. The authors in their review article Drawing immune-capacity of fish-derived antimicrobial peptides for aquaculture industry (2024), reported the possibilities of these peptides as universal antimicrobials, such as antifungal agents.

More so, proteomic and functional phenotypical profiling of peptides produced by fisheries by-catch showed that a *Paralonchurus brasiliensis* formed hydrolysate by Protamex had antifungal actions on Candida albicans by 81%, showing that chosen fish-derived peptide fractions may possess quantifiable antifungal activities. These observations but apply to food or microorganisms of the human type and are not directly extrapolated to the fungi pathogenic to the plant. Compared with the neem die-back *Phomopsis azadirachtae* (syn.). Drying off of neem shoots and neem branch *Diaporthe azadirachtae*) is among the pathogens that cause trouble regarding progressive drying of neem shoots and neem branch. The isolates of the pathogen were characterized by [27] based on various districts of Telangana and examined the management of the pathogen in terms of systemic fungicides and endophytic microorganisms. Their results maintain the necessity to have efficient and ecological disease-management mechanisms. Although the literature testifies to the nutritional, bio stimulant, antioxidant and antimicrobial profiles of fish-derived products, the effect of fermented fish amino acid on *P. azadirachtae* fermentation is not well studied.

Fish amino acid (FAA) is a fish-derived fermented agricultural input that contains amino acids, soluble peptides, nitrogenous compounds and other products of fermentation and has garnered attention due to its potential plant-growth-stimulating and bio stimulant properties. By-products of the fish-processing should not be overlooked as plant bio stimulants identified by [29-31] articulated that bio stimulants derived on the basis of amino acids and protein hydrolysates are beneficial in boosting the development of the roots, uptake of nutrients, and immune reaction and resistance to the environmental factors in the vegetation. [32] also reported that bioactive peptides are present in the fish protein hydrolysates and that are also antioxidant and antimicrobial, which suggests that they can be utilized in other fields, other than plant nutrition. Scientific reasoning was also reflected in the review of fish-derived antimicrobial peptides and their potential to be antifungal, which was also supported by scientific reasoning and justified the consideration of an investigation of fish-derived bioactive compounds against fungal pathogens [33]. The findings, though, pertain to fish protein hydrolysates or single antimicrobial peptides and might have absolutely no direct relation to antifungal activity of fermented FAA. [34] studied *Phomopsis azadirachtae* (syn) on the background of neem die-back. The use of systemic fungicides and endophytic microorganisms to control the episodes by *Diaporthe azadirachtae*) isolates collected in Telangana demonstrated the significance of environmentally friendly disease-control systems. In similar investigations, [35] provided the antifungal activity of antagonistic bacteria and antimicrobial extracts prepared during culture on *P. azadirachtae* over exploring this use of microbial metabolites as alternatives to control the disease. Concentration dependent antifungal activities of fermented papaya and banana fruit juices were also reported which would suggest the ability of naturally fermented agricultural products in research [36]. A combination of these studies has provided scientific explanation to the research of fish amino acid as a potential agricultural bio stimulant, antifungal formulation. Therefore, intensive research will be on the concentration-dependent antifungal activity of the fish amino acid against *P. azadirachtae* in *in-vitro*, effect on growth of the fungus, germination of the spores, possible morphological alterations, and effect of the amino acid on the health of the

neem plants and the guidelines of applications of amino acid at the available concentrations through phytotoxicity and greenhouse tests. A study like this would offer some initial scientific data with which to develop a fish waste based, value added agricultural input that can be incorporated in the integrated, sustainable management of neem die-back disease.

Material and methods

Collection and Authentication of Plant Material

The *Azadirachta indica* leaf samples were collected in the Arts and Science college, Dichpally, Nizamabad District in Telangana, India, in February-March 2024. My research supervisor, Dr. M. Mamatha, Professor of Forest Botany, Department of Basic and Social Sciences, Forest College and Research Institute, Mulugu, Hyderabad, Telangana, India authenticated the plant material.

Collection and Isolation of Fungal Pathogen

The *Azadirachta indica* trees in the Telangana University, Dichpally, Nizamabad District, Telangana, India was sampled by harvesting neem leaves with typical dieback symptoms (leaf chlorosis and tissue necrosis). The infected leaf tissues were cut into little pieces and sterilized on the surface with 0.2% solution of mercuric chloride ($HgCl_2$) 1 min. The segments were then washed three times with sterile distilled water to wash off any sterilizing agent and dried under aseptic conditions. Sterilized pieces of tissue, aseptically transferred, were put on potato dextrose agar plates (PDA) with streptomycin (50 µg/mL) to prevent the presence of bacteria in the sample. The plates with the inoculums were incubated at temperatures of 25-28C and allowed to incubate, over 7 days. The growth of colonies of fungi out of the affected layers of tissue was examined based on the culture and morphological characteristics. Possible colonies of *Phomopsis azadirachtae* were further sub cultured several times on fresh PDA media to obtain pure cultures. The isolates were further purified and transferred to PDA slants where they were kept at room temperature over 15 days and kept at 4 ^{0}C to be further studied. Initial identification of the fungal isolate was done on the basis of the cultural and microscopic features after lactophenol cotton blue staining and against the existing taxonomic descriptions (Fig:1A-D).



Fig: 1A-D: Dieback Diseased Neem plant with symptoms

Preparation of Fish Amino Acid (FAA)

The fish amino acid (FAA) was made by a traditional fermentation technique that is a variation of the Korean Natural Farming (KNF) technique, as reported by [37] subjected to modifications. Waste fresh fish including the heads, bones, skin, fins and viscera was obtained using locally available sea fish, including mackerel, sardine and skipjack tuna.

The fish waste (1kg) was also properly cleaned to get rid of any unwanted foreign substances and cut into small pieces to help in decomposition. The number of unrefined countries jaggery (1 kg) purchased in a local manufacturer without any added ingredients was powdered and served as a source of fermentable sugar. The fish waste and powdered jaggery were blended well at a ratio of 1:1 (w/w). The mixture was then poured into a clean, dry and food grade plastic fermentation container in five or six alternate layers, by making sure the fish material was well covered with jaggery. The last layer was sprinkled with powdered jaggery so as to have less direct exposure of the fish material to air. A clean and breathable cloth was used to cover the container or a loosely fitted lid was used in order to avoid contamination by insects and to release gases produced during fermentation. The container was kept in a well-ventilated, cool place, not exposed to direct sunlight, or to disturbance by animals. When the fish tissues eventually started to become soft and liquefied after about 3-5 days with the enzyme degradation, microbial action and the osmotic pressure of the added jaggery. The process of fermentation was extended to a period of about 2-6 months to get a fully fermented fish extract. The process of fermentation was evaluated by the formation of a fairly homogeneous dark-coloured liquid that had a typical sweet, slightly fishy smell and a significant amount of liquefaction of the fish matter. This mixture was then fermented and the mixture was then decanted and filtered using a clean nylon mesh to separate the liquid component of the mixture and the remaining solid matter. The filtrate, which was a preparation of fish amino acids (FAA), was pooled in clean and airtight labeled containers and stored under appropriate conditions awaiting further analysis and use in experiments. The solid fraction left was either composted or used independently. This process is based on the conventional fish-waste fermentation process whereby the sugars are used to enhance the extraction and preservation of moisture and enzymes generated endogenously and by microbes to decompose fish proteins and other organic components [39-41] (Fig:2).



Fig: 2. Preparation of Fish Amino Acid (FAA)

Culture Media and Composition

Preparation of Potato Dextrose Agar (PDA) Medium

The isolation, cultivation and maintenance of the fungal pathogen was done on potato dextrose agar (PDA) medium. Preparation of the medium was done in the traditional potato infusion procedure; in accordance with the composition as stipulated in FDA Bacteriological Analytical [41-42].

The medium was made up of 200 g of freshly peeled potatoes, 20 g of dextrose, 15 g of agar and 1,000 mL of distilled water per litre. In short, 200 g of peeled and cut potato tubers was boiled in 500 mL of distilled water till it was soft enough. Sterile cheesecloth was then used to filter the potato extract to remove the solid residues. Next 20 g of dextrose was added to the filtrate, and dissolved. A 15g of agar was warmed in 500 mL of distilled water and the agar solution put in the potato-dextrose mixture. The volume of the medium was then filled with distilled water to 1,000 mL. The PH was set to 5.6 +/- 0.2 by the use of 1 N hydrochloric acid (HCl) or sodium hydroxide (NaOH as necessary. The autoclave sterilization was done at 121 heat and 15 psi pressure and lasted 15 min. The medium was then allowed to cool down to about 4550 C and streptomycin was aseptically added at the necessary concentration, when necessary to avoid contamination by bacteria. The medium was then transferred into sterile Petri plates under aseptic conditions wherein the medium was left to dry [43].

Antifungal Activity Assay

Fish amino acid (FAA) was assessed as an antifungal agent against *Phomopsis azadirachtae* by the poisoned food method which was reported by [44] with appropriate changes. FAA preparation was dissolved in the sterile distilled water to obtain three concentrations of FAA (10%, 20% and 50% v/v). The desired final concentrations were obtained by adding FAA in proper amounts to the potato dextrose agar (PDA) medium of the desired strength. The medium used was changed by the FAA and mixed well to make the test material spread equally. The aseptic preparation of the FAA was followed by the sterilization of the PDA medium and then incorporation of the FAA aseptically was done after the medium had been chilled to approximately 45-50C. Media were amended after which they were transferred into sterile Petri plates (90 mm) and left to solidify. PDA medium in the absence of FAA was used as the untreated control. A 5-mm mycelial disc of a culture of *P. azadirachte* (7 days old) was cut out with a sterile cork borer and put in the middle of the solidified media in each Petri plate. All treatments (untreated control) were kept in triplicate and incubated at 25-28 C. Radial mycelial growth of the fungal pathogen was determined on 3rd, 5th and 7th days after the inoculation. The percentage inhibition in the growth of the mycelia was calculated using Vincent formula when compared to that of the untreated control [45].

The percentage inhibition was calculated using the following equation:

$$\text{Inhibition } (\%) = \frac{C - T}{C} \times 100$$

where C = radial growth in control (mm) and T = radial growth in treatment (mm).

Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$) in SPSS v26.0. Results are presented as mean ± standard deviation (SD) of triplicate experiments.

Results and observations

The fungal pathogen responsible for Neem dieback disease was successfully isolated from infected Neem leaves collected from Telangana University, Dichpally Nizamabad Telangana State.

The common symptoms of a disease in diseased leaves are browning, necrosis and progressive drying and these symptoms were a testimony that *Phomopsis azadirachtae* was actively infected. Surface sterilization of leaf samples followed by inoculation in potato dextrose agar (PDA) agar was done to isolate bacteria using streptomycin. Seven days incubation at room temperature resulted in the growth of fungal colonies initially whitish but then turning to darker due to sporulation, a common feature of *P. azadirachtae*. To obtain pure fungal isolates, sub-culturing was done that exhibited homogenous patterns of growth after incubation of 15 days. The presence of pycnidia, conidia, and other distinctive reproductive organs were confirmed with the help of the microscopic analysis, which confirmed the identity of the pathogen. Proper storage of the pure fungal cultures in the PDA slants and storage under low temperature (4 C) were also ensured to make sure that the cultures can be used in the following experimental research. The controlled model that was used to identify antifungal activity of fish amino acids was the successful isolation and identification of *P. azadirachtae*.

Fish amino acid (FAA) was prepared using fresh fish waste collected in a local fish market and prepared according to a traditional method of fermentation with respective modifications [46]. The weight of fish waste and unrefined country jaggery (1 kg each) were taken in the preparation. The fish waste collected was washed in order to eliminate any unwanted materials and then chopped into small bits to promote fermentation. The byproducts of the cut fish were stored in a clean food grade plastic, in a dry and clean condition and 1 kg of powdered jaggery was added. A thorough mixing was done to make sure that the fish waste and jaggery were in contact. A lid was put on top of the container to avoid contamination and the container was kept in a cool and dry environment, not exposed to direct sunlight. The mixture was then fermented after 45 days and this was followed by a filtering of the mixture using a clean nylon mesh to separate the liquid fraction and the solid material remaining out. The filtrate was taken in a clean container and utilized as the fish amino acid (FAA) preparation in further experimental research. The last was a thick liquid whose odour was typical of a sweet, fermented aroma that was similar to Panchamrutham (Fig; 3).



(A) (B)

Fig: 3AB. Isolation of Phomopsis azadirachtae

Evaluation of the Antifungal Activity of Fish Amino Acid (FAA) *Against Phomopsis azadirachtae*

Table 1: Effect of different concentrations of fish amino acid on the radial growth of P.azadirachtae over seven days.

Treatment	Day 1	Day 3	Day 5	Day 7
Control	0.50 ± 0.00	1.67 ± 0.29	4.17 ± 0.15	5.17 ± 0.15
10% FAA	0.50 ± 0.00	0.37 ± 0.23	1.00 ± 0.20	1.93 ± 0.12
20% FAA	0.50 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
50% FAA	0.50 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Values represent mean ± SD (n = 3). Measurements are expressed in centimeters.

The *in vitro* antifungal activity of fish amino acid (FAA) against the phytopathogenic fungus *Phomopsis azadirachtae* was determined at three different concentrations (10%, 20 and 50 percent). The growth of the fungi was recorded at four time points (Day 1, Day 3, Day 5, Day 7) the radial growth of the fungal colony was quantified using centimeters (cm). There was a control that remained untreated so as to discover the usual pattern of growth of the pathogen. All the treatments were repeated three times, and the data were presented as an average and standard deviation (SD). The results revealed that *P. azadirachtae* had an apparent concentration-dependent inhibition effect by FAA and a notable fungal growth was suppressed by 20 and 50 percent.

Control treatment

With the untreated control, the fungus showed uninterrupted and progressive growth of the mycelium over the seven days of incubation. The mean radial growth increased from 0.50 ± 0.00 cm on Day 1 to 1.67 ± 0.29 cm on Day 3. Afterward, there was a significant increase in fungal growth to 4.17 ± 0.15 cm on Day 5 and 5.17 ± 0.15 cm on Day 7. The active growth and viability of *P. azadirachtae* in the experimental conditions was confirmed by the gradual increase of colony diameter in the control treatment. Day 7 (5.17 cm) was taken as the maximum fungal growth in order to measure antifungal activity of the different FAA concentrations (Fig: 5A-D).

Effect of 10% FAA concentration

The 10% FAA activity against *P. azadirachtae* was moderate against fungi. The initial growth of fungi was recorded to be 0.50 cm 0.00 cm on day 1 with the control recording the same. However, on Day 3 fungi grew less and the mean diameter of the colonies was 0.37 ± 0.23 cm and it increased to 1.00 ± 0.20 cm on Day 5 and finally, it reached 1.93 ± 0.12 cm on Day 7.

Although the fungal growth was still present during this treatment the growth of the colony was greatly slowed compared to the growth of the fungi in the untreated control. When the average colony diameter was calculated based on the measured colony diameters, the colony diameter of the 10% FAA treatment was about 62.58% less than that of the control on Day 7. This means that 10% FAA was partly antifungal, but not so much that it could prevent the growth of the pathogen altogether (Fig:7: 1A, B, C; 2A, B, C; 3A, B, C and 4A, B, C).

Effect of 20% FAA concentration

A pronounced antifungal effect was observed when the concentration of FAA was increased to 20%. On Day 1, the fungal growth was 0.50 ± 0.00 cm. However, no fungal growth as measured after Day 3. On Day 3 and Day 5 and 7 the colony diameter was found to be smaller at 0.00 ± 0.00 cm. All three replicates of the experiment achieved the same response indicating that there was consistency in the inhibition in the experimental conditions. These observations have shown that 20 percent FAA was effective in inhibiting the growth of the fungal colonies that could be observed to grow during the remaining period of the incubation. The FAA treatment of 20% had completely inhibited a measurable fungal growth compared to the untreated control which had a mean colony diameter of 5.17 cm at Day 7 (Fig:7: 1A, B, C; 2A, B, C; 3A, B, C and 4A, B, C).

Effect of 50% concentration of FAA

The maximum concentration of FAA (50%), also showed total inhibition of the observable fungal growth. Day 1: the first the fungal growth was recorded to be 0.50 +0.00 cm and then no further growth of the fungi was observed on Day 3, Day 5 or Day 7. The three replicates had a steady zero quantifiable fungal growth up to the observation period after Day 1. The antifungal response at 50% FAA was similar to the response in the 20% FAA treatment. The two concentrations completely inhibited any fungi growth over the course of the experiment, but did not further inhibit any fungi growth as the concentration was increased to 50%. The findings reveal that 20% FAA was the lowest concentration that was experimented and this may completely suppress the development of the visible fungal colonies in the course of the seven days of the experiment (Fig: 7: 1A, B, C; 2A, B, C; 3A, B, C and 4A, B, C).

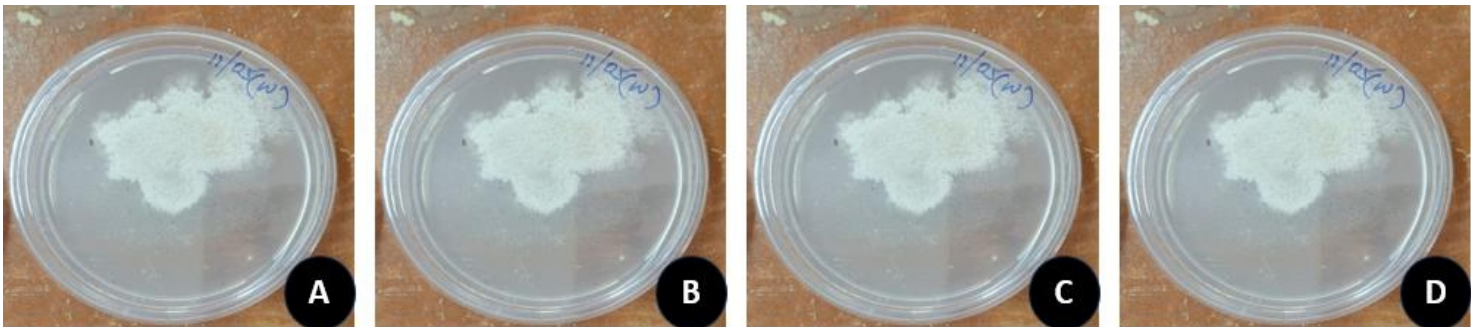


Fig: 5A-D: Control with no FAA treatment A-Day-1, B-Day-3 C-Day-5 D-Day-7



Fig:7: ***1 A, B, C:*** *Effect of 10% 20 % & 50 % concentration of FAA on pathogenic strain in Day-1;* ***2A,B,C:*** *Effect of 10% 20 % & 50 % concentration of FAA on pathogenic strain in Day-3;* ***3A,B,C:*** *Effect of 10% 20 % & 50 % concentration of FAA on pathogenic strain in Day-5;* ***4A,B,C:*** *Effect of 10% 20 % & 50 % concentration of FAA on pathogenic strain in Day-7.*

Antifungal activity Comparative analysis.

Comparative analysis of different concentrations of FAA revealed that concentration-dependent response against *P. azadirachtae* is evident. Even though the control was a continuous fungal growth, the 10 per cent FAA treatment did not fully stop the growth, but the 20 and 50 per cent FAA treatments completely stopped the fungal growth that was visible after Day 3. The findings indicate that the concentration of FAA had a positive correlation with the antifungal activity up to a concentration of 20 and no further testing has been done as the antifungal activity was not demonstrated to be increasing on the observed inhibitory response.

The absence of the growth of the fungal in the 20% and the 50% treatments at the remaining incubation time indicates that FAA has a strong potential of being used as a natural antifungal formulation against *P. azadirachtae*.

Table 2: Percentage inhibition of fungal growth (Using net radial growth on Day 7)

Treatment	Net fungal growth (cm)	Growth inhibition (%)
Control	4.67	0.00
10% FAA	1.43	69.29
20% FAA	0.00	100.00
50% FAA	0.00	100.00

Both 20% and 50% FAA exhibited 100% inhibition of net radial growth on Day 7 under the tested conditions. This current study distinctly shows that fish amino acid has a potential of being an antifungal agent against *Phomopsis azadirachtae*. Two out of three concentrations tested (20 percent and 50 percent FAA) had the highest inhibitory activities, and there was a complete inhibition of the growth of fungal colonies measured between Day 3 and Day 7. The 10 percent FAA solution was partially inhibited and this permitted the growth of the fungi to a certain extent during the incubation process. The control that was not treated allowed uninterrupted growth of fungi to confirm the adequacy of the experimental conditions to allow development of the pathogens. The better performance of 20% FAA than 10% FAA indicates that the effective concentration of bioactive constituents might be required to attain the antifungal activity. Preparations of fish amino acids can include a wide range of organic compounds, amino acids, peptides and other fish-derived metabolites, which might play a role in microbial growth inhibition. However, the individual antifungal compounds and their mechanism of action are yet to be determined using biochemical characterization and additional mechanistic studies. Notably, the similarities in the performance of 20% and 50% FAA suggest that the antifungal effect was not enhanced due to the concentration being increased above 20%. Thus, 20% FAA can be regarded as the most promising concentration of the ones tested, which could be effective in terms of fungal growth suppression with a lower concentration of application.

Discussions

Phomopsis azadirachtae, [56] is the causal organism of die-back of neem [48]. The diseased trees show common symptoms of twig blight, and inflorescence blight and rotting fruit. This disease results in a drastic reduction of evergreen canopy and in almost 100% loss of fruit production and thus affecting the availability of seeds [49]. The die-back of the neem is spreading rapidly diminishing the life span and seed production and hence the pressing need to come up with effective management strategies. *P. azadirachtae* can cause die-back of neem but this can be managed by use of a systemic fungicide Bavistin [50].

One potential solution to the reduction of inputs of agrochemicals in agriculture [51] and consequently avoid most of the related environmental and ecological issues is through biological control of plant diseases with the help of microorganisms. This can result in sustainable disease management approach. As a result of the above characteristics of the setup of a biocontrol strategy to die-back neem, in the present study amino acids of fish were selected on their antifungal action against *P. azadirachtae* during the in vitro experiment. The present study demonstrates the promise of the fermented fish amino acid (FAA) fermented with the help of fish waste and jaggery as a promising antifungal agent against the phytopathogenic fungus *Phomopsis azadirachtae*. The findings were that, there was a concentration-dependent inhibitory effect, 20 per cent and 50 percent FAA completely inhibited the visible growth of fungal colonies over the span of Day 3-Day 7, and 10 percent FAA exhibited an intermediate effect. These results suggest that fermented fish amino acid might be a potential source of naturally derived antifungal agent and may be employed as an alternative source of formulating sustainable agricultural formulations. The control that was not infected exhibited a steady growth of fungus throughout the incubation time with the average colony diameter varying with 0.50 cm on Day 1 and 5.17 cm on Day 7. This gradual growth substantiated the vigorous growth of *P. azadirachtae* in the experimental conditions. In comparison to this, fungal growth was greatly inhibited with treatment of 10% FAA resulting in an average colony diameter of 1.93 cm ± 0.12 cm on Day 7. This was a measure of about 62.58% colony diameter decrease compared to the control. At 20% and 50% FAA concentration, a stronger inhibitory effect was noted. There was no growth of fungal colonies in either of the treatment in the day 3, and this was suppressed up to the period of seven days of observation. The general response of the three replicates is that the two concentrations were both effective to prevent any visible fungal growth in the circumstances that were taken into account. Interestingly, the weakest concentration was 20% FAA which showed this effect and increasing the concentration to 50% did not provide any additional antifungal benefit. The antifungal effect that is of special interest is the one identified by the role that *P. azadirachtae* plays in the neem (*Azadirachta indica*) die-back disease. The fact that alternative biological means of the control of this pathogen should be developed is therefore of great importance. The current results are the initial indications that fermented fish-derived preparations could be useful in the inhibition of this phytopathogen. The antifungal activity that was obtained in the current study is comparable with that of the previous studies which investigate the effectiveness of naturally occurring compounds and biological agents against *P. azadirachtae*. [52] examined the antagonistic effect of different microorganisms against the neem die-back pathogen and found out that the microbial antagonists possessed an excellent antagonistic potential in regard to the pathogen. They found that the products they produced can be used as alternatives to control *P. azadirachtae.* Similarly, [53] compared five essential oils in terms of the effect on the pathogen and discovered that basils, camphor, lavender and rose oils at 400 ppm completely inhibited mycelial growth. As such, the results obtained led to the conclusion that the potential growth inhibitory effect of fungal pathogens using naturally occurring bioactive compounds was possible, the investigation that was conducted found that there was no fungal growth at all at 20% and 50% concentration of FAA.

The findings using different preparations and experimental concentrations were quite different than those of previous studies, but generally suggest that some promising directions might arise in controlling fungal diseases when using natural or biologically-derived materials. The current study contrasts with the studies which have examined essential oils or microbial antagonists and examine fermented fish waste as a source of agricultural antifungals. A second look on the use of organic wastes on materials to be used in controlling plant diseases. The antifungal activity reported in the current study is in accordance with previous studies which validated the efficacy of natural products against *P. azadirachtae*. [54] have reported the antifungal activity of microbial antagonists against neem die-back pathogen and [55] have reported complete inhibition of fungal growth with Basil, Camphor, Lavender and Rose essential oils at 400 ppm. Likewise, this study showed that detectable growth of the fungi was completely inhibited at 20% and 50% FAA concentrations. The present study is also different from the earlier studies that have been conducted on the microbial antagonists and plant essential oils, to explore the fermented fish waste as a potential alternative source of antifungal agents and their possible applications in developing the fish-waste based sustainable agricultural formulations. The complex composition of the FAA, such as amino acids, peptides, protein degradation products and fermentation derived metabolites, might explain its antifungal activity. [56] noted the biological properties of the fish protein hydrolysates such as antioxidant and antibacterial activities. These constituents could have been responsible for the inhibition of fungal growth in the present study, but their individual importance and mechanism of action have yet to be determined, as the chemical composition and the pH of the FAA was not analyzed. Further biochemical characterization of the compounds, responsible for the observed activity, should be performed and tested for activity against fungi. Use of fish waste for fermentation and the production of value-added agricultural products is promising. [57-58] converted fish waste (FW) into liquid fertilizer by using microbial biodegradation method, found the amino acid level to be 6.91 g/100g and it was comparable to commercial fertilizers that were used in hydroponic condition. In the present study, this concept was extended to study the antifungal activity of fermented fish waste against the fungus *P. azadirachtae*. Therefore, FAA could be a value-added agricultural formulation with combination of organic wastes utilization and preliminary antifungal activity. Ionic amino acids and peptides in preparations from fish are also new fish-related application areas in the field of agriculture, for example as plant nutrients and plant growth supports. The present work only investigated antifungal activity and not plant growth promotion and soil fertility/microbial activity. So, these uses need experimental validation prior to any recommendations being made at this stage. Based on the present results, 20% FAA, which inhibited any detectable fungal growth was the most promising concentration of the ones that were tested while 50% showed no additional benefit. Further studies to isolate the bioactive compounds, its antifungal mechanism, fungistatic/fungicidal activity by recovery tests on the fungus and phytotoxicity should be performed. These field and greenhouse trials also need to be conducted for confirmation of its application and safety, the production of fermented fish waste was successfully carried out in the production of a natural antifungal agricultural formulation, which has a potential use value.

Conclusions

The current study highlights the benefits of fish amino acid (FAA) as a cost effective, sustainable, and farmer friendly bio-input to improve the growth of plants, their soil and health. FAA may be applied to enhance plant metabolic, cell division and nutrient uptake and supplement an increase in plant height, leaf development, chlorophyll and photosynthetic rates. These physiological advantages can then be utilized to increase the yield properties and general crop yields. We have already established that the hydrolysates of fish protein in liquid fish amino acid preparations possess the capacity to promote phototropism, photosynthesis and carbon and nitrogen metabolism and used the nutrients, the fish amino acid extract using locally-ready fish waste and jaggery through a simple fermentation process is also cheap as compared to the agricultural products that are commercially obtained. It is an easy method to exercise, requiring a minimal input investment and farmers can draft in a way that can be executed using the local materials. FAA, besides its possible ability to promote growth in plants, might help enhance the soils frequently and beneficial microbial activity in the rhizosphere. Its future use as an insect-repellant might also have other uses in protecting crops. It is noteworthy that the present in vitro experiment revealed that FAA has tremendous antifungal capability against *Phomopsis azadirachtae*. The highest level of the test, i.e., 20 and 50 percent, inhibited the growth of the discernible fungal colonies in Days 3-7, but the lowest level of FAA (10 percent FAA) did it partly. The similarity in the antifungal activity of 20% and 50% of the concentration indicates that 20% FAA can be a valuable concentration in the experimental conditions used, fish amino acid made with the waste of fish-processing is a potential solution to transform the locally available organic waste into a beneficial agricultural input. It has also become a future of sustainable and environmentally friendly applications in crop production, as its potential has multiple functions such as promotion of plant growth, improving soil fertility and use as an antifungal agent.

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