EFFECT OF ENDOPHYTIC FUNGI ON *CRINUM JAGUS* LIQUID SUSPENSION CULTURES

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ABSTRACT

This study explored the impact of endophytic fungi on the biosynthesis of Amaryllidaceae alkaloids (AAs) in liquid suspension cultures of *Crinum jagus*, a perennial Amaryllidaceae species native to Africa with notable medicinal value. Due to the pharmaceutical industry's interest in AAs for their diverse biopotencies, including antiviral properties and anti-acetylcholinesterase activity for the treatment of Alzheimer's disease, and the high cost and low yield of current chemical synthesis methods, this research aimed to offer a sustainable, biotechnological approach to AA production. Utilizing *in vitro* cultures, the project investigated the stimulation of AA biosynthesis through biotic and abiotic stress, focusing on the elicitor activity of fungi and fungal secretions. Various endophytic fungi strains were isolated from *C. jagus* bulbs, and their effects were assessed in liquid suspension cultures. The investigation identified an optimal fungal elicitor for *in vitro* AA production, *Fusarium solani*, with the potential to contribute to more efficient and cost-effective pharmaceutical applications. These findings also provide a foundation for further investigation into the genetic expression and metabolic pathways associated with AA formation, highlighting the biotechnological potential of endophytic fungi in medicinal plant research.

Keywords: Endophytic fungi, *Crinum jagus*, Amaryllidaceae alkaloids, *Fusarium solani*, Plant-microbe interaction.

INTRODUCTION

For centuries, the Amaryllidaceae family has been recognized for its significant contribution to medicinal applications, largely due to its ability to produce a unique group of compounds known as Amaryllidaceae alkaloids (AAs)[1]. These alkaloids exhibit a wide range of biological activities, including anti-acetylcholinesterase, antiviral, antibacterial, antifungal, and cytotoxic effects[2]. Among the notable advancements in AA research is the approval of galanthamine for the treatment of mild cognitive impairments and Alzheimer's disease symptoms in over two dozen countries, attributed to its specific, competitive, and reversible inhibition of acetylcholinesterase[3]. Cherylline, another AA, has garnered attention for its antiviral properties[4].

The efficient production of AAs presents considerable challenges, primarily due to their low natural yield and the difficulties associated with cost-effective and environmentally friendly chemical synthesis[5]. In response, a biotechnological approach has been adopted, focusing on the laboratory cultivation of specific Amaryllidaceae species to explore and enhance their alkaloid biosynthesis pathways[6]. This approach includes the application of biotic and abiotic stresses[7], such as fungi and fungal secretions, to stimulate the production of these metabolites in liquid cultured-calli[8].

Crinum jagus, a perennial species native to Africa renowned for its traditional medicinal use and its production of cherylline, was chosen as the key subject in this research[9]. The study involved the development of *in vitro* callus cultures of *C. jagus*, utilizing various phytohormone combinations, and advancing to suspension cultures to investigate the biosynthesis of cherylline-type AAs. Furthermore, the isolation of endophytic fungal strains from *C. jagus* bulbs and their use as biological elicitors aimed to enhance AA production *in vitro*. The goal was to identify the most effective fungal elicitor for AA production and its impact on the genetic expression of key biosynthetic genes.

MATERIAL & METHODS

Initially, the preparation of the micro-organisms elicitors was carried out by growing, and isolating endophytic fungi from *C. jagus* bulbs. Fungal cultures were maintained in Sabouraud Dextrose Broth (SDB) solid medium, and grown in liquid SDB medium for several weeks before extraction through filtration. Extracts were earmarked for the main elicitor activity evaluation. Selected colonies were preserved for long-term study and aliquots stored for fungal identification via Direct Tissue PCR, using taxonomic markers specific to the Internal Transcribed Spacer (ITS) and Elongation Factors (EF) regions. This recognition process involved extracting fungal DNA, amplifying ITS and EF sequences of interest, and comparing these molecular sections against existing literature by phylogenetic analysis to pinpoint the fungal species involved accurately.

Concurrently, *C. jagus* calli were induced using a combination of 1-naphthaleneacetic Acid (NAA), 6-benzylaminopurine (BAP), and kinetin, with cultures established across a range of phytohormonal concentrations to optimize growth conditions. These calli were then meticulously maintained in growth chambers set to ideal conditions to ensure robust development. The *in vitro* suspension cultures of the plant subsequently underwent treatment with an array of fifteen different elicitor pre-candidates, including controls such as water, dimethyl sulfoxide (DMSO), melatonin, homoserine lactone, ions of cadmium and methyl jasmonate, to mainly evaluate their impact on alkaloid production. Cultures were kept under optimal growth conditions, with the experiment replicated twice to ensure reliability. After a ten-day treatment period, calli were processed for alkaloid extraction with

methanol. The metabolic profile of the extracts, revealing the diversity and quantity of AAs engaged, was analyzed using LC-MS and GC-MS techniques.

Afterwards, for those fungi strains classified as having promising elicitor activity, the possible metabolites from the explant cultures were again extracted and weighted at specified intervals ranging from 24 hours to four weeks post-treatment. The harvested cells were processed, and metabolites underwent the same extraction protocol, with samples subsequently stored in liquid nitrogen at -80°C. LC-MS and GC-MS were implemented to study the presence of AAs (with papaverine as internal standard), comparing the findings against established libraries and scientific literature to draw conclusions about the efficacy of fungal elicitors in enhancing AA production in the *Amaryllidaceae* discussed plant.

Finally, the study aimed to quantify the expression levels of genes involved in AA biosynthesis within *C. jagus* cultures. RNA was extracted from the treated cultures, transcribed into cDNA, and analyzed using specific primers for a set of known AA biosynthetic genes as for example *tyrosine/DOPA decarboxylase* (*TYDC)*, *norbelladine synthase* (*NBS)* and *O-methyltransferase (OMT)*. Gene expression analysis provided insight into the elicitor's effect on AA biosynthetic transcripts levels.

RESULTS & DISCUSSION

In the study of fungal taxonomy and phylogeny, the ITS sections are crucial for species barcoding, classification and leveraging the variability within these sequences across species. The primer pairs ITS1/ITS4 and ITS4/ITS5 target these regions, producing PCR amplicons that vary significantly in length due to the inherent diversity of the ITS regions among fungi. Specifically, ITS1/ITS4 amplifies both ITS1 and ITS2 regions, along with the 5.8S ribosomal RNA (rRNA) gene, yielding products approximately 350 to 700 base pairs (bp) in length. Conversely, ITS4/ITS5 focuses on the ITS2 region and adjacent rRNA gene segments, with products generally ranging from 200 to 500 bp[10]. Electrophoresis results provided additional insights into the fungal isolates' genetic makeup, for the ITS sections were properly pointed out with fragments around 500 and 600 bp.

Conversely, attempts to amplify EF genes as additional phylogenetic markers were unsuccessful, highlighting the ITS primer pairs' superior reliability and effectiveness in our fungal identification efforts. Certainly, EF markers usually exhibit less diversity compared to ITS sequences and may be challenging to replicate across a broad spectrum of fungi, which could reduce their application in extensive identification processes[11]. This contrast emphasizes the critical role of ITS regions in fungal studies.

In this project, a total of 34 fungal colonies were initially pre-isolated as candidates to elicit AAs production in *in vitro* cultures of *Crinum jagus*. Elicitors are molecules that, when recognized by the plant, can trigger a wide array of defense responses, including the production of specialized metabolites, such as alkaloids. Indeed, our analysis through HPLC-MS/MS brought to light the induction of specific alkaloids by fungal extracts. Notably, 11-hydroxyvittatine, a pivotal intermediate in the biosynthetic pathway of AAs, was detected. This compound is a key intermediate in the pathway leading to the synthesis of various bioactive alkaloids such as haemanthamine, pancracine and montanine[12]. The metabolic pathway involves several enzymatic steps that convert tyrosine and phenylalanine into a variety of intermediate compounds, which are then transformed into numerous alkaloids, including those with healthy properties such as galanthamine, lycorine, and narciclasine[13].

The detection of 11-hydroxyvittatine was particularly notable when one specific endophytic strain, characterized as *Fusarium solani*, was used as a treatment. The fungus was capable of specifically inducing the production of this metabolite in *C. jagus* cultures through the plant's defence response due to host-pathogen interaction[14]. The common specialized metabolites of *Fusarium* (such as phenols, flavonoids, alkaloids, saponins, and terpenes) have a wide range of biological properties comprising antioxidant, antidiabetic, antibacterial, antifungal, and cytotoxic activities[15]. The ability of *Fusarium solani* extracts to influence alkaloid biosynthesis in Amaryllidaceae liquid cultures points to its potential utility in enhancing the yield of pharmacologically important compounds.

CONCLUSION

In conclusion, this research successfully characterized the effect of endophytic fungal isolates on the enhanced production of AAs in *in vitro* cultures of *Crinum jagus*. While the modulation of gene expression involved in AA synthesis presents a promising avenue for further investigation, this aspect suggests a broader scope for continued research. During this study, 34 distinct potential fungal strains were isolated. Among these, *Fusarium solani* demonstrated an elicitor activity compared to others, indicating its potential role in stimulating AA production. The PCR identification of fungal species using ITS markers was effectively conducted with the ITS1/ITS4 and ITS5/ITS4 regions, highlighting the micro-organism's specificity. Metabolic profiling using HPLC-MS/MS revealed that this same strain, in association with *C. jagus* induced calli cultures, led to the production of the AA 11-hydroxyvitatine. The identification of additional metabolites linked to this biosynthetic route underscores the need for ongoing research. The outcomes of this study not only shed light on the potential of endophytic fungi in biotechnological and pharmacological applications but also pave the way for further studies aimed at exploring the full spectrum of metabolites producible through such symbiotic relationships and the underlying genetic mechanisms.

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