International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 16, Issue 9, 2024

Original Article

L-DOPA ELICITATION THROUGH CALCIUM ION MEDIATED CHANNEL IN CALLUS CULTURES OF *MUCUNA PRURIENS*

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Received: 08 Jun 2024, Revised and Accepted: 25 Jul 2024

ABSTRACT

Objective: The production of L-Dopa in callus cultures of *Mucuna pruriens* is influenced by substances that regulate calcium channels.

Methods: *M. pruriens* seeds were gathered from the Western Ghats area in Tamil Nadu. L-Dopa was obtained by the Brain (1976) method, *A. niger* cultures were used for fungal elicitor preparation, and the assay of Ca^{2+}ATPase was studied.

Results: The use of fungal triggers resulted in increased L-Dopa levels on the 9th day but reduced levels on the 24th day of culture. When calcium channel modulators like verapamil and chlorpromazine were added, it decreased the growth of calli and L-Dopa production, suggesting the role played by calcium ion channels in L-Dopa synthesis. The calcium ionophore A23187 enhanced calli growth and L-Dopa production, increasing Ca^{2+}ATPase activity. Particularly on the 12th d, Ca^{2+}ATPase was notably active, while the presence of calcium ionophore boosted L-Dopa biosynthesis.

Conclusion: The use of fungal elicitors in *in vitro* cultures of *Mucuna pruriens* is recommended as a potential method to increase the production of secondary metabolites.

Keywords: Calcium ionophore A23187, Calcium channel blockers, Ca^{2+}ATPase

DOI: https://dx.doi.org/10.22159/ijpps.2024v16i9.51727 Journal homepage: https://innovareacademics.in/journals/index.php/ijpps

INTRODUCTION

Plants are essential for food, shelter, and medicine due to their presence of phytochemicals, including primary metabolites like carbohydrates, amino acids, and lipids. Secondary metabolites, which contain biological activity against pathogens, play a crucial part in signaling and plant defence. Researchers have developed strategies to produce secondary metabolites from culture, aiming to enhance plant yield. Among them are elicitor therapy, signal molecules, and non-living stresses [1]. Productivity, however, is comparable for use in an industry. Industrialized processes that have proven successful for commercial purposes are taxol and shikonin. These methods involve the use of different elicitors, signaling molecules, and environmental stresses for treatment [2–8]. Various calcium influx and efflux proteins can identify these Ca^{2+} spikes, helping to regulate the balance of cytosolic Ca^{2+} [9]. Several treatments have proven effective in stimulating the production of different secondary metabolites in plants, both in laboratory settings and in living organisms. The use of elicitors and precursors in *in vitro* cultures enables the differential accumulation and synthesis of natural products. While industrially valuable secondary metabolites have been produced in plant cell cultures, the application of cell culture elicitation enhances the yield and amplifies the production of biologically active metabolites [10-13].

Fungal elicitation has been effective in enhancing secondary metabolite yield and understanding plant responses to biotic stress agents [14]. Calcium is crucial for the growth and development of plants as it helps transport elements and provides mechanical strength to cell walls. It is essential for plant metabolism, phosphorylation, ion transport, phytohormone signaling, cytoskeleton function, and protein folding. Ca^{2+} concentration is controlled in different compartments, and Ca^{2+}-pumping ATPases maintain Ca^{2+} homeostasis [15]. Ca^{2+} signals often depend on Ca^{2+}determinations in external media.

Mucuna pruriens is also known as Velvet Bean (L.). DC. (Fabaceae), also known as velvet bean, is a medically valuable plant in the Western and Eastern Ghats of India [16, 17]. Seeds have a high content of L-DOPA (3,4-dihydroxyphenyl alanine), a precursor of a neurotransmitter that is utilized in treating Parkinson's disease and mental disorders [18]. The herbaceous twining habit and presence of strident trichomes on the pods hinder the commercial production of L-Dopa from intact plants due to the strong itching sensation they cause, causing challenges in large-scale cultivation. Collecting a substantial amount of L-Dopa from natural sources is being considered; however, the commercial utilization of this method is hindered due to its restricted access.

The global pharmaceutical market's demand for *mucuna* is constantly increasing because the entire plant contains L-DOPA. Because conventional methods have limitations on the propagation and conservation of economically significant plants, *in vitro* organ culture and callus development may be viable alternatives [19]. There are fewer of these plants available in their natural habitats due to extensive L-DOPA extraction from wild populations.

The ongoing study aims to examine if a substance from *A. niger* triggers higher L-Dopa levels in *Mucuna* callus cultures. It also seeks to explore how calcium-channel blockers and ionophores impact L-Dopa production through elicitation.

MATERIALS AND METHODS

Collection of plant material

The *M. pruriens* seeds were gathered from the Western Ghats area in Tamil Nadu, India, and were cleaned and preserved. The callus was initiated from *M. pruriens* seedlings on a basal medium called MS, which was enriched with 3% sucrose for all *in vitro* studies. The pH of the medium was adjusted to 5.6±0.2, then agar (0.9% w/v) was added, and the mixture was autoclaved at 121 °C for 15 min. The cultures were kept at 22±1 °C under a 16/8-hour photoperiod using fluorescent tubes with 55–60% humidity. Plant growth regulators were sterilized through a 0.2 mm filter before being added to the culture medium. To introduce calcium-channel blockers on the 18th d of callus culture growth, filter-paper bridges were created using

Whatman No. 1 filter paper discs in 150 ml flasks with 40 ml of liquid media where the callus was inoculated.

Chemicals and reagents

In the experiment, calcium ionophore A23187, verapamil hydrochloride as a calcium channel blocker, and chlorpromazine as a calmodulin antagonist were utilized to study the influence of calcium channels on L-Dopa production in *Mucuna* callus cultures. The calcium ionophore and chlorpromazine were first dissolved in ethanol and filter-sterilized, while verapamil hydrochloride was dissolved in water before being added to the autoclaved medium. The addition of these channel modulators on day 21 aimed to assess their effect on the production of L-Dopa, which reached its peak accumulation on the 24th d of culture.

Analysis of L-dopa content

In line with Brain (1976) [20], L-dopa was obtained by extracting four milliliters (ml) of 0.1N HCl into 1.0 g of *Mucuna* callus tissue. The tissue was then finely mashed using a mortar and pestle and heated in boiling water for five minutes. After cooling, an equal amount of ethanol was added and vigorously mixed for ten minutes before being centrifuged for another ten minutes at 2000 rpm. The resulting supernatant was kept, and the residue was re-extracted with ethanol. The ethanol extract was combined with the supernatant and brought to a known volume. Subsequently, the L-Dopa levels were determined using a UV spectrophotometer.

Elicitor preparation

The study examined the impact of different fungal substances on plant growth and the level of L-Dopa. *A. niger* cultures were chosen for elicitation due to their positive effect on L-Dopa content. An elicitor based on the method by Rajendran *et al.*, (1994) [21] was used. Various concentrations of media filtrate (MF) and mycelial extract (ME) were tested, with 0.5% (w/v) ME and 5% (v/v) MF identified as the most effective concentrations for further experiments.

Assay of Ca^{2+}ATPase

The method described by Vambutas and Racker (1965) [22] was used to measure Ca^{2+}*ATPase* activity. The enzyme was isolated in pH 8.0 Tris buffer and activated for 5 min with 200 mg of trypsin. The addition of a trypsin inhibitor decreased trypsin-induced activation. The assay mixture, with a final volume of 1.0 ml, contained 50 µmol of Tris buffer (pH 8.0), 5 µmol of ATP, and 5 µmol of $CaCl_2$. The reaction was terminated with 0.1 ml of 20% trichloroacetic acid, and Pi was quantified.

In 1988, Baykov and colleagues [23] conducted a study where they quantified the inorganic phosphate released. They defined an activity unit as 10 mmol of Pi generated per milligram of protein within one hour, with the methodology Lowry and colleagues utilized.

Statistical analysis

L-Dopa content, Ca^{2+}*ATPase* activity, and fresh weight of *Mucuna* callus were measured in the samples; the average was then calculated. The method proposed by Freund and Perles was used to calculate the standard error [24].

RESULTS

Predominantly, *A. niger* elicitor ionophore mycelial extract and media filtrate are utilized both alone and in conjunction with the culture, and their impact on the development of *Mucuna callus* biomass has been investigated. The study examined the impact of different fungal substances on plant growth and the level of L-Dopa. *A. niger* cultures were chosen for elicitation due to their positive effect on L-Dopa content. Various concentrations of media filtrate (MF) and mycelial extract (ME) were tested, with 0.5% (w/v) ME and 5% (v/v) MF identified as the most effective concentrations for further experiments. Treatment with ionophore resulted in a slightly higher biomass accumulation (3.81±0.005 g/culture) on the 24th d when compared to control culture (2.17±0.08 g/culture). Treatments with ME (2.96±0.01 g/culture) and MF (2.95±0.017 g/culture) had a lesser biomass growth on the 15th d. In comparison to elicitor treatments alone, the biomass buildup was unaffected by the addition of the ionophore in conjunction with the elicitor. On the other hand, the biomass accumulation was further reduced by the addition of the channel blockers on day 21 (2.44±0.02 g/culture) (fig. 1).

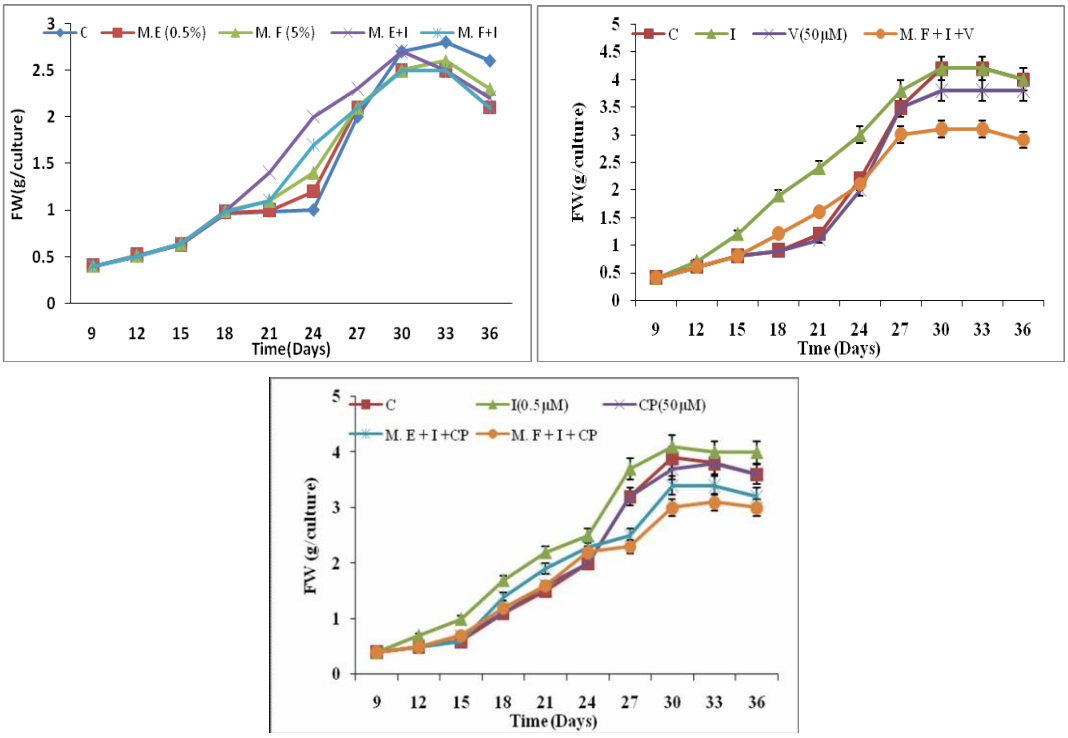

Fig. 1: Influence of elicitor, ionophore and channel blocker on the growth of callus of *Mucuna* C-control, I-Ionophore, CP-Chloropromazine, V-Verapamil, ME-mycelial extract with Ionophore and chloromazine MF-media filtrate with ionophore and Verapamil

Verapamil hydrochloride was administered in various concentrations in the culture medium to the 24th d culture (3.51±0.07 g/culture); the best results in terms of callus biomass accumulation were observed at a 50 µM concentration. In the study, *C. canephora* callus cultures were exposed to calcium, calcium ionophore, calcium chelator (EGTA), calcium channel blocker verapamil hydrochloride, and indoleamines SER and MEL at a concentration of 100 µM, as well as the indole inhibitor p-CPA at 40 µM, to investigate the effects of calcium.

There was an increase in L-Dopa levels on day nine, peaking on day twenty-four. Higher L-Dopa content was seen in treatments with ME (0.76±0.018%) and MF (0.29±0.016%) compared to the control on the 9th d. However, after day nine, the elicitor-treated cultures showed lower L-Dopa levels than the control cultures (fig. 2).

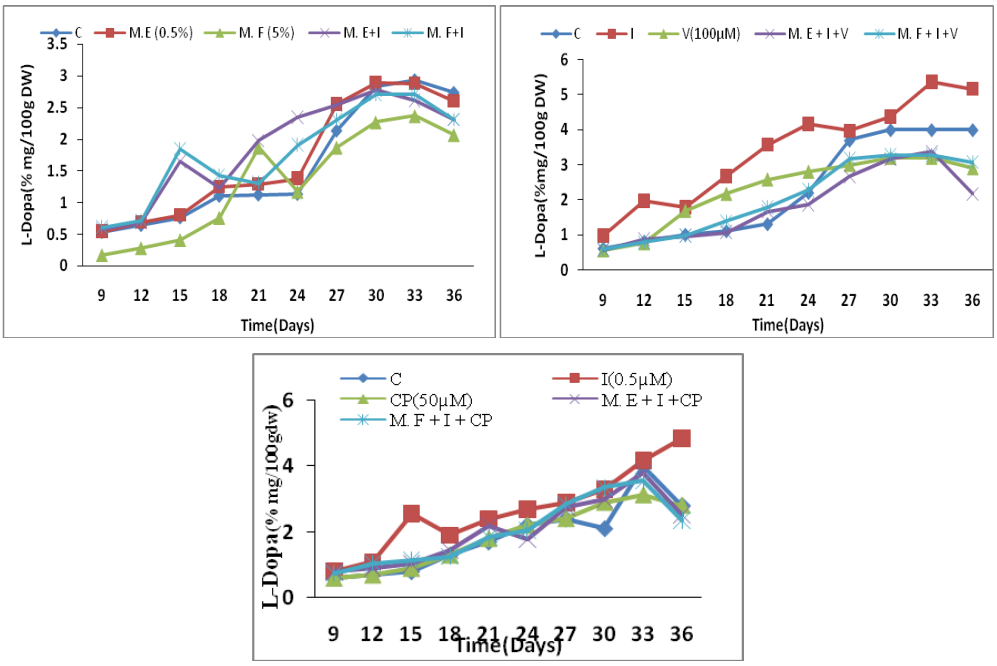

Fig. 2: L-Dopa content in callus culture of *Mucuna* under the influence of elicitor, Calcium ionophore and channel blocker, C-control, I-Ionophore, CP-Chloropromazine, V-Verapamil, ME-mycelial extract with Ionophore and chloromazine MF-media filtrate with ionophore and verapamil

There was a decrease on day nine, followed by a peak on day 36 in L-Dopa levels. The highest L-Dopa content was recorded in ionophore-treated cultures on the 24th d (3.96±0.01%), more than double that of the control cultures (2.14±0.001%). Verapamil and diltiazem, calcium antagonists, inhibited Ca^{2+}-ATPase and $Mg^{2+}Ca^{2+}$-ATPase activities in a dose-dependent manner.

The Ca^{2+}-*ATPase* activity peaked on the 12th day, showing the highest level (fig. 3).

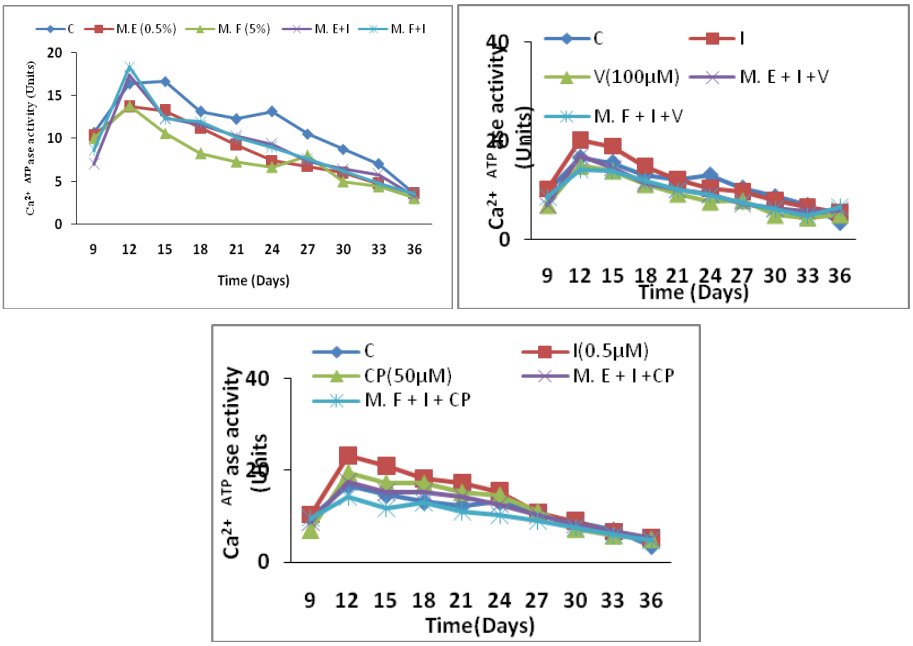

Fig. 3: In callus culture of *Mucuna*, the activity of Ca2+ATPase is infuenced by an elicitor, a calcium ionophore, and a channel blocker, C-Control, FW-Fresh weight, V-Verapamil, I-Ionophore, CP-Chlorpromazine, ME-Mycelial extract, MF-Media filtrate

When cultures were treated with ionophores, their Ca^{2+}ATPase activity increased to 20.26±0.005 units from the control level of 16.65±0.002 units. Elicitor-treated cultures had similar levels of Ca^{2+}ATPase activity compared to the control, but combining ionophore with elicitors resulted in a slight increase. This indicates a possible link between L-Dopa enhancement and calcium channel involvement. Using calcium channel blockers decreased Ca2+ATPase activity. The highest Ca^{2+}*ATPase* activity was observed on the 12th day, preceding peak L-Dopa production on the 24th day.

DISCUSSION

Researchers have previously discussed the significant influence of explant type on establishing *in vitro* cultures. Studies have shown that maize callus cultures treated with 2,4-D exhibited higher levels of phenolic compounds compared to other plant growth regulators. Phenolic acids and glycosides are associated with various health benefits, such as anticancer and antidiabetic properties, among others [25]. Ca^{2+}, through its interaction with the Ca2+-modulating protein and its targets, controls various cellular functions. While Ca2+ is primarily stored in the rough endoplasmic reticulum or vacuole, hypoxic Ca^{2+} signaling predominantly occurs in the mitochondria [26, 27]. Treatments with elicitors resulted in higher levels of L-Dopa accumulation compared to the control on the 9^{th} d, but by the 24^{th} d, L-Dopa levels were lower than the control.

Elicitors are commonly used to boost the production of secondary metabolites in plant cells, with their effectiveness dependent on plant varieties and targeted metabolites [28]. The concentration of elicitors is crucial in this process to prevent adverse effects like cell death due to hypersensitive responses. Plant growth regulators induce oxidative stress in plants, triggering the production of secondary metabolites through reactive oxygen species activation in callus cells [29]. Elicitors are substances that can induce physiological changes in organisms, triggering responses such as phytoalexin accumulation in plants. The effects of elicitors are typically observed within a few hours or days. The flux of Ca2+ induced by elicitors is crucial for the accumulation of secondary plant metabolites. Additionally, the activation of effectors transfers elicitor signals to second messengers, enhancing downstream reactions [30, 31]. Stimulation with specific signals leads to a comprehensive plant defense response involving various processes such as ion fluxes, oxidative bursts, and activation of defense-related genes. This highlights the significance of Ca^{2+} in regulating cellular responses, particularly in defense mechanisms against pathogens. Elicitors, like fungal elicitors, can influence the phosphorylation status of proteins in plant cells, leading to changes in cytosolic Ca2+ concentrations [32, 33]. Activation of Ca2+ channels in higher plants by natural triggers like plant hormones, light, and fungal substances is thought to start signal transmission processes [34–37]. Despite this, the specific elements making up these Ca^{2+}channels in higher plants are not yet identified. This study examines a protein from carrot cells that binds to blockers of membrane Ca^{2+} channels and, through various methods like solubilization and patch clamp analysis, aims to determine if this protein has a role in Ca2+ channel activity.

CONCLUSION

On day twenty-one of the experiment, the introduction of channel blockers impeded both growth and the synthesis of L-Dopa. Further investigation revealed that calcium is essential for ionophoretic treatment with L-Dopa, a finding that was confirmed through the use of channel blockers. It was observed that the presence of calcium had a greater impact on L-Dopa production compared to the stimulation caused by the fungal elicitor.

ACKNOWLEDGEMENT

We want to thank Dr. G. A. Ravishankar from Mysore for his valuable guidance. We sincerely thank Professor Dr. C. Muthamizchelvan, Director E and T, and Dr. M. Vairamani, Dean of SBE at SRM University, for their ongoing support and encouragement.

FUNDING

Nil

ABBREVIATION

C-Control, FW-Fresh weight, V-Verapamil, I-Ionophore, CP-Chlorpromazine, ME-Mycelial extract, MF-Media filtrate.

AUTHORS CONTRIBUTIONS

Uma completed the research work plan and Manuscript writing. Kavitha did the review of the literature collection, Gurumoorthi did the work plan and Manuscript corrections. All authors have read and agree to the manuscript's published version.

CONFLICTS OF INTERESTS

The authors declared no conflicts of interest.

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