

Endophytic Fungi Associated With *Cajanus Cajan*, Linn Produces Novel Bioactive Compound Cajaninstilbene Acid (CSA)

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Abstract: Plant kingdom is a rich source of structural biodiversity and offers a variety of natural products. Plants have been utilized to produce various types of medicines for thousands of years. Fungal endophytes from plants are well known for the production of bioactive metabolites that have wide prospects for use in industry and medicine. Endophytes are ubiquitous in nature found in virtually all plant species studied to date. The Present study aims isolation and screening of novel endophytic fungi producing Cajaninstilbene acid (CSA) from *Cajanus cajan*, Linn. Millsp. CSA has prominent pharmacological activities. Total 50 endophytic mycoflora were grouped into 6 genera on the basis of morphological characteristics. CSA producing fungi were identified as *Fusarium solani*, *Fusarium oxysporum*, *Fusarium proliferatum* and *Fusarium moniliformae* respectively.

Keywords: *Cajanus cajan*, Linn., Endophytic mycoflora, Bioactive compound, Cajaninstilbene acid (CSA), Isolation, Screening etc.

1. Introduction

Cajanus cajan (L.) Millsp. [Pigeon pea] also called as red gram, Congo pea, Gungo pea and no-eye pea respectively. It is a member of the family *Leguminosae*. It is mainly distributed in both tropical and subtropical countries. [Fujita K, Kai Y, Takayanagi M (2004)]. It has also been widely used as a traditional folk medicinal plant. Pigeon pea leaves have been reported to arrest blood flow, relieve pain and kill worms [Tang Y, Wang B, Zhou XJ (1999)]. They can be used to treat hepatitis, measles, dysentery, jaundice, diarrhea, sores, cough, bronchitis, bladder-stones, diabetes and many other illnesses [Grover JK, Yadav S, Vats VJ (2002)]. The extracts of pigeon pea leaves showed significant antioxidant, antibacterial, antiherpetic activities, hypoglycemic activity and potential in the treatment of postmenopausal osteoporosis. They are rich in flavonoids and stilbenes, which are known for their beneficial influence on human health [Wu N, Fu K, Fu YJ, Zu YG, Chang FR, et al. (2009)].

Cajaninstilbene acid (CSA), 3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid which is the main active constituent in pigeon pea leaves. [Cooksey CJ, Dahiya JS, Garratt PJ, Strange RN (1982)]. The chemical structure of CSA is shown as below:

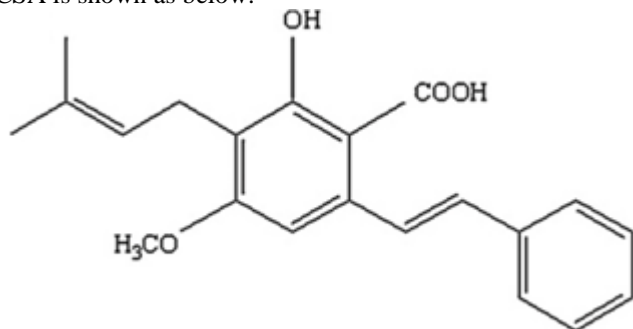


Figure: Chemical structure of Cajaninstilbene acid.

The general biosynthetic pathway of CSA in pigeon pea has not been studied in detail but the biosynthetic pathway of

plant stilbenes has been well studied [Chong JL, Poutaraud A, Huguency P (2009)]. Certain stimuli such as UV light, NO, H₂O₂, jasmonic acid and fungal infections induce the accumulation of stilbenes.

Endophytes in pigeon pea may contribute to CSA production. Endophytes infect living plants tissues without causing apparent disease symptoms in the host plant [Petrini O (1991)]. They are ubiquitous in nature with a mutualistic relationship with their host. They can improve plant adaptation to stress, increase biomass, or promote resistance to pathogen damage [Redman RS, Sheehan KB, Scout RG, Rodríguez RJ, Henson JM (2002)]. Some endophytic fungi are thought to prevent their host from attacks by fungi, pests and mammals by producing secondary metabolites. These endophytes are rich source of bioactive products [Li WK, Hu ZB (2005)]. They possess a broad synthetic capability and synthesize abundant secondary metabolites with potential economic significance. Some produce biologically active constituents that are similar to or the same as those produced by their host. The production of secondary metabolites with pharmaceutical relevance is relatively common in endophytes. Therefore, they have great potential value as a source of drugs [Strobel GA (2002)]. Therefore, investigating endophytes represents a promising strategy to identify novel nature compounds.

2. Materials and Methods

1) Isolation of fungal endophytes

Fungal endophytes were isolated from healthy roots, stems and leaves of pigeon pea [*Cajanus cajan* (L.) Millsp.]. Fresh tissue of pigeon pea were collected from local area which were deposited in the Laboratory. The plant materials were used to isolation of endophyte within 3 hours of harvest. The roots, stems and leaves of pigeon pea were washed with running tap water. They are sterilized with 75% ethanol for 60, 60 and 30 seconds then rinsed in sterile water and 5% sodium hypochlorite for 7, 5, and 3 minutes, rinsed in sterile water three times respectively. Finally these are cut into 1 cm long segments. Both edges of sterilized segments were

cut off, and the segments were incubated at $28 \pm 1^\circ\text{C}$ on potato dextrose agar (PDA) medium for 14–21 days until the colonies originating from the newly formed surface of the segments appeared. Penicillin ($20 \mu\text{g}/\text{mL}$) was added to culture medium to suppress bacterial growth. After that the purified fungal endophytes were numbered and transferred to fresh PDA slants separately, and these slants were incubated for 14–21 days and then kept at 4°C .

Five isolates of endophytes were identified according to their cultural and morphological characteristics as described by Nelson *et al.* (1983). The diameter and colour of colonies was measured and determined. The microscopic features such as size and shape of hyphae and conidia were examined and measured under a light microscope after maturation.

2) Preparation the endophytes samples and standard solutions

15 conical flasks of 500 mL capacity were used. Each endophyte isolate cultured in three parallel samples containing 200 mL Potato Dextrose Broth (PDB) medium. Fungal endophytes were grown in PDB medium and cultured for 5, 10, 15, 20 and 25 days at 28°C with shaking (180 rev/min) on a rotary shaker. The fungal culture liquid was filtered and extracted by ethyl acetate three times. Mycelium was rinsed in sterile water three times, oven for 48 h at 40°C , then ultrasonic processing 30 min in 80% ethanol and extracted by ethyl acetate three times. The evaporated ethyl acetate extracts were dissolved in chromatogram methanol and filtered with a $0.22 \mu\text{m}$ member filter, before being used for determination of CSA production.

A stock solution of reference sample CSA was prepared in methanol at a final concentration of $100 \mu\text{g}/\text{mL}$. The final concentrations of the standard curve samples were 10, 20, 50, 100, 200 500, 1000, 3000 and 6000 ng/mL. The standard stock solution and working standard solutions were all stored at 4°C . Standard calibration samples were stored at -20°C until analysis.

3) Quantitative determination of CSA by LC-MS/MS:

The determination of CSA based on LC-MS/MS methods described by Hua *et al.* (2010). Chromatographic separations were achieved on a HIQ Sil C18 column maintained at 20°C . The column effluent was monitored by mass spectrometer equipped with an electrospray ionization (ESI) source. The mobile phase consisted of water and methanol (9:91, v/v) containing 0.1% formic acid. The flow rate was 1.0 mL/min, and the sample injection volume was 10 μL .

The ion spray voltage was set at -4500 V . Compound parameters viz., declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CEX) were set at -60 , -25 , -10 , -5 V respectively for analysis of CSA. The mass spectrometer was operated in ESI negative ion mode, and the detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of m/z 337.1 precursor ion $[\text{M}-\text{H}]^-$ to the m/z 293.0 product ion for CSA. The compound of second peak were detected by LC-MS/MS in the same conditions as used to monitor the transition of m/z 337.1 precursor ion $[\text{M}-\text{H}]^-$ to the m/z 293.0 product ion.

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