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ISOLATION AND SCREENING OF ENDOPHYTIC FUNGI FOR THE PRODUCTION OF L-ASPARAGINASE ENZYME

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ABSTRACT
Life depends on the existence of powerful and specific biocatalyst. Virtually every biochemical reaction is catalyzed by an enzyme. Hence L-asparaginase is one of them, which catalyzes the conversion of L-asparagine into L-aspartic acid and ammonia and plays a vital role in the treatment of different forms of cancer. It is widely distributed enzyme among bacteria, fungi and plants. L-asparaginase enzyme from bacterial origin can cause hypersensitivity in the long term use, leading to allergic reaction and anaphylaxis. Hence, the search of L-asparaginase producing organisms from other sources can be useful for its production with less adverse effects. In recent studies, endophytic fungi present in different parts of plants have been recognised as an alternate source for L-asparaginase enzyme production. In the present study, endophytic fungi from the six medicinally important plants were isolated and screened for the production of L-asparaginase activity. Total 40 endophytic fungal isolates were isolated, out of which 14 showed positive L-asparaginase activity.

KEYWORDS: Endophytic fungi, L-Asparaginase, Acute lymphoblastic leukaemia.

INTRODUCTION
L-asparaginase enzyme is an antineoplastic agent, commonly used in the treatment of acute lymphoblastic leukaemia.[1] L-asparaginase enzyme usually catalyzes the conversion of L-asparagine into L-aspartic acid and ammonia. This anticancerous enzyme depletes tumor cells's L-asparagine and eventually cells die because of their inability to synthesise L-asparagine. It plays major role in the reduction of acrylamide formation in fried food stuffs.[2] It has been reported that L-asparaginase enzyme is present in many bacteria, fungi, molds, yeast, plants and other vertebrates. L-asparaginase derived from microrganisms are important for clinical uses.[3] Although L-asparaginase from *Escherichia coli* and *Erwinia chrysanthemi* have emerged as the most potent chemotherapeutic agents with minor side effects like thromboembolysis, hyperglycemia, weight loss, sweating, immune-suppression, acute pancreatitis and loss of consciousness.[4,5,6-8] Hence, endophytic fungi are an alternative source. Endophytes are usually chemical synthesizer in plants, which produce certain bioactive substances. Endophytic fungi live inside the living tissues of plants for a very short or prolonged time period, without visualizing any symptom on their respective host plants. In recent years, endophytic bioactive compounds were integrated in novel drug discoveries in various types of biological activities including antibiotics production and as anti inflammatory agents.[9] Several reports suggested that medicinal plants provide shelter or harbour the endophytes through different infectious agents and survival in harsh environmental conditions.[10] The discovery of taxol from endophytic *Taxomyces andrenae* was a great achievement.[11] *Taxomyces andrenae* was formerly extracted from pacific yew trees, without mass destruction of the plant. Hence, with this discovery many scientists hypothesize that endophytes from anticancerous plants have potential to synthesize various bio-active compounds which possess anticancerous activities: such as cajanol[12], maytanseni[13], campthothecin.[14] In order to search the potential and efficient endophytes as source of L-asparaginase, endophytes from various medicinal plants need to be screened. It is therefore, endophytes isolated from medicinal plants can also proved to be good source for L-asparaginase enzyme. In the present study, several endophytes have been isolated from various medicinally important plants and have been screened for the production of L-asparaginase enzyme.

MATERIALS AND METHODS
Isolation of endophytic fungi from the selected medicinal plant parts
The initial step, in dealing with endophytic fungi for the production of L-asparaginase enzyme is the selection of proper and promising medicinal plants for study. The

plant material was collected from the botanical garden of D.D.U. Gorakhpur university. The samples were brought to laboratory within 24 hrs. The collected medicinal plants were briefly washed under running tap water to remove adhered debris. The respective plant parts (such as roots, stem and leaves) of ***Solanum nigrum, Solanum lycopersicon, Capsicum annum, Murraya koengi, Flacourtia jangomas*** and ***Mangifera indica*** were cut into 2-3cm long pieces, and were surface sterilized.[15] These plant parts were rinsed in distilled water followed by surface disinfectation by soaking in 70% ethanol for 30 sec and 0.1% mercuric chloride solution for 2 min. The disinfected plants parts then again rinsed in distilled water and drained. Further, they were cut or scratch longitudinally with a sterile scalpel, with the exposed inner surface facing downwards on plates of Czapex dox agar (for the growth of endophytic fungi). As control, unscratched plant parts (surface disinfected) were placed on the Czapex dox agar media. All plates were incubated at 28ºC for 3-5 days depending upon the growth of endophytic fungi. All the chemicals used in the study were of analytical grade.

Screening of endophytic fungi for the production of L-asparaginase

The isolated fungal colonies were further screened for L-asparaginase production by rapid plate assay technique as the method described by **Gulati *et al.*, (1997).** The modified czapex dox agar medium (glucose 0.4g, L-asparagine 2.0g, potassium di hydrogen phosphate 0.3g, magnesium sulphate 0.1g, ferrous sulphate 0.002g, potassium chloride 1.0g, agar 4.0g for 200 ml water with phenol red 0.009% was used. The media was poured into plates and autoclaved. The plates were inoculated with respective endophytic fungal isolates and incubated at 28ºC for 2-3 days at pH 6.2. Colonies with pink zone were considered as positive L-asparaginase producers. The pink color indicates towards the amido hydrolytic activity of L-asparaginase which involves the conversion of L-asparagine into L-aspartic acid and ammonia. Due to accumulation of ammonia there is increase in pH, which turns phenol red from yellow to pink. It is considered as preliminary procedure for screening of L-asparaginase activity.

RESULTS AND DISCUSSION

In order to isolate endophytic fungi, six medicinal plants were selected, such as ***Solanum nigrum, Solanum lycopersicon, Capsicum annum, Murraya koengii, Flacourtia jangomas*** and ***Mangifera indica***. A total of 40 endophytic fungi were isolated from leaves, roots, fruits and stems of the above mentioned plants. Out of which 15 fungal isolates possess L-asparaginase activity. The color change from yellow to pink indicates positive L-asparaginase producers. While yellow medium indicates negative L-asparaginase producers.

Table 1: Screening of endophytes for L-asparaginase activity isolated from various parts of medicinal plants.

Plants	Parts	Fungal isolate	L-asparaginase activity
Solanum nigrum	Roots	Snr1, Snr2	Positive
	Stem	Sns1, Sns2	Negative
	Fruit	Snf1	Negative
		Snf2, Snf3	Positive
	Leaf	Snl1, Snl2	Negative
Solanum lycopersicon	Roots	Slr1	Positive
		Slr2, Slr3	Negative
	Leaf	Sll1, Sll2	Negative
	Stem	Sls1,Sls2	Negative
Capsicum annum	Fruit	Caf1, Caf2	Positive
		Caf3	Negative
	Stem	Cas1, Cas4	Positive
		Cas2, Cas3	Negative
	Leaf	Cal1, Cal3	Positive
		Cal2	Negative
Murraya koengii	Leaf	Mkl1	Positive
		Mkl2	Negative
	Stem	Mks1, Mks2	Negative
Flacourtia jangomas	Leaf	Fjl1,Fjl2	Positive
		Fjl3	Negative
	Stem	Fjs1	Negative
Mangifera indica	Leaf	Mil1,Mil2	Negative
	Stem	Mis1, Mis2	Negative
	Roots	Mir1, Mir2	Negative

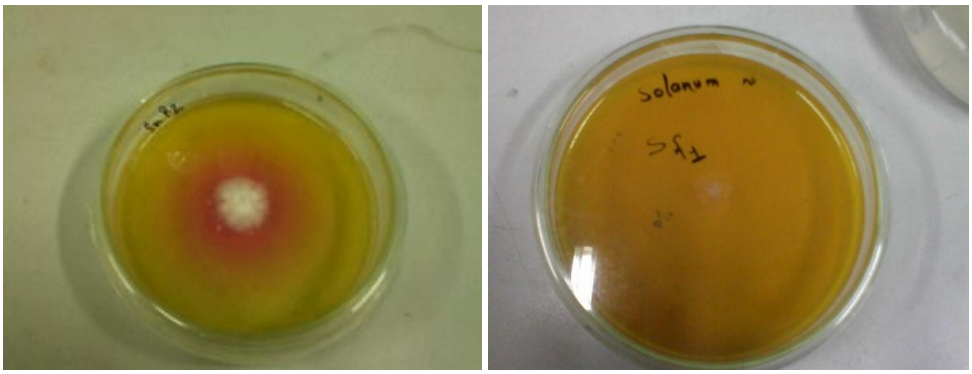
Isolation of endophytes and screening for L-asparaginase activity

(i) Solanum nigrum

Roots and fruits of *Solanum nigrum* were used for the growth of endophytic fungi. Total nine endophytes were isolated from S*olanum nigrum*. Two fungal endophytic isolates from its root (Snr1 &Snr2), two from stem (Sns1 &Sns2), three from fruits (Snf1, Snf2 & Snf3) and two from leaves (Snl1 & Snl2) were isolated. No endophytic fungal growth was observed in control. As shown in table (1) endophytic isolates from roots and fruits showed positive results for L-asparaginase activity, while isolates from stem and leaves did not show any L-asparaginase activity. L-asparaginase producing endophytes were detected by the formation of pink zone on agar plate as a result of hydrolysis of asparagine into asparatic acid and ammonia that converts phenol red dye indicator form yellow to pink.

(a) Control (b) Root (c) Fruit

Figure 1: Endophytic fungi from root (b), fruit (c) of *Solanum nigrum* and control (a).

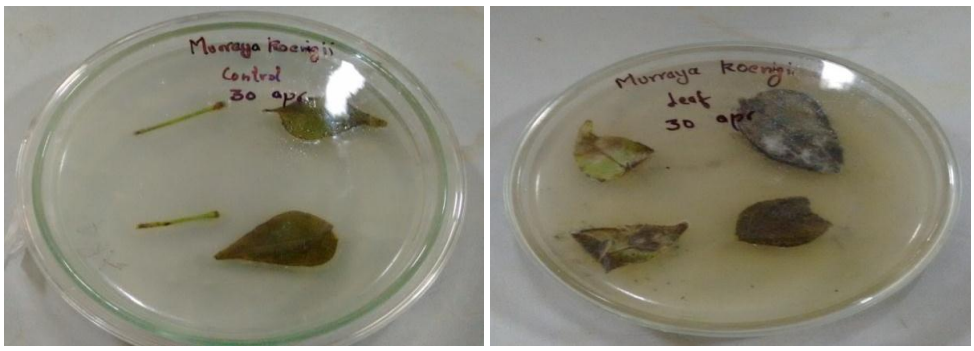


(a) (b)

Figure 2: Isolated endophytic fungi from *Solanum nigrum* showing L-asparinase activity (a) and showing no L-asparaginase activity (b)

ii) Murraya koenigii

From *Murraya koenigii*, total four endophytic fungal isolates, two from leaf (Mkl1 & Mkl2) and two from stem (Mks1& Mks2) were isolated. Only one isolate (Mkl1) isolated from leaf showed positive result for L-asparaginase enzyme while rest others did not show any L-asparaginase activity, as shown in fig.3.

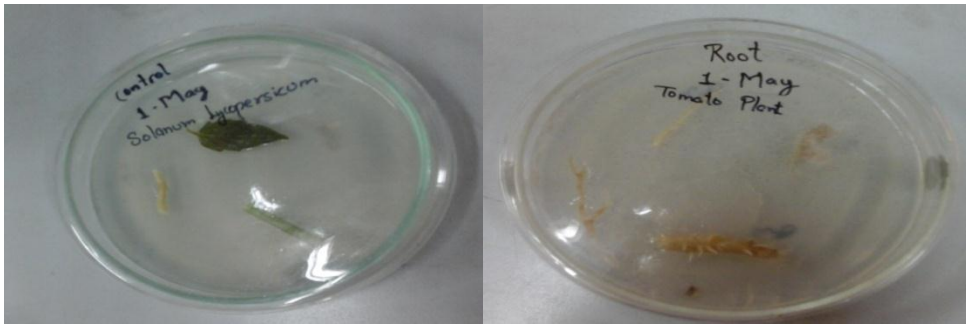


(a) Control (b) Leaf

Figure 3: Endophytic fungal isolates from leaf (b) and control (a).

(iii) Solanum lycopersicum

From *Solanum lycopersicum*, total seven endophytic isolates, three from root (Slr1, Slr2 & Slr3), two from leaf (Sll1 & Sll2) and two from stem (Sls1 & Sls2) were isolated. Out of all only one fungal isolate from root (Slr1) showed L-asparaginase activity while others did not show any L-asparaginase activity. Endophytic fungi isolated from root have been depicted in figure4.



(a) Control **(b) Root**

Figure 4. Fungal endophytes isolated from root (b) of S*olanum lycopersicum*, control (a).

(iv) Capsicum annum

Total ten endophytic fungi, three from fruit (Caf1, Caf2 & Caf3), four from stem (Cas1, Cas2, Cas3 & Cas4) and three from leaf (Cal1, Cal2 & Cal3) of Capsicum annum were isolated. Out of all isolated endophytes, total 6 isolates gave positive result for L-asparaginase activity. Two isolates from fruit (Caf1 & Caf2), two from stem (Cas1 & Cas4) and two from leaf (Cal1 & Cal3) showed L-asparaginase activity while rest others did not show any L-asparaginase activity.

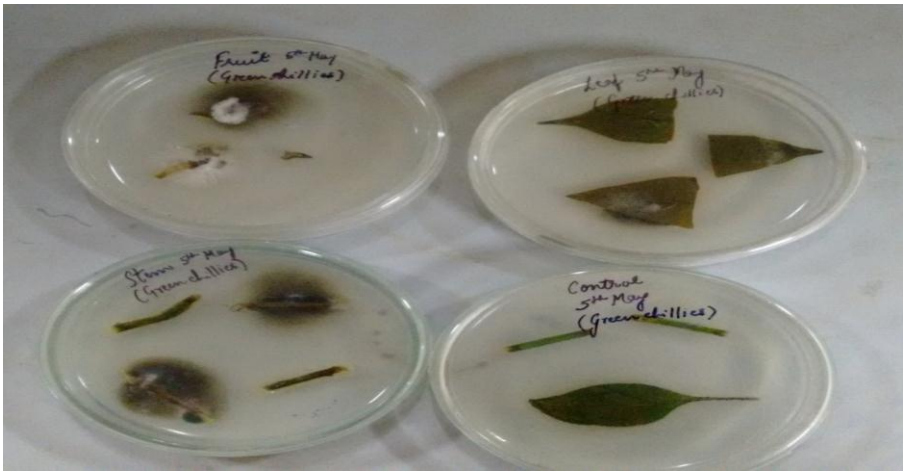


Figure 5: Fungal endophytes isolated from fruit, stem and leaf of *Capsicum annum*. Control is also shown in figure.

v) Mangifera indica

Six endophytic fungi were isolated from various parts of *Mangifera indica*. Two endophytes from leaf (Mil1 & Mil2), two from stem (Mis1 & Mis2) and two from root (Mir1 & Mir2) were isolated. But none of them showed L-asparaginase activity.

(a) **(b)**

Figure 6: Endophytic fungi from leaf (a) and stem (b) of *Mangifera indica.*

vi) *Flacourtia jangomas*

Three endophytic fungi from leaf (Fjl1, Fjl2 & Fjl3) and one from stem (Fjs1) of *Flacourtia jangomas* were isolated. Only two endophytes isolated from leaf (Fjl1& Fjl2) were found to have L-asparaginase activity, while one endophyte from leaf (Fjl3) and the only endophyte from stem (Fjs1) did not show any L-asparaginase activity.

(a) (b) (c)

Figure 7: Endophytic fungi from leaf (b) and stem (c) of *Flacourtia jangomas*, control (a).

CONCLUSIONS

From the above results, it may be concluded that endophytic fungi isolated from the above medicinal plants can be alternate good source for L-asparaginase enzyme production. These plants from which fungal endophytes have been isolated, are of great ethanobotanical history and also possess certain medicinal values. Although this preliminary study has shown many endophytes having L-asparaginase activity, further detail study is required to establish them as good source of better L-asparaginase enzyme. The enzyme being produced from the above endophytes needs to be characterized in order to analyse their efficacy. The isolated fungal endophytes may also be studied for their use as natural resource for production of other bioactive molecules.

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