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Isolation of endophytic fungi from *Cannabis sativa* and study their antifungal potential

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A systemic study of fungal endophytes associated with different plant parts of *Cannabis sativa* and their antifungal activity was investigated in the present study. A total of 281 plant segments, including 91 leaves, 93 stem and 97 petioles samples, were screened for the isolation of endophytic fungi. Totally, 212 (77.65%) segments were found colonised by different fungi. Highest colonisation frequency were observed in stem parts (84.94%), then leaves (82.41%) and lowest 59.79% in petiole. Total eight fungal genera belonging to 12 species were isolated. *Aspergillus* is recorded as the most frequently occurring genera with three species *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus nidulans* followed by *Penicillium* with two species *Penicillium chrysogenum* and *Penicillium citrinum*, while *Phoma*, *Rhizopus*, *Colletotrichum*, *Cladosporium* and *Curvularia* with single species. The antifungal potential of *A. niger* and *A. flavus* – two most frequently isolated endophytic fungi – was evaluated against two common plant pathogen, *Colletotrichum gloeosporioides* and *Curvularia lunata*. Different plant and fungal extracts individually and in combinations showed variations in antifungal activity against both the pathogens. The primary results obtained on antifungal activity of endophytes show their possible role in plant defence mechanism but it is a preliminary approach and more extensive research is still required.

Keywords: *Cannabis sativa*; endophytic fungi; poisoned food technique; antimicrobial activity

Introduction

Cannabis, a unique and a well-established plant genus, holds an important position in Ayurveda. The genus is well known for long time for its medicinal and ethnobotanical uses. There are three putative varieties namely, *Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis* in the genus having distribution in Central and South Asia. Earlier the *Cannabis* was placed in family Urticaceae or Moraceae, but included in Cannabaceae later (Schultes et al. 2001; Sohly and Mahmoud 2007).

C. sativa, known as Bhang in Hindi, is a member of the family Cannabaceae. The plant is annual, dioecious with palmate leaves and obtains a height in the range from 1 to 5 m. Interestingly, the life cycle of the male plant is completed soon after anthesis, but the female survives until full seed ripeness (Pate 1999). This plant occupies an exclusive place in Hindu religion. It is believed to be the favourite drink of Lord Shiva

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and used during his worship. All plant parts including leaves, stems, bark, flowers and seeds are valuable resources for folk medicine. The ideal distribution of the plant is found in temperate and tropical regions of the world. In India it is cultivated all over the country and commonly occurs in waste grounds, along road sides, along the canals used for irrigation of agricultural lands (Khare 2007).

Like other medicinal plants, *C. sativa* have many medicinally and industrially useful chemical compounds. The plant seems to be important in a virtual factory for the production of secondary metabolic compounds. A number of alkanes, nitrogenous compounds, flavonoids and terpenes have been identified from the plant. Cannabinoids are the major chemical compounds which comprise the active drug ingredients and are apparently unique to this genus (Adams and Jones 1973; Turner et al. 1973a, 1973b; Gellert et al. 1974; Hanus 1975a, 1975b; Hanus 1976a, 1976b). Chemical compounds are thought to be involved in plant defence mechanism, tolerance to environmental conditions and their production is believed to be linked with association of different endophytic microbes. Numbers of reports are available to illustrate the role of fungal endophytes in different plant mechanisms (Liu et al. 2001a; Arnold et al. 2003; Lewis 2004; Vu et al. 2006; Giménez et al. 2007; Kiraly et al. 2007; Dai et al. 2008; Ganley et al. 2008; Hung et al. 2008; Kuldau and Bacon 2008; Rodriguez et al. 2009; Gao et al. 2010; Ahmed et al. 2012). A hypothesis was framed that endophytes play an important role in plant defence mechanism, and the present study was carried out to check it with the objectives, to isolate endophytic fungi associated with *C. sativa* and study their antifungal potential.

Materials and methods

Collection of plant material

Healthy plants of *C. sativa* along with their leaves were collected from the local regions of district Mandi, Himachal Pradesh. The collected plant was identified, and a herbarium was submitted to the Department of Botany, Abhilashi Institute of Life Sciences, Mandi, Himachal Pradesh as record. Collected plants were carefully brought to the laboratory and processed within a few hours after sampling. Fresh plant material was used to reduce the chances of contamination.

Isolation of endophytic fungi

Healthy and mature plants parts, leaves, stems and petioles were carefully selected, washed gently in running tap water to remove dust and debris, and processed further under aseptic conditions. Leaves were cut into 3–4 mm diameter discs with and without midrib with the help of a sterilised cork borer. Stem and petioles were also cut into small segments (0.5–1 cm in length). All the plant parts were surface-sterilised with the procedure given by Hallmann et al. (2007). Each set of plant segments was treated firstly with sterilised distilled water for 1 min followed by immersion in sodium hypochlorite for 40 s. Later, the segments were again rinsed for 3–4 times with sterile distilled water. The plant pieces were blotted on sterile blotting paper in order to remove excessive moisture. Surface sterilised segments (5–6) of selected plant parts were inoculated in Potato dextrose agar (PDA), and sealed with parafilm wax and incubated at 27 °C ± 2 °C for 1–2 weeks in BOD incubator. Each sample was maintained in triplicates. After incubation, morphological and cultural characteristics of each growing fungi were observed. Hyphal tips from the growing colonies were transferred to fresh

PDA containing Petri plates with the addition of antibiotics to obtain pure cultures for identification.

The fungi were identified on the basis of their morphological and cultural characteristics (Gilman 2001). Fungal samples were placed on the slides, stained with lactophenol cotton blue and observed under compound microscope. Microscopic characteristics were noted and identification was done.

Colonisation frequency of each fungal endophyte was calculated as (Kumar and Hyde 2004):

$$\text{Colonisation frequency} = \text{number of segments colonised by fungi/total number of segments observed} \times 100.$$

Study of antifungal activity of fungal endophytes

To investigate the antifungal activity of fungal endophytes of *C. sativa*, two most frequently isolated fungi, namely, *Aspergillus niger* and *Aspergillus flavus* were selected. Different sets of combinations of plant and endophyte extracts were used against two common plant pathogen, *Colletotrichum gloeosporioides* and *Curvularia lunata*, using poison food technique.

Preparation of plant extracts

Fifteen grams thoroughly dried powder of leaf and stem of *C. sativa* macerated with 200 ml ethanol, was shaken well and left it for about four days with occasional shaking. Extract was then filtered through a muslin cloth for coarse residue and finally filtered through Whatmann No.1 filter paper. The resulting material centrifuged at full speed and supernatant was stored at 4 °C for further use (Kumar et al. 2011).

Preparation of extracts of entophytic fungi

Slightly modified methods as described by Tejesvi et al. (2007) and Vaz et al. (2009) were used to obtain the secondary metabolites of fungal endophytes. Pure culture of *A. niger* and *A. flavus* was maintained and inoculated in 250 ml potato dextrose broth in a conical flask. The fungal material was incubated at 27 °C for more than 15 days in BOD incubator. The broth with fungal culture was filtered through Whatmann No.1 filter paper. The resulting extract was centrifuged 10,000–12,000 rpm for 30 min. The pellet formed was discarded, and the broth was collected and stored at 4 °C for further use.

The fungal and plant extracts were mixed in different concentrations (Table 1) with PDA in sterilised Petri plates. Disc (5 mm) of *C. gloeosporioides* and *C. lunata* were

Table 1. Endophyte and plant extract combinations used.

S. no.	Fungal and plant extract combinations
1	*A. niger* (1.5 ml) + *A. flavus* (1.5 ml) + PDA (17 ml) = 20 ml
2	*A. flavus* (3 ml) + PDA (17 ml) = 20 ml
3	*A. niger* (3 ml) + PDA (17 ml) = 20 ml
4	*A. niger* (1.5 ml) + *A. flavus* (1.5 ml) + leaf (1.5 ml) + stem (1.5 ml) + PDA (17 ml) = 20 ml

inoculated in the Petri plates containing media and extracts. The plate containing media only was treated as control. The plates were incubated at 27 °C. The fungal growth diameter in each concentration was measured when the control attained its maximum growth. The percentage inhibition for each set combination was calculated by given formula (Alam et al., 2011).

$$\% \text{ age inhibition} = C - T/C \times 100$$

where

C =diameter of control

T =diameter of treatment

Statistical analysis

The data obtained were analysed statistically and the results are presented as mean ± standard deviation.

Results

Endophytic fungi isolated from C. sativa

A systemic study of fungal endophytes associated with different plant parts of *C. sativa* and their antifungal activity was investigated in the present study. A total of 281 plant segments, including 91 leaves, 93 stem, and 97 petioles samples, were screened for the isolation of endophytic fungi. Total 212 (75.44%) segments were found colonised by different fungi. Highest colonisation frequency was observed in leaves (84.94%) than in stem parts (82.41%) and lowest 59.79% in petiole (Table 2). The isolated endophytic fungi have been identified on the bases of their morphological, cultural and microscopic characteristics.

Analysis of fungi-colonised inoculated plant segments revealed that about eight fungal genera belonging to 12 species were isolated. *Aspergillus* is recorded as the most frequently occurring genus with three species followed by *Penicillium* with two while *Phoma*, *Rhizopus*, *Colletotrichum*, *Cladosporium* and *Curvularia* with single species. Three *Aspergillus* species namely *A. niger*, *A. flavus* and *Aspergillus nidulans*; two *Penicillium* species – *Penicillium chrysogenum*, *Penicillium citrinum*; and *Rhizopus stolonifer, C. lunata, Phoma* sp., *Colletotrichum* sp., *Cladosporium* sp. were isolated as fungal isolates isolated from leaf, stem and petioles segments of *C. sativa*. Fungi unable to produce fruiting bodies after one month of inoculation were treated as mycelia sterlia.

Highest 10 fungal species were found associated with stem parts, followed by nine with leaves and minimum seven with petiole. The *Aspergillus* genus with three species viz. *A. niger, A. flavus and A. nidulans* was found most frequently in stem. *A. nidulans* was not isolated from leaf and petiole. *P. chrysogenum, P. citrinum, C. lunata* was

Table 2. Colonisation frequency of different plant segments.

Plant parts (inoculated)	Parts colonised	Colonisation frequency (%)
Leaves (91)	75	82.41
Stem (93)	79	84.94
Petioles (97)	58	59.79

recorded only from stem. *P. citrinum* was isolated from stem and petiole while *Colletotrichum* from leaf and stem only. Similarly, *R. stolonifer* was isolated only from stem parts. The endophytes namely *Alternaria alternata* and *Cladosporium* sp. were found associated with all the plant parts. Detailed frequency of colonisation of endophytic fungi isolated from *C. sativa* is summarised in (Table 3).

By comparing the fungal percentage frequency of colonisation, *A. flavus* was recorded with highest (6.52 ± 2.42%), followed by *P. citrinum* (3.91%), *Cladosporium* sp. (2.84 ± 1.07%) and *A. alternata* (2.25 ± 2.08%). The percentage frequencies of remaining fungal isolates were found in the range of 1.42–0.35%.

Analysis of antifungal activity of plant and fungal extracts

Ethanol extracts of selected endophytic fungi viz. *A. flavus* and *A. niger* and plant segments were analysed against the growth of two common plant pathogens namely, *C. gloeosporioides* and *C. lunata*. Different concentrations (1, 2 and 3%) were tested and 3% combination of plant and fungal extracts found most effective against the mycelial growth of both the pathogens.

The ethanolic extract of *A. flavus* (39.76 ± 2.94) was more effective than *A. niger* (35.69 ± 7.30) alone, while the effect was 32.91 ± 2.26 when both were tested in

Table 3. Frequency of colonisation of endophytic fungi isolated from *C. sativa*.

S. no.	Fungi isolated	(%) Frequency of colonisation			Mean ± SD
		Leaf	Stem	Petioles	
1	*A. alternata*	4.62	1.42	0.71	2.25 ± 2.08
2	*A. niger*	1.42	1.42	2.49	1.77 ± 0.67
3	*A. flavus*	4.62	5.69	9.25	6.52 ± 2.42
4	*A. nidulans*	–	0.35	–	0.35 ± 0.00
5	*Colletotrichum* sp.	2.49	1.42	–	1.42 ± 0.00
6	*Cladosporium* sp.	2.84	3.91	1.77	2.84 ± 1.07
7	*C. lunata*	1.42	–	–	1.42 ± 0.00
8	*R. stolonifer*	–	1.42	–	1.42 ± 0.00
9	*P. chrysogenum*	3.20	1.77	5.33	3.43 ± 1.79
10	*P. citrinum*	–	7.47	0.35	3.91 ± 0.00
11	*Phoma* sp.	0.35	–	–	0.35 ± 0.00
12	Mycelia sterilia	4.62	3.20	0.71	2.84 ± 1.97

Table 4. Growth inhibition (%) of *C. gloeosporioides* at different concentration leaf and stem extracts.

S. no.	Biopesticides (3%)	3rd day	4th day	5th day	6th day	7th day	8th day	Mean ± SD
1	A.F + A.N + S	33.3	35.7	24.5	13.3	18.75	17.64	23.86 ± 9.01
2	A.F + A.N + L	41.6	35.7	27.85	20	18.75	17.64	26.92 ± 9.95
3	A.F + A.N + L + S	33.3	14.2	13.75	13.3	12.5	11.76	16.46 ± 8.29
4	A.F	41.6	42.8	41.4	40	37.5	35.29	39.76 ± 2.94
5	A.N	50	35.7	34.5	33.3	31.25	29.41	35.69 ± 7.30
6	A.F + A.N	33.3	35.7	34.5	33.3	31.25	29.41	32.91 ± 2.26

Note: A.F = *Aspergillus flavus*, A.N = *Aspergillus niger*, L = Leaf, S = Stem.

Table 5. Growth inhibition (%) of *C. lunata* at different concentration of leaf and stem extracts.

S. no.	Biopesticides (3%)	3rd day	4th day	5th day	6th day	7th day	8th day	Mean ± SD
1	A.N + A.F + S	53.84	35.71	37.85	40	37.5	35.29	40.03 ± 6.97
2	A.N + A.F + L	38.46	35.71	27.85	20	18.75	17.64	26.40 ± 9.06
3	A.N + A.F + L + S	61.53	21.42	20.71	20	25	23.52	28.69 ± 16.91
4	A.F	38.46	35.71	34.52	33.33	31.25	29.41	33.78 ± 3.22
5	A.N	69.23	64.28	62.14	60	62.5	58.52	58.82 ± 3.74
6	A.N + A.F	38.46	35.71	31.18	26.66	25	23.52	30.08 ± 10.08

Note: A.F = *Aspergillus flavus*, A.N = *Aspergillus niger*, L = Leaf, S = Stem.

combination against the growth of *C. gloeosporioides*. The combined extracts of both endophytic fungi were more effective along with leaf extract (26.92 ± 9.95) as compare to while in combination with stem (23.86 ± 9.01). When extracts of both endophytic fungi and plant parts tested in combination, surprisingly decreased (16.46 ± 8.29) effect was found (Table 4).

When we analyse the different combination of plant and fungal extracts against the growth of *C. lunata*, it was observed that ethanolic extracts of *A. niger* represent highest (58.82 ± 3.74) growth inhibition while it was about half (33.78 ± 3.22) in case of *A. flavus*. The combination of extract of both endophytic fungi was found less effective (30.08 ± 10.08) as compare to when used in combination with stem (40.03 ± 6.97). The combination with leaf (26.40 ± 9.06) was also found less efficient. As in the case of *C. gloeosporioides*, extracts of both endophytic fungi and plant extracts tested in combination, were found less (28.69 ± 16.91) effective (Table 5).

Discussion

C. sativa is a very popular herb and always occupied an exclusive position among Hindu religion, ancient rulers and other wings of society due its chemical composition and imparting properties since recorded history. The herb contains hundreds of pharmaceutical compounds and these are categorised into major groups namely, cannabinoids; flavanoidglycosides; carbohydrates, simple alcohols, aldehydes, ketones, acids, esters and lactones; non-cannabinoid phenols; nitrogenous compounds; and vitamins and pigments (Sethi et al. 1978; Turner et al. 1980; Hillig and Paul 2004; ElSohly and Slade 2005; Flores-Sanchez and Verpoorte 2008).

In the present investigation leaves, stems and petioles of *C. sativa* were used for isolation of endophytic fungi. The fungal colonisation was higher in leaves as compare to stem and petiole. Higher colonisation of endophytes in leaf and stem tissues, as compared to roots, was reported by Siegel and Latch (1991) and Clay and Schardle (2002) in grasses. About eight fungal genera belonging to 12 species were isolated, which indicated that the plant *C. sativa* is enriched with various fungal populations. Some fungi unable to produce fruiting bodies after one month of inoculation were also recorded, and treated as mycelia sterlia which means more extensive research is required to identify these isolates also. The fungal flora of this study showed that *A. niger, A. flavus, and A. nidulans*; *P. chrysogenum and P. citrinum;* and *R. stolonifer, C. lunata, Phoma* sp., *Colletotrichum* sp., and *Cladosporium* sp. were fungal isolates identified from *C. sativa*. Isolation of such isolates was also reported in previous studies (Suryanarayana et al. 2000, 2005; Ganley and Newcombe 2004; Rosa et al. 2009; Gazis

and Chaverri 2010; Liu et al. 2010; Marquez et al. 2010; Vega et al. 2010; González and Tello 2011) from different plants like *Cynodon dactylon, Pinus monticola, Chlorophytum borivilianum*, mangrove, *Zea mays*, *Cuscuta reflexa*, cactus and grasses. Endophytic diversity of pharmaceutically important *C. sativa* was investigated in USA (McPartland and Cubeta 1997) and Germany (Kusari et al. 2012).

Today, more research has been focused on endophytic fungi isolated from various medicinal plants and their antimicrobial activity. Many such studies on endophytic fungi, their metabolites and their role in plant metabolism were carried out (Huang et al. 2001; Liu et al. 2001b; Gunatilaka 2006; You et al. 2009). The research on antimicrobial activity is not only limited to study endophytes for finding new biopesticides but also to study their role in plant defence mechanism. We have investigated antifungal activity of isolated endophytes from *C. sativa* in the present study in order to assess their possible role in plant metabolism. When we analyse the antifungal potential of different extracts individually and in combinations against *C. gloeosporioides* and *C. lunata*, a variation in results was observed. Ethanolic extracts of both *A. niger* and *A. flavus* singly or in combination show variation in antifungal activities against both the pathogens tested, which shows their effective role in plant's antifungal activity. Similarly, when both endophytic fungal extracts tested in combinations of either leaf or stem extracts, the antifungal effect was recorded here also, but less as compared to the combination of all four extracts which justify the importance of both endophytes. Similar studies were carried out by Phongpaichit et al. (2007) on 65 crude extracts, of which 51 endophytic fungi were isolated from *Garcinia* plants and they assessed these extracts for various bioactivities. Wicklow et al. (2005) also isolated an endophyte *Acremonium zeae* from *Zea mays* and analysed its role in protective nature of the plant against pathogens. He observed that the *A. zeae* was inhibitory against the *A. flavus* and *Fusarium verticilliodes*, and recommended this fungus as biocontrol agent. Several studies on endophytic fungi and the biological activities of their metabolites for the isolation of new bioactive compounds were carried out by various researchers. Some of these compounds have been used for novel drug discovery (Strobel and Daisy 2003).

In our efforts of screening, *C. sativa* for fungal endophytic association and their variable antifungal activity strongly agree with the facts that fungal endophytes produce diverse and interesting metabolites. Although some primary results are obtained on antifungal activity of endophytes, which shows their possible role in plants defense mechanism, it is just a preliminary approach and more extensive research is still required.

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