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Diversity of endophytes associated with the medicinal plant *Pulicaria crispa*

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Abstract:

Pulicaria crispa is a medicinal and aromatic plant that is distributed around the world. In this study, a remarkable variety of endophytic fungi and bacteria were isolated from different parts of *P. crispa*. Fourteen endophytic fungal species belonging to seven genera (*Cladosporium, Chaetomium, Volutella, Emericella, Aspergillus, Penicillium,* and *Ulocladium*) were recorded. The relative abundance of genus *Aspergillus* was 40.5% (recorded the highest count of genera). *Volutella ciliata* registered the highest fungal species count with a relative abundant of 16.2% and it is the first record of this fungus in Aswan region. Five bacterial species belonging to the genus *Bacillus* including *Bacillus sianensis*, *B. safensis*, *B. subtilis*, *B. altitudinis*, and *B. vallismortis* were isolated. *B. sianensis* which colonized roots represented the highest relative abundance percent (32 %). This is the first study of the microbes' diversity inside the tissue of the medicinal plant *P. crispa*.

Keywords: Diversity, Endophytes, *Pulicaria crispa*, Fungi, Bacteria.

1- Introduction

Pulicaria crispa (Forssl.) Benth. ex Oliv. belongs to the family Asteraceae and is a medicinal and aromatic plant that is distributed around the world, especially in desert and semi-tropical regions including Egypt, Sudan, Senegal, Cameroon, and Saudi Arabia (Mirghani et al., 2020; Farag et al., 2015; Dar et al., 2017; Foudah et al., 2015). *P. crispa* has been used since ancient times in traditional medicine due to its bioactive phytochemicals such as tannins, lactones, flavonoids, alkaloids, and saponins, which play an important role in pharmacology and medicine (Rizk et al., 1985; Elshiekh and Abd-ElMoniem, 2015; Foudah et al., 2015; Manel et al., 2016; Albrahim et al., 2020).

The microbes found in the internal tissues of plants are called endophytes (Tadych and White, 2019). Initially, endophytes were defined as any organism that lives within the cells of a plant by De Barry, but later scientists added a more accurate description to the definition (Khare et al., 2018; Tadych and White, 2019). These endophytes are microbes, including bacteria and fungi, that live inside plants' parts like the stem, root, and leaf and don't cause damage to the plant (Wilson, 1995; Yadav, 2018).

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These creatures inhabit undamaged tissues of the host plant and perform a wide range of biological interactions ranging from symbiotic to slightly pathogenic (Strobel et al., 2005). The interaction between the plant and endophytes is a symbiotic relationship based on the benefit of both the plant and the microorganism (Polesi, 2011; Hardoim et al., 2008). As a result of this wonderful relationship, the microbes inside the plant produce a group of substances (secondary metabolites) with unique structures that cannot be obtained from a chemical reaction and which are of great importance to the plant (Carroll, 1988; Schulz et al., 1998; Parker, 1999; Redman et al., 2001; Schulz et al., 2002; Strobel and Daisy, 2003; Lee et al., 2004; Dudeja et al., 2012).

Fungi and bacteria that share the same environment (*i.e.*, the same food sources and the same dwelling) compete for resources and a number of relationships appear, including antagonism. This relationship organizes and protects a community in which microbes live, which leads to supporting the stability of this assemblage by limiting the excessive growth of these endophytes and by fighting foreign microorganisms that want to harm them and the environment in which they live (Bharati et al.,1982; Mille-Lindblom et al., 2006; Zettler et al., 2013; Wang et al., 2015; Kumar et al., 2015; Feichtmayer et al., 2017; García-Bayona and Comstock, 2018; Karunasinghe et al., 2020; Zhang et al., 2021). This study aims to screen the diversity of endophytic bacteria and fungi in the medicinal plant *Pulicaria crispa*.

2. Materials and methods

2.1. Collection of plant samples

Healthy plants of *P. crispa* were collected from Aswan University campus (24°5′26.95"N, 32°53′57.91"E). Samples were washed with running tap water to remove dust. Plant parts were surface sterilized with 70% ethanol (1 min), then with 4% sodium hypochlorite (3 min), and finally washed twice with sterilized distilled water (Vincent, 1970). The plant parts were cut into small pieces of 1cm (Rossman et al., 1998; Costa et al., 2012; El-Deeb et al., 2013).

2.2. Isolation and identification of endophytic fungi and bacteria

The plant segments were placed on the surface of potato dextrose agar (PDA) plates supplemented with chloramphenicol (0.5 g/L) for fungal endophytes and nutrient agar (NA) plates for bacterial endophytes. PDA plates were incubated at 28 °C for 3 weeks, while NA plates were incubated at 37 °C for 72 h. The growth was daily observed (El-Zayat et al., 2008).

Fungi were identified based on their morphological and microscopic characteristics, including the color and texture of the colony as well as the color and shape of the conidia and hyphae (Moubasher (1993). The bacterial isolates were molecularly identified by 16S rRNA gene sequencing using the universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-TCC TCC GCT TAT TGC TAT GC-3') (Frank et al., 2008). The obtained sequences were aligned with those deposited in the NCBI database to detect the similarity percentages using the Blast tool from the website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of the present isolates were submitted to the NCBI gene bank to gain accession numbers. A neighbor-joining phylogenetic tree was constructed by MEGA X software (Kumar et al., 2018).

3. Results and discussion

In the present study, fungi and bacteria inhabiting the medicinal plant *P. crispa* were screened. According to our knowledge, this is the first study to report the endophytic fungi and bacteria in *P. crispa*.

Fourteen endophytic fungal species belonging to 7 genera were isolated from different plant organs, including roots, stems, leaves, and flowers (Table 1). The roots had the highest fungal colonization where they recorded 15 colonies/segment, while the fungal colonization in the other organs of the plant, including the leaves, stems, and flowers, recorded 9, 8, and 5 colonies/segment, respectively (Table 1 & Fig. 1).

In the whole plant, the genus *Aspergillus* recorded the highest count of genera (15 colonies/segment and relative abundance 40.5%), followed by *Emericella* with colonization counts 7.0 colonies/segment and relative abundance 18.9%. *Volutella ciliata* recorded 6.0 colonies/segment and relative abundance 16.2%. This fungus represents the first record in Aswan region despite isolation of it from various parts of the world. *Volutella ciliata* protect plants against potentially toxic compounds in the soil Vaughan et al., 1993) and has a role in recycling and accumulation of organic matter in soil (Osono and Takeda, 2007). *Cladosporium* and *Chaetomium* count was 4.0 colonies/segment with relative abundance 10.8% and 3.0 colonies/segment with relative abundance 8.1 % respectively. The lowest colonization was recorded with both *Penicillium* and *Ulocladium* (only one colony/segment and relative abundance 2.7% for each) (Table 1 & Fig. 2).

The highest count of fungal species was *V. ciliata* (6 colonies/segment) with a relative abundant of 16.2%. The relative abundance of *A. niger, A. terreus, C. cladosporioides* and *E. quadrilineata* was 10.8%, and the total count of 4 colonies/segment while the remaining fungal species relative abundance was ranged between 2.7%-5.4%, and colonies count between 1-2 colonies/segment (Table, 1 & Fig. 3).

Root recorded the highest rate of endophyte isolation (Table 2 & Fig. 4), whereas *Aspergillus* rate of isolation was 57.14% by three species *A. carneus* (14.29%), *A. niger* and *A. terreus* (21.43%) for each. *Emercilla quadrillineata* represented 28.57% then *volutella ciliata* 21.43%. Other endophytes were not recorded in roots (Table 2). It was interesting that *A. carneus* and *E. quadrillineata* were isolated only from roots.

The second rate of isolation was recorded in flower at 18.75% by isolation of *V. ciliata* followed by leaf at 14.29% for two genera *Aspergillus* and *Cladosporium*. The first genus was represented by *A. flavus, A. fumigatus, A. sydowii,* and *A. terreus* (3.57% each). *Cladosporium* is represented by only one species *C. cladosporioides* and isolated only from the leaf. Also *A. sydowii* inhibited only leaves in this plant (Table 2 & Fig. 4).

Rate of endophytes isolation in the stem was in the next order 11.54% represented by the genus *Chaetomium* (*C. cochliodes* 7.7% and *C. globosum* 3.85%) which was also specific to stem, 7.70 % for *E. nidulans* and 7.69% for genus *Aspergillus* (*A. flavus* and *A. fumigatus*, 3.85% each). The rate of isolation (6.25%) was recorded in flowers for both *A. niger* and *U. chartarum* while 3.57% was noticed in leaves for *E. nidulans*. *Ulocladium chartarum* was isolated only from the flower and *P. duclauxii* from the stem (3.85%) (Table 2 & Fig. 4).

Endophytic fungi belonging to the genera *Aspergillus* and *Penicillium* were isolated from medicinal plants found in Saudi Arabia, including *Pulicaria crispa*, to know its antioxidant ability of the extracts extracted from these fungi. *Cladosporium* and *Ulocladium* were isolated from medicinal plants belonging to the family *Asteraceae*, as they produce the enzyme L - asparaginase, which is used in the treatment of cancer. *Aspergillus* and *Penicillium* were also isolated during the study conducted on medicinal plants belonging to *Asteraceae* and found in

countries with biodiversity: China, Thailand and Iran (Hatamzadeh et al., 2020; Caruso et al., 2020; Hassane et al., 2022). The genera *Cladosporium*, *Penicillium*, *Aspergillus* and *Chaetomium* has been isolated from plants other than this plant and does not belong to Asteraceae such as *Oryzae sativa* L. and *Ricinus communis* linn. plants (Sandhu et al.,2014). *Penicillium sp.,* and *Aspergillus niger* were isolated as endophytic fungi from medicinal plants in *Nigeria*, *Alstonia boonei*, *Enantia chlorantha* and *Kigelia Africana* (Adeyemi, 2015). *Aspergillus terreus* is obtained from the leaves of the *Bacopa monnien* plant (Soni et al., 2021). In addition endophytic fungi belonging to the genus *Penicillium* and *Aspergillus* were obtained from fruits of the *Chaenomeles* plant (Lykholat et al., 2021). *Phoma*, *Cladosporium*, *Alternaria* and *Bipolaris* were got from *Caralluma acutangula*, *Rhazya stricta* and *Moringa peregrina* plants. These microorganisms have been studied in order to know the types present in plants and their impacts on development of seeds and reducing oxidative processes (Khan et al., 2017).

Table 1: Counts, relative abundance and isolation rate (%) of isolated endophytic fungi/segment.

Fungal Species	**Count (colonies/segment)**				**Total Count (TC)**	**RA (%)**	**IR (%)**
	Flower	**Leaf**	**Stem**	**Root**			
Aspergillus	**1**	**4**	**2**	**8**	**15**	**40.5**	**17.9**
A. carneus	-	-	-	2	2	5.4	2.4
A. flavus	-	1	1	-	2	5.4	2.4
A. fumigatus	-	1	1	-	2	5.4	2.4
A. niger	1	-	-	3	4	10.8	4.8
A. sydowii	-	1	-	-	1	2.7	1.2
A. terreus	-	1	-	3	4	10.8	4.8
Chaetomium	**-**	**-**	**3**	**-**	**3**	**8.1**	**3.6**
C. cochliodes	-	-	2	-	2	5.4	2.4
C. globosum	-	-	1	-	1	2.7	1.2
Cladosporium cladosporioides	-	4	-	-	4	10.8	4.7
Emericella	**-**	**1**	**2**	**4**	**7**	**18.9**	**8.3**
E. nidulans	-	1	2	-	3	8.1	3.6
E. quadrilineata	-	-	-	4	4	10.8	4.8
Penicillium duclauxii	**-**	**-**	**1**	**-**	**1**	**2.7**	**1.2**
Ulocladium chartarum	**1**	**-**	**-**	**-**	**1**	**2.7**	**1.2**
Volutella ciliata	**3**	**-**	**-**	**3**	**6**	**16.2**	**7.1**
Total fungal population	**5**	**9**	**8**	**15**	**37**		
No., of genera (7)	**3**	**3**	**4**	**3**			
No., of species (14)	**3**	**6**	**6**	**5**			

*RA: Relative abundance (%) =TC (each genus or species) / TC (all population) X 100
*IR: Isolation rate (%) =TC (each genus or species) /total number of segments (84) X100

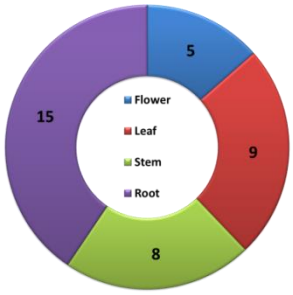

Fig. 1. variation of fungal total counts in different organs of *P. crispa.*

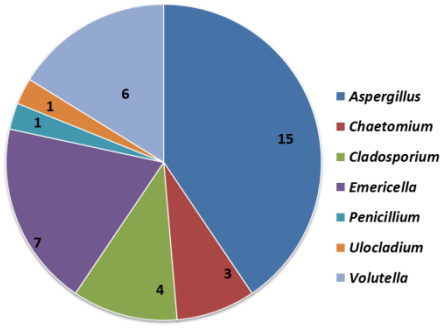

Fig. 2. Endophytic fungal genera counts isolated from *P. crispa*.

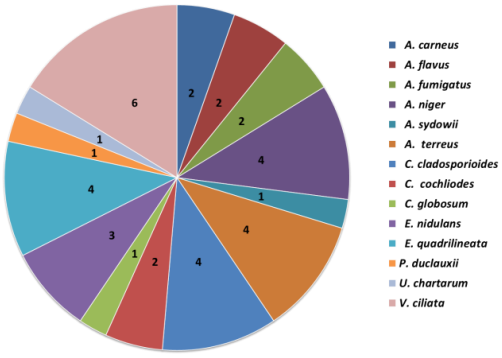

Fig. 3. Endophytic fungal species counts in *P. crispa*.

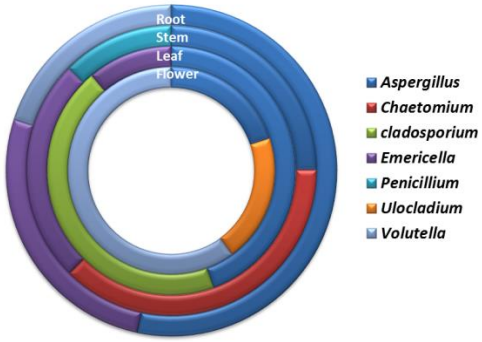

Fig. 4. The percentage of isolation of endophytic fungal genera associated with *P. crispa.*

Table (2): Isolation rate (%) of endophytic fungi of different organs of *p. crispa.*

Fungal Species	Plant organs			
	Flower	Leaf	Stem	Root
Aspergillus	**6.25**	**14.29**	**7.69**	**57.14**
A. carneus	0.0	0.0	0.0	14.29
A. flavus	0.0	3.57	3.85	0.0
A. fumigatus	0.0	3.57	3.85	0.0
A. niger	6.25	0.0	0.0	21.43
A. sydowii	0.0	3.57	0.0	0.0
A. terreus	0.0	3.57	0.0	21.43
Chaetomium	**0.0**	**0.0**	**11.54**	**0.0**
C. cochliodes	0.0	0.0	7.70	0.0
C. globosum	0.0	0.0	3.85	0.0
Cladosporium cladosporioides	**0.0**	**14.29**	**0.0**	**0.0**
Emericella	**0.0**	**3.57**	**7.70**	**28.57**
E. nidulans	0.0	3.57	7.70	0.0
E. quadrilineata	0.0	0.0	0.0	28.57
Penicillium duclauxii	0.0	**0.0**	**3.85**	**0.0**
Ulocladium chartarum	**6.25**	**0.0**	**0.0**	**0.0**
Volutella ciliata	**18.75**	**0.0**	**0.0**	**21.43**

Five endophytic bacteria were isolated from *P. crispa* (Table 3). The results of sequences alignments revealed that the isolates sp1, sp2, sp3, sp4 and sp5 were highly similar with *Bacillus safensis* strain NBRC100820 (99.75%), *Bacillus subtilis* strain DSM 10 (99.04%), *Bacillus altitudinis* 41KF2b (100.00%), *Bacillus vallismortis* strain NBRC 101236 (99.93%) and *Bacillus siamensis* KCTC 13613 strain PD-A10 (97.05%), respectively. The 16S rRNA gene sequences of the present isolates sp1, sp2, sp3, sp4, and sp5 were deposited in the NCBI under the accession numbers OQ874962, OQ874964, OQ874811, OR731260, and OQ874990 (Fig. 5).

Many endophytic bacterial species were isolated from plants belonging to the genus *Pulicaria*, including *Bacillus cereus*, *Agrobacterium farum*, *Bacillus substilis*, *Brevibacillus brevis, Acinetobacter rodioresistant*, and *Burkholderia cepacia* (Fouda et al., 2021).

Table 3: Counts, relative abundance (RA) and isolation rate (%) of isolated endophytic bacteria/segment

Endophytic Bacterial	Count/segment				Total Count (TC)	RA%	IR%
	Flower	Leaf	Stem	Root			
Bacillus altitudinis	-	3	-	-	3	9.67	3.57
Bacillus safensis	-	-	5	-	5	16	5.95
Bacillus sianensis	-	-	-	10	10	32	11.91
Bacillus subtilis	-	-	5	-	5	16	5.95
Bacillus vallismortis	-	8	-	-	8	25.8	9.52
Total	-	11	10	10	31		
No. of species (5 species)	-	2	2	1			

*RA: Relative abundance (%) = TC (each genus or species) / TC (all population) X 100
*IR: Isolation rate (%) = TC (each genus or species) /total number of segments (84) X 100

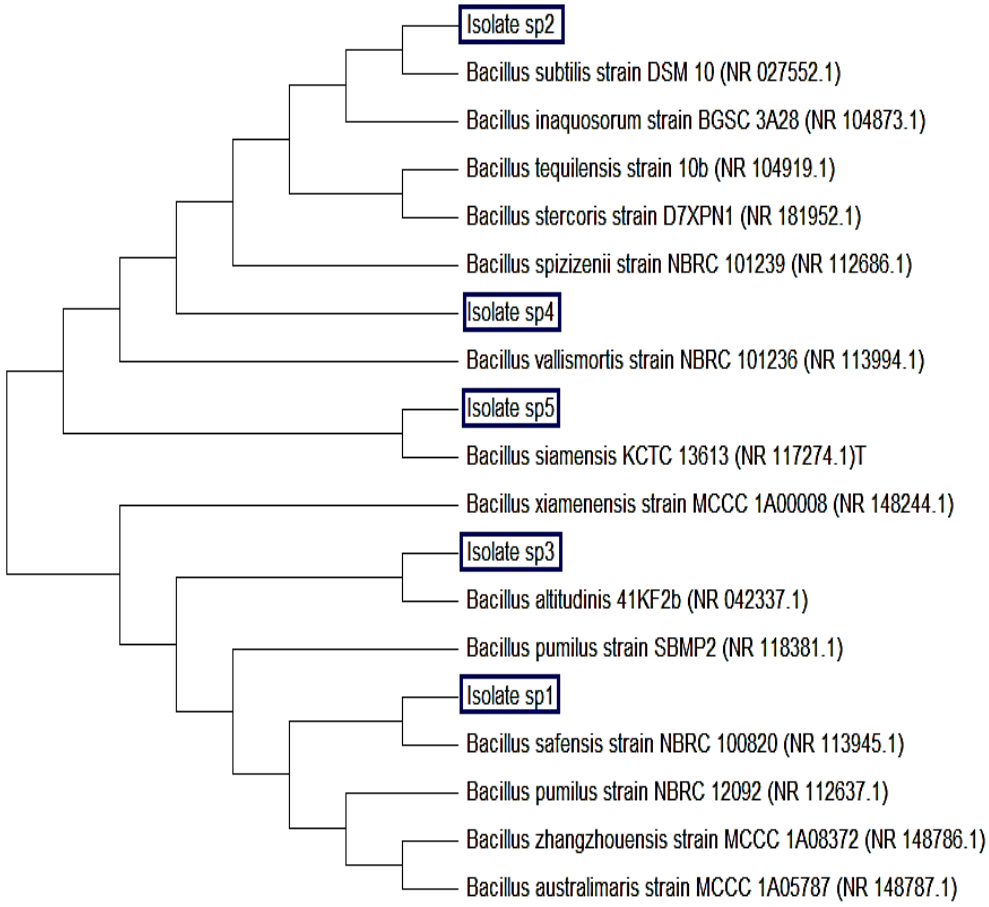

Fig. 5. A neighbor-joining phylogenetic tree with 1000 bootstraps shows the relatedness between the isolated strains and closely related members of the genus Bacillus derived from NCBI GenBank using the MEGA X software.

Pulicaria crispa is a desert plant found in multiple countries, including Egypt, Sudan, Saudi Arabia, and the Sultanate of Oman. It is considered an aromatic medicinal plant with a strong smell and has been used in the medical field since ancient times. The plant's medical benefits are attributed to its important compounds such as flavonoids (phenolic compounds found in the plant that help prevent mutations, and aid in cancer treatment) (Panche *et al.*, 2016), saponins (complex natural compounds in the plant with known for their anticancer properties and immune system enhancement) (Shi *et al.*, 2004), tannins (phenolic compounds that act as antioxidants by strengthening oxidative stability) and alkaloids (compounds used to treat malaria and cancer due to their aesthetic properties) (Tong *et al.*, 2022).

It was interesting to find that fourteen fungal species belonging to six genera and five bacterial species can be adapted to the antimicrobial compounds produced by this medicinal plant. Furthermore, these microbes can coexist with each other, fungi with fungi, bacteria with bacteria, or fungi with bacteria, without causing harm. However, further research is needed to understand the mechanisms behind their ability to live together. Consequently, our laboratory will be investigating the antagonistic reactions between these microorganisms.

Conclusion

This study screened the fungal and bacterial communities inhabiting the medicinal plant *P. crispa*. Endophytic microorganisms, including bacteria and fungi, were found to coexist within the plant. Despite the plant's production of many antimicrobial compounds, such as alkaloids, saponins, and terpenes, the isolated endophytes were able to reside within the plant tissue. Furthermore, these endophytes were able to coexist within the same tissue without causing harm to each other. Interestingly, certain bacteria and fungi were specific to particular parts of the plant. These findings have promoted further investigation into the mechanisms by which medical plant-associated microorganisms can coexist, with a focus on studying their antagonistic properties in further research.

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