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Original Research Article

Isolation and identification of growth promoting endophytic fungi from *Artemisia annua* L. and its effects on artemisinin content

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ABSTRACT

Artemisinin, a sesquiterpene lactone, is a well-known antimalarial drug isolated from *Artemisia annua* L. (Asteraceae). Semi-synthetic derivatives of artemisinin like arteether, artemether, artesunate, etc. have also been explored for antimalarial as well as other pharmacological activities. Endophytes are microorganisms which reside inside the living tissues of host plants and can form symbiotic, parasitic or commensalistic relationship depending on the climatic conditions and host genotype. In this study, endophytic fungi were isolated from the leaves of *A. annua* and were identified using the conventional as well as molecular taxonomic methods. Endophytes were identified as: *Colletotrichum gloeosporioides*, *Cochliobolus lunatus*, *Curvularia pallescens* and *Acremonium persicum*. Growth promoting activity of endophytes has been reported earlier. After treating potted plants of *A. annua* with elicitor extracts prepared from these endophytic fungi, we observed an increase in the plant biomass as well as artemisinin content.

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1. Introduction

Artemisia annua L. (Asteraceae), a perennial herb commonly known as sweet wormwood, is commonly used in folklore medicine. Traditional medicinal uses of *A. annua* include treatment of fevers, removal of intestinal worms, repellant of intestinal gases, gusher of menstruation, treatment of boils and hemorrhoids, etc. (Nageeb et al., 2013). Essential oil isolated from various species of *Artemisia*, including *A. annua*, exhibits strong insecticidal and bio-herbicidal activity (Nekoei et al., 2012; Mohammadhosseini et al., 2016; Pasha Zanousi et al., 2016; Mohammadhosseini, 2017; Pasha Zanousi et al., 2017). Pharmacological activities such as antipyretic, antiseptic, antifungal, antimalarial, antioxidant, anti-inflammatory, etc. associated with *A. annua* plants correlate well with its use in traditional medicine systems (Nageeb et al., 2013). *A. annua* is the primary source of artemisinin, a sesquiterpene lactone having antimalarial activity. Artemisinin based Combination Therapies (ACTs) comprising artemisinin, its derivatives like arteether, artemether etc. and few long acting drugs like amodiaquine and lumefantrine are effectively used worldwide for the treatment of cerebral malaria caused by *Plasmodium falciparum* (Dondorp et al., 2009; Nageeb et al., 2013; Dama et al., 2017; Steketee and Thomas, 2017). Artemisinin and its analogues exhibit antiangiogenic activity and cytotoxicity against cancer cell lines, demonstrating its possible utility in treatment of certain cancers (Wang et al., 2016; Wei and Liu, 2017). Schistosomiasis patients also respond well to artemether (Elmorshedy et al., 2016; Gold et al., 2017). A composition of arteether and other quinolones has been found to be effective in inhibiting the resistance developed due to mutation in the *gyrase A* gene of bacteria (Khanuja et al., 2002). Studies have shown that artemisinin semi-synthetic analogues inhibit *in vitro* Hepatitis C viral replication (Obeid et al., 2013). Moreover, phytotoxic effects of artemisinin have been reported, anticipating its potential as a natural herbicide

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(Pandey and Pandey-Rai, 2016).

Artemisinin is produced and stored in glandular trichomes (GLTs) of *A. annua* (Duke et al., 1994; Olsson et al., 2009; Czechowski et al., 2016) and its content in leaves ranges from 0.8%-1.3% (Gupta et al., 2002) while field grown crops of *A. annua* have reported 74.2 kg ha^{-1} as the highest average yield (Kumar et al., 2004). Environmental conditions and post harvest handling also affect artemisinin yield (Ferreira and Luthria, 2010; Thu et al., 2011; Poulson and Thai, 2015). So far, *A. annua* remains the popular source for artemisinin production throughout the world, but it is in short supply. Total synthesis of artemisinin is economically not viable (Farhi et al., 2013). Semi-synthetic production of artemisinin from the precursor artemisinic acid produced in engineered strains of *Saccharomyces cerevisiae* has also been successfully demonstrated (Ro et al., 2006; Paddon et al., 2013). Other approaches such as exploiting plant-microbe interactions that affect artemisinin production in *A. annua* are also being explored. Endophytes *Pseudonocardia* sp. and *Colletotrichum* sp. have been reported to stimulate artemisinin production (Wang et al., 2001; Li et al., 2012). Endophytes are fungal or bacterial microorganisms that colonize plant tissues intercellularly or intracellularly (Dennis Wilson 1995) and may produce compounds that promote plant growth or help them in adapting to the changing environment (Strobel and Daisy, 2003; Gandhi et al., 2015). Here, we have isolated and identified endophytic fungi from leaves of *A. annua* and studied the effect of extracts prepared from endophytic fungi on plant growth and artemisinin accumulation in treated plants.

2. Experimental

2.1. Plant material

A. annua L. plants were obtained from National Bureau of Plant Genetic Resources (NBPGR) regional station, Shimla (31.1048° N, 77.1734° E; India) and grown under greenhouse conditions at IIIM experimental farm, Jammu (32.7266° N, 74.8570° E; India). Voucher specimen (Accession no. 22399; Supplementary figure S1) was submitted to Janaki Ammal Herbarium (Acronym RRLH).

2.2. Isolation of endophytic fungi from *A. annua*

Endophytic fungi were isolated from surface sterilized leaves of *A. annua* at vegetative phase of the plant development. At least 5-7 healthy leaves (per plant, from three plants) from four weeks old plants were excised using a sterile scalpel. The leaves were washed thrice with autoclaved distilled water. The samples were then sterilized with 70% ethanol for 4-5 min, 0.1% $HgCl_2$ for one min and then washed with autoclaved distilled water 4 to 5 times. The final wash was spread plated onto PDA plate as control to ensure that surface microbes were removed from the leaves. For endophyte isolation, surface sterilized leaves were cut into segments of approximately 5×5 mm with the help of sterilized scalpel and two such segments per plate were placed on sterile water agar plates. The plates were incubated for 10-15 days at 28 ºC, following which pure cultures of endophytic fungi were isolated. Pure cultures were stored on potato dextrose agar (PDA) slants at 4 ºC.

2.3. Identification of endophytic fungi using spore morphology and ITS genotyping

Microscopic slides of pure isolates of endophytic fungi, at sporulation stage, were prepared; stained using lacto phenol cotton blue and examined under light microscope (Olympus, USA), for morphology based identification of fungi. Sequencing the ITS region of rDNA using universal primers (White et al., 1990) augmented the identification. Each endophytic fungus was inoculated in a 250 mL flask containing 50 mL of sterilised potato dextrose broth and grown at 28 ºC under shaking conditions (200 rpm) for four days. Mycelial mat was harvested and air dried. Total genomic DNA was extracted by CTAB method (Doyle and Doyle, 1987) and Polymerase Chain Reaction (PCR) was carried out using a thermal cycler (Mastercycler™ pro S, Eppendorf) in total volume of 20 µL reaction mixture containing 2.0 µL 10X buffer (with $MgCl_2$), 2.0 µL each primer (5 µM) (ITS5-F and ITS4-R; Refer supplementary table S1 for primer sequences), 2.0 µL dNTPs (2 mM), 1 unit of *Taq* DNA Polymerase (New England Biolabs, Inc.) and 50 ng of genomic DNA using following program: 5 min at 95 ºC, 35 cycles of 30 s at 95 ºC, 30 s at 55 ºC and 1 min at 72 ºC, and a final extension of 5 min at 72 ºC. Amplified PCR product was loaded on agarose gel (1.0%), bands were excised and eluted using Gel extraction kit (MinElute, QIAGEN) and sequenced using ABI 3130xl Genetic Analyzer (ABI, USA) as per manufacturer's protocol. BLAST search of the obtained sequences against the public database NCBI GenBank & preparation of phylogenetic trees was performed for aiding the identification of endophytic fungi. The sequences were submitted to NCBI GenBank and accession numbers were obtained.

2.4. Preparation of endophytic fungal elicitor extracts

The endophytic fungi were cultured in potato dextrose broth at 28 ºC for 10-12 days. The fungal biomass was sonicated and homogenised by microfluidizer (GEA Niro Soavi, Italy) into a fine powder. This fine powder prepared from fungal biomass was used to prepare elicitor solutions (henceforth called elicitor extract) using autoclaved distilled water. The elicitor extract was filter sterilised using 0.22 µm filters.

2.5. Treatment of potted plants with endophytic fungal elicitor extracts

For field treatment, plants were grown in pots for two weeks. Among these, plants with uniform height were selected for the study. 50 mL of elicitor extract solutions at the concentrations of 2.0%, 4.0% and 6.0% (w/v) were directly poured at the base of each plant on first day of every week for three consecutive weeks. Treatments were given in triplicates for each concentration of fungal elicitor extract and the pots were arranged randomly in the greenhouse to avoid any location bias. Control plants were treated with equal volume of distilled water only. Growth parameters in terms of plant height and leaf area were noted, on the seventh day of each week, for the period for three weeks after addition of elicitor extract. The readings were averaged and graphs were prepared in Excel 2007 (Microsoft Corporation).

2.6. Quantification of artemisinin in treated plants and chromatographic conditions

For quantification of artemisinin in the fungal elicitor treated plants, plants were sacrificed after three weeks of treatment. Leaves were shade dried, powdered, weighed and extracted thrice with dichloromethane with sonication for 30 min. The crude extracts were concentrated in rotary evaporator and dissolved in acetonitrile to obtain stock solutions of 1 mg/mL of each extract. Solutions, thus prepared, were filtered through 0.22 µm filters (Millipore, Billerica, MA) and subjected to HPLC analysis for quantification of artemisinin.

The HPLC system consisted of Waters instrument equipped with a binary pump (515), an auto sampler (717 plus), an automatic electronic degasser, an automatic thermostatic column oven and a photo diode array detector (2996). The system was operated using Empower software for data analysis. Chromatographic separation was achieved on a Lichrosphere RP-18 (4×250 mm) column (Merck) with a particle size of 5 µm. The column temperature was maintained at 45 ºC and the detector wavelength was fixed at 210 nm. Elution was achieved with a flow-rate of 0.6 mL/min using a gradient from 60:40 (acetonitrile:water) for 17 min, followed by a linear increase in organic mobile phase to 100% at 30 min, followed by a linear decrease in the organic percentage to 60% at 35 min, which was sustained for further 10 min with a total run time of 60 min. The mobile phase was filtered through 0.45 µm filter (Millipore) before use.

3. Results and Discussion

3.1. Isolation of endophytes and their identification

In the present study, four endophytic fungi were isolated from *A. annua* and their effects on plant growth were analyzed. Morphological identification of endophytic fungi isolated from leaves of *A. annua*, was carried out on the basis of external characteristics of the colony, hyphal morphology, characteristics of the spores and reproductive structures of the fungi (Fig. 1). ITS genotyping data further aided the morphology based identification of fungi. ITS DNA sequences were submitted to NCBI GenBank and accession numbers of submitted sequences are: *Curvularia pallescens*-KJ534376, *Acremonium persicum*-KJ534375, *Cochliobolus lunatus*-KJ534378, *Colletotrichum gloeosporioides*-KJ534377, *Curvularia pallescens* and *Cochliobolus lunatus* have earlier been reported as dominant endophyte species in various tissues of five medicinal plants sampled from Kudremukh range, Western Ghats (India) (Raviraja, 2005). *Colletotrichum gloeosporioides* has also been reported as an endophyte inhabiting several plant species like *Piper nigrum* L., *Forsythia suspensa* (Thunb.) Vahl, *Vitex negundo* L., etc. (Arivudainambi et al., 2011; Zhang et al., 2012; Chithra et al., 2013). Endophytic fungi belonging to *Colletotrichum* sp. have also been isolated frequently from *A. annua* (Zou et al., 2000; Wang et al., 2001) as well as other species of *Artemisia* (Huang et al., 2009). Endophytes of genus *Acremonium* have been isolated from grasses (Poaceae), *Mentha piperita* L., *Phoenix dactylifera* L., etc. (Lyons et al., 1990; Mucciarelli et al., 2002) and were found to provide protection to date palm against *Fusarium* wilt (El-Deeb and Arab, 2013).

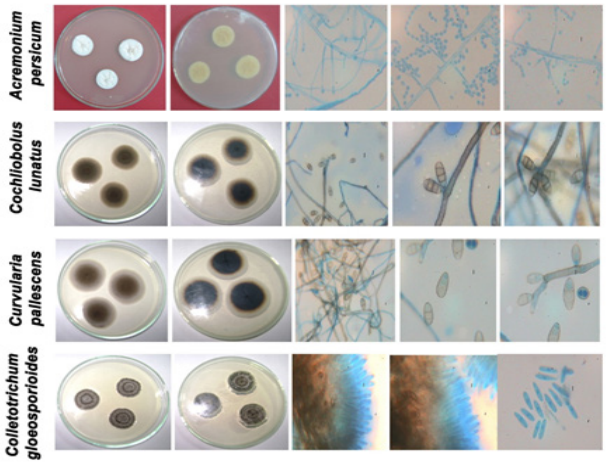

Fig. 1. Morphological characteristics of endophytic fungi isolated from *Artemisia annua* L. The endophytic fungi were identified on the basis of colony characteristics, hyphal morphology, characteristics of spores and reproductive structures.

3.2. Treatment of potted plants with endophytic fungal elicitor extracts

Endo-symbiotic associations are well known to regulate plant growth and development (Rodriguez et al., 2009). In order to study the effect of these endophytes on *A. annua* growth and development, potted plants of *A. annua* were treated with elicitor extracts prepared from four endophytic fungi: *Acremonium persicum*,

Cochliobolus lunatus, *Colletotrichum gloeosporioides* and *Curvularia pallescens*, in three different concentrations: 2.0%, 4.0% and 6.0% (w/v) each. Growth parameters observed in the study were plant height and leaf area. The treatments appeared to exhibit growth potentiation in *A. annua* plants. A substantial increase in height was observed in all the treated plants within first week of study. Plants treated with fungal elicitor extracts prepared from *Cochliobolus lunatus* and *Colletotrichum gloeosporioides*, attained maximum height in first week while those treated with elicitor extracts from *Acremonium persicum* and *Curvularia pallescens* attained maximum height in second week. However, plants treated with elicitor extracts prepared from *Curvularia pallescens* exhibited maximum increase in total height, which was 14.2% more compared to the control plants (Fig. 2a). 20.9% increase in leaf area was observed in plants treated with *Curvularia pallescens* elicitor extract at 2% (w/v) concentration. Surprisingly, leaf area appeared to decrease uniformly with further increase in the concentration of the elicitor extract. Plants treated with elicitor extract prepared from *Acremonium persicum* also showed an increase in leaf area compared to the control plants, however this treatment was not as efficient as that of *Curvularia pallescens*. Plants treated with elicitor extracts prepared from *Cochliobolus lunatus* and *Colletotrichum gloeosporioides* had leaf areas which were lesser compared to the control plants (Fig. 2b). Further, we observed that lateral branching also increased in plants treated with *Acremonium persicum* elicitor extract compared to the control plants (observation; data not recorded). Similar results have been reported in other medicinal plants also, for instance, enhancement in growth and asiaticoside content of *Centella asiatica* (L.) Urb. shoot cultures was observed on treatment with fungal elicitors (Prasad et al., 2013). Similarly, elicitor from endophyte *Trichoderma atroviride* has also been observed to promote hairy root growth and tanshinone biosynthesis in *Salvia miltiorrhiza* Bunge (Ming et al., 2013). Growth promoting effect of fungal elicitor extract could be attributed to some chemical substances produced by (or present in) endophytic fungi (Tan and Zou, 2001). Cell wall extract from an endophytic fungus has been reported to promote growth of *Arabidopsis thaliana* (L.) Heynh. seedlings (Vadassery et al., 2009). Fungal cell walls are rich in chitin and chitosan (Peter, 2005). Foliar application of chitosan was found to activate artemisinin biosynthesis in *A. annua* plants (Lei et al., 2011). It also induced plant growth in terms of height, biomass, leaf number, fruit yield, etc. in *Abelmoschus esculentus* (L.) Moench (Mondal et al., 2012). Further,

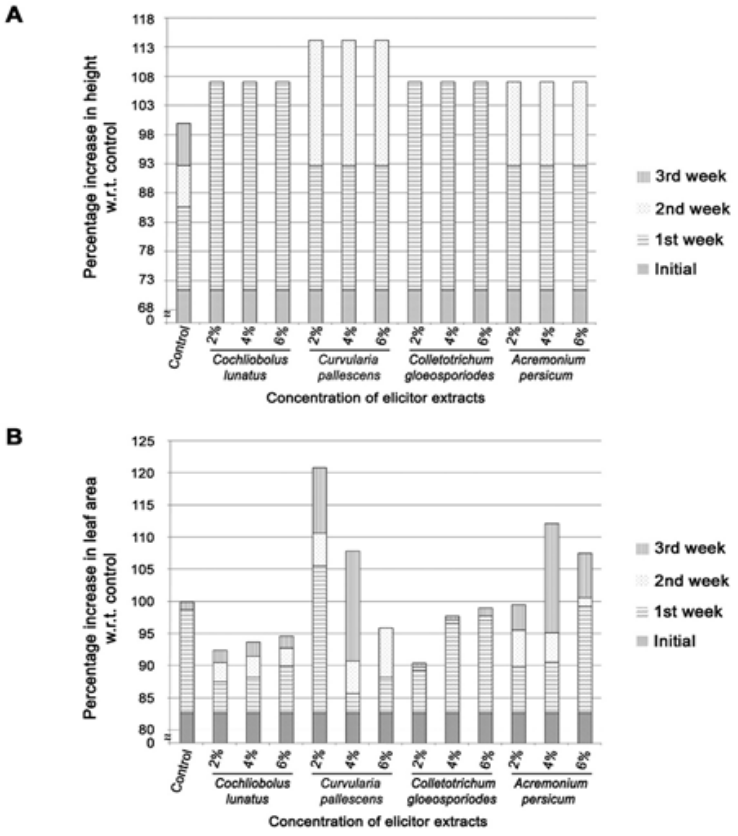

Fig. 2. Effect of elicitor extracts prepared from endophytic fungi on growth of potted *A. annua* plants. Elicitor extracts were prepared from the endophytic fungi *Cochliobolus lunatus*, *Curvularia pallescens*, *Colletotrichum gloeosporioides* and *Acremonium persicum* at three concentrations: 2%, 4% and 6% (w/v). Their effect on plant growth was studied based on percentage increase in (a) plant height (b) leaf area with respect to control plants.

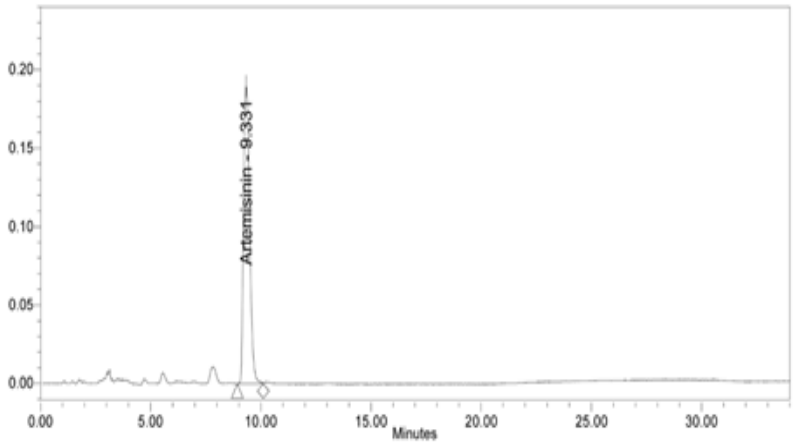

Fig. 3. HPLC chromatogram of artemisinin standard showing a single major peak at 9.331 min.

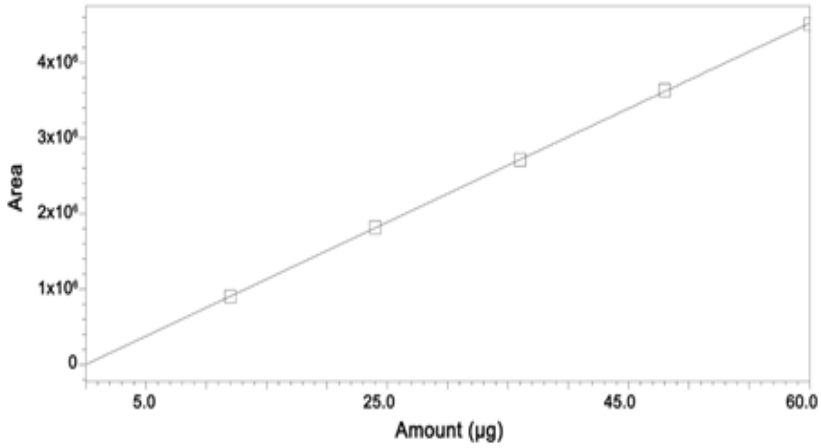

Fig. 4. Calibration curve of artemisinin standard (Dilutions of stock solutions of artemisinin standard: 1.0 mg/mL were prepared for further analysis by HPLC and construction of the calibration curve: r^2=0.999933).

the fungal elicitor extract may be inducing certain stress responses in the plant, rendering it more tolerant to pathogens and other environmental stresses, and thereby exhibiting better growth.

3.3. Quantification of artemisinin in treated plants

Amongst the tested endophytic fungi, elicitor extract prepared from *Curvularia pallescens* appeared to provide maximum growth promotion in potted plants of *A. annua* (Fig. 2), so we tested its affect on artemisinin accumulation in the treated plants at the end of three weeks, using HPLC. Pure artemisinin standard (1.0 mg) was accurately weighed and dissolved in HPLC grade methanol to obtain a stock solution of 1.0 mg/mL concentration. The stock solution was further diluted to obtain working calibration solutions. HPLC chromatogram of pure artemisinin is shown in Fig. 3. Quantification was carried out by external standard curve method. A plot of peak area versus concentration was used to prepare calibration curve. A 5-point calibration curve was obtained with a good linearity (r^2>0.999) over the concentration range of 5.0 µg to 60 µg (Fig. 4). Percentage of artemisinin with respect to dry weight of leaf sample was calculated for treated and control plants. Ratio of percentages was determined and it showed a consistent increase with increase in elicitor concentration used for treatment of plants (Table 1). Plants treated with 6.0% elicitor extract resulted in maximum accumulation of artemisisnin as compared

Table 1
Percentage artemisinin content in *A. annua* plants treated with fungal extract prepared from *Curvularia pallescens*[a].

S. No.	Sample Code	Ratio of % of artemisinin in dried sample versus control
1.	2% elicitor	1.21 ± 0.0092
2.	4% elicitor	1.30 ± 0.0180
3.	6% elicitor	3.47 ± 0.0661

[a]*Curvularia pallescens* was grown for 12 days, mycelia was sonicated and homogenized into a fine powder. Plants were treated with elicitor solutions (2%, 4% and 6% w/v) and artemisinin accumulation in treated vs. control plants (treated with distilled water) was quantified three weeks after treatment, using HPLC. *p value <0.01.

to control plants (3.47 times). Earlier, endophytic fungal extract from *Colletotrichum* sp. has been shown to enhance artemisinin production as well as the biomass of hairy root cultures of *A. annua* (Wang et al., 2001). Later, an oligosachharide elicitor component was purified after the chromatographic separation of crude mycelium of *Colletotrichum gloeosporioides*, which was found to stimulate artemisinin production (Wang et al., 2006). However, application of fungal elicitors in whole plants of *A. annua* was not reported earlier and our study appears to be the first such report.

4. Concluding remarks

Recent studies have showed that endophytes are equal partners in secondary metabolite production (Ludwig-Müller, 2015). Endophytes often produce plant growth effectors, such as auxins, which potentiate the increase in plant biomass (Pusztahelyi et al., 2015). Further, endophytic growth within the plant as well as elicitors prepared from the endophytic cultures have been shown to increase the secondary metabolite production in the host plants (Ming et al., 2013). Here, we have compared the effects on growth and artemisinin production on treatment of *A. annua* plants with elicitor extracts prepared from various endophytes that were isolated from *A. annua*. We observed that the elicitor extract prepared from *Curvularia pallescens* induced maximum growth (amongst the tested endophytic fungi) with respect to height, leaf area and lateral branching in potted *A. annua* plants, as well as induced accumulation of artemisinin. However it needs to be verified, if such treatments would help to increase biomass and yield of artemisinin in field grown *A. annua* plants as well. This study demonstrates that the elicitor extract prepared from fungal endophytes such as *Curvularia pallescens* could have immense utility as growth supplements in *A. annua* fields.

Conflict of interest

Authors declare that there is no conflict of interest.

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