Plant Physiology and Biochemistry 135 (2019) 61–68

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Cinnamic acid as an inhibitor of growth, flavonoids exudation and endophytic fungus colonization in maize root

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ARTICLE INFO

Keywords:
Sugars
Root colonization
Fusarium culmorum
Maize

ABSTRACT

Cinnamic acid (CA) is an allelochemical that inhibits the growth of root promoting soil microorganisms. To prevent the growth of soil microbes, CA modulates several metabolic pathways in host plants and soil microbes. The aim of the current study was to investigate the effect of CA on maize root growth, exudation of secondary metabolites and its interaction with beneficial endophyte Pz11. The endophyte Pz11 was isolated from the roots of drought stressed *Asphodelus tenuifolius* (wild onion). The Pz11 strain was identified as *Fusarium culmorum* by homology of the internal transcribed spacer (ITS) region of 18 S rDNA sequence. The *F. culmorum* Pz11 produced phytostimulants and signaling compounds, such as indole-3-acetic acid (IAA), flavonoids and sugars. Moreover, the strain have effectively colonized the roots of maize and subsequently enhanced the growth of its host plants. On the contrary, application of CA has reduced root growth in maize seedlings as well as root colonization ability of *F. culmorum* Pz11. Also, maize seedlings exposed to CA exude low quantities of flavonoids and polyphenols. In conclusion, CA reduces the maize root growth and exudation of secondary metabolites, which may affects its ability to attract plant growth promoting endophytic fungi.

1. Introduction

Cinnamic acid (CA) is a well-known allelochemical that is released by a number of plants to influence seed germination and root growth (Lupini et al., 2016). It has been shown that CA influence plasma membrane by inducing oxidative stress as a result of reactive oxygen species (ROS) production and/or a disturbed Ca^{2+} homeostasis (Yu et al., 2009). As an allelochemical, CA also influences the net uptake of nitrate and ATPase activity in the target plant (Abenavoli et al., 2010). It is also responsible for the enhanced lignification of plant cell by diverting the phenylpropanoid pathway in favor of lignin biosynthesis (Salvador et al., 2013). The phenylpropanoid pathway is a biosynthesis pathway shared for the production of flavonoids and lignin in plants. Lignification of root under the influence of CA may act as a physical barrier to reduce the leakage/exudation of different secondary metabolites. In addition, the ability of microbes to colonize a lignified root can be significantly reduced due to hardness of tissues and compromised release of chemical signals (Ryu et al., 2004).

Endophytic fungi can help the host plants to achieve fast growth, promote minerals absorption and provide protection against pests/pathogens (Sieber et al., 2002). Colonization of endophytic fungi also enhances the ecological adaptability of the host plants by improving its tolerance against the biotic and abiotic stresses (Schulz and Boyle, 2005). Rhizosphere is suggested as a rich source of endophytes because nearly all of the reported endophytes were isolated from this region (Sessitsch et al., 2002). Fungal endophytes enter the plant body either through degraded cell wall or fractures in root system (Gough et al., 1997; Waller et al., 2005). Before entering the plant root tissues, endophytic fungi establishes a chemical dialogue (in the form of released phytochemicals) with the host plants (Pathak and Nallapeta, 2014). Such signaling molecules are known as chemoattractant (Steinkellner et al., 2007). These chemical compounds includes polysaccharides,

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https://doi.org/10.1016/j.plaphy.2018.11.029
Received 31 August 2018; Received in revised form 23 November 2018; Accepted 25 November 2018
Available online 27 November 2018

sugars, aliphatic acids, amino acids, fatty acids, aromatic acids, flavonoids, sterols, enzymes, phenolic, vitamins, etc. (Neumann and Römheld, 2007). Besides, endophytic fungi secretes a number of different chemicals [gibberellic acids (GAs), indole-3-acetic acid (IAA) and cytokinins (CKs) to establish beneficial association in response to the root signals (Badri et al., 2009). The phytohormones (GAs, IAA and CKs) released by the endophytic fungi may promote the growth of host plants either directly or by enhancing the capacity of host roots to uptake nitrogen and phosphorus (Malinowski and Belesky, 1999). The ability of plant-associated microorganisms to produce free IAA provides them an opportunity to ameliorate the host's endogenous auxin pool (Pieterse et al., 2009). Auxin is often suggested to play a role in the cross-talk between plant and fungal signaling during ectomycorrhizal establishment (Felten et al., 2012).

A number of endophytic fungi have shown significant response towards the root exudates, but how exactly plants attract these endophytic fungi is still a mystery (Philippot et al., 2013). Though sugar and amino acids in the root exudates are considered the main bioactive compounds that can act as stimulants for the fungal growth (Nelson and Hsu, 1994). However, the knowledge is still limited that how the bioactive compounds of plants can trigger the growth of microbes in the rhizosphere (Bais et al., 2006). Flavonoids constitute another important class of compounds implicated in the establishment of root association with endophytes. During the nodulation process in leguminous plants, flavonoids act as signaling molecules. It promotes basidiospores germination in mycorrhizal fungi (Kikuchi et al., 2007), and chemo attraction, gene induction and growth enhancement in *Rhizobium* (Hirsch and Kapulnik, 1998). Currently, flavonoids have been reported to have great influence on root colonization by *Gigaspora* and *Glomus* species (Scervino et al., 2007). The role of flavonoids in root colonization by endophytic fungi, including *Aspergillus nodulias* and *Aspergillus orazyea* have also been reported in past (Qiu et al., 2010). Based on the aforementioned facts, the objectives of the current study were set to investigate the influence of CA on root growth, its ability to release secondary metabolites and interaction with endophytic fungi.

2. Materials and methods

2.1. Collection of plants

Generally the plants growing in dry areas accommodates more endophytes, hence healthy roots of *Asphodelus tenifolius* (wild onion) were collected from dry area of district Malakand. *A. tenifolius* was used for the first time in this study as a candidate for the isolation of novel endophytes. The plants were brought to Plant Microbe Interaction Laboratory, Department of Botany, Abdul Wali Khan University Mardan in sterile polythene bag. The plant roots were quickly processed to eliminate the risk of microbial contamination.

2.2. Processing of plant roots and isolation of endophytic fungi

The roots of the collected plants were rinsed with tap water to remove any visible dirt. After washing, the roots were surface sterilized by dipping in 5% (v/v) sodium hypochlorite solution for 5 s, followed by 95% (v/v) ethanol for 3 min. The residual ethanol was rinsed off by washing the roots (5-times) with autoclave double distill water. The clean sterilized roots were air dried under sterile condition to remove excess moisture. After drying, the roots were cut into 0.5 cm pieces, using a flame sterilized scalpel. About 5 to 6 segments were placed on Hegam medium plates (0.5% (w/v) glucose, 0.05% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$, 0.05% (w/v) NH_4Cl, 0.1% (w/v) $FeCl_3$, 80 ppm streptomycin and 1.5% (w/v) agar; pH 5.6 ± 0.2) for 1 week at 28 °C (Hamayun et al., 2010). The developing fungal plugs were then grown on potato dextrose agar (PDA) medium plates for purification. Purified fungus were then grown in 250 mL flask containing Czapek broth medium (50 mL; 1% (w/v) glucose, 1% (w/v) peptone, 0.05% (w/v) KCl, 0.05% (w/v) $MgSO_4.7H_2O$, and 0.001% (w/v) $FeSO_4.7H_2O$; pH 7.3 ± 0.2) for 7-days at 28 °C and 120 rpm in shaking incubator for the production of culture filtrate and biomass (Khan et al., 2008).

2.3. Determination of colonizing frequency

Colonization frequency (CF) of the Pz11 strain was determined by the method as described by Photita (2001) and Suryanarayanan et al. (2003). Briefly, root segments were plated on Hagem media plates. The plates were then shifted to a preset incubator at 28 °C and incubated for 7 days. After 7-days, the segments that were colonized by the Pz11 strain were counted and the colonization frequency was determined as:

2.4. Determination of indole-3-acetic acid in Pz11 culture filtrate

For the production of IAA, isolated strain was inoculated in Czapek broth media at 28 °C for 7 days in shaking incubator at 120 rpm (Hoffman et al., 2013). After incubation, the culture was harvested and filtered through Whatman's filter paper to collect the culture filtrate. About, 1.5 mL of the culture filtrate was then centrifuged at 11963 g for 2 min. The supernatant (1 mL) was collected and mixed with 2 mL of Salkowski reagent (150 mL concentrated H_2SO_4, 250 mL distilled water, 7.5 mL 0.5 M $FeCl_3.6H_2O$) and the mixture was incubated in dark for 30 min. IAA production was observed in the form of pink color and the absorbance was measured at A_{540} with a PerkinElmer Lambda 25 spectrophotometer. Pure IAA (10–300 μg/mL) from Sigma Aldrich was used to construct a standard curve.

2.5. Screening of the Pz11 for ACC deaminase activity

The endophyte Pz11 was screened for 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity by the well-established method as described earlier (Jia et al., 2000).

2.6. Determination of total flavonoids in Pz11 culture filtrate

The aluminium chloride method was used for the determination of the total flavonoid content in Pz11 culture filtrate with and without CA (Ahmad et al., 2014). Pz11 culture filtrate (1 mL) was added to a mixture consisting of 10% (w/v) aluminium chloride (100 μL) and 1 M potassium acetate (100 μL). Methanol (3.8 mL) was then added to the mixture. The mixture was shaken vigorously and the absorbance was recorded after 30 min at A_{415} nm. Quercetin (5, 10, 20, 25, 40, 50, 100, 120, 150, 200, 240, 300, 400 μg/mL) from Sigma Aldrich was used as a standard.

2.7. Determination of total sugars in Pz11 culture filtrate

The sugars in Pz11 culture filtrate was determined according to the method of Khan et al. (2017). Briefly, 500 μL of Pz11 culture filtrate was diluted with 10 mL of distilled water and centrifuged at 3000 g for 10 min. After centrifugation, 500 μL of the supernatant was mixed with 80% phenols and incubated for further 10 min. Finally, 5 mL of the concentrated H_2SO_4 was added to the mixture and incubated for 4 h at room temperature. The value was compared to standard curve obtained from different concentrations of glucose (Sigma Aldrich) at A_{420} nm.

2.8. Effect of cinnamic acid on dry biomass of Pz11 strain

For the determination of dry biomass the isolated strain was grown in 50 mL Czapek broth supplemented with CA (100 μL of 1 mM solution). The broth was inoculated with 100 μL spore suspension (10^6 spores/mL) and incubated at 28 °C for 7 d in shaking incubator at 120 rpm. Control treatment consisted of Czapek broth without any supplement. After 7 days, the Pz11 culture was harvested and filtered through Whatman's filter paper. The fungal biomass from the filter was cautiously shifted to pre-weighed sterilized falcon tubes. The tubes were shifted to an oven operated at 40 °C and kept there till constant dry weight for Pz11 biomass were achieved. Flavonoids, IAA and sugars secretions by the endophytes grown on inhibitors were also determined. The experiment was repeated three times and all the treatments were replicated at least three times.

2.9. Identification of Pz11

Fresh mycelium of Pz11 was collected and the genomic DNA was extracted using the SolGent Fungus Genomic DNA Extraction Kit (Cat No. SGD64-S120; SolGent Co., Daejeon, Korea) as described by (Waqas et al., 2012). The primers NS1 5′(GTA GTC ATA TGC TTG TCT C) 3′ and NS2 5' (AAA CCT TGT TAC GAC TTT TA) 3′ were used for the PCR. The PCR reaction was performed with 20 ng of genomic DNA as the template in a 30 μL reaction mixture by using a EF-Taq (SolGent, Korea). The conditions of PCR were: activation of Taq polymerase at 95 °C for 2 min, 35 cycles at 95 °C for 1 min, finishing with a 10-min step at 72 °C. The amplified products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

2.10. Plant microbe interaction under hydroponic conditions

Maize (*Zea mays* L.) seeds were surface sterilized using $HgCl_2$ solution (0.1%) and washed three times with distilled water. After surface sterilization, 5 seeds were put into autoclaved Petri plates with two fold filter papers. The seeds were allowed to germinate at 28 °C for 5 days. The uniform seedlings were transferred to a hydroponic solution and allowed to grow for two days (photoperiod 16/8 h and temperature 25 °C at midday and 15 °C at night; 390 ppm CO_2; 40% humidity). Hydroponic setup was composed of a 100 mL beaker containing 50 mL half strength Hoagland solution (Reversat et al., 1999; Barac et al., 2004). Truncated micropipette tips hold in sterilized cardboard were used to hold the seedlings in hydroponic solution (Dardanelli et al., 2010). The seedlings were aerated by using Hydrofarm active aqua air pump without disturbing the root growth (Bado et al., 2016). One set of seedlings received endophyte in the form of spore suspension (10^6 spores mL^{-1}) in culture media and labeled as E+. Similarly the seedlings in E-set did not receive any fungal spore. Both the set were further divided into three subsets:

Treatment 1: Received foliar spray of 1 mM CA (CAF).
Treatment 2: Received 100 μL of 1 mM CA in the culture medium (CAR).
Treatment 3: Received 100 μL of distilled water.

Each treatment was replicated three times, having 9 seedlings per replicate. The seedlings were allowed to grow for two weeks under conditions described above. Growth parameters, including root length, shoot length, root dry biomass and shoot biomass were recorded. Secondary metabolites including IAA, sugars and flavonoids were determined in root exudates and plant tissues by colorimetric method and LC-ESI-MS/MS.

To determine the effect of CA on endophyte colonization, the roots of the harvested seedlings were initially washed with tap water. The clean roots were then cut into small pieces and stained with lectophenol cotton blue dye for 20 min. After 20 min the segments were washed and observe under light microscope.

The level of colonization was also quantified by platting the root segments on PDA medium. For this purpose, roots were surface sterilized with 0.1% (w/v) $HgCl_2$, washed with distill water and cut into 1 cm segments. Six root segments were obtained for each plant, i.e. 2 segments from upper part (near to inoculum) two from middle part and two from lower part (near to stem). The obtained segments were kept on clean filter paper and inoculated on PDA plates and incubated for 7 days. After incubation, colonization percentage was determined as mentioned earlier.

2.11. HLPC fractionation of culture filtrates of pz11 and maize root exudates

For HPLC fractionation, the culture filtrate of Pz11 was extracted with ethyl acetate. Briefly, 5 mL of culture filtrate was mixed with equal volume of ethyl acetate and centrifuged at 1000 g for 5 min. After centrifugation, the collected supernatant was subjected to HPLC fractionation. Maize root exudates were centrifuged for 5 min at 4000 g and each sample was filtered through 45 μm pore size filter paper twice after centrifugation. The column was washed with methanol (60%) before samples were subjected to HPLC. The samples (100 μL) were then injected onto the C-18 reverse phase column using Flexer LC autosampler (PerkinElmer LC technology). The sample was eluted from the column through 60% methanol at a flow rate of 1 mL/min. The eluate was monitored at 250 nm and the peaks were collected with the help of fraction collector (FC 203 B USA). Each sample was injected five times to collect sufficient amount of different fractions.

2.12. LC MS/MS analysis

The HPLC fractions were subjected to ESI-MS/MS (LTQ XL, Thermo Electron Corporation, USA), using direct injection mode, for the identification of flavonoids, following the method of Steinmann and Ganzera (2011) with minor modifications. Briefly, a mixture of methanol and acetonitrile [80:20 (v/v)] was used as a solvent for the fractionations of both culture filtrates and root exudates. The mass ranges, in positive and negative ionization (wherever required) mode was chosen from 50 to 1000 m/z. The collision induced dissociation energy (CID) during MS/MS was kept in the range of 10–45, depending on the nature of parent molecular ion. The capillary temperature was kept at 280 °C and the sample flow rate was regulated to 8 μL/min. The MS parameters, e.g. sheath gas and auxiliary gas, for each compound were adjusted to confirm the maximum favorable ionization, ion transfer conditions and optimal signal of both the precursor and fragment ions. This was done via filling the analytes and physically rotating the parameters. The source parameters were same for all of the analytes. The acquired ESI-MS/MS data was analyzed using manual, Xcalibur (Xcalibur 3.0). ChemDraw (ChemDraw Ultra 8.0) software was used for structural elucidation and then compared with online published data.

2.13. Statistical analysis

Data analysis was done using SPSS 20 for Windows. In case of two groups, comparison was done by Student's *t*-test ($p = 0.01$) while in case of more than two groups comparison was done by analysis of variance (ANOVA) and Duncan multiple range test ($P = 0.05$).

3. Results

3.1. Isolation and identification of the selected strain Pz11

The selected strain Pz11 was isolated from the roots of *Asphodelus tenuifolius* along with 6 other strains. Healthy plants (5 in number) were used to obtain 25 root segments that yielded 7 endophytes after an incubation period of 7 days. Dominant strain was Pz14, sprouted out from 19 root segments with highest colonization frequency (76%) of the isolated strains (Table 1). The strains Pz11 and Pz16 were least

Table 1
Colonization frequency (%) of the isolated endophytic fungi in root of *Asphodelus tenuifolius* and their screening for plant growth promoting traits including IAA and ACC deaminase.

Endophytes	Colonization (%)	IAA	ACC deaminase
Pz11	20	+	+
Pz12	32	-	-
Pz13	28	+	-
Pz14	76	+	-
Pz15	48	-	-
Pz16	20	-	-
Pz17	60	-	-

abundant in the root of *A. tenuifolius* with 20% colonization frequency. Three fungal strains (Pz11, Pz13 and Pz14) were screened positive for IAA production, whereas strain Pz11 also produced ACC deaminase and have no disease symptoms. Production of IAA, ACC deaminase and nonpathogenic nature suggested Pz11 a possible candidate for plant growth promotion and hence selected for further study.

To identify the strain, its DNA was extracted for subsequent identification. Sequence of the ITS region near the 18 S rDNA was obtained and the obtained sequence was subjected to homology analysis, using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained sequence showed 99% homology and 100% query cover with *F. culmorum*. Its identity was further confirmed by carrying out phylogenetic analysis of the sequences of closely resembling endophytes retrieved from NCBI GenBank, using MEGA 7.0 software. The neighbor-joining tree (based upon ITS sequence homology) grouped our isolated starin with *Fusarium culmorum* (Fig. 1). Sequence was submitted to GenBank under the accession No. KY780172.

3.2. Effect of CA on the growth and metabolism of Pz11

Fungal growth was determined by measuring its dry biomass after incubation for 7 days. The results revealed that the growth of the endophyte was significantly reduced under the influence of CA. For instance, the dry biomass of CA exposed Pz11 was 36% as compared to the control (Fig. 2). Likewise, Pz11 was screened for the production of different metabolites, including IAA, flavonoids and sugars by colorimetric methods. Culture filtrate of the strain contained 33.2 μg/mL of IAA after 7 days of incubation. Also, the strain produced flavonoids and sugars in significant amounts (Fig. 2). Application of CA, on the other hand, has negatively influenced the level of IAA and flavonoids in the fungal culture filtrate (Fig. 2).

For further confirmation, LC-ESI-MS/MS was used to measure IAA and flavonoids in fungal culture filtrate. Flavonoids were structurally identified based on their data and fragmentation patterns previously reported in literature. The ESI-MS/MS analysis of those components was conducted in both positive and negative ion mode, and their properties are given in Table 2. Three flavonoids including calycosin, dihydroxyflavone and pratensein were identified in the culture filtrate of Pz11. However, exposure to CA inhibited the production of calycosin and pratensein, but dihydroxyflavone production continued even in CA exposed cultures of this endophyte (Fig. S1).

3.2.1. Effect of CA on the growth of maize seedlings

Maize seedlings were grown for 14 days in a hydroponic system, composed of half strength Hoagland's solution containing CA or endophyte Pz11 or their combinations. Control seedlings were grown on half strength Hoagland's solution without CA or Pz11. The Pz11 associated seedlings showed significantly higher root and shoot growth as compared to the control (Fig. 3A and B). However, the application of exogenous CA drastically retarded root length with no effect on shoot length. Interestingly, reduction in both shoot and root lengths were recorded in seedlings exposed to foliar application of CA (Fig. 3A and B). Similarly, dry biomass was significantly enhanced in seedlings inoculated with the Pz11 as compared to the control (Fig. 3C). Maize seedlings from CAF treatment have significantly lower biomasses, whereas from CAR treatments no observable differences were noticed as compared to the control. In fact, the presence of Pz11 didn't make any differences in the biomass of seedlings under CA stress (Fig. 3C).

3.2.2. Effect of CA on the exudation of secondary metabolites

The root exudates were collected and were screened for the quantification of IAA, flavonoids and sugars. Highest amount of IAA (11.6 ± 7.3 to 12.4 ± 3.0 μg/mL) was found in root exudates collected from endophyte associated seedlings (Fig. 4A). Application of exogenous CA (CAF or CAR treatments) did not influence IAA exudation by the maize roots. Exudates collected from endophyte *F. culmorum* PZ11 associated maize seedlings had significantly higher amount of IAA. However, its application in CAF or CAR treatments antagonistically interacted with the endophytes. As a result, it nullified the fungal factor that contributed towards IAA production by the host roots (Fig. 4A). Fungal isolate Pz11 also promoted the accumulation of sugar in maize root exudates by 21.8% (Fig. 4B). However, unlike IAA, sugars were not affected by CA application.

Flavonoids were checked in root exudates after 1, 3, 7, and 14 days of maize seedlings. A time dependent increase was recorded in the concentration root released flavonoids (Table 3). The first visible difference in flavonoids exudation among control and CA treated seedlings (CAF and CAR) was recorded at 3 days after treatment (Table 3). Seedlings in the two groups maintained this difference till the end of the experiment. A non-significant difference has been observed concerning the mode of AC application on flavonoids concentrations in the root

Fig. 1. Colony of endophyte Pz11 growing out from the root segments of *Asphodelus tenuifolius* on PDA medium (a) and its phylogeny shown through phylogenetic tree constructed by Neighbor-Joining method (b). Contig 1 is the sequence from Pz11 strain.

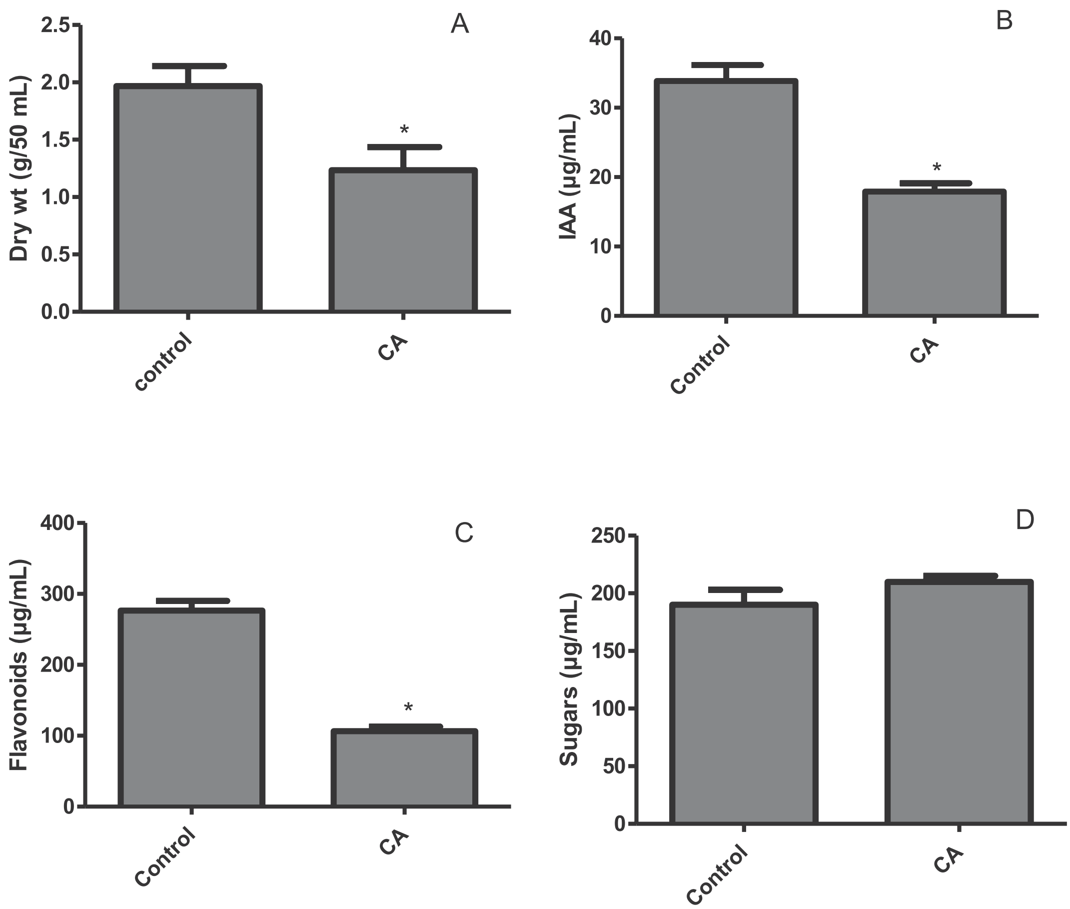

Fig. 2. Effect of cinnamic acid (CA) application on the growth and secondary metabolite production by the endophyte *Fusarium culmorum* PZ11. The isolate was grown inn 50 mL of Czapek broth in a 500 mL flask containing 100 µL of 1 mM solution of CA at 28 °C and 150 rpm for 7 days. Bars labeled with (*) shows significant difference between treatments (two tailed Student's *t*-test; $p < 0.01$).

Table 2
Identification of flavonoids in culture filtrate and root exudates of maize seedlings.

Sample	Rt (min)	[M-H] (m/z)	Ion mode	Ms2 (m/z)	Compounds with Reference
Pz11	7.5	283	-ve	268,265,255,239,221,200,182,167,150,124,114,96.9,96	Calycosin (Ye et al., 2012)
	11	255	+ve	237,223,213,197,195,	Dihydroxyflavone (Zhang et al., 2014)
	3.9	301	+ve	286,269,259,245,241,239,202,189,170,161,144,126,101	Pratensein (Zhang et al., 2014)
Pz11 + CA	12	255	+ve	237,223,211	Dihydroxyflavone (Zhang et al., 2014)
Root exudates	3.4	318	+ve	300,290,282,262,256,247,225,204,191,164	Quercetagatin
	3.5	301	+ve	283,269,256,245,239,206,188,170,153	Rhamnocitrin (Zhang et al., 2014).
Root exudates + CA	3.4	318	+ve	300,282,265,247,211,192,165,134,108	Quercetagetin
Root exudates + Pz11	5.7	301	+ve	283,269,245,240,218,188,170,162,148,130,118,94	Rhamnocitrin
	12.4	283	-ve	265,239,213,200,184,157,152,96	Calycosin
	2.12	318	+ve	300,282,265,256,245,216,188,178,146,128,97	Quercetegatin

Rt = retention time, (m/z) = mass to charge ratio.

exudates of seedlings. Endophyte associated seedlings released higher quantities of flavonoids as compared to the seedlings from all other groups. Conversely, the response of endophyte associated seedlings to CA exposure (in terms of flavonoids release) was similar to that of non-endophyte associated seedlings during the first three days of CA treatment. Interestingly, the amounts of flavonoids in the exudates of endophyte associated seedlings were non-significant to the control seedlings on the 7th day after CA treatment (Table 3). Maize roots released querecetagatin and rhamnocitrin under controlled conditions, whereas under CA treatment, the exudation of the later was inhibited (Fig. S2). Additionally, the endophyte associated maize seedlings exuded querecetagatin and rhamnocitrin. On the other hand, the CA exposed cultures of Pz11 released only calycosin (Fig. S3).

3.3. *Effect of CA on root endophyte interaction*

The endophyte showed remarkable potential to colonize maize root under hydroponic system. Fungal hyphae were not seen in the roots of control seedlings (Fig. 5A). However, roots of the endophyte associated seedlings were full of fungal hyphae (Fig. 5B) and exposure to CA largely compromised its root colonizing ability (Fig. 5C). The endophyte could colonize the root from the tip to the middle of root, leaving the root in proximity of stem (non-colonized). About 74.9% of roots segments (near root tips) from the endophyte associated maize seedlings were colonized by the Pz11 (Fig. 5D). Inhibition of flavonoids exudation by maize roots (CA application) also negatively influenced the root colonization efficiency of the endophyte Pz11. The colonization efficiency was reduced by 89% in CA treated maize seedlings. Foliar

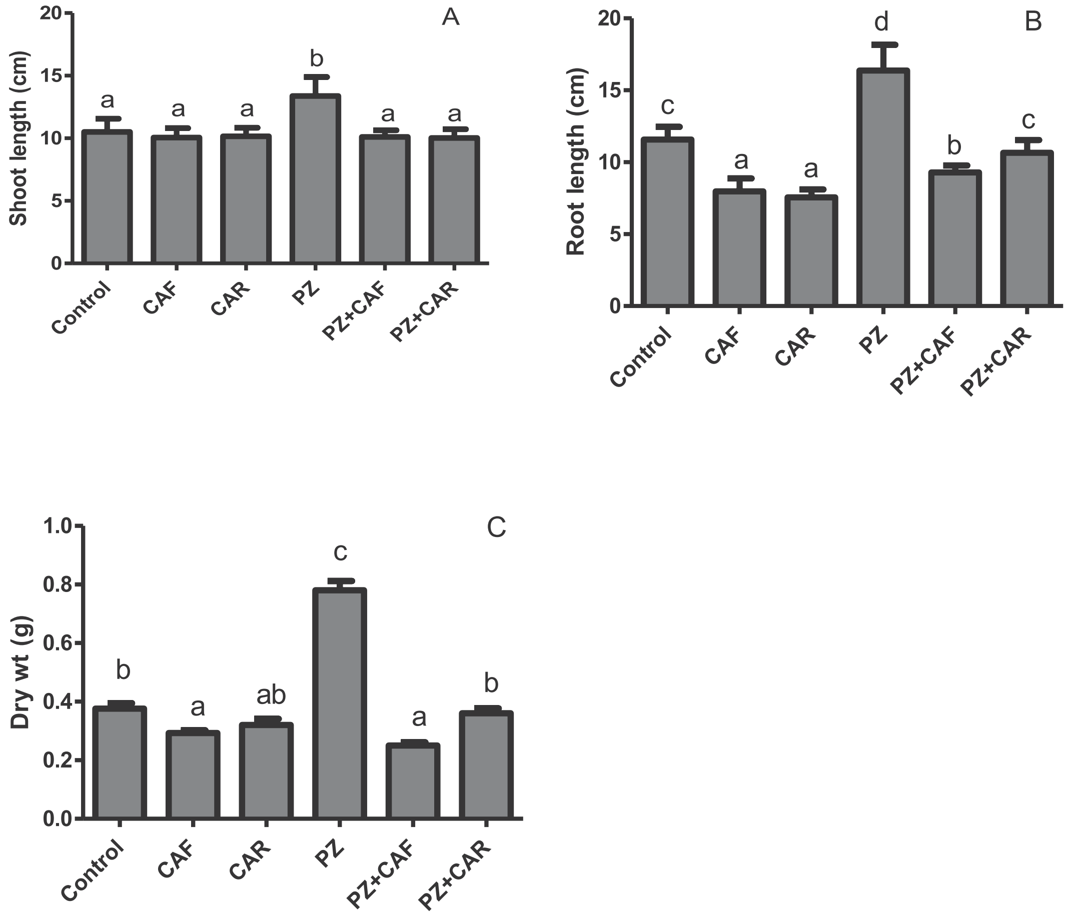

Fig. 3. Effect of CA and endophyte *F. culmorum* PZ11 on the growth of maize seedlings recorded as (A) shoot length, (B) root length and (C) dry weight. Seedlings were grown hydroponically on half strength Hoagland medium for two weeks under controlled conditions set at 25 °C (midday) and 15 °C (at night), 16/8 h (photoperiod) and 40% RH. Different labels on bars show significant difference among different treatments (Duncan test; $p < 0.05$).

application of CA was more devastating than its supply through nutrients solution.

4. Discussion

Plant interaction with endophytic fungi is a function of several factors, mainly depends on plant and microbial signals. Endophytic fungi are important group of microbes that interact with plant and promote their growth under normal and stressed environment. How allelochemical influence plant-microbe interactions have not been in focus. One of the important allelochemicals is CA, which has been known for plant growth retardation. CA interferes with metabolic pathways that leads to membrane damage and ROS accumulation (Ding et al., 2007). Current study has also demonstrated the important role of this allelochemical in maize growth modulation and exudation of secondary metabolites from roots. Furthermore, its interaction with an endophytic fungus (*F. culmorum* Pz11) isolated from *Asphodelus tenifolius* has also been explored. Interestingly, this allelochemical not only

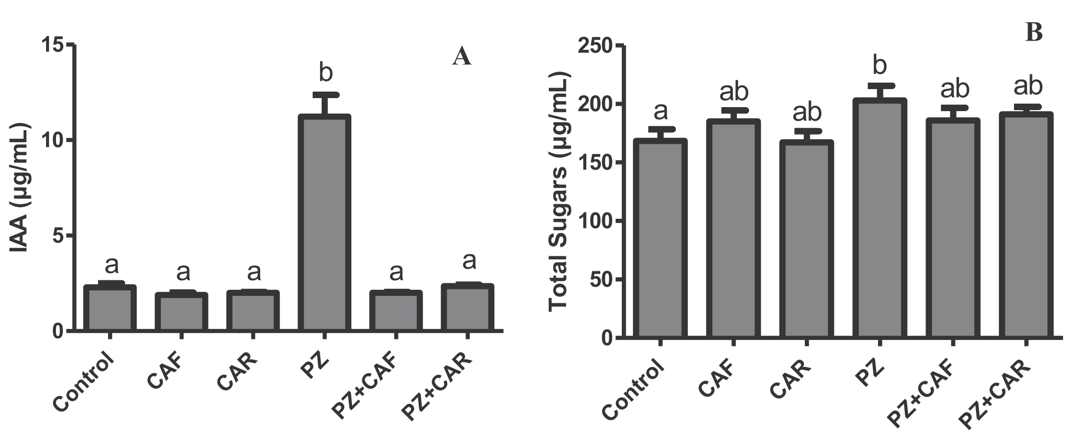

Fig. 4. Effect of CA and endophyte *F. culmorum* PZ11 on the exudation of secondary metabolites by roots of maize seedlings grown hydroponically on half strength Hoagland medium for two weeks under controlled conditions set at 25 °C (midday) and 15 °C (night), 16/8 h (photoperiod) and 40% humidity. Different labels on bars show significant difference among different treatments (Duncan test; $p < 0.05$).

Table 3
Effect of different treatments on the concentration of flavonoids in the root exudates of maize seedlings. Seedlings were grown for 14 d in Hogland' s solution under axenic conditions. Roots of one set of seedlings were inoculated with spore suspension 10^6 mL^{-1} of endophytic fungi and another set was left uninoculated. Of both sets three seedlings each were left untreated (control) and three seedlings were sprayed with exogenous IAA (10 µg/mL), CA (100 µL of 1 mM solution) and Yucasin, foliar and root entries. Roots exudates were collected 1, 3, 7 and 14 d after treatment and subjected to flavonoids determination.

Treatments	1 day treatment	3 days treatment	7 days treatment	14 days treatment
Control	86.4 ± 7.3 ab	174.5 ± 53.6c	208.5 ± 12.6cd	213.0 ± 25.0cd
CAF	81.4 ± 13.9 ab	84.8 ± 9.5a	90.9 ± 6.9a	104.1 ± 6.1 ab
CAR	82.8 ± 3.8a	96.4 ± 0.6a	108.0 ± 0.3 ab	111.1 ± 3.5 ab
PZ1	126.7 ± 74.0 ab	216.9 ± 60.7c	283.2 ± 14.0e	260.8 ± 38.8d
PZ1 + CA(Foliar)	73.9 ± 11.8a	52.0 ± 8.5a	150.8 ± 81.1bc	107.1 ± 48.1 ab
PZ1 + CA (root)	104.9 ± 7.6 ab	83.3 ± 4.4a	153.0 ± 11.9bc	156.0 ± 29.3bc

CA = cinnamic acid, IAA = indole-3-acetic acid.

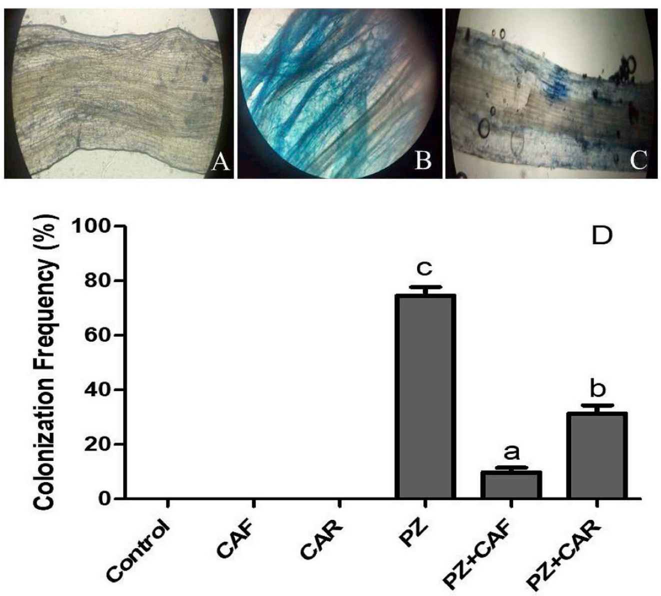

Fig. 5. Effect of CA on *F. culmorum* PZ11 colonization in maize roots. Colonization was assayed in roots using chitin specific lactophenol cotton blue dye in (A) control, (B) PZ11, (C) CA + PZ11 roots or plating surface sterilized toot segments on PDA (D). Different labels on bars show significant difference among different treatments (Duncan test; $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

reduced the root growth and dry biomass of maize seedlings, but also influenced the growth of the Pz11. Previously, it has been clearly demonstrated that CA has greatly influenced the fungal growth (Wu et al., 2008). Inhibition of root growth is taught to be due to the premature lignification of CA exposed root (Salvador et al., 2013). Growth inhibition in both host plant and endophyte was accompanied with reduction in the overall secretion of secondary metabolites, i.e IAA and flavonoids. This means that rhizosphere of CA stressed maize seedlings would have lower concentration of these two metabolites, which might reduce the number and diversity of microbes attracted for colonization (Hassan and Mathesius, 2012). CA stressed maize seedlings tried to compensate for this deficiency by increasing the amount of IAA and flavonoids released per gram dry biomass of seedling. However, full compensation was not possible as releasing extra amount of secondary metabolites overburden plant metabolism (Ouzounis et al., 2014).

Interestingly, release of different flavonoids was affected differentially by CA. In case of endophyte, secretion of calycosin and pratensein was completely abolished, but secretion of dihydroxyflavone continued in the presence of CA. Similarly, exudation of rhamnocitrin stoped and querecetagatin exudation continued in CA treated maize seedlings. This shows that CA might interfere and modulate flavonoids biosynthesis pathway in the target plants (Waśkiewicz et al., 2013). The most striking observation was about flavonoids profile of the root exudates obtained from endophyte associated maize roots exposed to foliar CA, where three flavonoids including rhamnocitrin, querecetagatin and calycosin were found. These observations suggest that rhamnocitrin was important for maize root to establish symbiotic interaction with *F. culmorum* Pz11 and cope with the stress. Microbes that can utilize flavonoids are highly competitive and can grow at least 100-fold better than their auxotrophic mutants on plant roots with high flavonoids (Narasimhan et al., 2003). Plant roots are known to modify their roots exudates in order to cope with stresses (Chaparro et al., 2013). Phytohormones, such as IAA is also of great significance in establishing root interations with microbes (Hussain et al., 2015). Reduction in the amount of IAA might be attributed to the induction of IAA oxidase under the influence of CA (Salvador et al., 2013). However, release of sugars by maize root was not influenced by CA application. On the other hand, total sugars secretion was increased in CA exposed culture of *F. culmorum* Pz11. Taken together, there is a strong evidence that CA stress reduce the overall release of IAA and flavonoids, but the ratio of these metabolites to dry weight of seedlings is increased. This phenomenon has been confirmed in the past, where root secreted higher amounts of secondary metabolites in response to environmental stresses (Chaparro et al., 2013).

5. Conclusion

Cinnamic acid application has been an effective antagonist of maize and *F. culmorum* Pz11 growth. Besides, CA application can inhibit the release of flavonoids by plant and fungus, whereas the release of IAA and sugars can't be affected by this growth inhibitor. The endophytic fungus *F. culmorum* Pz11 has the ability to colonize maize root effectively and promote its growth. However, in the presence of CA, PZ11 was unable to colonize maize root effectively and alleviate the CA induced stress.

Conflicts of interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Asif Mehmood: Investigation, Methodology, Writing – original draft. **Anwar Hussain:** Conceptualization, Project administration, Supervision, Writing – review & editing. **Muhammad Irshad:** Supervision, Writing – review & editing. **Muhammad Hamayun:** Resources, Visualization. **Amjad Iqbal:** Writing – review & editing. **Hazir Rahman:** Software. **Abdul Tawab:** Formal analysis. **Ayaz Ahmad:** Data curation. **Sultan Ayaz:** Validation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

doi.org/10.1016/j.plaphy.2018.11.029.

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