IAA and flavonoids modulates the association between maize roots and phytostimulant endophytic Aspergillus fumigatus greenish

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ABSTRACT
The association between microbes and plant roots involves a complex chemical dialogue featured by IAA and flavonoids in addition to other signals. Current study is focused on the role of IAA and flavonoids as essential elements of the chemical dialogue between an endophytic fungus and maize roots. The strain was isolated from the leaves of Withania somnifera and was identified as Aspergillus fumigatus greenish by 18S rDNA sequence. Culture filtrate of the strain contained phytostimulant (IAA, ammonia and ACC) and signaling compounds including IAA, flavonoids and avonoids. The strain effectively colonized the maize roots and enhanced its growth. In order to determine the effect of IAA and flavonoids on the ability of the endophyte to colonize maize roots, we inhibited the release of IAA and flavonoids in maize separately which reduced the colonization of endophyte in maize root to 66% and 55% of the untreated control. Similarly, application of IAA in combination with endophyte enhanced root colonization by 90% in zone of cell division, 66% in the zone of elongation and 30% in the zone of maturation. These findings determined that IAA and flavonoids are among the key players of the complex chemical dialogue between plant root and fungal endophyte.

1. Introduction
Endophytic fungi colonize the living plant tissue without causing significant damage, and include a very diverse group of fungi with different life history. Mutualistic endophytic fungi have been the focus of increasing interest worldwide, mainly because of their beneficial effects on plant suitability, such as tolerance to biotic and abiotic stresses, nutrient availability and increased growth (Prado et al. 2012). Endophytic fungi drive three essential aids to host plants, such as nutrition, body protection, and adverse replication (Hyde and Sot Yong 2008). Plants provide shelter and staple food for nutrition, while in return endophytes produce bioactive secondary metabolites and enzymes (Schulz and Boyle 2005). These secondary metabolites can range from various types of phenolic compounds to hormone-like compounds. In the past decade, it has been reported that different kinds of endophytic fungi produce auxin (IAA) and gibberellins (GA3, GA4 and GA7) (Khan et al. 2015). Fungal endophytes produce bioactive metabolites that promote the plant–endophyte interaction (Strobel 2003). Promoting plant growth is the most important effect of fungal symbiosis (Hassan et al. 2013), however endophytic fungi promote plant growth by producing various secondary metabolites, including ammonia and plant hormones, particularly IAA (Jain and Pundir 2017). Plants and microorganisms, including bacteria, algae and fungi, can produce IAA (Spaepen et al. 2007; Khan et al. 2018a). The role of microbial IAA in plant–microorganism interactions has received increasing attention recently (Khan et al. 2017). In addition, some studies show that IAA is a signaling molecule in microorganisms because it affects gene expression in several microorganisms (Yuan et al. 2008). IAA produced by fungi can induce lateral root formation and root hair development (Contreras-Cornejo et al. 2009). Improved root development results in enhanced nutrient absorption by the relevant plants. The role of fungal produced IAA in different plant-fungal interaction systems recommends that fungi can be associated to plant for pathogenesis or symbiotic approach using IAA and related compounds leading to plant growth promotion and changes in the basal defense mechanisms of the plant. In addition, IAA produced by fungi can defeat pathogenic strains and disease progression by enhancing the plant’s immune response (Ludwig-Muller 2015). Among fungi, the indole-3-pyruvic acid (IPA) pathway is the most commonly used pathway for IAA biosynthesis (Hilbert 2012), although the tryptamine pathway (TAM) and bacterial indole-3-acetamide (IAM) pathways are also reported in Ustilago, the IAM pathway in Colletotrichum spp has also been reported (Hilbert 2012). In addition, tryptophan (TRP) independent IAA biosynthetic pathways have recently been shown in yeast (Rao et al. 2010). Flavonoids are an important class of secondary metabolites found in various plants and their microbial partners (Khan et al. 2018a). The role of flavonoids as signal molecules in modulating the interaction between plant roots and nodulating bacteria is well understood (Hassan and Matthesius 2012). Flavonoids constitute another important class of compounds implicated in the establishment of root...
association with AM. Flavonoids that are present in large amount nearly in all parts of plants perform a number of physiological and ecological functions in plants (Wink 2003). Root flavonoids are perceived by the bacterial cells releasing node factor which is believed to initiate root nodulation (Spank 2000). Flavonoids are found not only in plants but also in most root exudates (Cesco et al. 2010). Flavonoid exudation in the rhizosphere is not well explained, although some efforts to identify transporters have been concluded. Frequently, in response to the stimulant, flavonoids may drastically exude from the roots (Weston and Mathiesius 2014). In the current study, an important medicinal plant, *Withania somnifera*, was explored for the existence of endophytic fungi. *W. somnifera* is an erect, evergreen, downy shrub, 30–150 cm tall, with thick, fleshy rhizomes. The leaves are simple, ovate and glabrous (Khan et al. 2010).

Keeping the above background in view present study was conducted to isolate IAA and flavonoids producing endophytic fungi from leaves of wild plant *W. somnifera* and to assess the role of these compounds and maize root–endophyte interactions and plant growth promotion.

2. Materials and methods

The experiment was conducted in plant–microbe interaction laboratory Abdul Wali Khan University Mardan, Pakistan. Maize variety GAUHER was obtained from national agriculture research center (NARC) Islamabad. The seedlings were allowed to grow for 14 days inside growth chamber (DAIHN Lab Tech) under controlled conditions (photo-period 16/8 h and temperature 25°C at midday and 15°C at night; 390 ppm CO2; 40% humidity). The endophyte was isolated from a leaf of a 30 cm tall, 150 cm tall, with thick, fleshy rhizomes. The leaves are simple, ovate and glabrous (Khan et al. 2010).

2.1. Isolation of endophytic fungi

The leaves of *W. somnifera* were collected from dry area of district Mardan. The plant materials were brought to laboratory in sterile polythene bag and were processed within 24 h to eliminate the risk of microbial contamination. To isolate the endophytic fungi, the selected leaves were washed to remove the dust and soil particles with tap water. The leaves, of selected plants were separated inside laminar flow hood. The leaves were then cut into 0.5 cm segments with the help of sterilized knife and dipped in 70% ethanol for 30 s followed by 2 min treatment with 0.5% sodium hypochlorite and finally washed three times with sterile distilled water (Khan et al. 2008). Internal tissues of the sterilized segments were then exposed by firmly against glass slide and the injured segments were kept on Hagam agar (0.5% glucose, 0.05% KH2PO4, 0.05% MgSO4·7H2O, 0.05% NH4Cl, 0.1% FeCl3, 80 ppm streptomycin and 1.5% agar; pH 5.6 ± 0.2) at the rate of 5 pieces/dish. Imprints of the surface sterilized explants on Hagam medium were used to test the efficiency of surface sterilization protocol (Lubna et al. 2018). Control plates received surface sterilized leaves segments that were not injured. The plates were then incubated at 25°C till fungal colonies appeared from the plant segments (Khan et al. 2009). After the appearance of fungal plugs, the distinctly different fungal plugs were sub-cultured on to Hagem and potato dextrose agar (PDA) medium plates for 7 days at 25°C. Axenic cultures was established following repeated sub-culturing and confirmed through light microscopy. Stock cultures of the isolated endophytes were maintained at −20°C on PDA slants under mineral oil. For production of culture filtrate and biomass the isolated strains were grown in 250 mL Erlenmeyer flask containing 50 mL czapek medium (50 mL; 1% glucose, 1% peptone, 0.05% KCl, 0.05% MgSO4·7H2O and 0.001% FeSO4·7H2O; pH 7.3 ± 0.2) for seven days at 28°C and 120 rpm in shaking incubator (Khan et al. 2008). After a week, fungal biomass was separated from the culture filtrate by filtering fungal cultures through Whatman paper No. 1 and fresh biomass was dried in an oven at 60°C to determine dry weight of the isolate.

2.2. Screening of isolates for plant growth promoting characters including ammonia, IAA and ACC

The isolated strains were screened for the production of ammonia as described by (Chadha et al. 2015). Endophyte was grown in 15 mL Czapek broth medium contained in test tubes, under previously described conditions. After 7 days, culture filtrate (CF), was obtained as mentioned above and 0.5 mL of Nessler’s reagent (Potassium iodide 50 g/50 mL cold water, mercuric chloride 22 g in 350 mL water and 5 N NaOH 200 mL dilute to 1 L) was added after it. Appearance of brown color indicated the presence of ammonia in the CF. The production of IAA was assayed calorimetrically using Salkowski reagent (150 mL concentrated H2SO4, 250 mL distilled water, 7.5 mL 0.5 M FeCl3·6H2O) and was named as Aspergillus fumigatus.

2.3. Morphological identification of isolated strains

The isolated strain was identified morphologically by slide culture method. A block of sterilized PDA was placed on sterilized slide and inoculated from 4 sides. After 72 h of incubation at 28°C the block was removed carefully and slides was stained with lactophenol cotton blue dye (Cotton blue 0.05 g, phenol crystals 20 g, glycerol 40 mL, lactic acid 20 mL, distilled water 20 mL). The stained slides were observed under a light microscope (B-350 OPTIKA), noting hyphae morphology, conidiophores structure and spores morphology. Also, fungal colony morphology on agar plates was observed for the purpose of identification.

2.4. DNA extraction and molecular characterization of the isolated strain

Fresh mycelium was collected and fungal genomic DNA was extracted using the SolGent Fungus Genomic DNA Extraction Kit (Cat No. SGGD4-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 GAT TTG 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) as follows: activation of Taq polymerase at 95°C for 2 min, 35 cycles of 95°C for 1 min, 55°C and 72°C for 1 min each were performed, finishing with a 10-min step at 72°C. The amplification 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).

2.5. Determination of Indole-3-acetic acid in culture filtrate by High Performance Liquid Chromatography

The isolated strain greenish was screened for IAA production by subjecting the CF to High Performance Liquid Chromatography (HPLC) analysis (Khan and Bano 2016). The strain was cultured in czapke broth media containing different concentrations of tryptophan (0, 100, 500 and 1000 µg/mL) at 28°C and 120 rpm in shaking incubator for seven days. After seven days, the cultures were harvested and filtered through Whatman No 1 filter paper. The CF was assed for the presence of IAA with the help of HPLC system, equipped with a differential ultraviolet (UV) detector (set at 280 nm) and a C18 (5 µm; 25 × 0.46 cm) column. Mobile phase was methanol and water (80:20 [v/v]) at a flow rate of 1.5 mL/min. The sample injection volume was 20 µL. Retention times for the analyte peaks were compared to those of authentic internal standards added to the medium and extracted by the same procedures used with fungal cultures. Quantification was done by comparison of peak area (Shahab et al. 2009).

2.6. Effect of yucasin on release of IAA in culture filtrate and biomass production

To check the effect of yucasin (5-(-4-chlorophenyl)-1,2-dihydro-1,2,4-triazole-3-thione), an auxin biosynthesis inhibitor, the isolated endophyte was grown in Czapek medium containing (50 µM) of yucasin under conditions mentioned above. After a week, the cultures was harvested and sieved with Whatman No 1 filter paper. Filtrate was screened for IAA production as described earlier and biomass was collected and kept in 50 mL falcon tube at 60°C in an oven for 5 days. After five days dry biomass was determined as described by (Klamer and Baath 2004).

2.7. Determination of total flavonoids

Colorimetric method based on aluminum chloride reagent was adopted for the screening of the total flavonoids in CF of the isolated endophyte greenish. The endophyte was grown under conditions described above in the presence or absence of cinnamic acid (CA), a flavonoids inhibitor (Ahmad et al. 2014). The CA was added to the culture media (100 µL of 1 mM solution). After 7 days of growth, fungal CF (1 mL), was mixed with 100 µL of 10% aluminum chloride solution and 100 µL of 1 M potassium acetate solution. To the mixture 3.8 mL methanol was added making the final volume up to 5 mL. The test solution was shaken vigorously and absorbance was recorded after 30 min at 415 nm against the blank containing 100 µL of 1 M potassium acetate solution and 3.8 mL methanol. Flavonoids quantification was done by using the straight line equation $Y = 0.0105X + 0.1024$ derived from a standard curve obtained from different concentrations (5, 25, 50, 100, 125, 150, 200, 250, 300, 350 and 400 µg/mL) of quercetin (Sigma Aldrich).

2.8. Determination of total flavonols in culture filtrate

The content of total flavonols in isolated strain greenish was determined as described by Djeridane et al. (2006). To a 500 µL CF 500 µL of aluminum chloride (20%) and 1.5 mL of sodium acetate (50%) were injected. The reaction was established for two hours at 25°C and the optical density checked at 440 nm. The total flavonols were quantified by using standard curve equation $Y = 0.0105X + 0.1024$ as used above for total flavonoids obtained from different concentrations of standard quercitin.

2.9. Effect of cinnamic acid and yucasin on dry biomass of isolated strains

For the determination of dry biomass, the isolated strain was grown in Czapek broth supplemented with 100 µL of 1 mM solution of cinnamic acid (Merck) or 100 µL of (50 µM) yucasin (Sigma–Aldrich) at 28°C and 120 rpm for 7 days. Control treatment was Czapek broth without no cinnamic acid and yucasin. After 7 days the culture was harvested and sieved over Whatman’s No. 1 filter paper to obtain the fungal biomass which was cautiously moved to pre-weighed sterilized falcon tube. The fungal biomass was oven dried at 60°C and its dry biomass was recorded. Flavonoids, IAA and flavonols secretions by the endophyte grown in the presence of inhibitors were also determined. The experiment was repeated three times with three replicates of each treatment every time.

2.10. Root colonization assay

To perform root colonization assay, 40 mL half-strength Hoagland’s solution (Reversat et al. 1999; Barac et al. 2004) was taken in 100 mL beakers with truncated micropipette tips hold in sterilized cardboard and sealed with squash tape. The uniform seedlings were transferred into hydroponic setup (Dardanelli et al. 2010). Maize seeds were surface sterilized using HgCl2 solution (0.1%) and washed three times with distilled water as described above. After surface sterilization, five seeds were put into autoclaved petri plates with two-fold filter paper and incubated at 28°C for 5 days and allowed to germinate. The uniform seedlings were transferred into hydroponic set up and after two days of growth the seedlings were inoculated with endophytes spores suspension in water adjusted to an inoculum density of $10^6$ mL$^{-1}$ spores.

2.11. Inoculum preparation

Fungal spore suspension was prepared by mixing loopfull spores with distill water. Spores density was determined by adding 10 µL of the spore solution to each side of the hemacytometer and counting number of spores on both sides of hemacytometer under a light microscope.

2.12. Determination of fungal colonization in maize roots

To determine fungal colonization in maize root plants were collected after 14 days, washed with tap water, cut into small pieces and stained with lactophenol cotton blue dye for 20 min. After 20 min, the segments were washed and observe under light microscope. The level of colonization
was quantified by plotting the root segments on PDA. Shortly, the root were surface sterilized with 0.1% HgCl2 and washed with distill water and cut into 1 cm fragments. Six root segments for each plant, two from upper part (near to inoculum), two from middle part and two from lower part (near to stem) were kept on clean filter paper and inoculated on PDA plates for one week. After incubation for a week, colonization percentage was determined by dividing number of root segments positive for colonization by total number of root segments studied and multiplied by 100. For comparison and validation of surface sterilization procedure, non-injured root segments were plated on PDA. Also, the root exudates of inoculated and non-inoculated seedlings were collected and screened for the determination of different signaling compounds viz flavonoids (with different time intervals i.e after 1, 3, 7 and 14 days) IAA, and flavonols followed standard procedures as discussed previously.

2.13. Inhibition of flavonoids secretion by plant root with cinnamic acid application

For the inhibition of flavonoids biosynthesis in plants, 100 µL of 1 mM cinnamic acid was applied aerially to each seedling grown in liquid medium. To inhibit flavonoids production by the endophyte, CA was applied in the nutrient solution. Flavonoids were checked in root exudates at different time intervals viz after 1, 3, 7 and 14 days. Endophyte colonization in the roots was determined as described earlier.

2.14. Inhibition of IAA biosynthesis by yucasin

Biosynthesis and exudation of IAA by the root was inhibited by applying yucasin (50 µM) (Nishimura et al. 2014) in the form of foliar spray. Root colonization by the endophyte was assayed in the presence of yucasin to assess the role of IAA in root–endophyte association.

2.15. Exogenous application of Indole-3- acetic acid

For the exogenous application of IAA plants were grown in 40 mL half strength of Hoagland’s solution in 100 mL beaker. After two days of growth in liquid solution, standard IAA at a final concentration of 10 µg/mL was applied through aerial spray on plants (Vandeleur et al. 2014). The seedlings were then inoculated with fungi as described earlier and uninoculated seedlings were used for comparison. After 14 days of growth the seedlings were harvested and various growth constraints via shoot length, root length and dry weights were measured and roots exudates were screened for IAA, flavonoids and flavonols.

2.16. Statistical analysis

The data were analyzed using SPSS for windows 16.0 (SPSS Inc., Chicago, IL, USA). An ANOVA was performed to determine the effect of treatments and error associated with the experiment with three replications and treatments as random effects. To identify significant differences among treatments, a mean comparison was carried out by using Duncan multiple range test ($p = .05$) where the error mean square was used to estimate the standard error of differences between mean.

3. Results

3.1. Isolation and preliminary screening of endophytic fungi

A total of four endophytes were isolated from the leaves of W. somnifera. Out of these isolates, only the strain greenish was capable to produce all the three tested metabolites i.e. IAA, ammonia and ACC (Table 1). The production of IAA, ACC and ammonia hints at growth promotion ability of the strain and hence was selected for further study.

3.2. Morphological and molecular identification of strain A. fumigatus greenish

The isolated strain A. fumigatus greenish had grey or greenish colonies with smooth surface and entire margins (Figure 1(a)). The strain showed rapid growth on PDA. Under light microscope, conidial heads are typically columnar and uniseriate. Conidiophore stalk are short, smooth-walled. Conidia are produced in basipetal succession forming long chains and are globose to subglobose (Figure 1(b)). On the basis of apparent morphological similarity with A. fumigatus, the strain was presumed to be A. fumigatus. This was confirmed.
by molecular identification of the strain using NCBI online tool BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for sequence homology analysis of ITS region of 18S rDNA that revealed 99% sequence similarity with A. fumagitus and 100% query coverage (Figure 2). Furthermore, phylogenetic analysis of ITS sequences of closely resembling endophytes making neighbor-joining tree also grouped our sequence with A. fumagitus. The sequence was submitted to the gene bank under accession number MF503666.

3.3. Determination of IAA in culture filtrate of A. fumigatus

Culture filtrate of the strain A. fumigatus greenish contained 41.6 µg/mL of IAA. The amount of IAA released by the endophyte proportionally increased when subjected to different concentrations of tryptophan (100, 500 and 1000 µg/mL). The culture of endophyte exposed to yucasin (IAA biosynthesis inhibitor) produced significantly lower amount of IAA than the culture grown in the absence of this inhibitor (Figure 3) with a maximum of 31% reduction in IAA amount. Application of CA (flavonoids inhibitor), on the other hand, also negatively influenced the level of IAA in culture filtrate of endophyte. However, its influence on IAA was comparatively lesser than the effect yucasin on IAA revealed by 19% reduction in the amount of IAA produced by the strain under CA treatment as compared to 31% reduction under yucasin treatment.

3.4. Effect yucasin and cinnamic acid on growth of A. fumigatus

Exposure to CA was inhibitory to endophyte growth, while exposure to yucasin also adversely affect growth of endophyte (Figure 4). Upon exposure to CA, 57% loss was evident in dry biomass of the endophyte than that of control. Likewise, yucasin exposed culture also had significantly lower biomass as compared to biomass of untreated culture. For instance, yucasin exposed culture of greenish had dry weight reduced to 22% of their control.

3.5. Determination of total flavonoids and flavonols in culture filtrate of A. fumigatus

Culture filtrate of the isolated strain contained 218 µg/mL flavonoids and 20.9 µg/mL flavonols. Application of CA in culture media inhibited the synthesis of flavonoids and flavonols by 59% and 51% of their respective controls (Figure 5).
3.6. Effect of CA, yucasin, IAA and A. fumigatus on maize growth

Exogenous application of CA in the form of foliar spray retarded shoot length. Also, yucasin treatment (foliar) reduced shoot length of maize seedlings. Yucasin was more potent inhibitor of shoot growth than the CA. Exogenous application of CA in the form of foliar spray reduced shoot length by 27% in comparison to control (Figure 6(a)). Similarly exogenous application of yucasin (IAA inhibitor) in the form of foliar spray reduced shoot length by 32% in comparison to control. Root growth of maize seedlings was negatively influenced by exogenously applied of CA or yucasin either as foliar spray or root entries (Figure 6(b)). For instance, foliar spray or root application of CA retarded root length by 38% and 50%, respectively in comparison to control. Likewise, exogenous application of yucasin in the form of foliar spray caused 30% reduction in root length whereas root treatment of yucasin reduced root length by 31% in comparison to control. Application of both foliar and root entries of CA and yucasin reduced seedlings dry biomass in comparison to control (Figure 6(c)). However, root application of CA inhibited seedlings dry biomass the most leaving it by 33% of the control. On the other hand, yucasin was more hazardous to maize seedlings reducing the dry biomass by 38% of the control seedlings. As we expected exogenous application of phytohormone IAA and endophyte greenish significantly improved growth parameters of maize in comparison to control. For instance, exogenously applied IAA enhanced shoot length by 40%, root length by 22% and dry weight by 19% whereas endophyte greenish improved shoot length by 29%, root length by 37% and dry weight by 61% as compared to control (Figure 6(a)). As noted above, shoot growth declined in seedlings exposed to exogenously applied CA or yucasin. However, endophytes associated seedlings were resistant to these inhibitors and showed normal growth even in the presence of yucasin and CA. As renowned above, seedlings growth was promoted by exogenous application of IAA. However growth of maize seedlings was enhanced further in media containing endophyte in binary combination with IAA or endophyte in binary combination with IAA and yucasin.

3.7. Effect of IAA, yucasin and CA on maize root colonization with A. fumigatus

The endophyte A. fumigatus greenish exhibited remarkable potential to colonize maize roots under hydroponic system (Figure 7). Colonization frequency of greenish in maize root varied among different root zones. Root zone of active division attracted the endophyte with highest efficiency showing a colonization frequency of 80%. However, colonization frequency dropped in the root above this zone where colonization frequency was 65% in zone of elongation and only 20% in the zone of maturation near the stem root junction. Application of IAA in combination with the endophyte had even more pronounced effect on root colonization by the endophyte with colonization frequency to 90% in the zone of cell division, 66% in the zone of elongation and 30% in the zone of maturation. Conversely, foliar and root application of exogenous CA (a flavonoids inhibitor) reduced root colonization frequency to 65% and 57% of the roots not exposed to this inhibitor. Application of yucasin (IAA inhibitor) also negatively influenced the root colonization of greenish strain reducing it colonization frequency to 56% (under foliar application) and 52% (under root application) of the seedlings receiving no yucasin. Interestingly, the foliar mode of application of both the inhibitors proved to be more effective in terms of their effect on root colonization as compared to their root application.

3.8. Effect of CA, yucasin, IAA and A. fumigatus on exudation of flavonoids in maize root exudates

A sharp increase in the accumulation of flavonoids in root exudates was observed after 24 h of inoculation of roots with the endophyte greenish and the increase was maintained during the course of experiment (Table 2). An increase of 70% in exudation of flavonoids was recorded 24 h after inoculation of roots with endophyte greenish in comparison to control. Similarly, higher flavonoids content in comparison to control was recorded 24 h after application in seedlings exposed to exogenous application of IAA. However, exposing seedlings to CA and yucasin (foliar or root) for 24 h did not influence flavonoids concentration in the root exudates (Table 2). However, endophyte associated maize seedlings that received CA and yucasin was different from control seedlings in flavonoids exudation and enhanced exudation of flavonoids when received these inhibitors either in the form of foliar spray or as root treatment. However, 3 days post CA exposure, the seedlings ability to exude flavonoids was drastically reduced in comparison to the control (Table 2). Interestingly, both foliar and root application of CA was inhibitory to the release of flavonoids. The endophyte greenish associated maize seedlings were resistant to the inhibitory effect.
of CA, where more quantity of flavonoids was found in the root exudates as the control. Exposing seedling to CA for 7 days negatively influenced exudation of flavonoids in comparison to control (Figure 6(c)). Interestingly, both foliar and root application of CA was inhibitory to the release of flavonoids by roots. However, inhibitory effect of CA on flavonoids exudation was not more in seedling associated with endophyte greenish. Likewise exposing seedlings to foliar IAA and associated with endophyte greenish had more flavonoids as compared to control.

3.9. Effect of CA, yucasin, IAA and endophyte on exudation of IAA in maize root exudates

Seedlings associated with endophyte greenish or exposed to exogenous IAA as foliar spray exuded significantly increased amount IAA as compared to control. On exposure to CA and yucasin (foliar or root), the seedlings ability to exude IAA was drastically reduced (Table 3) For instance, Exposing seedlings to CA (foliar or root) reduced concentration of IAA 43% and 39% respectively in the root exudates as compared to control.

Figure 6. Effect of endophyte association greenish, IAA (foliar), CA (foliar, F and root applied, R) and yucasin (Foliar or root) on the (a) shoot length, (b) root length and (c) dry biomass of maize seedlings grown hydroponically in Hogland’s solution for 2 weeks. Data are mean of nine replicates from three independent experiments with standard error bars. Bars labelled with different letters are significantly different (Duncan test; p < .05).
In the same way, exposing seedling to yucasin (foliar or root) caused 73% and 71% reduction in exudation of IAA as compared to control. However, IAA production by seedlings treated with CA and associated with A. fumigatus was significantly higher than the ones not associated with endophyte A. fumigatus. Likewise, seedlings association with endophyte relaxed the inhibitory effect of yucasin on maize producing IAA in amount comparable to untreated control. The effect of endophyte association and exogenous application of IAA on maize seedlings was synergistic increasing IAA exudation by 29% as compared to control.

4. Discussion

The growing population has put pressure on the agricultural sector to increase crop productivity and satisfy food needs. To avoid the constant depletion of natural resources and the achievement of sustainable agricultural development, eco-friendly substitutes are available. Among them symbiotic relationship between fungi and crops shows considerable promise because of their effectiveness, specific mode of habitation and their ability to provide multiple benefits (Shulz and Boyle 2005). In present study we isolated an endophytic fungus from leaves of W. somnifera and was identified as A. fumigatus by comparative homology of ITS region near the 18S rDNA gene (Khan et al. 2009). A. fumigatus has been previously reported as symbiotic non-pathogenic class 2 endophyte from different host plants and found to confer resistance in its hosts against environmental stresses (Khan et al. 2011). Fungal endophytes yield bioactive metabolites that promote the plant–endophyte interaction (Strobel 2003). Promotion of plant growth is the most important effect of fungal symbiosis (Hassan et al. 2013). Endophytic fungi promote plant growth by producing various secondary metabolites, including ammonia and plant hormones, particularly IAA (Fouda et al. 2015). Important secondary metabolites including IAA, flavonoids and flavonols, were determined in the culture filtrate of the selected endophyte A. fumigatus greenish. Culture filtrate of the strain contained 41.6 µg/mL of IAA which was comparatively higher than previously reported (Khan et al. 2011). Production of this phytohormone is common among plant growth promoting endophytes and bacterial pathogens (Khan et al. 2018b). For instance, bacterial pathogens including Pseudomonas and Xanthomonas are known to synthesize IAA. However, there are reports that IAA production is an important tool of biocontrol microbes against fungal pathogens including Colletotrichum spp (Yue et al. 2000). Beside this, exogenous IAA has been proved to improve plant growth under normal and stressed conditions (Kaya et al. 2013). Tryptophan is

Figure 7. Effect of (a) yucasin (Y) and CA on the mean percent colonization of maize roots by endophyte A. fumigatus. The inhibitors were applied in the form of foliar spray (F) or in the culture media (R). Other treatments included exogenously applied IAA. The level of colonization was quantified by plotting the root segments on PDA after 14 days of co-culturing. Six root bricks for each plant, two from superior part (near to inoculum) two from middle part and two from inferior part (near to stem) were kept on clean filter paper and inoculated on PDA plates and incubated for one week. After a week colonization percentage was recorded according to the expression. Percent colonization of endophytic fungi = Number of positive segments/ total number of root segments studied * 100.

### Table 2. Effect of different treatments on the concentration of flavonoids in the root exudates of maize seedlings.

| Treatments          | 1 day post treatment | 3 day post treatment | 7 day post treatment | 14 day post treatment |
|---------------------|----------------------|----------------------|----------------------|-----------------------|
| Control             | 91.5 ± 6.3b          | 174.5 ± 30.9d        | 208.5 ± 7.2f         | 213.0 ± 14.4ef        |
| IAA                 | 137.2 ± 22.1cd       | 197.9 ± 32.4e        | 214.2 ± 13.9f        | 222.1 ± 14.3f         |
| Greenish            | 155.9 ± 52.2cd       | 241.5 ± 27.0f        | 275.6 ± 28.7g        | 284.1 ± 8.2g          |
| CA (F)              | 78.2 ± 4.9a          | 88.0 ± 2.9a          | 100.5 ± 2.4ab        | 103.1 ± 6.1ab         |
| CA (R)              | 82.8 ± 3.8a          | 96.4 ± 0.6a          | 108.0 ± 0.3ab        | 111.1 ± 3.5ab         |
| Y (F)               | 91.1 ± 3.4b          | 140.8 ± 17.4bc       | 179.7 ± 6.0cd        | 188.0 ± 3.8cd         |
| Y (R)               | 90.4 ± 2.7b          | 156.6 ± 8.9cd        | 187.5 ± 13.4e        | 195.1 ± 9.7cd         |
| Greenish + CA (F)   | 102.6 ± 4.3b         | 107.5 ± 9.9b         | 136.0 ± 10.5c        | 155.9 ± 37.6c         |
| Greenish + CA (R)   | 108.0 ± 2.7b         | 134.7 ± 12.5bc       | 153.7 ± 32.5c        | 175.3 ± 16.5cd        |
| Greenish + Y (F)    | 103.4 ± 2.7b         | 163.0 ± 9.5cd        | 186.2 ± 3.8e         | 189.1 ± 8.4d          |
| Greenish + Y (R)    | 108.7 ± 0.6b         | 180.4 ± 0.1e         | 184.9 ± 4.3de        | 194.4 ± 7.0d          |
| Greenish + IAA      | 185.2 ± 3.8e         | 245.8 ± 1.0f         | 251.6 ± 0.9fg        | 266.4 ± 1.2g          |

Notes: Seedlings were grown for 14 days in Hoagland’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), Cinnamic Acid (CA) (100 µL of 1 mM solution) and Yucasin, foliar and root entries. Roots exudates were collected 1, 3, 7 and 14 days post treatment and subjected to flavonoids determination.
considered as a precursor for IAA biosynthesis and its addition of in culture medium enhances IAA production (Ahmad et al. 2005). In our isolate, IAA production occurred via both tryptophan independent and tryptophan-dependent pathways as the production of this phytohormone was significantly enhanced in the media containing tryptophan. Among fungi, the IPA pathway is the commonly used pathway for IAA biosynthesis (Hilbert 2012), although the TAM and bacterial IAM pathways are also reported in Usitago and Colletotrichum sp. (Hilbert 2012). In addition, TRP-independent IAA biosynthetic pathways have recently been shown in yeast (Rao et al. 2010). Exposure of our isolate to yucasin induced remarkable reduction in IAA production indicating the presence of IPyA pathway. Also, yucasin inhibited the growth of the endophyte. The reduction in IAA concentration is due to inhibition of IAA biosynthesis by yucasin in endophyte A. fumigatus.

The isolate was also producing a significant amount of flavonoids, a class of phenolic compounds some of which have been implicated in signaling during plant–microbe interaction (Peters and Verma 1990; Hassan and Mathesius 2012). The function of flavonoids as signals is well established for legumes and Rhizobium symbiosis (Vierheilig and Piché 2002; Sugiyama et al. 2007).

Flavonoids protect plants from various biological and abiotic stresses and play an important role in the interaction between plants and the environment (Pourel et al. 2007). Flavonoids play an important role as signal molecules in symbiosis process (Dixon and Steele 1999). The plant control development of fungus in the arbuscular mycorrhizal (AM) symbiosis before and after the contact and infiltration of the fungus inside the root. Flavonoids can influence the pre infective state of the AM fungus (Akiyama et al. 2002; Steinkellner et al. 2007), and some of these compounds can increase the level of AM colonization of roots. Our isolate released a greater amount of flavonoids (218 ± 8.7 µg/mL) in the culture filtrate than previously reported in the cultures of Aspergillus nidulans (11.62 ± 1.4 µg/mL) and Aspergillus oryzae (12.6 ± 3.7 µg/mL) (Qiu et al. 2010). Metabolite profiling of mycorhizal roots of Medicago truncatula based essential accretion of flavonoid at different phases of mycorrhization (Schlemann et al. 2008). Moreover, high levels of phenylammoniylase (PAL) and chalcone synthase (CHS) transcripts were identified in the root of M. trunculata colonized by Glomus versiforme (Harrison and Dixon 1994). In Trifolium repens, the different arrangement of the flavonoids extracted from shoots and roots grown with and without the AM fungus clearly shows that the metabolism of these molecules is strongly affected when the plant is AM colonized. Quercetin, acacetin and rhamnetin accumulated in roots of inoculated plants, whereas they were not detected in non-inoculated plants (Ponce et al. 2004). The flavonoids daidzein and genistein were presenting root and seed exudates of soybean respectively (Graham 1991; Dardanelli et al. 2010). The isolated strain was able to colonize maize roots and enhanced shoot and root length and biomass. Flavonoids have been shown to accumulate in the plant host during the process of mycorrhization. It was observed that c-glycosyl flavone accumulates in phosphate-deficient melon roots and it was observed that this compound increased mycorrhization, suggesting thus that this c-glycosyl flavonoid is involved in the regulation of mycorrhizal colonization. The application of exogenous IAA in combination with fungus significantly increased the shoot length and root colonization by isolated strain. Additionally, the efficiency of root colonization by the isolated strain was higher in part of root near to inoculum sprayed with IAA and inoculated with endophyte and also in roots inoculated only by endophyte.

Cinnamic acid inhibits root growth by enhancing the biosynthesis of lignin at the expense of flavonoids in plant resulting in enhanced lignification of root cells making root recalcitrant to growth. We hypothesized that exogenous application of this organic acid will reduce the biosynthesis and/or exudation of flavonoids either by shifting flavonoids biosynthesis pathway to lignin biosynthesis or hardening root to check flavonoids secretion (Ferro et al. 2015). Interestingly, exogenously application of cinnamic acid on the aerial parts of maize seedlings reduced root growth and exudation of flavonoids as well as IAA. Additionally, the ability of such roots to attract A. fumigatus for colonization was greatly impaired as evident from a low number of fungal hyphae colonized on them.

Inability of the endophytic A. fumigatus to colonize in the root exuding lesser amount of flavonoids and IAA and failure of non-induced maize flavonoids would suggest that fungal flavonoids and IAA has no role in the endophyte–root interplay and subsequent colonization. However, this assumption was not true. Inability of endophyte to secrete flavonoids also negatively influenced root–endophyte interaction as evident by the low abundance of endophyte in the root tissue. Also, flavonoids exudate by endophyte associated root (induced roots) are often remarkably different than the flavonoids species released by the non-endophyte roots (Steinkellner et al. 2007).

Application of IAA in the form of aerial spray on corn seedlings enhanced the ability of roots to attract fungus and their subsequent colonization in the roots. The flavonoids contents increased in root exudates sprayed with IAA and inoculated with endophyte. Previously, it was shown that exogenous application of IAA restored in vitro growth as well as the efficiency of colonization in rice roots by mutant Nostoc spp (Hussain et al. 2015). To verify the role of IAA in root–endophyte interaction, IAA biosynthesis was inhibited in plant by foliar application of yucasin (Nishimura et al. 2014). Seedlings exposed to yucasin released significantly low amount of IAA confirming the inhibition of its biosynthesis. Interestingly, low IAA exuding maize roots were least colonized by the endophyte A. fumigatus greenish pointing a role of this phytohormone in

### Table 3. Effect of different treatments on the concentration of IAA in the root exudates of maize seedlings.

| Treatments | IAA (µg/mL) |
|------------|-------------|
| Control    | 6.9 ± 0.8c  |
| Cinnamic acid (Foliar) | 3.9 ± 0.7b |
| Cinnamic acid (Root entries) | 4.2 ± 0.7b |
| Yucasin (Foliar) | 1.8 ± 0.0a |
| Yucasin (Root entries) | 2.0 ± 0.0a |
| IAA         | 1.0 ± 1.6d  |
| greenish    | 11.8 ± 2.1e |
| greenish + Cinnamic acid (F) | 8.3 ± 0.3c |
| greenish + Cinnamic (R) | 9.1 ± 0.2c |
| greenish + Yucasin (F) | 4.5 ± 0.1b |
| greenish + Yucasin (R) | 5.4 ± 0.6b |
| greenish + IAA | 13.1 ± 2.1f |
| greenish + IAA + Yucasin | 9.6 ± 1.1d |
plant–endophyte interaction. To further dissect the mechanism, IAA biosynthesis was inhibited in the endophyte by applying yuca in the root microbe interface in the Hoagland’s solution and colonization was determined. Again, colonization was reduced significantly indicating that IAA crosstalk is involved in the establishment of plant–endophyte association.

5. Conclusion

The endophyte A. fumigatus greenish promote the growth of maize seedlings under hydronic condition. Its ability to generate and perceive flavonoids and IAA is responsible for colonizing maize roots and establishing beneficial endophytic associations. Inhibition of flavonoids and IAA by either of these two partners limits their ability to interact and carry out symbiotic associations.

Disclosure statement

No potential conflict of interest was reported by the authors.

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