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# In Vitro Maize Growth Promotion by Endophytic *Fusarium Oxysporum* WLW

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## ABSTRACT

Fungal endophytes are well-unknown for their potential to improve plant growth and health by production of bioactive compounds including indole-3-acetic acid (IAA) and ammonia. In current study we isolated an endophytic fungus wlw from leaves of *Watheniasomnifera* growing under xeric conditions. The isolate was identified as strain of *Fusarium oxysporum* by sequencing internal transcribed spacer regions (ITS) of 18S rDNA and phylogenetic analysis. Screening of culture filtrate (CF) revealed that the strain was capable to produce IAA and ammonia in considerable amount. Application of CF of the strain significantly enhanced maize growth demonstrated by higher shoot and root length and fresh and dry plant biomass as compared to the control plants supplied with endophyte free medium or water. In conclusion, *F.oxysporum* wlw has significant potential of plant growth promotion and hence recommended for field trials as potential candidate for use as bio fertilizer to improve plant growth and yield in sustainable agriculture.
**KEY WORDS:** endophytic fungi, plant growth promotion, *Fusariumoxysporum* IAA, ammonia

## INTRODUCTION

Endophytic fungi are found asymptomatically in plant tissues in virtually all plant species [1]. The interaction between endophyte and host is mutualistic or neutral and possibly on different hosts and according to environmental conditions [2]. Endophytes are microorganisms (fungi or bacteria) that are widespread in plant tissues, inhabiting their host asymptomatically. A symbiotically relationship is maintained between host and endophyte throughout the life cycle of the host, beginning with germination of the seed and persist till seed set[3]. These fungal symbionts can profoundly affect plant ecology, adaptability and evolution [4], and are important for community structure and associated biodiversity [5]. Endophytic fungi provide a number of benefits to the host plants in various ways, such as promotion of growth, protection against diseases and pests, and augmenting absorption of minerals**.** Host colonization by endophytic fungi enhances the ecological adaptability of the plant by improving its tolerance against the biotic and abiotic stresses [1]. Such benificial endophytes are the members of a large family of organisms known as plant growth promoting microorganisms (PGPMs). Ability of these endophytes to mobilize insoluble soil phosphorus, improve nitrogen availability and produce phytohormones including IAA make them excellent candidates to be used as bio-fertilizers [6]. Plants and microorganisms, including bacteria, algae and fungi, can yield IAA [7]. The role of microbial IAA in plant-microbe communications has recently received increasing attention. In addition, several studies have shown that IAA is a signaling molecule in microorganisms affecting gene expression in numerous microorganisms [8]. They can affect vital physiological features of plant protecting it against biotic and abiotic stresses[9,10]. Under extreme environmental conditions, endophytes may secrete biologically active compounds such as plant hormones that can bring greater benefits to host plants [9], while on the other hand they are environment friendly and can be introduced for commercial application. Fungi may play an important role in plant survival by enhancing nutrient uptake and producing growth promoting metabolites such as gibberellins and auxins. *Penicillium* and *Trichoderma* strains are known to produce a number of beneficial compounds to inhibit pathogens [11] and stimulate plant growth by producing plant hormones [12] and/or degradation of complex substrates [13]. Likewise, *Penicillium* and *Aspergillus* have been reported to produce gibberellins, which are growth regulators in higher plants [12,14]. The current study was aim to isolate endophytic fungi from leaves of *Watheniasomnifera* and to screen the isolates for production of plant growth promoting substances (IAA and ammonia) and their potential for *in-vito* plant growth promotion.

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## MATERIALS AND METHODS

### Plant material and isolation of endophytic fungi

The healthy leaves of *Watheniasomnifera* were collected from drought stress area of district Mardan. The plant materials were brought to laboratory in sterile polythene bags and were processed within 24 hours to diminish the risks of contamination. The collected leaf materials were rinsed with tap water and were then surface sterilized with 5% sodium hypochlorite solution for 5 seconds followed by 95% ethanol for 3 min and finally rinsed 5 times with autoclave double distill water and were air dried under sterile condition to remove excess moisture. After surface sterilization, the root materials were cut approximately into 0.5 cm pieces using a flame sterilized scalpel. About 5 to 6 segments were placed on Hegam medium plates (0.5% glucose, 0.05% $KH_2PO_4$, 0.05% $MgSO_4.7H_2O$, 0.05% $NH_4Cl$, 0.1% $FeCl_3$, 80ppm streptomycin and 1.5% agar; pH 5.6±0.2) for one week (Hamayun et al., 2010). For purification the developing fungal plugs were grown on potato dextrose agar (PDA) medium plates. For production of culture filtrate and biomass, the purified fungus was then grown in 250 mL flask containing czapekbroth 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 [15]. Colonization frequency (CF %) of the isolated strain was determined as described previously [16,17]. Colonization frequency was equal to number of segments colonized by endophyte/total no of segments observed*100.

### Screening of isolates for plant growth promoting characters including ammonia and IAA

The isolated strains were screened for the production of ammonia as described earlier [18]. Endophytes were grown in 15 mL czapek broth medium contained in test tubes, under previously described conditions. After 7 days, CF was obtained as mentioned above and 0.5 mL of Nessler's reagent was added after to it. Appearance of brown color indicated the presence of ammonia in the CF. For IAA production salkowski reagent tests was adopted [19].

### 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. SGD64-S120; SolGent Co., Daejeon, Korea) as described previously [20]. 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. In a 30 µL reaction mixture, PCR was performed using 20 ng of genomic DNA as a template using EF-Taq (SolGent, Korea) as follows: Taq polymerase was activated at 95°C for 2 minutes and 35 cycles of 95°C for 1 minute each, 55°C and 72°C for 1 minute, completing the 10-minute 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).

### Plant growth promotion assay

Fungal inocula were prepared by growing the endophytes in Czapek broth under previously described conditions. The culture was harvested after seven days by centrifugation to separate the pure CF and mycelium. Maize seeds were dipped in water for 30 min and then surface sterilized with 0.1% mercuric chloride solution and finally rinsed 5 times with sterilized distilled water. Filter paper was cut according to the shape of petri plates. Two-fold filter paper was placed in petri plates and autoclaved. To avoid contamination, surface sterilized seeds were placed in autoclaved petri plates with the help of sterilized forceps and were allowed to germinate. After germination, the seedlings were transferred to autoclaved petri plates as described earlier [21]. Five plants per treatment were grown for two weeks. The freeze-dried CF was diluted with 1 mL autoclaved distilled water. Each plant received 10 µL of CF at two leaf stage.For comparison, two kinds of control plants received (i) of endophyte-free medium and (ii) distilled water. After two weeks of growth different growth characteristics such as shoot length, root length and fresh and dry weight was determined.

### Statistical analysis

Software SPSS for windows 16.0 (SPSS Inc., Chicago, IL, USA) was used to compare means by one-way analysis of variance (ANOVA) and Duncan multiple range test ($p = 0.05$).

## RESULTS AND DISCUSSSION

### Isolation and preliminary screening of endophytic fungi

A total of 3 endophytes were isolated from the leaves of the selected plants. two plants were used to obtain 15 leaves segments yielding 3 endophytes after an incubation period of 7 days. Dominant strain was wlw1, sprouted out from 9 root segments showing highest colonization frequency (65%) of the isolated strains (Table 1). The strains wlw, wlw2 were least common showing (15%) colonization frequency. Only the wlw strain wasfound positive for production of both IAA ammonia. The production of IAA, ammonia suggested that wlw may be suitable candidates for plant growth promotion and was therefore selected for further study. Fungal endophytes yield bioactive metabolites that promote the plant endophyte interaction [22]. Promoting plant growth is the most important effect of fungal symbiosis [23], where endophytic fungi promote plant growth by producing various secondary metabolites, including ammonia and plant hormones, particularly IAA [24].

### Molecular identification of the selected strain

To identify the strain, its DNA was extracted for subsequent amplification and sequencing ITS region. Sequence of the ITS region near the 18 S rDNA was obtained and 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 that of *Fusariumoxysporum*. Identification of the strain was 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 strain with *Fusariumoxysporum* (Figure 1). Sequence was submitted to GenBank under the accession No MH005071.

### Plant growth promotion assay

Maize seedlings grown in Petri plates for one week were exposed to 10µL of filter sterilized fungal culture filtrate (obtained by growing endophytes in Czapek broth for 7 days). For comparison, maize seedlings were treated with same volume ofendophytes free czapek medium and $dH_2O$ [14]. Maize seedlings in all the sets were allowed to grow for 7days and growth parameters were then recorded. All the growth parameters including shoot and root length, fresh and dry weight were significantly enhanced by endophyte wlw in comparison to endophytes free czapek medium and water applied controls (Figure 2a and b). The strain wlw improved shoot growth by two fold in comparison to endophyte free medium control. Previously [25] reported thatendophytic*F. oxysporum* increase plant height 11.3% which was lower than increase in shoot length caused by our isolate.Also root growth was further enhanced by the endophyte and maize seedlings exposed to their culture filtrate had roots which 26% longer than the media control respectively. Similarly, Czapek media showed stimulatory effect on the production of fresh biomass by maize seedlings. The stimulatory effect of Czapek medium on maize fresh biomass was further enhanced by the secondary metabolites of endophyte. For example, CF of wlw caused maize seedlings to produce 31%, more fresh biomass than the media control. BlankCzapekmediumalsoenhancedmaizedryweightthanthewatercontrol (Figure 2b). Indole acetic acid as an essential compound for the growth and development of shoot and roots, many microorganism including plant growth promoting rizobacteria (PGPR) produce IAA [26]. Similarly seedling dry biomass was further enhanced by endophyte wlw by 62% in comparison to endophytes free czapek medium.Previously [27] also observed the positive effect of endophyte on banana growth, they reported that endophytic fungi *Fusarium oxysporum* increased height of plant, pseudostem diameter and number of leaves. Enhanced growth through endophytes may be the result of phytohormones produced by fungal endophytes such as in maize [28].

**Table 1. Characterization of endophytic fungi isolated from *W. somnifera* for plant growth promoting characters including IAA and ammonia production along with their colonization frequency.**

| Plant | Strains | IAA | Ammonia | Colonization % |
|---|---|---|---|---|
| ***Watheniasomnifera*** | Wlw1 | + | - | 60 |
| ***Watheniasomnifera*** | Wlw | + | + | 15 |
| ***Watheniasomnifera*** | Wlw2 | + | - | 15 |

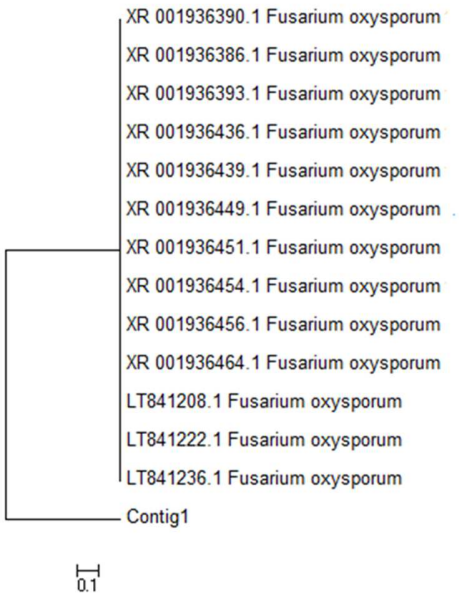


**Figure 1. Phylogenetic tree constructed with neighbor joining method with 18S rDNA sequence (ITS region) of *Fusariumoxysporum*. Fungal isolate wlw formed a sub clade with *Fusariumoxysporum*.**

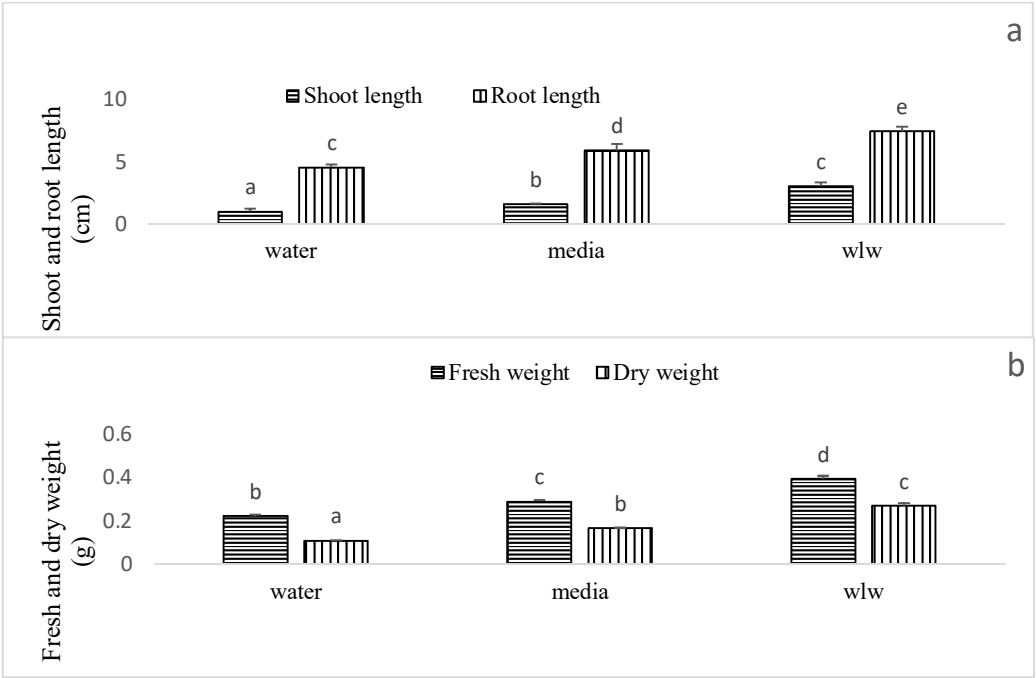


**Figure 2. Effect of endophyte culture filtrate on the a) shoot length and root length (b), fresh biomass and dry biomass of maize seedlings grown in petri plates for 2 weeks. Data are mean of 9 replicates from 3 independent experiments with standard error bars. Bars labelled with different letters are significantly different (Duncan test; $p<0.05$).**

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