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Quantitative Confrontation Test: Effect of Atlantis® OD 42 on *Trichoderma asperelloides* and *Fusarium graminearum* Interactions

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Abstract: Plant residues are important in the integrated disease management against fusarium head blight (FHB). This study was performed to predict the *in situ* effects of Atlantis® OD 42 herbicide on the interaction between a biological control fungus (*Trichoderma asperelloides* Samuels T-92) and the mycotoxigenic pathogen (*Fusarium graminearum* Schwabe) by *in vitro* confrontation test. Collectively, Atlantis® OD 42 was found positively effective in pathogen control. Here, the modified confrontation test was applied with new parameters to analyze the detailed effect of Atlantis® OD 42) on the fungus-fungus interactions. The study revealed the negative effect of Atlantis® OD 42 on the pathogen growth and gave a clue to its negative impact on mycotoxin efflux (an important determinant of the pathogen virulence). The effect of herbicide on the biological control of the pathogen was discussed. This is the first introduction of the quantitative confrontation test after its modification.

Keywords: Biocontrol; Mycotoxin; Sulfonylureas; Toxin; Virulence

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1. Introduction

Fusarium head blight (also known as fusarium ear/ head scab) is one of the most important diseases of small grain cereals throughout the world. The disease caused by *Fusarium* spp., not only results in reduced quantitative yield but also in the contamination of cereal grains to the range of mycotoxins of various biochemical origins: trichothecenes such as nivalenol (NIV), deoxynivalenol (DON), their more toxic acetylated derivatives, estrogenic mycotoxins such as zearalenone and its derivatives, fumonisins such as fumonisin B1, and others. Different roles in the defense of the territory (Pitt, 2002), pathogenicity (Desjardins and Hohn, 1997, Ismaiel and Papenbrock, 2015), and virulence (Zeilinger et al., 2015) have been attributed

to these toxins. Hyphal tip, although not the only active part of the hypha (Read, 2011), is metabolically the most active part actively involved in fungal growth and the efflux of fungal metabolites (Sen et al., 2013). So, any factor that affects fungal growth can also potentially affect fungal efflux of mycotoxins. However, a reduction in primary metabolism and fungal growth may result in increased secondary metabolism and efflux of mycotoxins (Calvo et al., 2002). There is little information on the antifungal activity of herbicides and the impact of HRAC B herbicides on plant diseases and pathogens. One of interesting research subjects in this field is the direct antifungal potential of some herbicides. Such herbicides can play a partial role in the integrated management of plant diseases. *Fusarium* spp. are saprobically highly potent fungi. At least some *Fusarium* species degrade and use various sources of polymerized organic matters, grow fast, secret a range of toxins, and produce different types of asexual and sexual propagules. Antifungal herbicides can help in the management of the debris of herbicide-treated weeds that otherwise could serve as frequently accessible substrate for pathogen saprobic growth and reproduction required for the subsequent outbreak of the disease. The fungi are of multinucleate hyphal compartments, colonial growth and pseudoparenchymatous fungal tissue, high saprobic potential reflective of their rich arsenal of extracellular enzymes, hyaline thalli and uni, bi-, and multi-cellular hyalospores, and at least some species can produce sexual spores, chlamydospores, and/ sclerotia. Therefore, *Fusarium* spp. are also among the fungal pathogens that can fast adapt or develop resistance to fungicides. Thus, antifungal herbicides can help to fungicide resistance management. Despite of the importance of the subject, there is little information on the antifungal activity of HRAC B herbicides. One of the commercial herbicidal products applied for the control of wheat field broad-leaf as well as grass weeds is Atlantis® OD 42 from Bayer Crop Company. The product is based on two active ingredients mesosulfuron (10 g ai L^{-1}) and iodosulfuron (2 g ai L^{-1}) immunized for wheat by the addition of mefenpyr-diethyl (30 g L^{-1}) compound (http://www.syria.cropscience.bayer.com/en/Products/Herbicides/Atlantis-OD-42.aspx). As another part in the integrated control of FHB, *Trichoderma* spp. are regarded as valuable biological control fungi (BCFs). *Trichoderma* spp. display a complete range of BC activities that includes indirect impacts exerted by competition for nutrients and space, modification of environmental conditions, or promotion of plant growth and plant defensive mechanisms, as well as by direct effects through antibiosis and mycoparasitism (Benítez et al., 2004). *Trichoderma* spp. can turn on plant resistance pathways responsible for systemic acquired resistance (Vitti et al., 2016), and induced systemic resistance (Djonović et al., 2007). Additionally, *Trichoderma* spp. are known as the repressors of *Fusarium* toxin production (Błaszczyk et al., 2017; Rojo et al., 2007), and effective detoxifiers of *Fusarium* toxins via glycosylation (Tian et al., 2016) or reduction and conversion to sulfated forms (Tian et al., 2018). Therefore, the present study was performed in order to study the possible impact of Atlantis® OD 42 as the representative of HRAC B herbicides on the interaction of *Trichoderma asperelloides* T-92 and *F. graminearum* Schwabe.

2. Materials and Methods

2.1. Fungal isolates

Iranian isolate of *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* [Schweinitz] Petch) and *Trichoderma asperelloides* Samuels (T-92) were obtained from the collection of Plant Pathology Laboratory, Faculty of Agriculture, Agricultural Sciences and Natural Resources University of Khuzestan (ASNRUKH), Mollasani, Iran. *F. graminearum* isolate had already been isolated from scabbed wheat ear, morphologically identified and then confirmed by molecular studies in Lorestan University, Khorram Abad, Iran. The isolate of *T. asperelloides* had been isolated from terragon (*Artemisia dracunculus subsp. sativa*) roots, Azarbaijan, Iran, kindly identified and confirmed as *T. asperelloides* by macroscopic, microscopic and molecular studies by Dr. Irina S. Druzhinina at the Technical University of Vienna.

2.2. In vitro confrontation test

Four-day-old colonies of *T. asperelloides* T-92 and *F. graminearum* isolates grown on potato dextrose agar (PDA) plates and incubated at 26°C in dark conditions were used in the inoculation of PDA plates considered for the quantitative confrontation test (Pakdaman et al., 2013). The treatments included A0 (PDA as the basal medium and with no addition of Atlantis® OD 42), A50 (Atlantis® OD 42 amended to the final ratio of 50 parts per million or 50 mg L^{-1}), A500 (Atlantis® OD 42 amended to the final ratio of 500 mg L^{-1}), and A5000 (Atlantis® OD 42 amended to the final ratio of 5000 mg L^{-1}). Four replicates were considered per treatment, and the experiment was repeated. Each treatment was of its own fungal controls (inoculated with only a single fungal isolate) as in the method previously described (Pakdaman et al., 2013). All plates were incubated at 26°C under dark conditions. Here in this research, new parameters were additionally defined, calculated, and compared. The parameter *D* was defined as the difference between the *Z* and *C* values (Equation 1; in day; Pakdaman et al., 2013) obtained per plate per treatment:

$$D = Z - C \quad (1)$$

It was the temporal period since contact till the end of complete overgrowth of the dominant fungus as revealed by its sporulation in the farther edge of the colony of the defeated fungus. Accordingly, the parameter implicating the resistance of a pathogenic fungus to a BC fungus, *R* (Pakdaman et al., 2013) was broken to R_1 and R_2 sub-parameters in order to analyze the rate of resistance of the pathogen exhibited in pre-contact and post-contact phases. R_1, the pre-contact resistance, was calculated as follow (Equation 2):

$$R_1 = \frac{C}{M} \quad (2)$$

Similarly, R_2, post-contact resistance, was calculated as follow (Equation 3):

$$R_2 = \frac{D}{M} \quad (3)$$

The parameter *X* (in mm) was defined as the linear equatorial growth of the BCF in the single cultures (only including the BCF) on each treatment medium in the temporal point of contact between the BCF and the pathogenic fungus taken placed in dual confrontation cultures of the same treatment.

The other parameter, *T* (in mm) was defined as the linear equatorial growth of the BCF measured in dual confrontation cultures in the temporal point of contact between two fungi. The parameter O_{BCF} was defined in order to investigate the negative impact of a pathogen on a BCF in the pre-contact phase of a fungus-fungus interaction, where the fungi were mostly affected by environmental conditions such as medium composition, and the metabolites produced and secreted by themselves and by another fungus in a confrontation culture. The parameter was defined and calculated as follow (Equation 4):

$$O_{BCF} = \frac{T}{X} \quad (4)$$

Therefore, O_{BCF} was just a ratio without dimension and unit. Indeed, O_P is the simplified form of the following formula (Equation 5):

$$O_{BCF} = \left({}^{T}/_{C}\right) \Big/ \left({}^{X}/_{C}\right) \quad (5)$$

The parameter A_P (Equation 6; in mm day^{-1}) was defined in order to get more information on the antibiotic effect of *F. graminearum* on the BCF:

$$A_P = \frac{X-T}{C} \quad (6)$$

Similarly, to get more detailed information on the pathogen, and the antibiotic effect of the BCF on the pathogen, other parameters were introduced here in this research: The parameter *G* was introduced as the linear equatorial growth of the pathogen in the plates similarly inoculated and incubated as axenic control culture of pathogen that was measured in the time point when two fungi came into contact to each other in confrontation plates. The antibiotic effect of *T. asperelloides* T-92 on the pathogenic fungus was calculated by the introduction of another parameter A_{BCF} (in mm day^{-1}) defined as follow (Equation 7):

$$A_{BCF} = \frac{G-p}{C} \quad (7)$$

The calculated data were normalized by the following formula (Equation 8), and applied in analysis of variance:

$$\text{Normalized } A_{BCF} = \cos A_{BCF} \quad (8)$$

Another parameter to investigate the pre-contact phase of a fungus-fungus interaction, O_P was defined as a ratio with no unit and calculated for the pathogenic fungus as follow (Equation 9):

$$O_P = \frac{p}{G} \quad (9)$$

Indeed, O_P is the simplified form of the following formula (Equation 10):

$$O_P = \left({}^{p}/_{C}\right) \Big/ \left({}^{G}/_{C}\right) \quad (10)$$

Two parameters were defined in order to study the pre-contact inhibitory effect of either fungus on the mycelial growth of another, pathogen growth inhibition percentage in the temporal point of contact between both fungi (PGI_C calculated as in Equation 11; in percent), and BCF growth inhibition percentage in the temporal point of the contact between both fungi ($BFGI_C$, in percent) calculated as in following lines:

$$PGI_C = \frac{100\,(G-p)}{G} \quad (11)$$

The values calculated for PGI_C were transformed by following formula (Equation 12) and the normalized data were then used in analysis of variance:

$$\text{Normalized } BFGI_C = \left(\frac{180}{3.14}\right) Arc\sin\sqrt{\frac{|25+BFGI_C|}{100}} \quad (12)$$

The values of *BFGIC* were calculated by following formula (Equation 13) and the calculated data were then applied in the analyses of variance:

$$BFGI_C = \frac{100\,(X-T)}{X} \quad (13)$$

Finally, the percentage of pathogen growth inhibition (*I*, in percent) as it is traditionally calculated, was calculated following the Equation14:

$$I = \frac{100\,(\emptyset-p)}{\emptyset} \text{ or } \frac{100\,(T)}{(T+p)} \quad (14)$$

, where *Ø* was the equatorial linear distance between the internal edges of two inoculation discs applied in confrontation plates. The experiment was planned as a completely random design, and the analysis of variance was made using SAS software, version 9.2 (SAS, 2009). Tukey's honestly significant difference (Tukey's HSD) test was applied in order to compare the mean of the treatments.

3. Results

Amendment of PDA media with the herbicide Atlantis® (applied at the final rate of 5000 mg L^{-1}) totally inhibited colonial growth of both fungi, indicating the strong antifungal activity of the herbicide in high concentrations. Therefore, the data obtained from only three treatments were statistically analyzed. With the parameters *C, Z, M, D, R, R_1, R_2,* the calculated rate of coefficient of variances were 0.00, therefore for further information, the reader is referred to the Table 1. The analysis of variance of *G* values indicated highly significant differences among *G* values in the studied treatments ($F_{2,9}$ = 47.26***; CV = 12.31%; P < 0.0001). While there was no significant difference between mean *G* values obtained with A0 (control) and A 50, a considerably higher average *G* value was obtained with the A500 plates of axenic *F. graminearum* cultures applied as controls of the pathogen (Table 2). There were also statistically highly notable discrepancies among *p* values obtained in confrontation plates of the investigated treatments ($F_{2,9}$ = 42.68***; CV = 17.26%; P < 0.0001). Comparison of mean *p* values by Tukey's studentized range (HSD) test (α = 0.01) indicated that very significantly higher *p* values were obtained with A500 (Table 2). The analysis of variance of the values calculated for the applied parameter of O_P indicated that there were significant differences among O_P values in the studied treatments ($F_{2,9}$ = 8.76**; CV = 9.01%; P = 0.0077). The highest mean O_P value was calculated for A500, and the lowest mean O_P value was obtained with A0 (control), while A50 resulted in an intermediary average O_P value statistically indifferent from either A500 or A0 (Table 2). The analysis of *X* values indicated highly significant differences among the growth rates in different ratios of the herbicide ($F_{2,9}$ = 27.98***; CV = 4.59%; P = 0.0001).

Table 1. Average values of the parameters *C* (time required for two colonies to come to contact on the equatorial line interconnecting the centers of two inoculation discs), *Z* (time required to fulfill equatorial overgrowth of the dominant fungus on an inner part of the colony of a failed fungus), *M* (time required after inoculation for the full growth of the dominant fungus on a control axenic culture plate), *D* (*D* is calculated as *Z* - *C*), O_{BCF} (the negative impact of the failed fungus on a the dominant fungus growth in the pre-contact phase of a fungus-fungus interaction), A_{BCF} (the antibiotic effect of the dominant fungus on the failed fungus), *R* (resistance of the failed fungus calculated as $Z\,M^{-1}$), R_1 (Pre-contact resistance calculated as C M^{-1}) and R_2 (Post-contact resistance calculated as D M^{-1}).

Treatment	**Mean *C* value (day)**	**Mean *Z* value (day)**	**Mean *M* value (day)**	**Mean *D* value (day)**	**Mean O_{BCF} value**	**Mean A_{BCF} value (%)**	**Mean *R* value**	**Mean R_1 value**	**Mean R_2 value**
Atlantis® OD 42 (500 mg L^{-1})	9 (±0)*	25 (±0)	13 (±0)	16 (±0)	0.93 (±0.0200)	-0.33 (±0.06)	1.92 (±0)	0.69 (±0)	1.23 (±0)
Atlantis® OD 42 (50 mg L^{-1})	2 (±0)	5 (±0)	3 (±0)	3 (±0)	0.97 (±0.0047)	0.00 (±0.20)	1.67 (±0)	0.67 (±0)	1.00 (±0)
Atlantis® OD 42 (0 mg L^{-1})	2 (±0)	5 (±0)	3 (±0)	3 (±0)	0.94 (±0.0048)	0.38 (±0.24)	1.67 (±0)	0.67 (±0)	1.00 (±0)

* Standard error was indicated in parenthese by ±.

Comparison of mean *X* values by Tukey's studentized range (HSD) test (α = 0.01) indicated that the herbicide Atlantis® was of no considerable effect on *X* value when it was applied at the final rate of 50 mg L^{-1}, however, the herbicide led to more reduced *X* value at the final rate of 500 mg L^{-1} (Table 2). Furthermore, the analysis of the measured values of the parameter *T* indicated highly considerable differences in the growth levels of dual cultured BCF, *T. asperelloides* T-92 measured in the temporal point of the contact taken place in confrontation plates ($F_{2,9}$ = 99.78***; CV = 2.64%; P < 0.0001).

Table 2. Tukey's studentized range test-based comparison (α = 0.01) of average values of the parameters *X* (the linear equatorial growth of the dominant fungus in the control axenic cultures in the temporal point of contact between two fungi in dual confrontation cultures), *T* (the linear equatorial growth of the dominant fungus measured in dual confrontation cultures in the temporal point of contact between two fungi.), *p* (the radial distance of failed fungus colony growth between the edge of the inoculation disc and the marginal point of the colony located on the presumed diagonal line connecting centers of two discs in a confrontation plate), *G* (the linear equatorial growth of the failed fungus in the plates similarly inoculated and incubated as axenic control culture of failed fungus measured in the time point of contact between two fungi in confrontation plates), O_P ($p\ G^{-1}$, a ratio to investigate the inhibitory effect of the dominant fungus in the pre-contact phase of a fungus-fungus interaction), $BFGI_C$ (dominant fungus growth inhibition percentage in the temporal point of the contact between both fungi), PGI_C (the failed fungus growth inhibition percentage in the temporal point of contact between both fungi), A_P (the antibiotic effect of *the* failed fungus on the dominant fungus), *I* (the percentage of pathogen growth inhibition) and PBCI (Pakdaman's Biological Control Index calculated as $M\ Z^{-1}p^{-1}$)

Treatment	Mean *X* value (mm)	Mean *T* value (mm)	Mean *p* value (mm)	Mean *G* value (mm)	Mean O_P value (mm)	Mean $BFGI_C$ value (%)	Mean PGI_C value (%)	Mean A_P value (mm day^{-1})	Mean *I* value (%)	Mean PBCI value (mm^{-1})
Atlantis® OD 42 (0 mg L^{-1})	56.00^{a} (±0.41)*	52.50^{a} (±0.29)	7.50^{b} (±0.50)	8.25^{b} (±0.25)	0.91^{b} (±0.06)	-6.24^{b} (±0.48)	-9.03^{a} (±5.9)	1.75^{a} (±0.14)	87.52^{a} (±0.71)	0.081^{a} (±0.01)
Atlantis® OD 42 (50 mg L^{-1})	52.50^{a} (±0.29)	50.75^{a} (±0.25)	8.25^{b} (±0.25)	8.25^{b} (±0.25)	1.00^{ab} (±0.05)	-3.33^{a} (±0.47)	0.35^{ab} (±4.8)	0.88^{b} (±0.13)	86.02^{a} (±0.42)	0.073^{a} (±0.00)
Atlantis® OD 42 (500 mg L^{-1})	44.00^{b} (±1.96)	40.75^{b} (±1.03)	19.25^{a} (±1.65)	16.25^{a} (±1.11)	1.18^{a} (±0.03)	-5.44^{ab} (±1.04)	18.05^{b} (±2.5)	0.36^{b} (±0.11)	67.99^{b} (±2.40)	0.028^{b} (±0.00)

* Standard error was indicated in parenthesis by ±.

Means in each column followed by similar letter(s) are not statistically significant differences (P≤0.05).

The analysis of variance of the values calculated for the applied parameter of O_{BCF}, indicated no significant differences among O_{BCF} values in the studied treatments ($F_{2,9}$ = 2.68 ^{ns}; CV = 2.58%; P = 0.1218). The analysis of the variance of the calculated values of the applied parameter A_P implicated highly significant differences in the antibiotic activity of *F. graminearum* against the BCF ($F_{2,9}$ = 29.84***; CV = 25.83%; P = 0.0001). The comparison of mean A_P values by Tukey's studentized range (HSD) test (α = 0.01) indicated that the herbicide Atlantis® OD 42 significantly reduced the antibiotic activity of *F. graminearum* against the BCF, however, this effect was statistically equal with both A50, and A500 treatments (Table 2). The most

negative value of the parameter A_P, reflecting the highest antibiotic activity of the tested isolate of *F. graminearum* was obtained with A0, i.e. the control with no herbicide amendment (Table 2). The analysis of variance of the values calculated for the applied parameter of BCF growth inhibition percentage in the temporal point of the contact between two fungal colonies ($BFGI_C$) indicated significant differences in $BFGI_C$ values calculated under different conditions of antagonism provided by different concentrations of the herbicide ($F_{2,9}$ = 4.40*; CV = 28.66%; P = 0.0464). The analysis of variance of the values calculated for the applied parameter of A_{BCF}, indicated that there were no significant differences among A_{BCF} values in the studied treatments ($F_{2,9}$ = 0.17^{ns}; CV = 6.13%; P = 0.8496). Although statistically not significant, a positive mean A_{BCF} value of 0.38 was obtained with A0 (control), and a negative mean A_{BCF} value of -0.33 was calculated with A500, while the intermediate average A_{BCF} value of 0.00 was calculated with A50 (Table 1). The analysis of variance of the values calculated for the applied parameter of pathogen growth inhibition percentage in the temporal point of the contact between two fungal colonies (PGI_C) indicated significant differences in PGI_C values calculated under different conditions of antagonism provided by different concentrations of the herbicide (F2,9 = 10.55**; CV = 25.33%; P = 0.0044). A0, and A500 treatments were of significantly different impacts on $PGIP_C$, while the effect of A50 was intermediary and not different from either of above treatments (Table 2). The pathogen was of higher growth in the presence of the BCF in A0 control plates, while its growth was decreased by an insignificant level in A50, and to a significantly less level in A500. Finally, the analysis of variance of *I* values revealed statistically highly significant differences in the effect of herbicidal treatments on this traditional parameter ($F_{2,9}$ = 55.07***; CV = 3.64%; P < 0.0001). The average *I* value obtained with A500 was highly significantly (α = 0.01) less than that calculated for either A50 or A0, both placed in a single Tukey's group (Table 2). PBCIs were statistically highly different ($F_{2,9}$ = 51.14***; CV = 13.32%; P<0.0001), varying highly considerably as the consequence of the herbicide concentration applied. Comparison of mean PBCI values by Tukey's studentized range (HSD) test (α = 0.01) indicated that the herbicidal treatment A50 was of no considerable effect on PBCI, however, A500 led to reduced PBCI values (Table 2).

4. Discussion

The experiment was performed because no information was found on the antifungal activity of widely used HRAC B herbicide Atlantis® OD 42 (a product from Bayer CropScience ltd). The active ingredients in the commercial product, mesosulfuron (10 g L^{-1}) and iodosulfuron (2 g L^{-1}) are known to inhibit the activity of acetolactate synthase (ALS; EC 4.6.3.8) enzyme (also known as acetohydroxyacid synthase, AHAS). The enzyme found in plants, fungi, algae, and bacteria, but not in animals (Gedi and Yoon, 2012) is involved in the catalysis of the first step in the synthesis of branched-chain amino acids (isoleucine, leucine, and valine). Therefore, a systemic herbicide like Atlantis® OD 42 may exert an antifungal impact via the inhibition of the enzyme activity in fungi. Just recently, it was indicated that AHAS inhibiting herbicides inhibited the enzyme activity in *Aspergillus fumigatus* and exerted an inhibitory effect on the fungus growth (Low et al., 2021). Former works have indicated the antifungal activity of some HRAC B herbicides against *F. graminearum* (Liu et al., 2014). Such an impact would be very beneficial in plant debris management after post-emergence chemical control of weeds. Furthermore, it has already been indicated that the deletion of the genes encoding branched-chain amino acid biosynthetic enzymes resulted in the reduced production of DON and reduced virulence in wheat (Liu et al., 2014, 2015).

With such a view, the present study was performed in order to have a predictive study on the effect of Atlantis® OD 42 on the interaction between the BCF, *T. asperelloides* T-92 and the mycotoxigenic pathogen, *F. graminearum*. Atlantis® OD 42 totally inhibited the colonial growth of both fungi when it was applied at

the final ratio of 5000 mg L^{-1}. With the temporal parameters (*C*, *M*, and *Z*) and the resulted time-dependent parameters (*R*, R_1, and R_2) the coefficient of variance was 0.00, then no analysis of variance was performed. This accuracy was resulted from the precision rate applied in the determination of temporal parameters (in day), as the practically useful and sufficiently precise unit of time. This precision was found in agreement with the findings published elsewhere (Pakdaman et al., 2013).

The herbicide reduced *T. asperelloides* T-92 growth when it was applied at the ratio of 500 mg L^{-1}, but it was of no significant impact on the isolate growth when the applied ratio was decreased to 50 mg L^{-1}. A similar trend was observed with *C* values (Table 1) and *Z* values (Table 1). Atlantis® OD 42 at the ratio of 500 mg L^{-1} significantly raised the *R* value. Compared with A0 (with no Atlantis® OD 42), however its application at the ratio of 50 mg L^{-1} had not a significant effect on *R* value. Also, the mean *R* value (1.667) calculated for A50 treatment was not different from that in the control (A0) (Table 1). The *R* value more than 1 means the resistance of *F. graminearum* to the BCF (Pakdaman et al., 2013). The *R* values more than 1 are not unexpected with toxigenic fungi like *Fusarium* spp. In contrast, an increase in the final ratio of Atlantis® OD 42 up to 500 mg L^{-1} led to a higher *C* and to a statistically increased *R* value of 1.923 (Table 1). The pre-contact parameter O_{BCF} was not so informative in the analysis of the effect of herbicidal treatments on the interaction between two fungi (Table 2). The growth of *T. asperelloides* T-92 was not considerably affected by *F. graminearum* secretions. The parameter A_P was found more informative. The comparison of the mean A_P values calculated for the treatments indicated that the least antibiotic activity by *F. graminearum* was found in the treatment A500 (Table 2). This meant that either the production or the efflux of *Fusarium* toxins had decreased in this treatment. Reduced biosynthesis of trichothecene toxins has already been reported as the result of interrupted biosynthesis of branched chain amino acids (Liu et al., 2014: Subramanian et al., 2015). Comparatively, based on the comparison of mean values of O_P (Table 1), the pre-contact parameter related to the effect of *T. asperelloides* T-92 on *F. graminearum*, T-92 could not effectively inhibit the mycelial growth of *F. graminearum* in A500 plates. The comparison of mean O_P values (Table 1) indicated the increased growth of *F. graminearum* in dual cultures compared with that in the axenic cultures. All these results implied to more linear mycelial growth of *F. graminearum* in dual cultures in A500 plates in the temporal point of contact compared with that in axenic control cultures in A500 plates. Considering the results from A0 and A50 treatments, this increased growth could not be resulted from either the collaborative activity of extracellular enzymes involved in the digestion of polymerized organic materials in the medium or the induction of the pathogen growth by the BCF, *T. asperelloides* T-92. It might be because of the mycoremediation of the toxic herbicide by both fungi. The reduced but still positive antibiosis of *F. graminearum* gave a clue to the reduced production and/ efflux of *Fusarium* toxins in A500 plates. It has been indicated that the specific inhibition of branched-chain amino acids by amino-oxyacetic acid (Su et al., 2012) leads to the complete abolishment of 15-acetyl DON (15-ADON) biosynthesis (Subramanian et al. 2015).

The parameter A_{BCF}, indicating the antibiotic effect of T-92 on *F. graminearum* was statistically equal in all three treatments, however, its rate came down from a positive figure in A0, to null in A50, and to a negative figure in A500 (Table 1). These results all revealed the effect of Atlantis® OD 42 in the reduction of antibiotic activity of T-92 isolate in pre-contact phase. This is an example of the cases where statistics fails to explain biological phenomena. Also, the reduced growth of T-92 (revealed by higher *C* values in A500 plates; Table 1) indicated the reduced competitiveness of T-92 for food sources. The negative antibiotic and the reduced competitiveness of T-92 together with the reduced but still positive antibiotic activity and the increased growth of *F. graminearum* in A500 plates can mean the increased pre-contact resistance of the

pathogen, that is well reflected by the increased R_1 value in A500 (Table 1). In post-contact phase, the time required for growth of T-92 over each longitudinal unit of *F. graminearum* colony was significantly higher in A500 (data not shown). The increased resistance of *F. graminearum* in A500 plates was well confirmed by R_2 values calculated with A500 plates (Table 1). Considering that the reduced growth and primary metabolism of *F. graminearum* could be compensated by increased production of *Fusarium* toxins, and considering the positive value of the parameter A_P (Table 2), it seems that Atlantis® OD 42 at the final ratio of 500 mg L^{-1} interfered with the secretory system responsible for the secretion of *Fusarium* toxins. Thus, the accumulation of mycotoxins as the result of interrupted efflux of these metabolites was considered as the reason of the increased R_2 value (Table 1). *TRI101* and *TRI12* are known to provide some degree of the fungus resistance to its own trichothecene mycotoxins (Kimura et al., 1998a,b; Alexander et al., 1999; McCormick et al., 1999). TRI101 acetylates trichothecene to a hypotoxic form by catalyzing the transfer of an O-acetyl group at the C3 position (Garvey et al. 2008). According to the data available from National Center of Biotechnology Institute (NCBI), the protein, trichothecene 3-O-acetyltransferase (Sequence ID: BAA24430.1) is made of 451 aa of which 91aa are branched chain amino acids (20.18%). TRI12 is a major facilitator system (MFS) transporter involved in DON efflux (Menke et al., 2012) and the disruption of this gene in *F. sporotrichoides* and *F. graminearum* has led to the increased sensitivity of the mutants to diacetoxyscirpenol (DAS) toxin (Alexander et al., 1999), and endogenous mycotoxins (Menke et al., 2012), respectively. TRI12 of *F. graminearum* (Sequence ID: AXP33197.1) includes 623 aa of which 154 aa are branched chain amino acids (24.72%) and conserved leucine residues are found in substrate binding pocket (L_{97}) as well as in dimer interface (L_{117}, L_{139}, and L_{148}) of MFS transporter protein. Thus, it is expected that Atlantis® OD42 can interfere with the efflux of mycotoxins and their conversion to hypotoxic forms. Transporters involved in DON efflux play an important role in *F. graminearum* self-defense against self-produced mycotoxins (Wang et al., 2018). Also, it has already been indicated that a *Fusarium* toxin, such as DON affects the chitinolytic activity of *Trichoderma atroviride* (Lutz et al., 2003). This is in contrast to the results with *T. gamsii*, where the studied genes from chitinase subgroups *A*, and *C* were upregulated before/ and during contact with *F. graminearum* and *F. culmorum* (Mataresse et al., 2012). Interrupted efflux of *Fusarium* mycotoxins can be translated to reduced virulence of the pathogen (Menke et al., 2012). Anyway, the increased resistance of *F. graminearum* together with the reduced antibiosis, reduced competitiveness, and the reduced mycoparasitic activity of *T. asperelloides* T-92 resulted in the reduced BC potential of T-92 (reduced mean PBCI; Table 2), however, T-92 was still able to compensate for the effect of Atlantis® OD 42 in a longer period (greater *Z* value; Table 1). To get better understanding on the pre-contact interactions between two fungi, two parameters $BFGI_C$ and PGI_C were defined and applied to calculate the rate of pre-contact inhibition imposed by a fungus against another one. It was found that despite significant differences among average A_P values (Table 2), the differences among the calculated average $BFGI_C$ values were statistically insignificant (Table 2). Therefore, A_P seems more sensitive and informative in the interpretation of the fungus-fungus interactions. An inverse situation was found with average A_{BCF} and PGI_C values (Table 2), where insignificant differences among average A_{BCF} values was compensated with significantly different PGI_C averages in the treatments. More studies are required to know more about usefulness of these parameters.

Based on whole findings of this research, Atlantis® OD 42 seems as an herbicide enough suitable for the integrated management of fusarium head blight disease. The herbicide inhibited pathogen growth at the dose near to that recommended for field applications, apparently interfered with *Fusarium* toxin efflux, however, its unwanted effects on *T. asperelloides* T-92 could not prevent the final failure of *F. graminearum*.

The reduced efflux of *Fusarium* toxins can lead to the gradual replacement of the population of these harmful fungi (known as biological indicators of soil conduciveness) by other soil inhabitants such as *Trichoderma* spp. The results are in good agreement with the reduced incidence of fusarium head scab of wheat observed in Atlantis® OD 42- treated fields in Provinces Ardabil, East Azarbaijan, and West Azarbaijan (Personal communication with Eng. Siamak Pakdaman Sardrood, the former regional expert of Bayer CropScience ltd.). Despite studies performed on the role of branched chain amino acids and on the importance of ALS enzymes in pathogenesis and virulence of fungi (Du et al., 2013; Liu et al., 2014, 2015; Subramanian et al., 2015), more-detailed studies are still required at molecular level in order to analyze the effect of the herbicide. This may lead to a new generation of fungicides that target fungal ALS enzyme. Pathogen growth inhibition percentages (*I*s) and PBCIs were positively correlated and exhibited similar results, however, the new method was found more informative, comprehensive and useful in the detailed analysis of a fungus-fungus interaction under *in vitro* experimental conditions.

5. Conclusions

This study showed that quantitative confrontation test is a very simple, cheap, potent, and informative method. The method can differentiate the resistance of the pathogenic fungus in pre-contact and post-contact phases. Also, the antibiotic effect of both fungi can be calculated. All the parameters can be calculated and statistically analyzed and compared. The effect of extrinsic (as well as intrinsic) factors can be studied. The conscious use of the method can lead to the clues on the changes in the growth, production and secretion of the enzymes and toxins. Quantitative confrontation test allows more detailed analysis of *in vitro* mutual culture experiments. Additionally, this study suggests that Atlantis® OD42 as a HRAC B herbicide is potentially useful in the integrated management of fusarium head blight of wheat, however, more studies are needed under field conditions. Acetolactate synthase inhibitors may provide clues to the development of a new generation of fungicides that inhibit fungal growth and possibly reduce the secretion of *Fusarium* toxins.

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