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TIME SCENARIOS OF INTERACTION IN "TOMATO SEEDLINGS - *Alternaria* - FUNGAL ENDOPHYTES"

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Abstract

In biological control one of the most common approaches is selecting microorganisms with antagonistic traits. Yet, indirect mechanisms of plant growth promotion may give advantages in biotic stress by allowing plants to overcome the colonization of the pathogen. Rapid methods of combined antagonistic and plant growth promotion traits are scarce. Here we propose a method for screening potentially bioactive fungal endophytes in a tripartite interaction "endophytes - Alternaria - tomato seedlings". Endophytes are known to interact with pathogens chemically inhibiting their growth or development by means of specific biologically active metabolites, mycoparasitism, or by competition for nutrients, or indirectly by inducing resistance mechanisms in the host. The proposed method here could substitute the conventional assays of antagonism in dual culture (i.e. fungus – fungus), having introduced from the beginning the central biological actor, the plant. Results indicate that the order of arrival of each actor determines if seedling colonization or enhancement of seedling growth occur as the antagonistic interaction between endophyte and pathogen may precede the latter beneficial interactions.

Key words*: biological control agents, screening, in-planta assay.*

INTRODUCTION

The genus *Artemisia*, with over 400 hardy herbaceous plant and shrub species, has gain attention over the years, being an important resource, contributing to medicine, chemistry, agriculture, industry, and ecology.

The early exploration of the fungal endophytic communities indicates an ecological diversity with a good prospect for plant protection like biological control agents or sources of specific metabolites with biological activity (Cosoveanu & Cabrera, 2018). Biological activity against bacteria and fungi was sought among species of *Artemisia* following the "medicinal plant harbouring active fungal endophytes" model (Cosoveanu et al., 2019; Cosoveanu & Cabrera, 2018; Lu et al., 2000; Zhang et al., 2012; Zou et al., 2000). The present study was meant to obtain an easy, feasible method for screening potential bioactive fungal endophytes against plant pathogen - *Alternaria* sp.

The workflow design (i.e. strategy and resources) regarded that laboratories with limited resources in terms of equipment, reagents and researchers can easily filter the active endophytic strains (i.e. seedlings growth stimulation and protection against pathogen). Therefore, we have proposed the following factors: i) instrumental constraints, ii) high number of potentially active strains; iii) juxtaposed data interpretation (i.e. plant growth stimulation and protection against pathogen); iv) moment of arrival (i.e. multiple scenarios of time shifts for each biological partner and duration of interaction). These factors will be described elsewhere in this manuscript.

The study has two objectives: i) to evaluate which of the endophytes is the most efficient and ii) which of the time scenarios is most propitious to give best results.

The study hypothesis on arrival shifts was that if the endophytes are inoculated first on the seeds, before the pathogen, results would be

favourable in terms of i) seedlings growth and ii) protection against pathogen.

MATERIALS AND METHODS

Study design

The tripartite interaction "tomato seedlings - *Alternaria* - fungal endophytes" was evaluated under multiple time scenarios. The factors depending outcomes were: i) who comes first and ii) how much time do they interact. For this, five interaction scenarios were put in place: i) T1A: At moment zero inoculate the endophyte, after two days inoculate the pathogen and after another two days lay the seeds. Evaluation was made after 14 days from the moment zero. ii) T1B: At moment zero inoculate the endophyte, after two days lay the seeds and after 14 days inoculate the pathogen. Evaluation was made after 23 days from the moment zero. iii) T2: At moment zero inoculate endophyte and pathogen and lay the seeds. Evaluation was made after 18 days. iv) T3A: At moment zero lay the seeds, after seven days inoculate the endophyte and after seven days inoculate the pathogen. Evaluation was made after 21 days from the moment zero. v) T3B: At moment zero lay the seeds, after seven days inoculate the pathogen and after seven days inoculate the endophyte. Evaluation was made after 16 days from the moment zero. The evaluated indicators were: i) length of radicle and plumule (shoot), ii) number of leaves, iii) index of seedlings development (ID), v) and index of necrosis (IN). Developmental stages (ID) were scored using a 0-3 grade scale (0 - no germination; 1 - root emergence; 2 - cotyledon emergence; 3 - well-developed seedling). Disease severity (IN) was scored on seedlings using a 0-9 grade scale (0 - no disease symptoms; 1 - 1-2 mm necrosis on seedling stem; 3 - extension of necrosis all around the stem; 5 - a third of the radicular system or hypocotyl affected; 7 - the whole radicular system or aerial part affected and covered with spores; 9 - not germinated seed/seedling killed and covered with spores).

Variables were the responses of: i) endophyte + pathogen + seedlings (T1), ii) endophyte + seedlings (C1), iii) pathogen and seedlings (C2) and iv) seedlings (C3). For the first objective (i.e. to evaluate which of the endophytes is the most efficient), all indicators were averaged, compared among endophytes and finally scores were obtained. So, for index of development (idem for all indicators), following questions and conditions were addressed:

a) which of the endophytes improves index of development when pathogen is present? Answer: compare averaged values of T1 and C2, where T1 > C2 as a positive effect due to the tripartite interaction;

b) is there any difference between presence of pathogen or endophyte? Answer: compare values between C1 and C2, where C1 > C2 as a positive effect due to endophyte;

c) which of the endophytes influences positively index of development compared to standard (i.e. seeds only). Answer: compare averaged values of C1 and C3, where C1 > C3 as a positive effect due to endophyte (this also demonstrates the innocuous effects of the endophyte on new hosts).

*For index of necrosis, we are interested in the smallest values of the variables T1 and C1, therefore interpretation is inversed. **For the cases where values were equal no scores were given but the case was considered for the total sum of the cases.

Questions "a, b and c" were addressed among time scenarios (T1A, T1B, T2, T3A and T3B) and scores were summed, so to get a final score.

For the second objective (i.e. which of the time scenarios is most propitious to get best results) same indicators were evaluated, this time all endophytes were combined and following questions were addressed (given example of index of development):

a) which of the time scenarios improves value of index when also the pathogen is present? Answer: compare averaged values of T1 and C2 obtained from all endophytes to give scores for each scenario, where T1 > C2 as a positive effect due to tripartite interaction;

b) in which of the time scenarios differences between presence of endophyte or pathogen on seeds were observed? Answer: compare averaged values of C1 and C2 obtained from all endophytes to give scores for each scenario, where C1 > C2 as a positive effect of the endophyte;

c) in which of the time scenarios did the endophytes positively influence seeds?

Answer: compare averaged values of C1 and C3, where C1 > C3 as a positive effect due to endophyte (this also demonstrates the innocuous effects of the endophyte on new hosts). d) which of the time scenarios is the optimum one (considering a, b and c)? Answer: sum scores obtained from "a, b and c".

*For index of necrosis we are interested in the smallest values of the variables T1 and C1, therefore interpretation is inversed.

Data analysis

Raw data were registered for each fungal endophyte in the assessments of germination %, pairs of leaves, index of development and index of necrosis, for each variable (T1, C1, C2, C3) throughout all time scenarios (T1A, T1B, T2, T3A and T3B). Descriptive analysis; normality of data distribution and mean comparison and dispersion; test for equality of variances (Levene's) and post hoc test (Tukey) were calculated with ANOVA. Analysis were conducted with JASP v. 0.14 (computer software © University of Amsterdam).

Selected statistical instruments were used to validate the following hypotheses used for variables:

H1: seedlings alone (C3) would have better results than i) pathogen and seedlings (C2) and ii) all three biological partners (T1).

H2: endophyte + seedlings (C1) would have better results than i) pathogen and seedlings (C2), ii) seedlings (C3) and all three biological partners (T1).

H3: endophyte + pathogen + seedlings (T1) would have better results than pathogen and seedlings (C2).

*Otherwise stated: C1>C3>C2, T1 and T1<C2.

Therefore, values of pairs of variables were compared (i.e. C3 versus C2, C3 versus T1, C1 versus C2, C1 versus T1, C1 versus C3, T1 versus C2) and were questioned how they responded to the hypotheses above stated (acceptation/rejection). Significantly different cases were coded "1" and vice versa for $p > 0.05$. Per endophytic fungus, for all evaluated parameters, among time scenarios, there were 150 possible cases. Only significantly different cases were further considered to select a highly potential bioactive fungal endophyte. Time scenarios (objective 2) were evaluated using same system of comparing variables according to hypotheses per parameter of evaluation, irrespective of the identity of the endophyte. A total of 1050 possible cases were given and only the significantly different ones were used to rank best time scenarios.

Venn diagrams were performed using the web-based tool InteractiVenn (Heberle, Meirelles, da Silva, Telles, & Minghim, 2015).

Tripartite interaction setup with details about biological partners

Seven strains of fungal endophytes previously isolated at Universidad de La Laguna (Cosoveanu et al., 2017) were used in this study, namely *Fusarium tricinctum* (strain HRO8), *Thielavia arenaria* (strain HCV15), *Preussia* sp. (strain HLP14), *Neoplatysporioides aloicola* (strain HLP46), *Fusarium* sp. (strain HCV9), *Epicoccum nigrum* (strain HRO169) and *Neoplatysporoides aloicola* (strain HLP44).

Isolation and identification of fungal endophytes was described elsewhere (Cosoveanu et al., 2018). Fungal endophytes were isolated from *Artemisia* spp., namely HRO8 & HRO169 - *A. austriaca*, HCV15 & HCV9 - *A. gorgonum*, HLP14, HLP44 & HLP46 - *A. thuscula*. The targeted phytopathogenic fungus was *Alternaria alternata*, strain Aa100, isolated from *Lycopersicon esculentum* (Department of Botany, Ecology and Plant Physiology, Universidad de La Laguna, Tenerife). Fungal endophytes were selected due to their antagonist capacity observed previously in dual culture assays against the pathogenic strain. Assessments were made on commercial tomato seeds "Buzau 1600" (Research and Development Station for Vegetables Buzau). Tomato seeds were surface sterilized to supress epiphytic microorganisms: firstly, washed with sterile water, then immersed in 70% ethanol for 1 min, followed by an immersion in 15% sodium hypochlorite for 1 min, again in 70% ethanol for 1 min and lastly were washed with sterile distilled water. Subsequently, the seeds were dried on filter sterile paper under cabinet flow (approx. one hour). Tomato seeds were cultivated *in vitro* on agar Hoagland's nutrient solution in Petri plates (9 cm diameter). In each Petri plate biological partners were placed equidistantly; three seeds were laid in the middle, 3 cork borer plugs of endophytic fungus mycelium in the bottom and 3 cork

borer plugs of pathogen mycelium in the top of plate, each with 0.4 cm diameter. Environmental conditions were: light/dark regime of 22±1ºC for 8 h and 18±1ºC for 16 h; RH = 55% and 60%, respectively; day photon flux of 120 (+/- 10) µmol m^{-2} s^{-1} (light step setting 4 - ls4). Plates were incubated vertically, on trays, in a Sanyo versatile environmental test chamber. At five days interval, the plates were opened for oxygen requirement, at the laminar flow. Three plates were used per variable with three biological repetitions. Under the system conditions, four endophytes can be screened in a series for all five time scenarios and four variables, in a biological repetition (Figure 1).

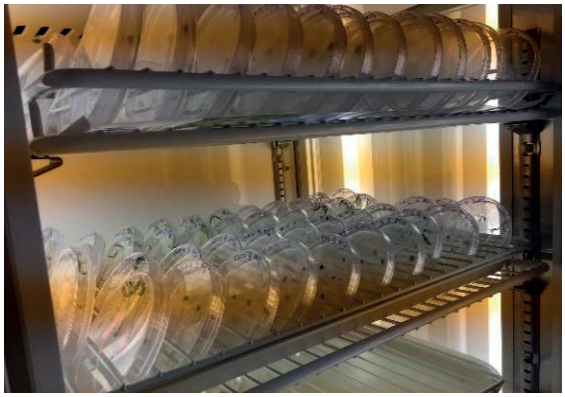
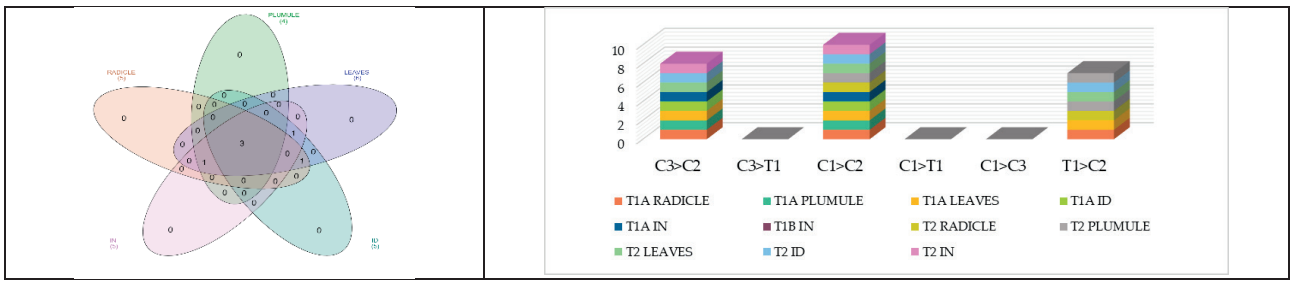


Figure 1. Example of arranging plates in two trays in a Sanyo environmental chamber

RESULTS AND DISCUSSIONS

To evaluate which of the endophytes is the most efficient in terms of seedlings growth stimulation and protection against pathogen, all sets of parameters data were compared. Significantly different ($p < 0.05$) hypothetical cases of variables cases (i.e. T1 > C2 and C1 > C2, T1) were registered for all tripartite interactions along time scenarios. Of 150 hypothetical cases for each endophytic fungus among five time scenarios results showed significant cases for HLP44 - 35, HCV15 - 32, HRO8 - 30, HLP14 - 26, HCV9 - 25, HRO169 - 21, HLP46 - 19. The bioactive potential of endophytic fungi cannot be demonstrated only by summing significant cases but also by i) their convergence per studied parameter among multiple time scenarios and by ii) the weight of H2 among all parameters where inoculated seeds with endophyte would have better results than seedlings alone/seedlings + pathogen/seedlings + pathogen + endophyte. Hence, for seedlings growth stimulation (H2) following percentages of the total significant cases were obtained: HRO8 - 56.67%, HLP44 - 53.85%, HLP46 - 50.36%, HLP14 - 51.43%, HCV9 - 40%, HCV15 - 37.50%, HRO169 - 47.62%. For the antifungal biological effect (H3) only HRO8 registered significant cases (40%). For H2, HRO8 registered 10 significant cases of 25 in total, followed by HLP44 (9 cases). Least number of significant cases was registered for HRO 169 (5 cases). These results indicate that endophytes are innocuous to seedlings and positively stimulate their growth, compared to when pathogen is present. Endophytes + seedlings were positively influenced by the presence of endophytes when compared to the presence of both endophytes and pathogen, especially for HCV9 (9 cases) and HLP14 (10 cases), $p < 0.05$. Only HLP46, HLP44 and HCV15 showed one significant case each for seedlings growth stimulation, compared to seedlings alone. For H3, the most active endophytic strain against *A. alternata* was HRO8 with 7 significant cases when endophytes + pathogen were compared to pathogen.



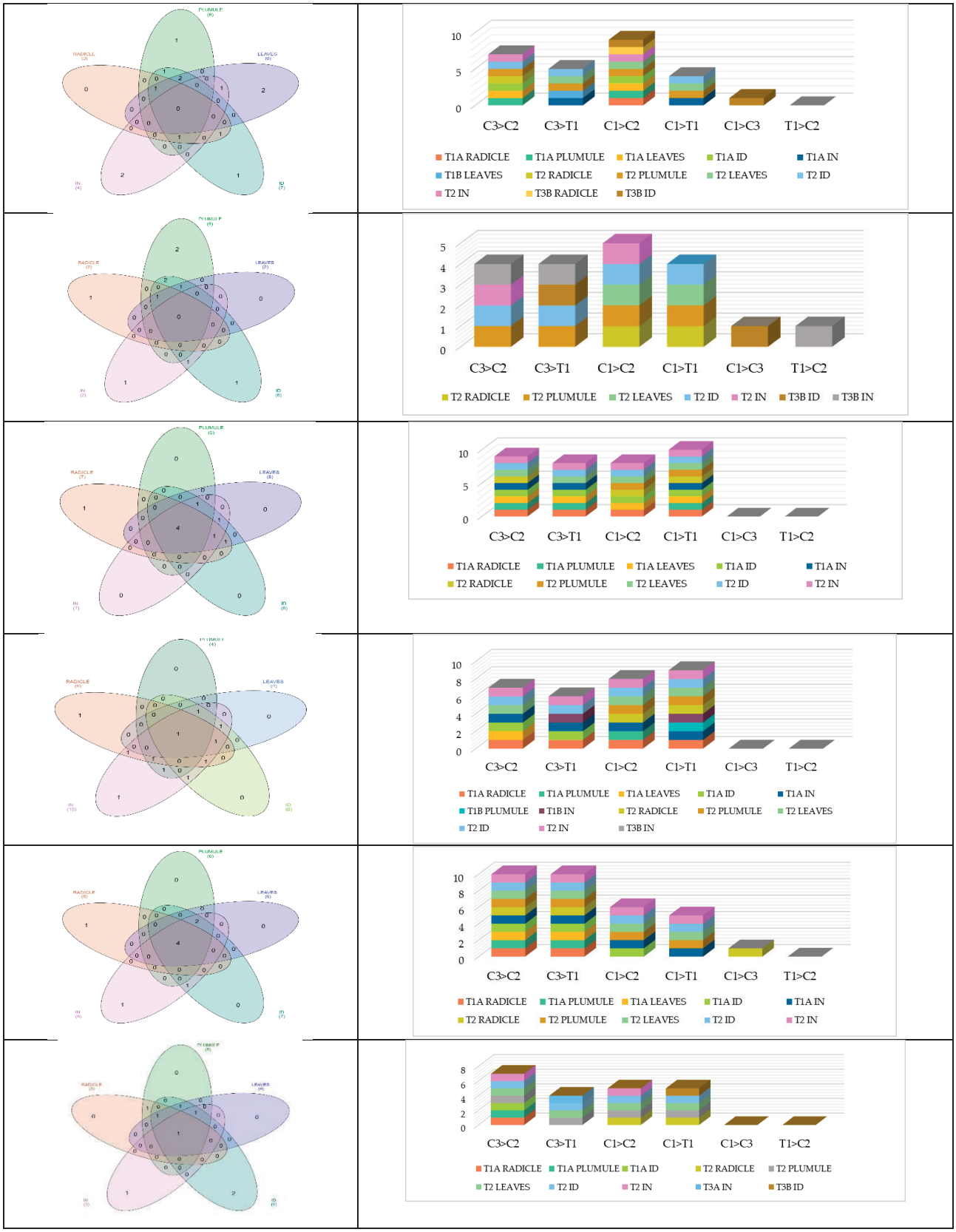


Figure 2. Vertically from up to bottom: HRO8; HLP44; HLP46; HLP14; HCV9; HCV15; HRO169. On the left Venn diagram showing the number of significant cases ($p < 0.05$) per observed parameter and coincidences of cases among parameters. On the right, columns represent number of cases (C1: seedlings+endophytes; C2: seedlings+pathogen; C3: seedlings; T1: seedlings+ endophytes+pathogen) responding according to work hypotheses ($p < 0.05$) identified among time scenarios (T1A, T1B, T2, T3A, T3B) using the values of parameters (root, shoot, index of necrosis, index of development and leaves)

To evaluate which of the time scenarios is most propitious to positively influence growth and protection of seedlings of 1050 possible cases according to the planned hypotheses only 188 cases were validated ($p < 0.05$).

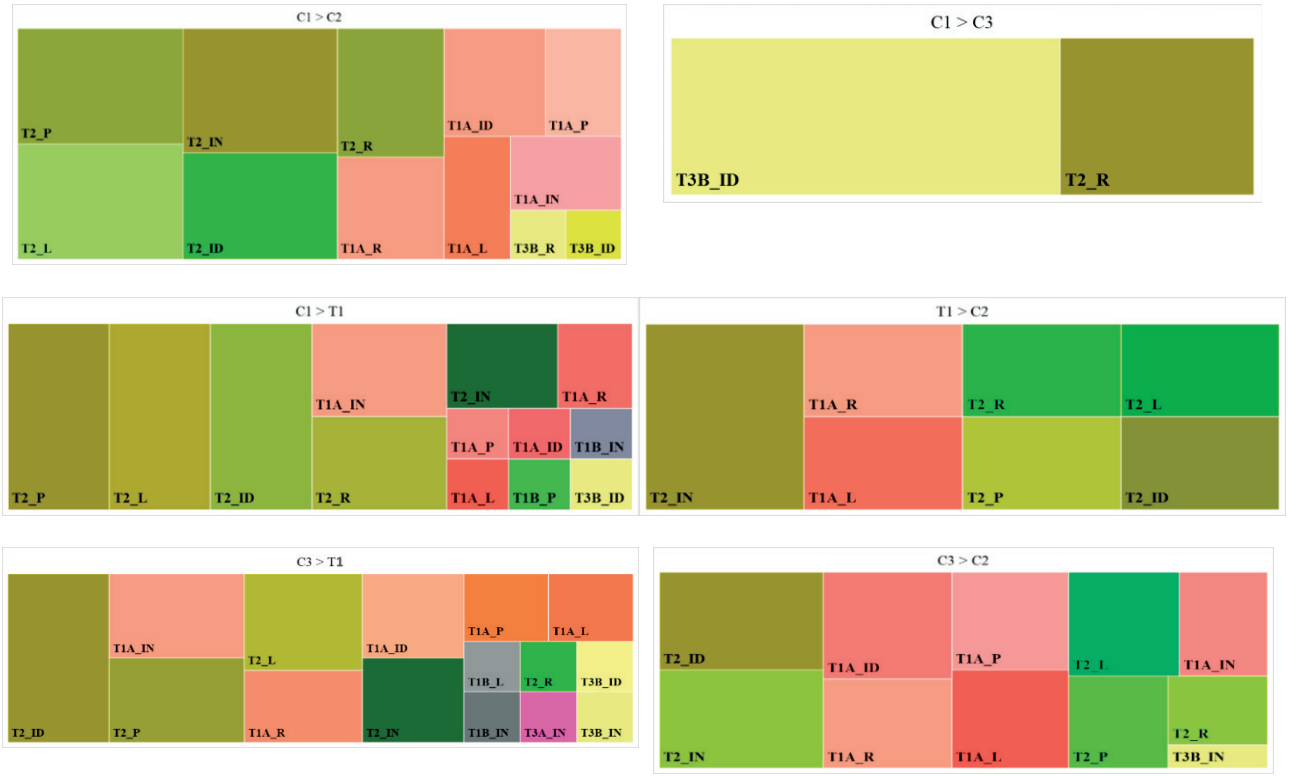


Figure 3. Tree map of significantly different cases ($p < 0.05$) among scenarios, using values obtained from seven fungal endophytic strains. Each window corresponds to a demonstrated hypothesis ($p < 0.05$). Blocks dimensions correspond to the number of significantly different cases. Codes for blocks: T1A/T1B/T2/T3A/T3B - time scenarios, L = leaves, R = roots, P = plumule, IN = index of necrosis, ID = index of development

Best time scenarios were T2 where all biological partners were added at same time and T1A where endophyte precedes pathogen and seeds are added at the end.

Figure 4. Images of all variables in the T2 time scenario (all biological actors were introduced at the same time). From left to right: C1 - seedlings + endophyte (HRO8); C2 - seedlings + pathogen; C3 - seedlings alone; T1 - seedlings + pathogen + endophyte (HRO8)

Both time scenarios converge into a single probable explanation that is the antagonistic interaction between endophyte and pathogen may prelude other beneficial interactions like seedling colonization or induced resistance (T1B). Direct confrontation of endophyte and pathogen at the same stage - T2 or with 2-days advantage for endophyte - T1A results in an inhibition of the pathogen previously observed in dual culture assays. Therefore, the interaction with seeds may have a secondary place in terms of used resources being preceded by competition of space. It also indicates that seedlings may be positively influenced in terms of growth stimulation by the presence of both pathogen and endophyte. Plants have complex veracity of endophytic-pathogenic lifestyles, which are transposable and get triggered by chemical and molecular factors (Cosoveanu et al., 2021). It is tempting to speculate that modulation of stress hormones like salicylic acid (Yan & Dong, 2014), abscisic acid (Adie et al., 2007) or developmental hormones like gibberellins and indole-3 acetic acid contributed to mitigating stress on seedlings (Waqas et al., 2012). Several studies showed that plants treated with endophytes are often healthier (Khan et al., 2008; Larriba et al., 2015). When endophytic *Fusarium verticillioides* strains were inoculated onto maize seedlings before, simultaneously, or after inoculation with the pathogen *Ustilago maydis*, smut disease severity was significantly decreased only when *F. verticillioides* was simultaneously inoculated with *U. maydis* (Lee et al., 2009). Similarly, Adame-Álvarez et al. (2014) observed that pathogenic bacterial titers were significantly lower when endophyte was inoculated same day or before the pathogen in lima bean plants. Significant effect of endophytes on reducing *Fusarium* head blight was observed when endophytes were applied at least two days before pathogen (Rojas et al., 2020). In tomato plants, *Verticillium dahlie* was excluded from roots when endophytic strain was inoculated before or simultaneously with the pathogen (Shittu et al., 2009). In the present study the hypothesis on arrival shifts where if endophytes arrive first, before the pathogen, results would be favourable in terms of i) seedlings growth and ii) protection against pathogen was partially demonstrated. Of 188 possible cases time scenario T1A registered 67 significant cases where endophytes responded according to rules for hypotheses. Yet, the time scenario which was least considered to be successful (T2), revealed 108 significant cases. The proposed method could substitute the conventional assays of antagonism in dual culture having introduced from the beginning the central biological actor, the plant. The study is only a preliminary screening which cannot foreseen the complex interactions in the field where host genotype, tissue type and abiotic factors influence significantly fungal communities (Latz et al., 2021). Yet, this screening method could eliminate the drawbacks from dual culture confrontation assays where potential biological control agents are hidden (Rojas et al., 2020). A higher number of parameters can be evaluated in the same set like i) host compatibility, ii) seedling growth promotion, iii) seedling protection against pathogen; iv) active volatile/soluble compounds, v) colonization of host.

CONCLUSIONS

Here we propose a combined method to screen fungal endophytes with plant growth promotion and plant protection traits. Technical procedure and data analysis are suitable for laboratories with limited resources, therefore making it a feasible tool to research biological control agents.

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