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TECHNICAL NOTE

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A robust hydroponic-based system for screening red clover (*Trifolium pratense*) for *Fusarium avenaceum*

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Abstract

Red clover (*Trifolium pratense*) is an important forage legume crop that suffers like most perennial crops from attacks by soil-borne pathogens. *Fusarium* root rot is one of the most serious diseases and at the same time problematic to identify resistance because of its hidden life in the soil. Current screening methods are laborious and hampered by limited reproducibility. To remedy this situation, we aimed to establish a simple and reliable hydroponics-based screening system to facilitate studies of red clover–*Fusarium avenaceum* interactions. First, the fungal spore concentrations were balanced toward the development of red clover plants grown hydroponically. We found that the optimum concentration was 30,000 spores in 2 L of hydroponic medium to ensure infection during the plant growth period in this system. The procedure was scaled-up to screen plants from 25 populations to identify red clover individuals with the improved resistance to *F. avenaceum*. Susceptible plants had approximately two-fold higher amounts of fungal DNA than resistant plants, demonstrating a correlation between the disease readings of the plants and pathogen DNA. We foresee this screening procedure meeting the needs of both applied breeding work and in-depth molecular studies of responses between this pathogen and its host plant. This method could be applied for the screening of other plant species for resistance to *Fusarium* spp. or to other root microbes.

KEYWORDS
Fusarium avenaceum, hydroponics, red clover, rhizobium, root rot

1 | INTRODUCTION

Red clover (*Trifolium pratense*) is an important forage legume crop in temperate agriculture, where it plays a key role in sustainable livestock farming systems (Sullivan & Quesenberry, 2015). It is widely cultivated within Europe and worldwide. In Scandinavia, particularly in Sweden (www.scb.se), red clover is the number one forage legume. Red clover offers high-value feed to livestock because of its high protein and micronutrient contents and its easy digestibility. Red clover replaces soybean in regions where the latter cannot be grown (Ferreira et al., 2021; Lindström et al., 2014), which applies for most central and northern European countries. Red clover can adapt to a wide range of soils and is rather drought tolerant because of its taproot, which can extend to a depth of more than 1 m (Taylor & Quesenberry, 1996). Additional ecosystem services are associated with its ability to fix atmospheric nitrogen through bacterial symbiosis with *Rhizobium leguminosarum* bv. *trifolii*, its beneficial effects on soil structure, and the promotion of genetic diversity through insect pollination (McKenna et al., 2018). Concerns regarding climate change and the rising importance of sustainable agriculture and organic

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farming have revived interest in increasing the use of red clover as a cover crop, forage, and/or green manure (Ferreira et al., 2021; Taylor, 2008). Red clover is a perennial crop and is expected to provide good biomass yield for 2 to 3 years but rarely does so. This limited persistence has been attributed to several biotic and abiotic stress factors (Annicchiarico et al., 2015; Taylor, 2008; Taylor & Quesenberry, 1996).

Soil-borne pathogens commonly pose threats to perennial crops, and red clover is no exception. The long-standing root system makes it vulnerable to infection by soil-borne pathogens particularly fungi. Root rot caused by *Fusarium* spp. and clover rot caused by *Sclerotinia trifoliorum* are major problems (Yli-Mattila et al., 2010). *Fusarium avenaceum* may lead to complete plant loss in red clover (Stoltz & Wallenhammar, 2012; Yli-Mattila et al., 2010) and other legumes (Baćanović-Šišić et al., 2018). The ubiquitous genus *Fusarium* is a member of the family Nectriaceae comprising genera with complex taxonomy where consensus of phylogenetic, morphological, and other characteristics is lacking (Crous et al., 2021). *Fusarium avenaceum* is a cosmopolitan pathogen reported to cause damage to both monocot and dicot plant species (Desjardins, 2003; Kovačikova & Kůdela, 1982; Leach & Hobbs, 2013). This fungal species has a genome enriched with genes encoding enzymes known to be active in mycotoxin production. To date, no reports are available on toxins in *Fusarium*-diseased red clover plants, which contrasts with the situation in cereals (Inbaia et al., 2023). Obviously, any enrichment of *Fusarium* in farm soils is not desirable. The picture is complicated because of possible exchange of traits between different *Fusarium* strains or species which could make strains more virulent (Geiser et al., 2021; Lysøe et al., 2014; Summerell, 2019).

Root rots cause substantial yield loss and impose extra stress on the plants, leading to poor persistence. There are no chemicals available that target these pathogens. Thus, identification of improved *Fusarium* resistance is an important aspect of red clover breeding programs. Previous *Fusarium* disease screening methods relied mainly on field evaluations or experiments in pots containing soil or peat-sand potting compost conducted under greenhouse conditions (Coulman & Lambert, 1995; Skipp et al., 1986; Venuto et al., 1999; Yli-Mattila et al., 2010). A common challenge in all these methods is to have an even disease pressure enabling comparisons between repeated experiments. Although field testing results in infection under natural conditions, field trials are problematic with variable weather conditions and limited knowledge of pathogens (species and quantity) in the soil. The method is time-consuming and labor intensive, making it challenging to screen several populations at the early stages of a breeding program (Farias Neto et al., 2008; Jat & Ahir, 2013). Screening in greenhouse conditions is also affected by many constraints: limited reproducibility, large space requirement, being time-consuming, and interactions with secondary contaminants. Thus, there is a wish to establish a cleaner, more rapid, and reproducible screening method under controlled environment conditions that provide the precise timing of infection, consistent inoculum distribution, leading to minimized experimental variations.

In our long-term research on red clover improvement, it is of utmost importance to generate detailed information on the plant interaction with *F. avenaceum*, the most pathogenic *Fusarium* species on red clover in Sweden (Jambagi et al., 2023). The main objectives of this study were to establish a rapid and reproducible screening system in red clover to *F. avenaceum*, and investigate the effect of pathogen on nodule development. Here, we describe a simple hydroponic-based system under controlled conditions where the addition of the pathogen and its infection process could be more easily monitored on any chosen red clover variety or breeding materials. Furthermore, because we lack red clover varieties with improved resistance to *F. avenaceum*, we decided to screen plants from 25 breeding populations utilizing the new hydroponics-based method. This was performed with the goal to identify superior individual plants in each population that could form new sub-populations to be integrated in near future breeding work.

2 | MATERIALS AND METHODS

2.1 | Fungal strain confirmation and preparation of inoculum

The *F. avenaceum* CBS 115699 strain, obtained from the Westerdijk Fungal Biodiversity Institute, The Netherlands, was grown on potato dextrose agar (Sigma–Aldrich) at 21°C in darkness for 10 days. Three mycelium plugs (5-mm diameter) were transferred into 100 mL of potato dextrose broth (Becton, Dickinson and Company, USA) and incubated at 21°C in darkness with occasional shaking. After 10 days, mycelia were harvested, air-dried, ground into fine powder in liquid nitrogen using a mortar and pestle, and stored at −20°C until DNA extraction (Möller et al., 1992). The internal transcribed spacer (ITS) region ITS1–ITS4 was polymerase chain reaction (PCR) amplified using the universal primer pair ITS1–ITS4 (ITS1: 5′ TCCGTAGGTGAACCTGCGG 3′, ITS4: 5′ TCCTCCGCTTATTGATATGC 3′) (White et al., 1990) followed by Sanger sequencing and BLAST (Basic Local Alignment Search Tool) search using full length ITS sequences in the GenBank database to confirm species identity. This *F. avenaceum* strain CBS 115699 was then used for all red clover inoculations.

For hydroponic inoculum preparation, three agar plugs (5 mm) with actively growing hyphae were transferred into Erlenmeyer flasks containing 200 mL of Czapek Dox (CD) broth (Sigma–Aldrich). The inoculated CD broth was incubated at 25°C with rotatory shaking at 180 rpm under soft white fluorescent light for 10 days to produce spores. After filtration through two layers of Miracloth (Millipore, USA), the spores were washed by centrifugation twice and resuspended in sterile distilled water. The spore concentration was determined using a hemocytometer. An average concentration of 3×10^6 spores/mL was prepared and diluted to the desired concentrations in the hydroponic boxes. The virulence of the fungus was maintained by regular infection of 3-week-old sterile red clover seedlings and re-isolation of the fungi growing out of the root tissues on fresh potato dextrose agar plates.

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2.2 | Hydroponic system for growing red clover plants

Seed from 25 red clover breeding populations, provided by Lantmännen, Sweden, were used in the hydroponic-based screening. Approximately 500 mg seeds from each population were placed in 50 mL Falcon tubes, surface sterilized with 70% (v/v) ethanol for 15 s, washed with sterile water, sterilized with 10% (v/v) Klorin (commercial bleach) for 10 min with occasional shaking, washed 6–7 times, and imbibed in sterile water for 72 h at 4°C for vernalization. Sterile 200 µL tip boxes with transparent lids were used as a mini-greenhouse for germination. Sterile 200 µL tips (Sarstedt, Germany) were filled aseptically with molten (50–60°C) half strength hydroponic solution (Table 1) containing 0.7% Bacto agar (Saveen and Werner, Sweden). The bottoms of the tips were first sealed by adding 10–20 µL droplets of agar solution and left to solidify before they were filled completely. After vernalization, surface-sterilized seeds from the Falcon tube were transferred to a Petri dish containing 0.1% sterile agarose to decrease the seed density, followed by sowing. Single seed were placed in the center of each tip using a spatula (Figure 1a). Filled boxes were closed and sealed with 3 M tape (Micropore, Germany). The sealed boxes were transferred to the growth chamber for germination at 22 ± 1°C with a 16 h photoperiod. After 4 days, when seedlings had reached the two-leaf stage (Figure 1b), one-third of the pipette tips were cut to allow root growth, returned back to the tip boxes and transferred to the growth chamber without sealing the boxes. Twenty tips containing 5-day-old plants with well-growing roots (Figure 1c) were then transferred into a perforated lid of black painted and surface-sterilized polyethylene 2 L boxes (IKEA, Sweden) filled with 2 L of full-strength growth solution (Figure 1d). Plants were further grown until they were 3-weeks old (Figure 1e).

TABLE 1 Hydroponic medium composition.

1. Prepare the following stock solutions and store them at 4°C		
Solutions	**Concentration**	**Molecular weight**
KNO_3	1 M	101.0
$CaCl_2$	1 M	147.0
$MgSO_4{\cdot}7H_2O$	1 M	246.5
KH_2PO_4.	1 M	136.1
$NH_4\ NO_3$	1 M	236.0
Fe-EDTA	70 mM	367.1
2. Prepare the micronutrient stock solution and store at −20°C		
H_3BO_3	50 mM	61.8
$MnSO_4{\cdot}H_2O$	10 mM	169.0
$ZnSO_4{\cdot}7H_2O$	1.5 mM	287.5
$CuSO_4{\cdot}5H_2O$	1 mM	249.7
Na_2MoO_4	0.58 mM	241.9
3. Prepare 1 L of full-strength hydroponic solution, mix		
Stock solutions	**Volume (mL)**	
KNO_3	2.5	
$CaCl_2$	1	
$MgSO_4{\cdot}7H_2O$	0.75	
KH_2PO_4.	0.5	
$NH_4\ NO_3$	0.5	
Fe-EDTA	1	
Micronutrients	1	
	Make up to 1 L in distilled water	

Note: Hydroponic media formulation is as adapted from (Bindschedler et al., 2008; Norén et al., 2004; Tocquin et al., 2003).

2.3 | Hydroponic-based *in planta* inoculation

Three-week-old hydroponically grown red clover plants were challenged with the appropriate *F. avenaceum* spore concentration. To identify an optimal balance between the infection rate and plant growth under the given growth conditions in the growth chamber, three different final spore concentrations, 15,000, 30,000, and 60,000 spores/mL, were examined at different time points: 1-, 3-, 5-, and 7-days post inoculation (dpi). The breeding population no. 17 was chosen to optimize spore concentration as it is an intermediate flowering type plant material with good germination capacity and considered to have the moderate persistence in Sweden based on the breeder's former experience. The root symptoms were scored on a scale from 0 to 3 (Table 2). A disease index (DI) was calculated according to the formula $DI = [(0 \times N_0) + (1 \times N_1) + (2 \times N_2) + (3 \times N_3)]/(N_0 + N_1 + N_2 + N_3)$, where N_n = number of plants in the respective class (Happstadius et al., 2003). DI values between 0 and 1.0 were considered low, values between 1.0 and 2.0 were considered moderate, and values between 2.00 and 3.00 were considered high. Plants that were severely wilted or dead reached a DI value of 3.0. Twenty red clover plants/box were used for testing different spore concentrations. Next, the screening procedure was performed on plants from 25 diploid red clover populations. Boxes with 3-week-old plants with and without 30,000 fungal spores/mL were prepared. For without pathogen treatment, instead of spore suspension, an equal volume of sterile water was added to the boxes. The course of infection was examined daily, and the phenotypic responses were turned into a DI. Twenty red clover plants for each red clover population were used for fungal infection. Three replicates were maintained for all the screening experiments.

2.4 | Quantification of fungal DNA in red clover roots

The roots of control and treated red clovers were harvested 3, 5, and 7 days after fungal infection, rinsed thoroughly with sterile water and patted dry on a clean paper towel. Roots from three individual plants were pooled, snap-frozen in liquid nitrogen, and stored at −70°C until DNA extraction. Genomic DNA was prepared using a cetyltrimethylammonium bromide (CTAB) procedure (Möller

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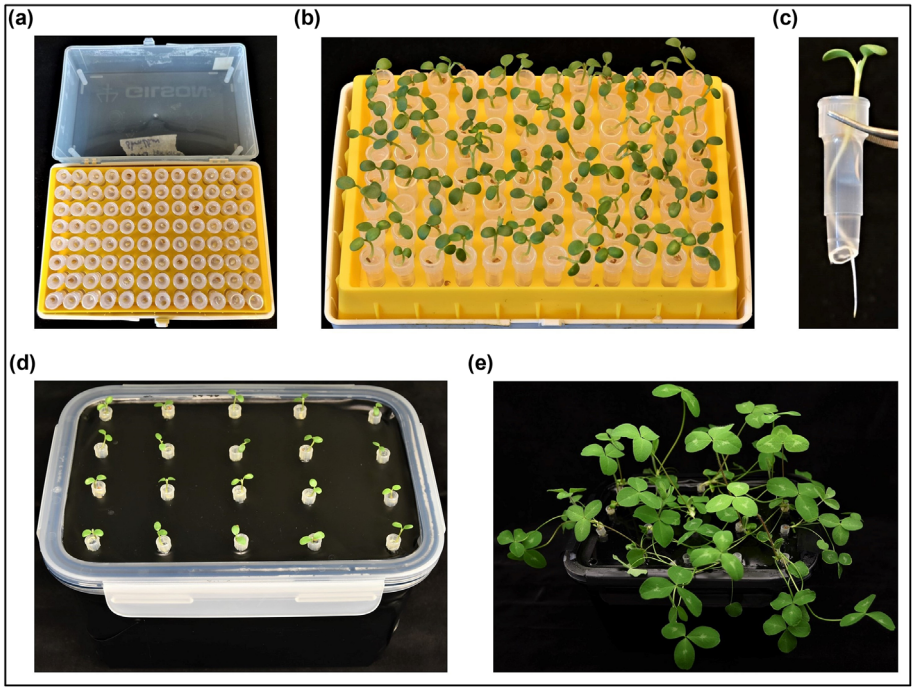

FIGURE 1 Hydroponic system for growing red clover plants. **(a)** Sterilized red clover seeds transferred to a 200-µL pipette tip containing half strength hydroponic solution with 0.7% Bacto agar, (b) 4-day-old seedlings (two-leaf stage), (c) roots growing through the excised pipette tip, (d) healthy seedlings transferred into the holes of sterilized black painted plastic boxes (length – 21 cm, width – 15 cm, and height – 12 cm) containing 2-L full-strength hydroponic solution, and (e) 3-week-old plants growing in hydroponic media.

TABLE 2 Disease assessment scale for screening of inoculated plants.

Disease development	Scale
Healthy/white roots	0
Discoloration of roots – brown roots	1
Blackening of roots	2
Rotting of roots/wilting/plant dead or dying	3

et al., 1992). In brief, 500 µL of 3% CTAB extraction buffer was added to 100 mg of ground powder, followed by RNaseA treatment. Samples were first incubated at 37°C for 30 min and then at 65°C for 30 min. After centrifugation at 13,000 rpm for 10 min, the supernatant was purified using chloroform extraction. The final aqueous phase was mixed with one volume of isopropanol, and DNA was precipitated at −20°C for 30 min and centrifuged at 13,000 rpm for 20 min. The pellet was washed with 70% ice-cold ethanol (approximately 0°C), dried, and resuspended in sterile water. The extracted DNA was quantified using Qubit (Invitrogen) and stored at −20°C until further use. Genomic DNA of *F. avenaceum* was extracted using the same procedure that was used as reference material to generate standard curve in the root infection DNA quantification assay.

The amount of fungal DNA present in red clover root samples was quantified using a SYBR green qPCR assay with the *F. avenaceum*-specific primer pair EF-1 forward primer (5′- ATG GGT AAG GAR GAC AAG AC -3′) and EF-2 reverse primer (5′- GGA RGT ACC AGT SAT CAT G – 3′) targeting the translation elongation factor 1-alpha (*TEF1*) gene region (Pollard & Okubara, 2019). Three replicates for each sample were prepared. The standard curve technique was applied with a tenfold dilution series of DNA from *F. avenaceum* dissolved in sterile water ranging from 100,000, 10,000, 1000, 100, and 10 pg DNA, with three replicates of each dilution. The slope of the standard curve was used to determine the PCR efficiency ($E = 10^{-1/\text{slope}} - 1$). The amount of fungal DNA in each red clover root sample was estimated by comparing the obtained data to the standard curve. The result was converted into nanogram *F. avenaceum* DNA per 100 mg red clover root biomass. All calculations and statistical analyses were performed as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems, USA) as previously explained (Martin et al., 2011; Tzelepis et al., 2017). The statistical significance of fungal DNA amounts in different samples was calculated using Student's *t* test.

2.5 | *R. leguminosarum* bv. *trifolii* experiments

Rhizobium strains were isolated from nodules of Swedish red clover as earlier described (Lilja, 2023). *R. leguminosarum* bv. *trifolii* (GenBank accession number MW980046) grown on yeast extract-mannitol (YEM) agar plates were used to inoculate 200 mL of sterile YEM broth. The inoculated YEM medium was incubated at 28°C under dark with rotatory shaking at 120 rpm for 72 h. Rhizobial cell density was measured by automated cell counter (Invitrogen Countess™). Rhizobial suspension of approximately 10^3 cells/mL prepared in nitrogen-free modified Fåhraeus solution (Lodeiro et al., 1995) was used as inoculum. For rhizobium treatment, plants from most resistant and susceptible breeding population were used. Box lids with two-week-old red clover seedlings were transferred to a square Petri dish containing 50 mL rhizobial suspension (10^3 cells/mL) (Lodeiro et al., 1995; Mongiardini et al., 2008) (Figure S1). Incubation was carried out for 2 h at 28°C with

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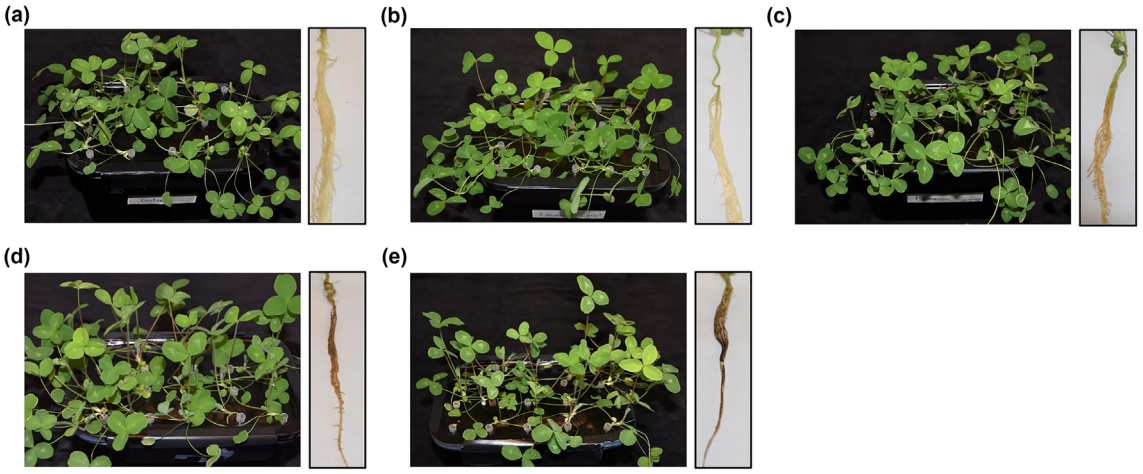

FIGURE 2 Phenotypic responses of plants from red clover breeding population no. 17 grown in hydroponic media with 15,000 spores/mL final concentration of *Fusarium avenaceum*. (a) Noninoculated plants, (b) wilting and root symptoms at 1-day post inoculation (dpi), (c) 3 dpi, (d) 5 dpi, and (e) 7 dpi.

TABLE 3 Disease index (DI) values 1-, 3-, and 7-days post inoculation of the population no. 17.

Spore concentration/mL	3 dpi	5 dpi	7 dpi
15,000	0.2	0.7	1.0
30,000	0.8	1.5	2.5
60,000	2.9	3.0	3.0

occasional shaking under dark conditions. Next the roots with adsorbed rhizobia were washed multiple times in the same manner by transferring the box lids to a new container containing sterile water. The lids were transferred back to the boxes containing hydroponic solution adjusted to one third of the initial nitrogen concentration. After 1 week of *Rhizobium* inoculation, *F. avenaceum* was added at a final concentration of 30,000 spores/mL as earlier described. The experimental design was; non-inoculated (no fungal or bacterial inoculant), *F. avenaceum*, rhizobia + *F. avenaceum*, and only rhizobia inoculated. The root responses were monitored daily. Twenty red clover plants/box were used for each treatment. Three replicates were prepared for all combinations.

3 | RESULTS

In our work, we first tried greenhouse-based and paper roll (Liljeroth et al., 1993) methods to screen red clover to its root rot pathogen *F. avenaceum* but encountered problems with secondary contaminants and reproducibility. Therefore, we established hydroponic-based screening system in red clover. To establish the method, responses of

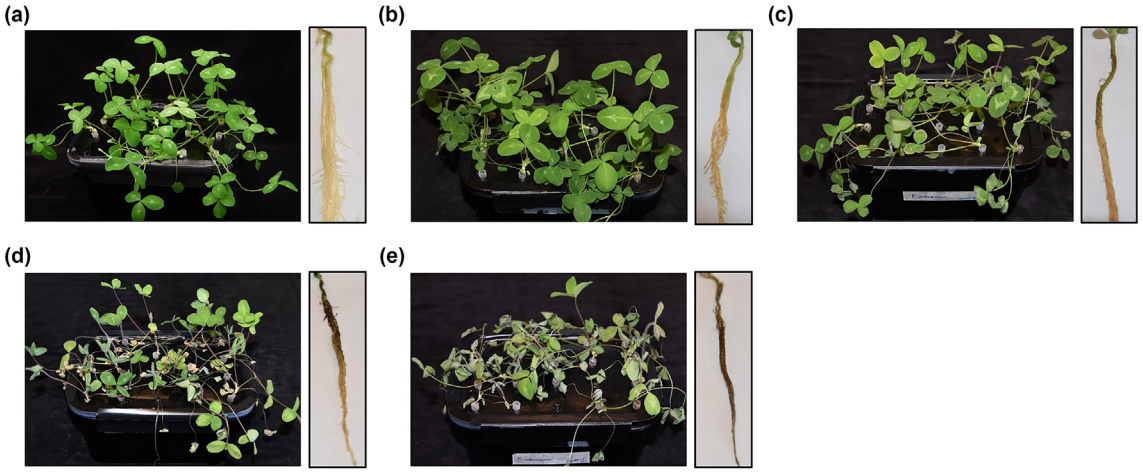

FIGURE 3 Phenotypic responses of plants from red clover breeding population no. 17 grown in hydroponic media with a final concentration of 30,0000 spores/mL *Fusarium avenaceum*. (a) Noninoculated plants, (b) wilting and root symptoms at 1 day post inoculation (dpi), (c) 3 dpi, (d) 5 dpi, and (e) 7 dpi.

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different fungal spore concentrations were first monitored. There was no visible difference between plants challenged with different spore concentrations at 1 dpi. Plants challenged with 15,000 spores/mL had low DI values at 3, 5, and 7 dpi, all with limited symptom development of bright yellowing, some wilting of older leaves and initiated discoloration of roots (Figure 2a–e and Table 3). Plants challenged with 30,000 spores/mL generated a low DI at 3 dpi, moderate DI at 5 dpi, and high DI at 7 dpi (Figure 3a-e and Table 3). When using the highest spore concentration in the boxes, a high DI was achieved at 3 dpi (Figure 4a–e and Table 3). Based on these results, a spore concentration of 30,000 spores/mL was found to be the most appropriate to balance the speed of infection by *F. avenaceum* in this cultivation regime.

Next, we applied the screening method on plants from 25 diploid red clover populations. Disease symptoms could first be observed on the roots as early as 3 dpi. Typical symptoms of *F. avenaceum* infection such as brown to black rot of roots and wilting of aerial parts (Figure 5) were observed, and many plants died between 5 and 7 dpi. Individuals from four breeding populations with DI values between 1.8 and 2.3 were identified, indicating that complete resistance was not present among the material evaluated. Individual plants from a total of 10 diploid breeding populations were completely susceptible,

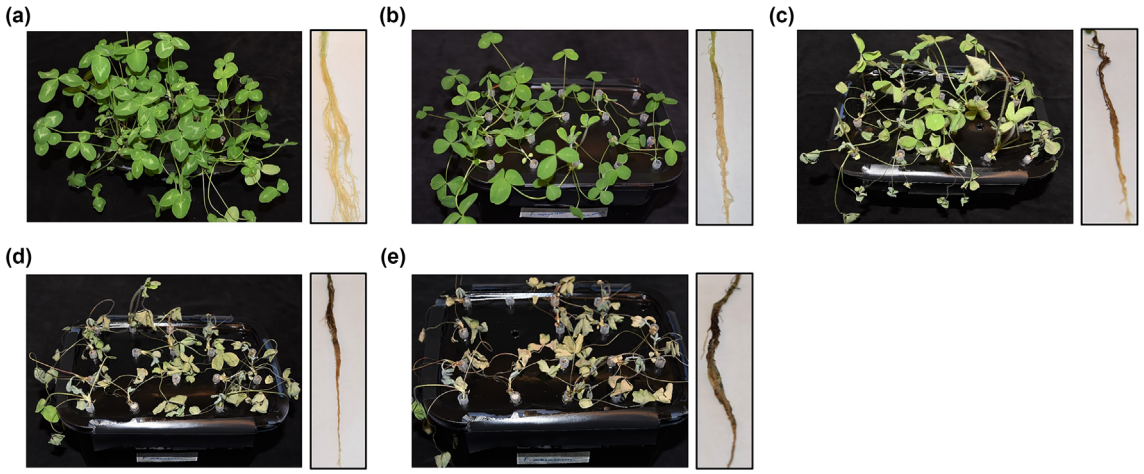

FIGURE 4 Phenotypic responses of plants from red clover breeding population no. 17 grown hydroponically with a final concentration of 60,000 spores/mL *Fusarium avenaceum*. (a) Noninoculated plants, (b) wilting and root symptoms at 1 day post inoculation (dpi), (c) 3 dpi, (d) 5 dpi, and (e) 7 dpi.

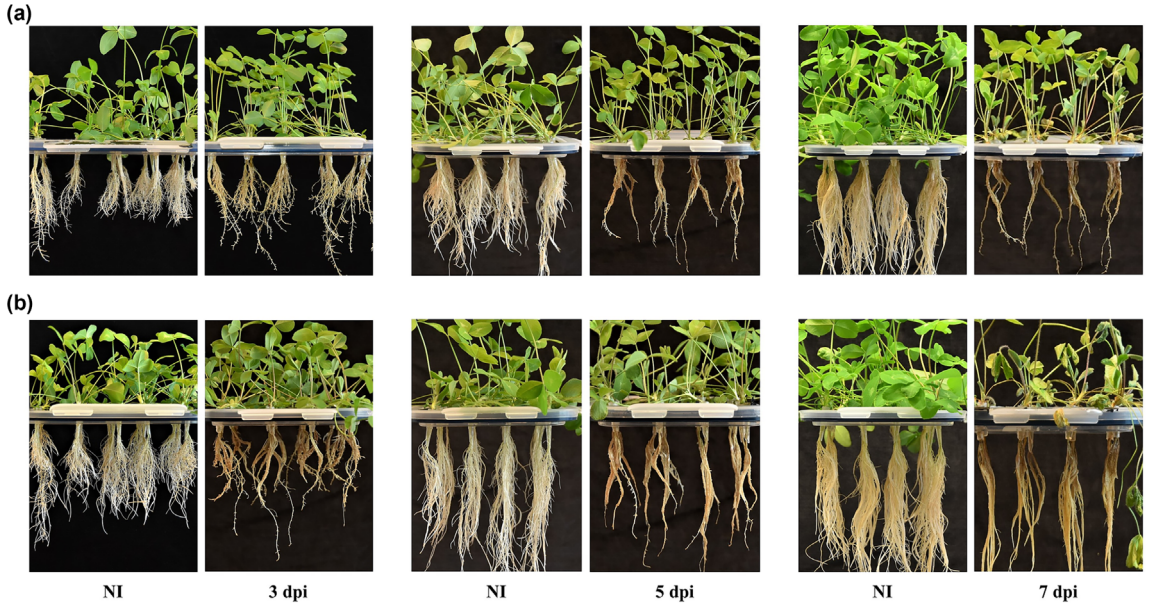

FIGURE 5 Phenotypic responses of resistant and susceptible red clover plants grown in hydroponics media with 30,000 spores/mL final concentration of *Fusarium avenaceum*. Root and foliage symptom development on (a) resistant (population no. 3) and (b) susceptible red clover (population no. 18) plants at 3-, 5-, and 7-days post inoculation (dpi). NI indicates noninoculated plants at different time points.

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with a DI of 3.0 at 7 dpi (Table 4). The screening procedure was repeated on plants from the four most resistant breeding populations and on one of the most susceptible populations to evaluate the reproducibility of the observed results. The results were consistent with the previously generated data (Table S1). Next, we selected individuals from the breeding population no. 3 with a DI of 1.80 as the most resistant and from the breeding population no. 18 with a DI of 3.0 as the most susceptible (Figure 5 a and b) for fungal DNA quantification. The amount of *F. avenaceum* DNA was significantly higher in the samples from the susceptible plants at 5 and 7 dpi than in the samples from the more resistant plants ($P < .05$; Figure 6). The fungal DNA content detected in the more resistant samples at 3, 5, and 7 dpi ranged between 28.6 and 46.5 ng, whereas in the susceptible material, fungal DNA varied between 38.2 and 87 ng per 100 mg infected root materials. Overall, the amount of fungal DNA in the susceptible root materials was approximately two-fold higher than that in the resistant samples at 5 and 7 dpi.

The effect of pathogen on nodule development in the most resistant and susceptible red clover plants was monitored using our hydroponic system. Based on the phenotypic observation at 7 dpi of *F. avenaceum*, *R. leguminosarum* bv. *trifolii* protected the roots from pathogen establishment and reduced root rot severity (Table 5 and supporting information Video S1). In rhizobia + *F. avenaceum* treated plants, nodules were not observed (Video S1). However, new roots started to develop in these plants which under other types of growth regimes may eventually lead to survival of the plants.

TABLE 4 Disease index (DI) values 7 days post inoculation on red clover breeding populations.

Breeding populations	DI
1	**2.3**
2	3.0
3	**1.8**
4	2.6
5	2.9
6	3.0
7	2.6
8	3.0
9	3.0
10	2.9
11	2.9
12	3.0
13	3.0
14	**2.1**
15	2.4
16	2.8
17	**2.3**
18	3.0
19	3.0
20	3.0
21	3.0
22	2.8
23	2.5
24	2.9
25	2.8

Note: Bold numbers indicate most resistant breeding populations.

TABLE 5 Disease index (DI) values 7-days post inoculation of *Fusariun avenaceum* on most resistant and susceptible red clover breeding population with and without *Rhizobium leguminosarum* bv. *trifolii* treatment.

	DI	
Breeding populations	Without rhizobia	With rhizobia
Most resistant breeding population no. 3	1.9	0.7
Most susceptible breeding population no. 18	3.0	0.9

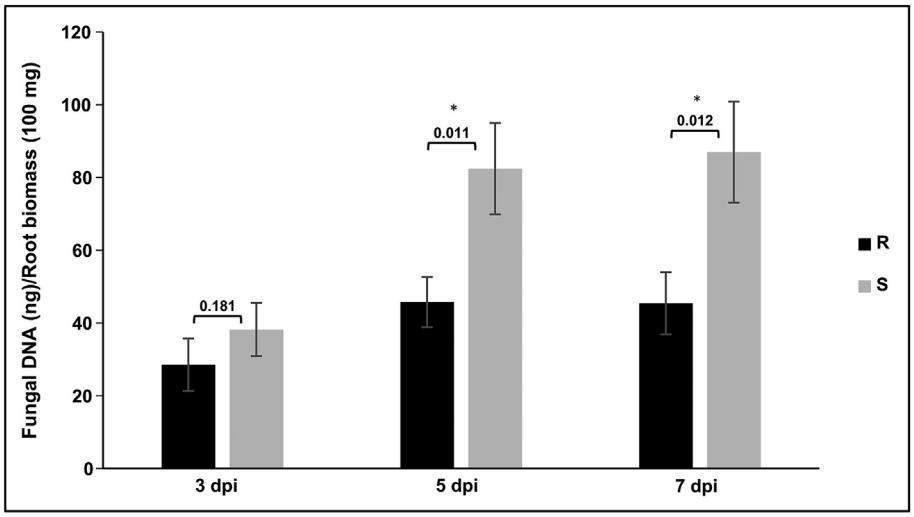

FIGURE 6 Absolute quantification of *Fusarium avenaceum* DNA in resistant (R) and susceptible (S) red clover plants grown in the hydroponic system. Error bars indicate standard deviations. The value above the bars shows the corresponding *P* values obtained by Student's *t* test. Asterisks indicate significant differences between resistant and susceptible individual plants from different breeding populations at the $P < .05$ significance level. The experiment was repeated twice each with three biological replicates.

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4 | DISCUSSION

The method described in the current study was successfully applied to screen plants from multiple red clover breeding populations for *F. avenaceum* resistance in a limited space over a short period of time. It required 28 days to complete the experiment. For earlier greenhouse- and growth chamber-based screening methods, a duration of more than 70 days has been reported in red clover (Skipp et al., 1986) and more than 60 days in alfalfa (Deng et al., 2007; Ding et al., 2011). For field screening methods, nearly 9 months are required to select for root rot resistance in red clover (Coulman & Lambert, 1995). Moreover, because screening via the hydroponics method was conducted in a growth chamber, it was less affected by external environmental factors, and precise growth conditions were easier to maintain.

To date, hydroponic screening systems have been documented for use in several other crop studies, such as those on bean root rot (Anderson & Guerra, 1985), chickpea *Phytophthora* root rot (Amalraj et al., 2019), soybean *Phytophthora* root rot (Lebreton et al., 2018), *Fusarium* in alfalfa (Cong et al., 2018), and *Fusarium* in banana (Li-sha et al., 2015). Compared to these studies, the hydroponic screening system developed here in red clover is simpler, requires low maintenance, and is cheaper because basic laboratory materials and plastic boxes, which can at least partly be recycled, are used to build this system. One of its specific advantages is the nature of the support used for germination and seedling growth (200 μL tip boxes used as a mini-greenhouse). Furthermore, because both the solution container and the cover are made of black plastic, algae growth is prevented and the solution remains clear. However, simple and basic equipped laboratory and access to controlled environments are required.

Rhizobia are known to protect legume hosts against soil-borne pathogens (Jack et al., 2019). Thus, we were curious to know whether such effects could also be seen using our system. We took advantage of the mobile seedling holder (box lid) that easily allowed the transfer of plants to another container. Phenotypic observation demonstrated that although nodule development was affected in presence of the fungus, *R. leguminosarum* bv. *trifolii* decreased root rot severity. Whether this phenomenon is related to exopolysaccharide production (Acosta-Jurado et al., 2021) or any other metabolite would be interesting to explore. Likewise, if growth and host establishment of *R. leguminosarum* bv. *trifolii* somehow could be promoted, use of such microbes as biocontrol agent may prove of great benefit in reducing losses because of *F. avenaceum*. Taken together, these results indicate that the hydroponic screening method described in this study is a valuable option for germplasm evaluations and in-depth studies of this pathosystem.

5 | CONCLUSION

In this study, we developed a simple, cost-effective, and reproducible hydroponics-based screening method for *F. avenaceum* in red clover. The system allows easy access to the roots for measurement, sampling, and monitoring of infection responses without destructive tissue treatments. The latter will be utilized in near future work on fungal and red clover gene interactions based on transcriptomic data. Further, this method is not restricted to red clover. We tested other crops, such as timothy that demonstrated high susceptibility to both *F. avenaceum* and *F. graminearum* (Athukorala, 2021). Several potential pathogens have also been screened for their pathogenicity in red clover using this hydroponics-based method (Jambagi et al., 2023). In conclusion, this hydroponic-based procedure has potential to generate valuable information in multiple areas.

AUTHOR CONTRIBUTIONS

Experiments were designed and planned by SJ and CD. Work was conducted and data acquired by SJ. The paper was written and edited by SJ and CD. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the Swedish University of Agricultural Sciences.

DATA AVAILABILITY STATEMENT

The materials used and data generated/or analyzed in this current study are available from the corresponding author upon request.

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