

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

Shridhar Jambagi (✉ shridhar.jambagi@slu.se)

Swedish University of Agricultural Sciences

Christina Dixelius

Swedish University of Agricultural Sciences

Method Article

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Abstract

Background

Red clover (*Trifolium pratense*) is an important forage legume crop that suffers like most perennial crops from attacks by soilborne pathogens. *Fusarium* root rot is one of the most serious diseases and at the same time problematic to identify resistance to due to 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 more undemanding and reliable system to facilitate studies of red clover-*Fusarium avenaceum* interactions.

Results

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 twenty-five populations to identify red clover genotypes with the most contrasting responses to *F. avenaceum*. Susceptible plants had approximately 2-fold higher amounts of fungal DNA than resistant plants, demonstrating a correlation between the disease readings of the plants and pathogen DNA.

Conclusions

A hydroponics-based screening system was established to facilitate reproducible and rapid screening of *Fusarium* root rot disease in red clover. 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.

Background

Red clover (*Trifolium pratense*) is an important forage legume crop in temperate agriculture, where it plays a key role in sustainable livestock farming systems [1]. It is widely cultivated within Europe and worldwide. In Scandinavia, as well as 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, and red clover replaces soybean in regions where it cannot be grown [2, 3]. Red clover can adapt to a wide range of soils and is rather drought tolerant due to its taproot, which can extend to a depth of more than 1 m [4]. 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 [5]. Concerns regarding climate change and the rising importance of sustainable agriculture and organic farming have revived interest in increasing the use of red clover as a cover crop, forage, and/or green manure [3, 6]. Red clover is a perennial crop and is expected to provide good biomass yield for two to

three years but rarely does so. This limited persistence has been attributed to several biotic and abiotic stress factors [4, 6, 7].

Soilborne pathogens commonly pose threats to perennial crops, and red clover is no exception. *Fusarium avenaceum* causes root rot, which may lead to complete plant loss in red clover [8, 9] and other legumes [10]. 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 [11]. *Fusarium avenaceum* is a cosmopolitan pathogen reported to cause damage to both monocot and dicot plant species and trees [12–14]. This fungal species has a genome enriched with genes encoding secondary metabolites known to be active in mycotoxin production. Furthermore, it has the capacity to enter a sexual stage, which suggests that the exchange of traits is possible between different *Fusarium* strains and possibly species [15]. To date, no reports are available on toxins produced in *Fusarium*-diseased red clover plants, which is in contrast to the situation in cereals [16].

In our work on red clover improvements, 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 [17]. To accomplish this goal, the infection process must be studied more closely, including functional evaluations of altered fungal genes [18], along with nonsoil-based inoculation procedures. Here, we describe a simple and reliable hydroponic-based system under controlled conditions where the addition of the pathogen of choice and its infection process could be more easily monitored on any chosen red clover genotype. Furthermore, since we lack red clover genotypes with clear contrasting responses to *F. avenaceum*, we decided to screen 25 genotypes utilizing the new hydroponics-based method.

Methods

Fungal strain confirmation and preparation of inoculum

The *F. avenaceum* CBS 115699 strain 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 [19]. The internal transcribed spacer region ITS1-ITS4 was PCR amplified [20] and then Sanger sequencing was performed to confirm species identity. This *F. avenaceum* strain was then used for all red clover inoculations. The generated *F. avenaceum* DNA was also used as reference material in the root infection DNA quantification assay.

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 2 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 three-week-old sterile red clover seedlings and reisolation of the fungi growing out of the root tissues on fresh potato dextrose agar plates.

Hydroponic-based *in planta* inoculation

An *in planta* inoculation method was established to screen red clover for *Fusarium* root rot resistance using hydroponically grown seedlings inoculated with *F. avenaceum* spore suspension culture. The red clover seeds (approximately 500 mg) 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 seeds were placed in the center of each tip using a spatula (Fig. 1a). Filled boxes were closed and sealed with 3M tape (Micropore, Germany). The sealed boxes were transferred to the growth chamber for germination at $22 \pm 1^\circ\text{C}$ with a 16 h photoperiod. After 4 days, when seedlings had reached the two-leaf stage (Fig. 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 (Fig. 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 (Fig. 1d). Boxes with three-week-old plants (Fig. 1e) with and without 3×10^6 fungal spores/mL were prepared. The course of infection was examined daily. The root symptoms were scored on a scale from 0 to 3 (Table 2). A disease index 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 [21]. Twenty red clover plants for each red clover genotype were used for fungal infection, and the experiments were replicated three times.

Table 1
Hydroponic medium composition

1. Prepare the following stock solutions and store them at 4°C		
Solutions	Concentration	Molecular weight
KNO ₃	1M	101.0
CaCl ₂	1M	147.0
MgSO ₄ ·7H ₂ O	1M	246.5
KH ₂ PO ₄ ·	1M	136.1
NH ₄ NO ₃	1M	236.0
Fe-EDTA	70mM	367.1
2. Prepare the micronutrient stock solution and store at -20°C		
H ₃ BO ₃	50mM	61.8
MnSO ₄ ·H ₂ O	10mM	169.0
ZnSO ₄ ·7H ₂ O	1.5mM	287.5
CuSO ₄ ·5H ₂ O	1mM	249.7
Na ₂ MoO ₄	0.58mM	241.9
3. To prepare 1 L of full-strength hydroponic solution, mix		
Stock solutions	Volume (mL)	
KNO ₃	2.5	
CaCl ₂	1	
MgSO ₄ ·7H ₂ O	0.75	
KH ₂ PO ₄ ·	0.5	
NH ₄ NO ₃	0.5	
Fe-EDTA	1	
Micronutrients	1	
Make up to 1 L in distilled water		
Hydroponic media formulation is as adapted from [42–44]		

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/plant dead or dying	3

Quantification of fungal DNA in red clover roots

The roots of control and treated red clovers were harvested 3, 5 and 7 d after fungal infection, rinsed thoroughly with sterile water, patted dry on a clean paper towel, snap-frozen in liquid nitrogen and stored at -70°C until DNA extraction. Genomic DNA was prepared using a cetyl trimethyl ammonium bromide (CTAB) procedure [19]. 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, dried and resuspended in sterile water. The extracted DNA was quantified using Qubit (Invitrogen) and stored at -20°C until further use.

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 [22]. The standard curve technique was applied with a tenfold dilution series of DNA from *F. avenaceum* dissolved in sterile water ranging from 100000, 10000, 1000, 100 and 10 pg DNA. 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 ng *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 [23, 24]. The statistical significance of fungal DNA amounts in different samples was calculated using Student's t test.

Results

This hydroponic-based screening of red clover to its root rot pathogen *F. avenaceum* was initiated since no other methods tried were reproducible. 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 in the boxes (15,000 spores/mL, 30,000 spores/mL and 60,000 spores/mL) were examined at different time points 1, 3, 5 and 7 days post inoculation (dpi), and the phenotypic responses were turned into a disease index (DI). 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.

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 (Additional file 1: Fig. S1 and Table S1). 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 (Additional file 1: Fig. S2 and Table S1). When using the highest spore concentration in the boxes, a high DI was achieved at 3 dpi (Additional file 1: Fig. S3 and Table S1). Repeated experiments did not change these observations. 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 screened a smaller collection of 25 diploid red clover genotypes in this hydroponic system. When plants were challenged with *F. avenaceum* in the hydroponic solution, disease symptoms could first be observed on the roots as early as 3 dpi. Typical symptoms of *F. avenaceum* infection were observed both on roots and aerial parts, and many plants died between 5 and 7 dpi. Four genotypes with elevated levels of resistance with DI values between 1.8 and 2 were identified, indicating that complete resistance was not present among the germplasm evaluated. A total of 10 diploid genotypes were completely susceptible, with a DI of 3.0 at 7 dpi. The screening procedure was repeated for the four most resistant genotypes and one of the most susceptible genotypes to evaluate the reproducibility of the observed results. The results were consistent with the previously generated data. Thus, we selected the genotype with a DI of 1.80 as the most resistant and the genotype with a DI of 3.0 as the most susceptible (Fig. 2) for qPCR analysis. The amount of *F. avenaceum* DNA was significantly higher in the samples from the susceptible genotype at 5 and 7 dpi than in the samples from the more resistant genotype ($P < 0.05$; Fig. 3). The fungal DNA content detected in the more resistant samples at 3, 5 and 7 dpi ranged between 28.6 ng and 46.5 ng, whereas in the susceptible material, fungal DNA varied between 38.2 ng and 87 ng per 100 mg infected root materials. Overall, the amount of fungal DNA in the susceptible root materials was approximately 2-fold higher than that in the resistant samples at 5 and 7 dpi.

Discussion

In red clover breeding programs, disease resistance to soilborne pathogens is of prime importance to extend the persistence of the plants to at least two years. For the selection of resistant materials, field- and/or greenhouse-based screening methods have been applied [8, 25–28]. Those approaches commonly encounter many constraints; they have limited reproducibility, require large spaces, and are labor-intensive and time-consuming. In our work, we also tried greenhouse-based and paper roll [29]

methods to screen red clover for *F. avenaceum* but encountered problems with secondary contaminants and reproducibility.

To date, hydroponic screening systems have been documented for use in several other crop studies, such as those on bean root rot [30], chickpea *Phytophthora* root rot [31], soybean *Phytophthora* root rot [32], *Fusarium* in alfalfa [33], and *Fusarium* in banana [34]. Compared to these studies, the hydroponic screening system developed here in red clover is much simpler, requires low maintenance and is cheaper since 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. Furthermore, since both the solution container and the cover are made of black plastic, algae growth is prevented and the solution remains clear. However, laboratory and controlled environments in climate chambers are required only for the use of sterile materials.

The method described in the current study was successfully applied to screen multiple red clover genotypes 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 [25] and more than 60 days in alfalfa [35, 36]. For field screening methods, nearly 9 months are required to select for root rot resistance in red clover [26]. Moreover, since 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.

Red clover is an outbreeding plant species with high genetic diversity and cultivars representing populations [37]. When applying this hydroponic system, superior plants can be easily recognized within individual cultivars and replanted in soil to form smaller populations whose offspring could be implemented in ongoing breeding work. In that context, our hydroponic system offers speed breeding attributes.

Rhizobia can protect legume hosts against soilborne pathogens [38]. 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. For *Rhizobium* treatment, we transferred box lids with two-week-old red clover seedlings to a square Petri dish containing rhizobial suspension [39, 40] (Additional file 1: Fig. S4). Multiple washing steps were performed in the same manner by transferring the box lids to a new container containing sterile water, and then the lids were transferred back to the boxes containing hydroponic solution adjusted to 1/3 of the initial nitrogen concentration. After one week of *Rhizobium* inoculation, *F. avenaceum* was added at a final concentration of 30,000 spores/mL as before. Based on the phenotypic scoring at 7 dpi of *F. avenaceum*, *Rhizobium* protected the roots from pathogen establishment (Additional file 2: Movie). Whether this phenomenon is related to exopolysaccharide production [41] or any other metabolite would be interesting to explore. Likewise, if growth and host establishment of *Rhizobium leguminosarum* bv. *trifolii* could be promoted and used as a biocontrol agent to reduce losses due to *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.

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 sampling. The system is not restricted to red clover. We tested other crops, such as timothy and oilseed rape, that could be grown and screened for soilborne pathogens in similar ways. Additionally, in our recent work [17], this hydroponics-based screening system was used to screen several potential pathogens on the susceptible red clover genotype. Moreover, this soil-free system is well suited for advanced omics analysis, allowing for insights into important processes regarding pathogen virulence, Rhizobium responses and plant defense genes.

Abbreviations

CTAB

cetyl trimethyl ammonium bromide

DI

Disease index

dpi

days post inoculation.

Declarations

Acknowledgments

All red clover seeds were provided by Lantmännen, Sweden. The CBS 115699 strain was obtained from the Westerdijk Fungal Biodiversity Institute, the Netherlands.

Authors' 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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Availability of data and materials

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

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.

References

1. Sullivan ML, Quesenberry KH, Clover. Red (*Trifolium pratense*). In: Wang K, editor. Agrobacterium Protocols. Methods in Molecular Biology. New York: Springer; 2015. p. 1223.
2. Lindström BE, Frankow-Lindberg BE, Dahlin AS, Watson CA, Wivstad M. Red clover increases micronutrient concentrations in forage mixtures. Field Crops Res. 2014;169:99–106.
3. Ferreira H, Pinto E, Vasconcelos MW. Legumes as a cornerstone of the transition toward more sustainable agri-food systems and diets in Europe. Front Sustain Food Syst. 2021;5:694121.
4. Taylor NL, Quesenberry KH. Red clover science. Current Plant Science and Biotechnology in Agriculture 28. Dordrecht, the Netherlands: Kluwer Academic Publishers; 1996.
5. McKenna P, Cannon N, Conway J, Dooley J, Davies WP. Red clover (*Trifolium pratense*) in conservation agriculture: a compelling case for increased adoption. Int J Agric Sustain. 2018;16:342–66.
6. Taylor NL. A century of clover breeding developments in the United States. Crop Sci. 2008;48:1–3.
7. Annicchiarico P, Barrett B, Brummer EC, Julier B, Marshall AH. Achievements and challenges in improving temperate perennial forage legumes. Crit Rev Plant Sci. 2015;34:327–80.
8. Yli-Mattila T, Kalko G, Hannukkala A, Paavanen-Huhtala S, Hakala K. Prevalence, species composition, genetic variation and pathogenicity of clover rot (*Sclerotinia trifoliorum*) and *Fusarium* spp. in red clover in Finland. Eur J Plant Pathol. 2010;126:13–27.
9. Stoltz E, Wallenhammar AC. Micronutrients reduce root rot in red clover (*Trifolium pratense*). J Plant Dis Prot. 2012;119:92–9.
10. Baćanović-Šišić J, Šišić A, Schmidt JH, Finckh MR. Identification and characterization of pathogens associated with root rot of winter peas grown under organic management in Germany. Eur J Plant Pathol. 2018;151:745–55.

11. Crous PW, Lombard L, Sandoval-Denis M, Seifert KA, Schroers HJ, Chaverri P, Gené J, et al. *Fusarium*: more than a node or a foot-shaped basal cell. *Stud Mycol.* 2021;98:100116.
12. Kovačikova E, Kúdela V. *Fusarium* species associated with root rot of red clover. *Zentralblatt für Mikrobiologie.* 1982;137:407–14.
13. Desjardins AE. Gibberella from A (venaceae) to Z (eae). *Annu Rev Phytopathol.* 2003;41:177–98.
14. Leach MC, Hobbs SL. Plantwise knowledge bank: delivering plant health information to developing country users. *Learn Publ.* 2013;26:180–5.
15. Lysøe E, Harris LJ, Walkowiak S, Subramaniam R, Divon HH, Riiser ES, Llorens C, Gabaldon T, Kistler HC, Jonkers W, Kolseth AK. The genome of the generalist plant pathogen *Fusarium avenaceum* is enriched with genes involved in redox, signaling and secondary metabolism. *PLoS ONE.* 2014;9:e112703.
16. Inbaia S, Farooqi A, Ray RV. Aggressiveness and mycotoxin profile of *Fusarium avenaceum* isolates causing *Fusarium* seedling blight and *Fusarium* head blight in UK malting barley. *Front Plant Sci.* 2023;14:1121553.
17. Jambagi S, Hodén KP, Öhlund L, Dixelius C. Red clover root-associated microbiota is shaped by geographic location and choice of farming system. *J Appl Microbiol.* 2023;134:1–17.
18. Sørensen LQ, Lysøe E, Larsen JE, Khorsand-Jamal P, Nielsen KF, Frandsen RJ. Genetic transformation of *Fusarium avenaceum* by *Agrobacterium tumefaciens* mediated transformation and the development of a USER-Brick vector construction system. *BMC Mol Biol.* 2014;15:1–15.
19. Möller EM, Bahnweg G, Sandermann H, Geiger HH. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. *Nucleic Acids Res.* 1992;20:6115.
20. White TJ, Bruns T, Lee SJ, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications.* 1990;18:315–22.
21. Happstadius I, Ljungberg A, Kristiansson B, Dixelius C. Identification of *Brassica oleracea* germplasm with improved resistance to *Verticillium* wilt. *Plant Breed.* 2003;122:30–4.
22. Pollard AT, Okubara PA. Real-time PCR quantification of *Fusarium avenaceum* in soil and seeds. *J Microbiol Methods.* 2019;157:21–30.
23. Martin T, Biruma M, Fridborg I, Okori P, Dixelius C. A highly conserved NB-LRR encoding gene cluster effective against *Setosphaeria turcica* in sorghum. *BMC Plant Biol.* 2011;11:1–11.
24. Tzelepis G, Bejai S, Sattar MN, Schwelm A, Ilbäck J, Fogelqvist J, Dixelius C. Detection of *Verticillium* species in Swedish soils using real-time PCR. *Arch Microbiol.* 2017;199:1383–9.
25. Skipp RA, Christensen MJ, Biao NZ. Invasion of red clover (*Trifolium pratense*) roots by soilborne fungi. *New Z J Agric Res.* 1986;29:305–13.
26. Coulman BE, Lambert M. Selection for resistance to root rot caused by *Fusarium* spp. in red clover (*Trifolium pratense* L.). *Can J Plant Sci.* 1995;75:141–6.

27. Venuto BC, Smith RR, Grau CR. Selection for resistance to *Fusarium* wilt in red clover. *Can J Plant Sci.* 1999;79:351–6.
28. Vleugels T, Cnops G, Van Bockstaele E. Screening for resistance to clover rot (*Sclerotinia* spp.) among a diverse collection of red clover populations (*Trifolium pratense* L.). *Euphytica.* 2013;194:371–82.
29. Liljeroth E, Jansson HB, Schäfer W. Transformation of *Bipolaris sorokiniana* with the *GUS* gene and use for studying fungal colonization of barley roots. *Phytopathol.* 1993;83:1484–9.
30. Anderson AJ, Guerra D. Responses of bean to root colonization with *Pseudomonas putida* in a hydroponic system. *Phytopathol.* 1985;75:992–5.
31. Amalraj A, Taylor J, Sutton T. A hydroponics based high throughput screening system for *Phytophthora* root rot resistance in chickpea (*Cicer arietinum* L.). *BMC Plant Methods.* 2019;15:1–15.
32. Lebreton A, Labbé C, De Ronne M, Xue A, Marchand G, Bélanger R. Development of a simple hydroponic assay to study vertical and horizontal resistance of soybean and pathotypes of *Phytophthora sojae*. *Plant Dis.* 2018;102:114–23.
33. Cong LL, Sun Y, Wang Z, Kang JM, Zhang TJ, Biligetu B, Yang QC. A rapid screening method for evaluating resistance of alfalfa (*Medicago sativa* L.) to *Fusarium* root rot. *Can J Plant Pathol.* 2018;40:61–9.
34. Zeng LS, Lv S, Du CX, Xia L. Identification of banana resistance to *Fusarium* wilt by method of hydroponics inoculation. *J South Agr.* 2015;46:817–22.
35. Deng R, Xiang QH, Zhang DH. In vivo whole plant inoculation test of alfalfa root rot. *J Anim Husb Vet Med.* 2007;31:3.
36. Ding SY, Li MQ, Zhao H. Identification of disease resistance of different varieties of alfalfa to *Fusarium* spp. *Plant Prot.* 2011;37:110–4.
37. Jones C, De Vega J, Lloyd D, Hegarty M, Ayling S, Powell W, Skøt L. Population structure and genetic diversity in red clover (*Trifolium pratense* L.) germplasm. *Sci Rep.* 2020;10:1–12.
38. Jack CN, Wozniak KJ, Porter SS, Maren L, Friesen ML. Rhizobia protect their legume hosts against soil-borne microbial antagonists in a host-genotype-dependent manner. *Rhizosphere.* 2019;9:47–55.
39. Lodeiro AR, Lagares A, Martinez EN, Favelukes G. Early interactions of *Rhizobium leguminosarum* bv. *phaseoli* and bean roots: Specificity in the process of adsorption and its requirement of Ca²⁺ and Mg²⁺ ions. *Appl Environ Microbiol.* 1995;61:1571–9.
40. Mongiardini EJ, Ausmees N, Pérez-Giménez J, Julia Althabegoiti M, Ignacio Quelas J, López-García SL, Lodeiro AR. The rhizobial adhesion protein RapA1 is involved in adsorption of rhizobia to plant roots but not in nodulation. *FEMS Microbiol Ecol.* 2008;65:279–88.
41. Acosta-Jurado S, Fuentes-Romero F, Ruiz-Sainz JE, Janczarek M, Vinardell JM. Rhizobial exopolysaccharides: Genetic regulation of their synthesis and relevance in symbiosis with legumes. *Int J Mol Sci.* 2021;22:6233.
42. Norén H, Svensson P, Andersson B. A convenient and versatile hydroponic cultivation system for *Arabidopsis thaliana*. *Physiol Plant.* 2004;121:343–8.

43. Tocquin P, Corbesier L, Havelange A, Pieltain A, Kurtem E, Bernier G, Périlleux C. A novel high efficiency, low maintenance, hydroponic system for synchronous growth and flowering of *Arabidopsis thaliana*. BMC Plant Biol. 2003;3:1–10.
44. Bindschedler LV, Palmblad M, Cramer R. Hydroponic isotope labelling of entire plants (HILEP) for quantitative plant proteomics; an oxidative stress case study. Phytochem. 2008;69:1962–72.

Figures

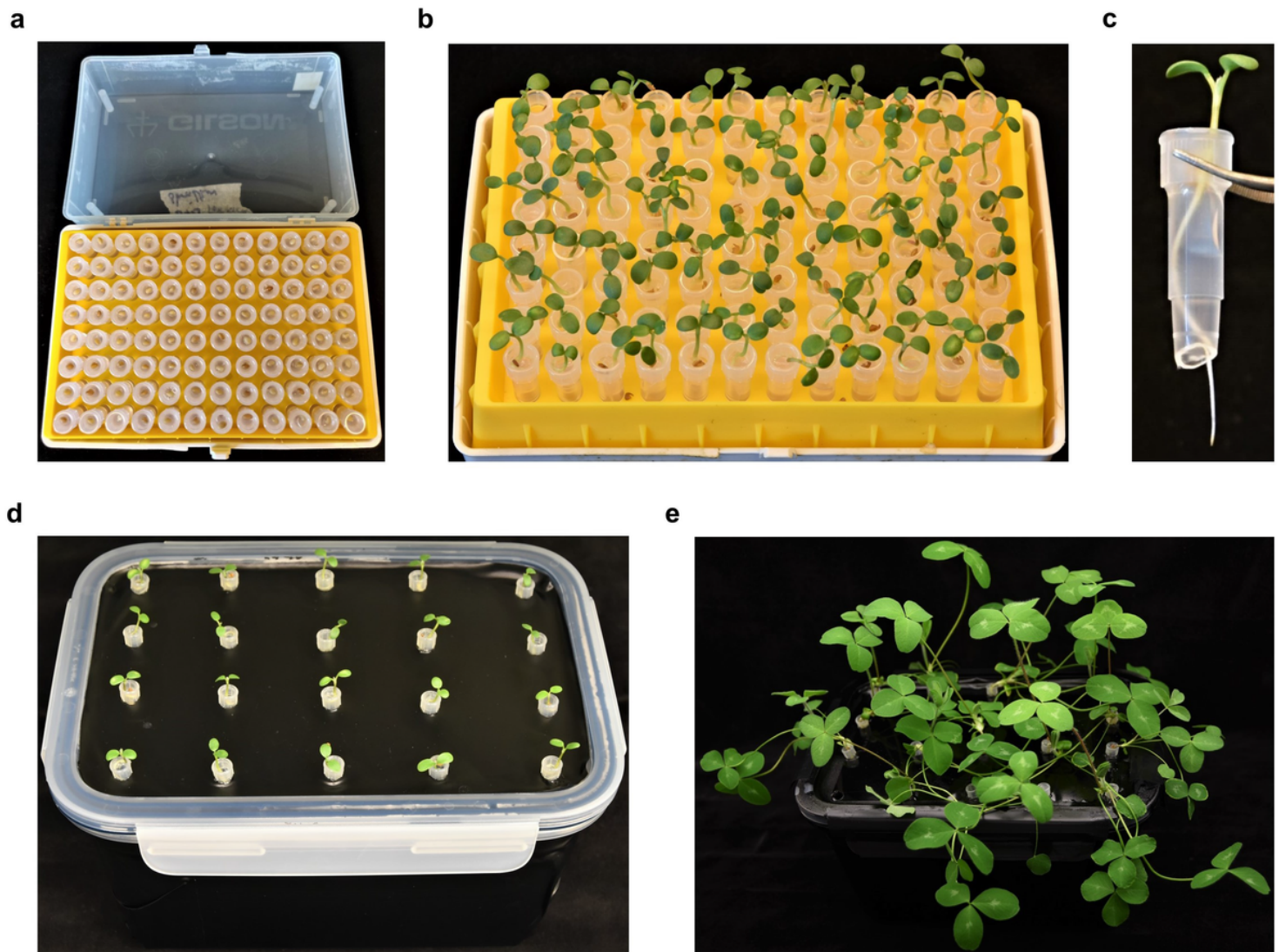


Figure 1

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) four-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, (e) three-week-old plants growing in hydroponic media.

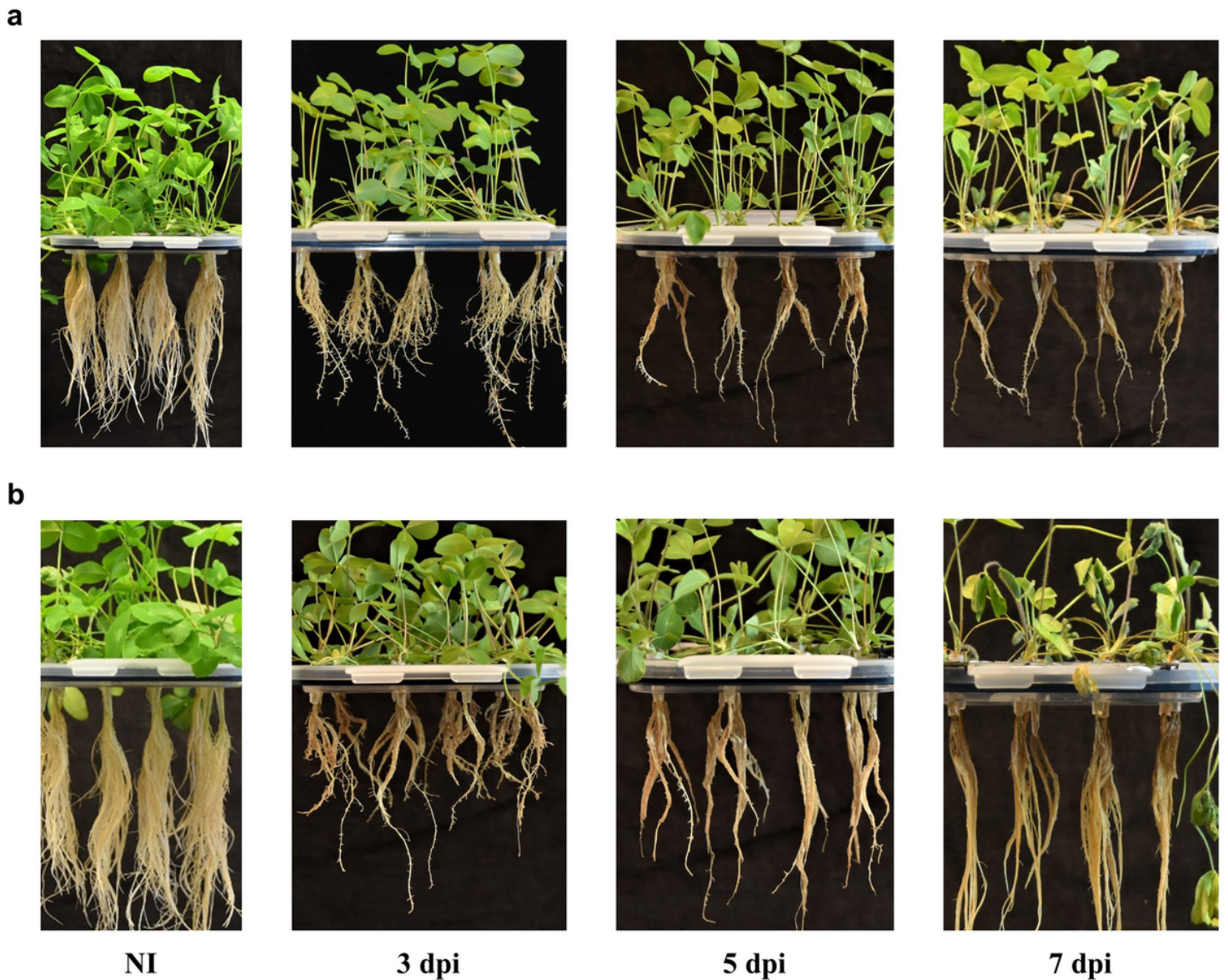


Figure 2

Phenotypic responses of resistant and susceptible red clover plants grown in hydroponics media with 30,000 spores/mL final concentration of *F. avenaceum*. Root and foliage symptom development on (a) resistant and (b) susceptible red clover plants at 3, 5 and 7 days post inoculation (dpi). NI indicates noninoculated.

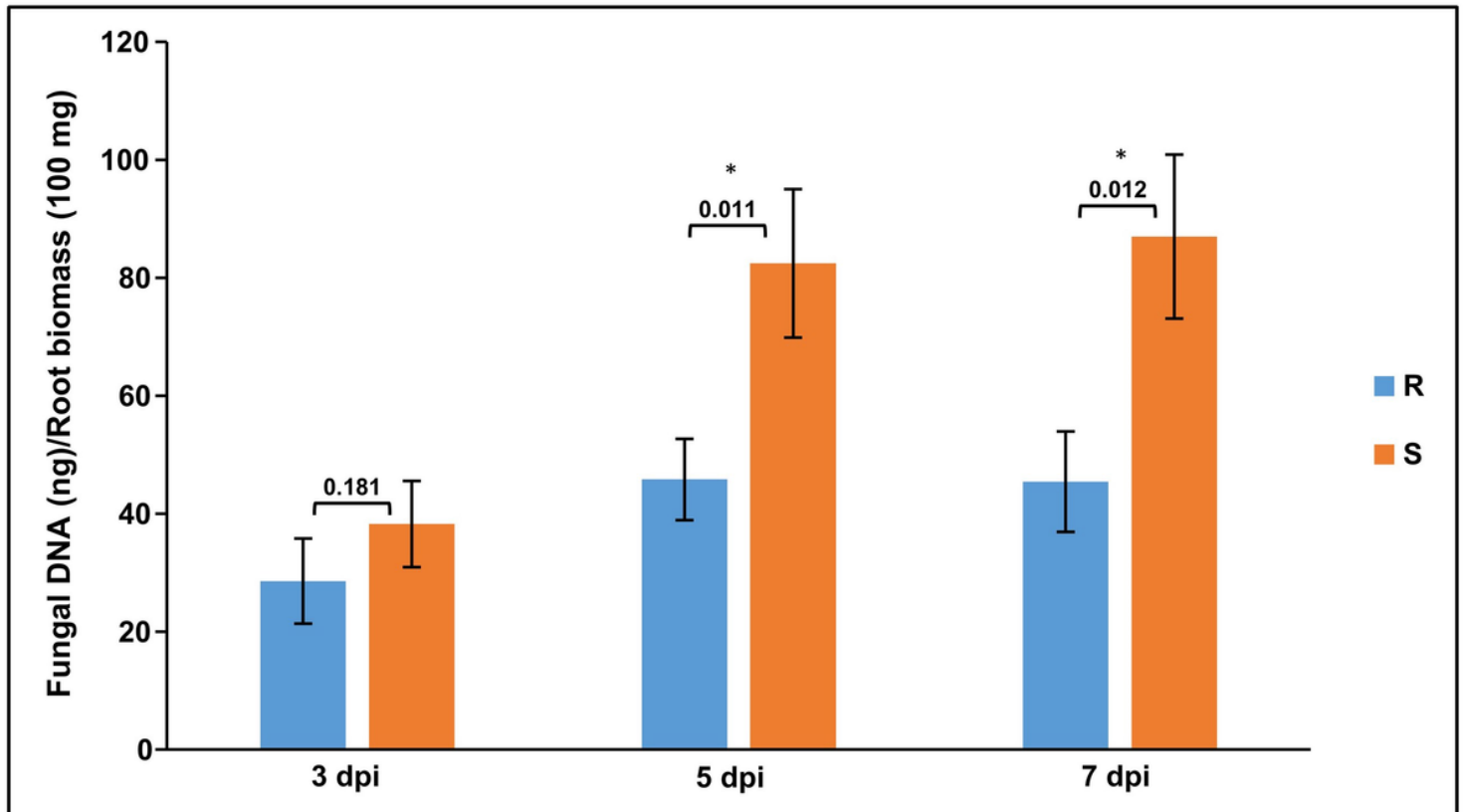


Figure 3

Absolute quantification of *F. 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 genotypes at the $P < 0.05$ significance level. The experiment was repeated twice each with four biological replicates.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1SupplementaryfiguresandtablesFinal.docx](#)
- [Additionalfile2Movie.mp4](#)