Effects Of Cereal Cyst Nematode Infection on Resistant and Susceptible Barley

By

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List of abbreviations

CCN- cereal cyst nematode

cM- centiMorgan

cMX- central metaxylem

d- day

DAI- days after inoculation

ER- endoplasmic recticulum

Ho- hydraulic conductance

Hpr- hydraulic conductivity

HPTS- 8-hydroxyprene-1, 3, 6- trissulfonic acid trisodium salt

J2- second stage juvenile

PCN- potato cyst nematode

RKN- root-knot nematode

RSA- root surface area

SCN- soybean cyst nematode

UV- ultraviolet light

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Abstract

Cereal cyst nematodes (CCN) are obligate biotrophs that affect the yield of cereals including barley. This parasite is one of the biotic factors that affect plant-water relations and imposes early stress that reduces yield irrespective of the availability of water and nutrient. Invasion, migration, feeding site formation, and feeding by this nematode alter host root anatomy and interfere with water and nutrient transport in the plant.

To better understand the effects of CCN infection on water transport, susceptible and resistant barley cultivars were studied using exudation and suction methods to measure hydraulic conductivity (Lpr). Hydrostatic Lpr obtained using the suction method was assessed in the presence of xylem tension which was exerted at the shoot base using a vacuum while osmotic Lpr obtained using the exudation method was determined in the existence of osmotic gradient and water flow. Control plants showed higher hydraulic conductivity in the suction experiment (mean $5.47 \pm 0.8 \times 10^{-7}$ $ms^{-1}MPa^{-1}$) than in two exudation experiments (mean $2.99 \pm 0.2 \times 10^{-7}$ $ms^{-1}MPa^{-1}$ and $1.46 \pm 0.06 \times 10^{-7}$ $ms^{-1}MPa^{-1}$). CCN infection reduced growth, exudation flow rate, hydraulic conductance, and hydraulic conductivity of both resistant and susceptible barley. Both techniques showed the effect of CCN infection on hydraulic properties, but the effect was more evident in the suction experiment (70.1% reduction in Lpr in inoculated plants) than in the exudation experiments (21.5% and 19% reduction in Lpr in inoculated plants).

To further investigate how water and solutes travel through seminal roots of barley that are parasitised by CCN, 8-hydroxyprene-1, 3, 6-trisulfonic acid trisodium salt (HPTS) was used as an apoplastic tracer dye. The flow of HPTS (presumably carried with water) through seminal roots was temporarily interrupted in infected regions. The duration of this interruption was longer in a CCN-susceptible cultivar Sloop than in its CCN-resistant derivative Sloop SA.

Another CCN-resistant cultivar, Galleon for which resistance is conferred by the *Rha4* locus on chromosome 5H, was confirmed to exhibit more robust seedling growth in hydroponics than the CCN-susceptible cultivar Sloop. Similar differences were also observed between resistant and susceptible early-generation Galleon × Sloop backcross families, making it seem possible that seedling vigour plays a role in CCN resistance. However, comparisons of seedling vigour between resistant (*Rha4*+) and susceptible (*Rha4*-) progeny within advanced generation backcross families revealed no significant differences. It was concluded that the CCN resistance locus *Rha4*+ is not the cause of the seedling vigour.

1 Chapter 1: Introductory background

1.1 Introduction

Nematodes are non-segmented roundworms of the phylum Nematoda. They are the world's most abundant life forms and exist in all ecosystems (Jenkins 2005; Bohlmann 2015). Most species of these roundworms live freely and are essential in organic matter recycling, but some exist as parasites of animal or plant species.

Plant-parasitic nematodes are either sedentary or migratory. Among the sedentary nematodes are the cyst nematodes (*Betulodera*, *Paradolichodera*, *Vittatidera*, *Dolichodera*, *Punctodera*, *Cactodera*, *Globodera*, and *Heterodera* species) (Turner and Subbotin 2013) and the root-knot nematodes (*Meloidogyne* species). Notable members of the genus *Heterodera* include the cereal cyst nematodes (CCN) (*Heterodera avenae*), soybean cyst nematode (*H. glycine*), and sugar beet cyst nematode (*H. schachtii*). The cereal cyst nematode *Heterodera avenae* is an important endoparasite of cereal crops such as wheat, barley, and oat causing great destruction in many parts of the world with extensive cereal farming. It is also a minor parasite of maize (Nicol et al. 2011).

Barley, *Hordeum vulgare*, is an important grain crop in Australia with an estimated gross value of AUD 2.3 billion (ABARES 2019). The total production area of barley in Australia in 2018-2019 was about 4 million ha with an estimated production of 8.82 million tonnes (ABARES 2018). This crop is highly adaptable and grown over a large geographical area in Australia. The environmental conditions of the southern growing regions in Australia are suitable for *Heterodera avenae*.

Global yield losses due to plant-parasitic nematodes have been estimated to be worth over USD 80 billion annually (Handoo 1998). *Heterodera avenae* can cause about 20-50% yield loss to cultivated cereals in temperate regions and is more prominent in areas with drought (as

reviewed by Nicol 2002). According to Murray and Brennan (2010), the estimated yield loss of barley caused by CCN in three growing regions (Western, Southern, and Northern) in Australia was AUD 25 million with a potential loss increasing the amount to AUD 153 million if uncontrolled. However, the use of CCN resistant crops and clean fallows in rotation with CCN resistant cereal cultivars has contributed to the reduction of CCN populations in the soil (Nicol and Rivaol 2008; Riley and McKay 2009; Dababat et al. 2015).

In Australia, a single pathotype of CCN (Ha13) has been identified and is widely distributed in the southern regions and is detected in red-brown earth, grey clay, heavy clay, brown soils, and sandy soils (Brown 1969; O'Brien and Fisher 1979). In barley, four CCN resistance loci have been mapped: *Rha1*, *Rha2,* and *Rha3* on chromosome 2H (Cotten and Hayes 1969; Andersen and Andersen 1970; Kretscher et al. 1997) and *Rha4* on chromosome 5H (Barr et al. 1998). *Rha2* and *Rha4* are known to confer effective resistance against the Australian pathotype Ha13 (Barr et al. 1998; Williams and Fisher 1993).

In work conducted in the University of Adelaide, Dr. Pradeepa Bandaranayake (unpublished data), noticed that plants of Galleon (*Rha4* resistant cultivar) had more robust root systems than plants of Sloop (a susceptible cultivar) after being grown for 7 days in a hydroponic system. Could *Rha4* resistance simply be due to greater root vigour?

Recent research on CCN infection on wheat, revealed alterations of the anatomy of the metaxylem vessels, causing those vessels to be shorter and wider than in non-inoculated plants (Levin et al. 2020). Similar changes were also observed in barley (Pradeepa Bandaranayake unpublished data). With the application of a fluorescent dye 8-hydroxyprene-1, 3, 6- trissulfonic acid trisodium salt (HPTS) at the tips of seminal roots of CCN-inoculated wheat plants, it was observed that dye transport (and presumably water transport) was blocked or inhibited near the

feeding site (Levin 2020). Further investigation is needed to confirm to this observation and to determine how CCN infection affects the hydraulic properties of cereal plants.

This thesis reports on experiments to understand the flow of water through the roots of CCN-infected and non-infected control barley plants to estimate water flow rates for resistant and susceptible barley cultivars. The thesis also reports on an investigation of whether the *Rha4* region on chromosome 5H affects seedling vigour.

2 Chapter 2: Literature review

2.1 Barley and its economic importance

Barley is an important and versatile crop that ranks fourth after wheat, maize, and rice globally and second to wheat in Australia. Due to the adaptation of the crop to a wide range of environment, it can strive in areas where rice and maize cannot grow. The production of barley is mostly in areas with Mediterranean climate and stretches to the artic and sub-artic zones and in areas with continental and oceanic climate (Zhou 2009). The global production of barley in 2017-2018 was 145.28 million metric tonnes (FAOSTAT 2020). In Australia, barley is grown as a winter and spring crop in a crop rotation with legumes. Barley is principally used for malting and animal feed. This crop is rich in dietary fibre, minerals, and vitamins. Barley also serves as a model crop for biotechnology, plant breeding methodology, pathology, and genetics studies (Saisho and Takeda 2011).

2.2 Cyst nematodes

2.2.1 Biology and morphology of cyst nematode

Cyst nematodes are obligate biotrophs and consist of eight genera (*Betulodera, Paradolichodera Vittatidera, Cactodera, Heterodera, Globodera, Punctodera,* and *Dolichodera*) in the subfamily Heterodeninae (Turner and Subbotin 2013). The two genera considered to have the most species with agronomic importance globally are *Heterodera* and *Globodera*. *Heterodera* is significantly larger with 82 species compared to 12 species of *Globodera* (Bohlmann 2015). The most destructive species of agricultural importance are the potato cyst nematode (*Globodera pallida* and *G. rostochiensis*), soybean cyst nematode (*Heterodera glycine*), beet cyst nematode (*H. schachtii*), and the cereal cyst nematodes (*H. avenae*) (Turner and Subbotin 2013). Cyst nematodes have a predominantly indistinguishable morphology. The juveniles are vermiform, with a dome-shaped head and tapering conical tail, ranging in size between 330 to 700 µm in length. The adult males are also vermiform and range from 450 to 1700 µm in length. The adult females are spherical or lemon-shaped (300-900 µm

in length and 200-810 µm in width) (Stone 1985; Bohlmann 2015). Since nematodes lack a skeleton, the presence of the cuticle and hydroskeleton maintain their shape and aid in feeding, digestion, movement, and reproduction (Basyoni and Rzik 2016).

2.2.2 Life cycle and host penetration of cereal cyst nematode

Cysts can contain hundreds of eggs. Moulting from first-stage juveniles (J1) to second-stage juveniles (J2) starts in the egg. Hatching of second-stage juveniles (J2) is in response to secretion of exudates from host plant roots (such as sugars, carbon dioxide, and amino acids) (Turner and Subbotin 2013) at appropriate soil temperatures and soil moisture levels. In southern Australia and other regions with Mediterranean climates, these conditions occur in autumn (10-15^{o}C soil temperature) while in the northern ecotype regions, they occur in spring (5-10°C soil temperature) (Kerry and Jenkinson 1976; Rivoal 1978; Rivoal 1986; Stanton and Eyres 1994; Baklawa et al. 2017). In southern Australia, the emergence of J2 begins in late May with hatching peaking in July. Under favourable conditions, approximately 80% of eggs hatch from cyst annually with the other 20% remaining dormant until the next season. Under dry conditions, about 50% remain dormant (Hollaway 2013), potentially increasing the population of larvae in the next season (Meagher 1970).

Hatched J2 in the soil are attracted to the roots of their host plants and penetrate host cells in the elongation zone behind the root tip, using their stylet and cell-wall degrading enzymes and other biomolecules (Curtis 2007, Gheysen and Jones 2006) produced in their sub-ventral glands. They move intracellularly through the root cortex towards the vascular cylinder where they form syncytial feeding sites (Wyss and Zunke 1986; Tytgat et al 2002). These sites are large and metabolically active multinucleated cells, formed by the nematode-induced fusion of multiple uninuclear cells. They become a sink where water and nutrients are drawn to nourish the nematode for their development (Seah et al. 2000; Hofman and Grundler 2007).

The J2 nematodes feed and moult into J3 and J4 and develop into males or females. The adult males become motile and leave the root, and later fertilise the sedentary female guided by a sex pheromone produced by the female. After fertilisation, the females' bodies are swollen with eggs and as the root matures, the female dies. The Mediterranean climate of hot dry summers with wet winters in southern Australia favours the rapid development of the female *H. avenae* to complete its life cycle in late spring and form a cyst around the eggs for survival in summer (Meagher 1970).

The generational life cycle of nematodes is influenced by the prevailing environmental conditions. During favourable conditions, more than one generation per season could be attained. Cereal cyst nematodes complete one generational life cycle in the host crop season. However, there have been reports of *H. schachtii* completing two or more generational life cycle in the host crop season depending on the prevailing temperature (Lewis 1971; Kakaire et al 2015).

2.2.3 Cereal cyst nematode distribution and symptoms

Cyst nematodes are among the most adapted and specialised plant-parasitic nematode pests of agricultural importance. According to Nicol et al. (2011), *Heterodera avenae, H. latipons* and, *H. filipjevi* are the three main species of economic importance in the Mediterranean, West Asia, and North Africa. Populations of *H. avenae* are widespread and have been found in countries including northern Africa, South Africa, USA, Asia, Canada, Australia, Europe, Japan, China, and Israel (Nicol et al. 2011). In Australia, a single pathotype of CCN (Ha13) has been identified and is widely distributed in South Australia, southern New South Wales, Victoria, and Western Australia (Brown 1969; O'Brien and Fisher 1979).

2.2.4 Symptoms and damage to crops

Symptoms of CCN infection in a cereal field appear as patches of yellowed stunted chlorotic growth, and wilting. Root growth is impaired, and roots have a knotted appearance. White cysts

the size of a pinhead become visible on the roots about the time of head emergence in barley. Above-ground symptoms are similar to other diseases, water stress, and soil nutrient deficiency. A feature of host-root response to CCN infection is the formation of lateral roots in the region of infection. Williams and Fisher (1993) also observed dense proliferation of root hairs behind the apical meristematic region of the root and attributed it to impediment of root elongation by the nematode. The region swelled into galls and produced lateral roots within 14 days.

Assessing the impact of damage caused by nematodes is very difficult since the infection can be misdiagnosed due to the similarity of symptoms with those of other diseases (Nicol et al. 2011). The feeding activities (mechanical damage) of the plant-parasitic nematodes sometimes initiate secondary infections to the host plant with other pathogens such as viruses, bacteria, fungi, and other nematode species causing the host plant to be susceptible to other plant diseases (Trudgill et al. 1975; Manzanilla-López and Starr 2009; Nicol et al. 2011). For example, studies on *Meloidogyne* species have shown disease complexes with *Fusarium oxysporum* which increased the susceptibility of tomato and cotton to vascular wilt disease (Porter and Powell 1967; Atkinson 1892; Starr and Martyn 1991).

2.2.5 Current management of cereal cyst nematode

In Australia, management of this pest has been achieved using break crops (legumes) and resistant varieties in crop rotations of two or more years to reduce the numbers of CCN in the soil. Also, early sowing with optimal soil fertility enhances root development and plant growth, and this reduces the effect of CCN on the crop (Nicol and Rivoal 2008). The use of CCN-resistant cereal cultivars has long been considered the most cost-effective and leading approach to minimise crop losses to nematodes (Trudgill 1991).

2.3 Plant responses to cyst nematode infection

Upon entry into the roots of the host plant, J2 nematodes migrate to the stele where they explore several neighbouring procambium or cambium cells to establish their initial syncytial cell. The

release of effector proteins through the stylet of the J2 nematodes can induce typical root cells to transform into specialised feeding sites (Hewezi and Baum 2012). However, if a nematode encounters a defence reaction of the plant cell such as deposition of callus-like material on the stylet or collapse of the protoplasm it can redraw its stylet to probe other cells or move through the cell to probe other suitable cells (Wyss 1992; Golinoswki et al. 1997; 1999). According to Seah et al. (2000), differentiation of cells induced to form syncytia, is more advanced in resistant barley (*Rha2* and *Rha4*) and wheat than in susceptible cultivars in the early days (2-5 d) of infection by CCN. Syncytia in all resistant plants were extensively vacuolated while susceptible plants had reduced vacuoles. Although the resistant barley cultivars had highly vacuolated syncytia, their syncytia were less metabolically active as compared to the resistant wheat cultivars (Seah et al. 2000). Williams and Fisher (1993) also observed the ultrastructure of syncytia in both resistant and susceptible wheat. In susceptible wheat, syncytia were active in appearance with dense cytoplasm, numerous organelles (plastids, mitochondria, free ribosomes, endoplasmic reticulum), reduced vacuoles, and membrane-bound cell wall ingrowths adjacent to vascular elements. Conversely, the resistant cultivar had the cytoplasm of its syncytia reduced to a thin layer with compressed cell organelles. At the final stage of the infection, the syncytia of the resistant cultivars degenerated with scattered cytoplasm and chromatin.

Resistance responses of white mustard (*Sinapsis alba*) to beet cyst nematode (*Heterodera schachtii*) have been reported by Golinowski and Magnusson (1991) to include necrosis of the xylem parenchyma, which inhibits the formation of the feeding site near the xylem. While necrosis of cells was also observed in both resistant (+*Cre1* and +*Cre3)* and susceptible (-*Cre1* and -*Cre3*) wheat (Williams and Fisher 1993), it did not affect the separation of the feeding site from the xylem. This necrosis could be the result of mechanical damage caused by the migration of the J2 nematodes through the plant. Williams and Fisher (1993) suggested that *Cre1* and

Cre3 resistance responses are induced by elicitors produced by the female nematode at a stage of its development. These elicitors activate a hypersensitive response of the host plant which degrades the syncytia.

In contrast, Levin et al. (2021) reported differences in the position of syncytia relative to xylem vessels between CCN-resistant (+*Cre8*) and susceptible (-*Cre8*) wheat plants. In +*Cre8* plants, syncytia were usually at a distance from the metaxylem vessels with no direct connections to those vessels. In -*Cre8* plants, syncytia were directly adjacent to metaxylem vessels, often with direct connections through xylem walls, with some xylem vessels elements seeming to have been incorporated into feeding sites. Furthermore, the xylem vessels of the resistant cultivars had greater secondary thickening (lignin) which may have prevented the connection of the syncytia to the xylem. These observations may indicate that resistance mechanisms differ among resistance genes, and they highlight the potential for CCN infection to interfere with water transport.

2.4 CCN resistance loci in barley

CCN resistance loci have been mapped in earlier studies in barley. Three of these loci (*Rha1*, *Rha2*, and *Rha3*) are on chromosome 2H. *Rha2* and *Rha3* are closely linked with each other, but distant from *Rha1* (Cotten and Hayes 1969; Andersen and Andersen 1970).

Kretscher et al. (1997) mapped *Rha2* relative to RFLP markers in two mapping populations, Chebec/Harrington, and Clipper/Sahara 3771. In both linkage maps, the *Rha2* locus was flanked by AWBA21 and MWG694, with the intervals being 11.4 cM in Chebec/Harrington and 13.2 cM in Clipper/Sahara 3771. More recently Van Gansbeke et al. (2019) fine mapped the *Rha2* locus to a 978 kbp region and discussed three genes as possible candidates for *Rha2*.

Using a Haruna Nijo/Galleon mapping population, Barr et al. (1998) mapped a new resistance locus, *Rha4* on chromosome 5H, within an 18.7-cM interval between RFLP markers (XYL)

and BCD298. Recently, the *Rha4* region has been further narrowed to ~542 kbp on the IBSC Morex assembly Version 2 (Kelvin Khoo, unpublished). Within this region of the assembly, there are 14 high confidence predicted genes and 5 low confidence predicted genes.

Seah et al. 2000 determined that *Rha2* and *Rha4* confer resistance by degrading the syncytia in barley in 13 and 10 days respectively after CCN infection. A similar observation was made by Williams and Fisher (1993) in wheat where resistance was by syncytia degradation in the root stele and stopping of female nematode development at the J4 stage at about 15 days after the infection.

During a recent visit to our laboratory, Dr. Pradeepa Bandaranayake noticed that plants of Galleon (a *Rha4* resistant cultivar) had much more robust root systems than plants of Sloop (a susceptible cultivar) after being grown for 7 d in a hydroponic system. Could *Rha4* resistance simply be due to greater root vigour? To investigate this possibility, there is the need to determine whether the difference in root vigour can be attributed to the *Rha4* locus.

2.5 Nematode-host water and nutrient transport

Cyst nematodes are wholly reliant on their hosts for nutrients and water to complete their lifecycle. The host xylem supplies the syncytia with inorganic solutes and water while amino acids and sugars are obtained from the phloem (Hofmann and Grundler 2007). Although there is no published data on nutrient use of *H. avenae*, Sijmons et al. (1991) indicated that the J4 females of *H. schachtii* consume 0.4 - 0.6 times the syncytia volume whilst adult females can take up to four times the syncytium volume of solute per day from the host Arabidopsis. Studies conducted by Golinowski et al. (1996) showed that there is hypertrophy of phloem components that promote high nutrient transfer into the syncytia. Additionally, Jones and Northcote (1972), observed projections of the cell wall of the syncytia which squashed sieve elements and further

acted against xylem elements indicating high demand of nutrients by the nematode, causing a bottleneck of water and inorganic solutes in potato roots infested with *Globodera rostochiensis*.

The discovery of CCN infection altering the anatomy of the metaxylem vessels of both wheat and barley (Levin et al. 2020; Pradeepa Bandaranayake, personal communication) and blockage of the apoplastic tracer dye (8-hydroxyprene-1, 3, 6- trissulfonic acid trisodium salt (HPTS)) near the feeding site when applied at the tips of seminal roots of wheat (Levin 2020), need further investigation to confirm or determine how infection by CCN affects water transport in cereal plants.

2.6 Water and solute transport

The transport of water and solutes in plants is thought to involve transcellular transport across plasmalemma, symplastic transport across plasmodesmata, and apoplastic transport within and between cell walls in the vascular cylinder (Steudle et al. 2000). Cell-to-cell transcellular and symplastic pathways involve transport across membranes and can involve aquaporins. The apoplastic pathway involves transport within the cell walls, intercellular air spaces, and middle lamella (Steudle 2000a; Knifer and Fricke 2010).

The principal pathway of water movement in roots varies in response to the environment, root developmental stages, induced changes in root anatomy due to stress, day/ night, and the prevailing driving force (osmotic, hydrostatic) (Steudle 2000, Steudle and Peterson 1998). The hydraulic barrier to water uptake by the root is mainly between the root epidermis and xylem (radial transport path) rather than the axial transport path along the xylem conduit (Steudle and Peterson 1998; Frensch and Steudle 1989). Apoplastic barriers (Casparian bands and suberin lamellae, which are impermeable to water and solutes) limit the transport of water through the root exodermis and endodermis and necessitates a switch to symplastic flow (the cell-to-cell pathway) creating a local water flow equilibrium/ balance between the two pathways (Steudle 2000). Studies conducted on hydraulic conductivity and water transport in barley have shown

that the leading radial transport pathways are the cell-to-cell (symplastic and transcellular) pathways at all stages of development (Steudle and Jeschke 1983, Suku et al. 2014).

Root hydraulic properties change with the magnitude of water flow that is induced across the root (Passioura and Munns 1984). This makes it important to determine the contribution of individual roots to water uptake dependent on the main radial transport pathway and the induced driving force (hydrostatic and osmotic). Knipfer and Fricke (2011), analysed the hydraulic conductivity of barley grown hydroponically dependent on the main driving force and reported that 92% of water uptake is through the seminal roots whereas 8% is contributed by the adventitious roots. From the observations made, both types of roots had similar radial hydraulic conductivity with little dependence on the prevailing driving force. This suggested a transport involving the crossing of membranes consistent with reports that water transport in barley is mainly symplastic. The wide difference in water uptake between adventitious roots and seminal roots was attributed to the difference in root numbers, surface areas, and axial hydraulic conductance.

2.6.1 Connection of syncytia to the symplastic and apoplastic pathways

In moving from phloem to nematode feeding structures, organic solutes are transported through both symplastic, and apoplastic short distance pathways (Hofmann and Grundler 2007). The mechanism that activates solute transport into nematode-induced sink tissues is uncertain. Böckenhoff and Grundler (1994) studied nutrient uptake by *Heterodera schachtii* in the feeding structure of *Arabidopsis thaliana* using microinjection of fluorescent dyes, fluorochrome lucifer yellow CH (LYCH) and fluorochrome-labelled dextrans. The tracer dyes were not detected in the root vascular elements or in cells adjacent to the nematode-induced structure indicating that syncytia are symplastically isolated. Hofmann et al. (2007) supported this claim with findings that transporters play a significant role in the early stages of syncytia development when functional plasmadesmata to the phloem have not been established. However, symplastic

connections were observed at day 14 when phloem-mobile dye carboxyfluorescein (CF) (460 Da) was used to study the occurrence of functional plasmadesmata. This is consistent with results reported by Hofmann and Grundler (2006) that green fluorescent protein (GFP) was transported to the syncytia from the phloem late in nematode development. It, can therefore be, concluded that syncytia are symplastically isolated at the early stages of nematode development and later symplastically connected to the phloem through plasmadesmata. Also, at later stages, transporters retrieve leaking sucrose from the apoplast to the syncytia (Hofmann et al. 2007).

2.6.2 Relationship of cellular root tissue of the host and the induced syncytia

Research on root tissue infected by cyst nematodes show abnormal enlargement of the host cell, tissues, and organs, creating multinucleated and highly metabolic feeding sites (Jones and Northcote 1972; Golinowski et al. 1996; Williams and Fisher 1993; Seah et al 2000) for their nourishment and development. Also, remodelling of the central cylinder in wheat has been observed (Levin et al. 2020). In studying feeding sites induced by *H. schachtii* in the root of white mustard, Magnusson and Golinowski (1991) observed that a significant portion of the syncytia volume arose from xylem parenchyma cells, which were the first cells to differentiate to form the spearheads of the syncytia. Lateral roots were found to increase the functional base of the syncytia as the syncytia in the main root extended into the lateral roots.

While cereal cyst, potato cyst, and soybean cyst nematodes initiate their feeding site in the stele, beet cyst nematodes induce syncytia in the pericycle that extend longitudinally to the central cylinder and the xylem parenchyma (Endo 1964; Jones and Northcote 1972; Magnusson and Golinowski 1991; Bohlmann 2015). Nevertheless, there are many features that are similar among syncytia induced by all cyst nematodes including dense cytoplasm, tubular structures, plastids, mitochondria, smooth endoplasmic reticulum, an enlarged nucleus with a reduced vacuole and invagination of cell wall adjacent to the vascular element and syncytia (Magnusson and Golinowski 1991; William and Fisher 1993; Golinowski et al. 1996).

Examination of the ultrastructure of *H. schachtii* in Arabidopsis roots showed a direct connection of the feeding tube to the endoplasmic reticulum (ER). Owing to the function of ER (transfer of sugars, the formation of lipids and transfer to membranes, and processing of proteins), it could contribute significantly to the nourishment of the nematodes by supplying activated nucleotide sugars, proteins, and unsaturated fatty acids that could serve as an energy source and guard against oxygen radicals (stress factor) in the nematode (Sobczak et al 1999; Hofmann and Grundler 2007). Further, the abnormal enlargement of the xylem parenchyma, graniferous tracheary elements, and extension of the syncytia into the lateral roots contribute significantly to the syncytia showing the extensive demand for solutes and water (Magnusson and Golinowski 1991).

2.7 Nematode nutrient requirements

Adequate nutrient uptake and a balanced distribution of nutrients within plant tissues are required for proper plant growth. Nematodes require amino acids, carbohydrates, lipids, vitamins, and mineral elements for their survival and reproduction. The nutrient requirements and consequences of nematodes on nutrient uptake differ among species, host type, and stage of the infection (Trudgill et al. 1975; Blevins et al. 1995; Goheen et al. 2013;). According to Blevins et al. (1995), root concentration of magnesium and potassium decreased upon treatment of soybean with two populations of soybean cyst nematodes (PI 209332 and PI 89772) at day 35 whereas root calcium and phosphorus increased. Translocation of calcium and magnesium to the leaves was higher with lower potassium concentrations. Nutrient element concentrations of the isolated cyst had higher nutrient element concentrations in decreasing order of calcium > phosphorus > potassium > sodium or magnesium. Trudgill et al. (1975) found that infection by the potato cyst nematode (PCN) reduces nutrient concentrations such as phosphorus, potassium, and nitrogen in the foliage of infected potato plants. Although a significant quantity of nitrogen was removed by the PCN, it was not a major growth-limiting factor and stunted

growth was attributed to loss of potassium. De Ruijter and Haverkort (1999) also made similar observations. They concluded that depletion of phosphorus is the cause of total biomass reduction.

2.8 Conclusion

Crop improvement for pathogen resistance is important for increased yield and quality to meet global population increases and ensure food security. Understanding of the interactions between host plants and communities of microorganisms has been a major area of concern for many years. Studies on CCN infection and resistance in barley and wheat (Cotten and Hayes 1969; Trudgill et al. 1975; Williams and Fisher 1993; Blevin et al. 1995; Seah et al. 2000; Goheen et al. 2013; Siddique and Grundler, 2018, Levin et al 2020; 2021) have broadened the understanding on the complex interaction of CCN and their host plants. These studies have given insight into how the infection affects the availability of water and nutrients to the host plant, root cellular response to the infection process, and how resistance loci affect the pathogen. However, little is known about how the infection affects the hydraulic conductivity of host plants. In-depth knowledge, particularly concerning water use efficiency could inform pathological management systems on barley and could provide useful insights for dealing with other plant species parasitised by nematodes. Findings from this work will enhance knowledge in the field of plant physiology in understanding the effects of the physiological changes induced by nematodes on water transport.

Therefore, the work presented in this thesis focuses on the use of hydraulic conductivity and an apoplastic fluorescent dye protocol to study the flow of water through the root system of CCN-infected and control barley plants to estimate water flow rates for resistant and susceptible barley cultivars. It also addresses whether the *Rha4* region on chromosome 5H of barley is associated with increased root vigour and whether that could help in conferring resistance against CCN.

2.9 Research questions

A. How does CCN infection affect water transport in barley roots?

B. Do CCN effects on water transport differ between resistant (*Rha2* and *Rha4*) and susceptible lines?

C. Does the *Rha4* region on chromosome 5H affect root growth in barley? If so, does this correspond to resistance?

2.10 Aims/ Objectives of the project

In the research conducted for this thesis, I aimed to describe and evaluate the functional consequences of cereal cyst nematode infection in barley roots and to relate these findings to previous observations of anatomical alteration of the root vascular tissue.

To address question A, the specific objectives were:

1. To use hydraulic conductivity measurements and tracer dyes to investigate the effects of CCN infection of barley root on water transport through whole root system.

To address question B, the specific objective was:

2. To compare the effect of CCN infection between resistant (*Rha2* and *Rha4*) and susceptible barley sister lines to establish differences or similarities in water transport.

To address question C, the specific objective was:

3. To compare the seedling vigour between *Rha4* barley lines and susceptible sister lines.

3 Chapter 3: Effect of CCN infection on hydraulic conductivity of resistant and susceptible barley

Summary

The experiments described in this chapter were conducted to evaluate the effects of CCN on hydraulic conductivity in barley and compare these effects between resistant and susceptible cultivars.

Sloop, Sloop SA, and Galleon barley were inoculated with second stage J2 CCN and their controls were mock inoculated with water. They were then grown hydroponically for 9 d followed by shoot removal and root hydraulic conductivity measurements. Root suction and exudation methods were used to estimate hydrostatic (daytime) and osmotic (night-time) hydraulic conductivity (Lpr), respectively.

The two techniques resulted in different hydraulic conductivity values with control plants exhibiting higher hydraulic conductivity values (means $5.47 \pm 0.8 \times 10^{-7}$ $ms^{-1}MPa^{-1}$) in the suction experiments than in two exudation experiments (means $2.99 \pm 0.2 \times 10^{-7}$ $ms^{-1}MPa^{-1}$ and $1.46 \pm 0.06 \times 10^{-7}$ $ms^{-1}MPa^{-1}$). Infection by CCN impeded growth, exudation flow rate, hydraulic conductance, and hydraulic conductivity in both resistant and susceptible cultivars. Both techniques detected these effects, but the effects were more pronounced with the suction method.

3.1 Introduction

Plant-parasitic nematodes including the cereal cyst nematode *Heterodera avenae* affect plant-water relations. Their infection decreases water use efficiency, declines water potential, and reduces plant biomass (O'Bannon and Reynolds 1965, Meon et al. 1978, Rahi et al. 1988), due to withdrawal of large amounts of water and inorganic solutes (sugars and amino acids) from transport systems of the host plant.

Cyst nematodes convert host plant cells into multinucleate syncytia (feeding sites) in the central vascular cylinder (in proximity to the xylem). These sites become the main source of nutrients and water for nourishment and development of the nematodes (Golinowski et al. 1996; Seah et al. 2000). According to Sijmons et al. (1991), an estimate of 0.4-0.6 times the syncytial volume of solute is consumed daily by a J4 female *H. schachtii* in Arabidopsis while an adult female can consume about four times the syncytial volume per day. Hofmann and Grundler (2007), Golinowski et al. (1996), and Jones and Northcote (1972) observed abnormal enlargement of phloem components, and the extension of syncytial cell walls that squashed sieve elements and acted against xylem elements all indicating high demand for nutrients by the nematode.

Recently, Levin et al. (2020) observed shortening of elements in the central metaxylem (which carries about 80% of water) in CCN-inoculated wheat but not in non-inoculated controls. According to Carlsbecker et al. (2010), certain fine-tuned mechanisms are coordinated for xylem formation. And as observed by Jones and Northcote (1972) these mechanisms can be hampered by the projections of syncytial cell walls. Hence, transport of water is reduced in the affected regions, creating a bottleneck of water flow through the xylem towards the shoot of the host plant. These changes (syncytium formation and activities, abnormal enlargement of phloem components, and disruption of xylem vessels) occurring in the host plant result in a physiological alteration that affects the inherent signalling system of the host causing the plant to have a pathological reaction that affects its yield (Hofmann and Grundler 2007). The

interference of water transport by nematodes and the recent discovery of xylem disruption shows the importance of studying how the parasitic activities of root pathogens and parasites affect the hydraulic conductivity and conductance of roots of host plants.

Hydraulic conductivity is a property of vascular plants that reflects how easily water can move through membranes and pore spaces. It is defined as water flow rate/ (unit driving force × root surface area) (Knipfer and Fricke 2010). Hydraulic conductance reflects the efficiency of bulk flow of water through a medium. It is defined as water flow rate/ unit driving force. These hydraulic properties are affected by root anatomy and development, day/night cycles, stress, and driving forces (Steudle and Jeschke 1983). Root hydraulic properties can be experimentally assessed, but this is complicated by the complexity of water transport (with apoplastic, symplastic, and transcellular components) and the hydraulic barrier (epidermis, cortex, and endodermis) (Steudle and Jeschke 1983).

Despite the complexity of water transport in plants, Steudle and Jeschke (1983) were able to quantify effects of pressure relaxations, initial water flow, constant water flow, and driving forces (osmotic and hydrostatic pressures) on water transport in whole roots and through cell membranes in barley. They reported higher hydraulic conductivity (between 0.3×10^{-7} and 4.3×10^{-7} cm s^{-1} bar^{-1}) for whole roots than for cell membranes (between 0.8×10^{-7} and 2.2×10^{-7} cm s^{-1} bar^{-1}). This indicates significant cell-to-cell water transport in the roots. Knipfer and Fricke (2010; 2011) and Suku et al. (2014) also studied water uptake in barley roots. They reported similar values for hydrostatic and osmotic hydraulic conductivity (Lp_r). Accordingly, cell-to-cell transport seemed to be the main factor underlying the radial transport water pathway in barley roots at all stages of development.

During the early vegetative stages of barley, the water potential gradient that drives water uptake doubles and can contribute to increases in water flow. Increased water flow increases

the hydrostatic and osmotic Lp_r, which are positively correlated with the transpiration rate of barley (Suku et al. 2014). However, the overall developmental increase in barley (~2-fold) is smaller than increases (~10- 40-fold) in root surface area and water loss during transpiration (Suku et al. 2014).

Although much work has been carried out on water transport in barley, little is known about how CCN infection affects water transport in barley roots. In the research that is reported here, established hydraulic conductivity protocols (Knipfer and Fricke 2011; Meng et al. 2016) were used to study the flow of water through the root systems of hydroponically grown CCN-infected and control roots of resistant and susceptible barley.

3.2 Materials and methods

3.2.1 Plant material

Plant materials used for this research included the CCN- susceptible barley cultivar Sloop, and two CCN-resistant cultivars (Sloop SA, with resistance conferred by the *Rha2* locus, and Galleon, with resistance conferred by the *Rha4* locus).

3.2.2 Seed germination

Seeds were surface sterilised using 3% sodium hypochlorite solution (NaOCl) (v/v) on a shaker for 10 and 5 minutes and rinsed thrice with sterile autoclaved water after each shake. After the final rinsing, seeds were plated on sterilised 2% agar (w/v) in sterile Petri dishes (140 mm) and incubated in the dark at room temperature. In each Petri dish, 16 seeds were arranged, all oriented in the same manner so that their roots would emerge in the same direction (Figure 3.1). After 3 d, germinated seeds were transferred to a growth room at a temperature of 20°C, at 80% relative humidity, and with a 12-h day/night cycle.

3.2.3 Nematode inoculum and inoculation

A silk bag (100 µm mesh) containing a mixture of *H. avenae* brown cysts and organic matter (infested soil sampled from the Yorke Peninsula, South Australia) was placed in water and kept

in a refrigerator at 5°C. After between six and eight weeks, J2 nematodes could be collected. One day prior to when nematodes were required for inoculation, the "nematode farm" was washed to flush away nematodes that already hatched. On the following day, the farm was washed again to harvest freshly hatched J2 nematodes. Water from the nematode farm was collected into a beaker and sieved through a 38 µm mesh sieve. The retained nematodes were resuspended in a small volume of clean water and poured into a 50 mL beaker. A 20 µL aliquot of the resulting nematode suspension was examined under a light microscope and the number of J2 nematodes counted. Depending on the number of J2 nematodes, the nematode suspension was further diluted to obtain a concentration of 2000 nematodes per mL.

Inoculation was carried out in a laminar fume hood once the seminal roots of barley seedlings on agar had reached between 1 and 3 cm in length, with the tip of each seminal root inoculated with 20 µL of nematode suspension. Control seedlings were mock inoculated with 20 µL of water at the root tips. Inoculated and mock-inoculated seedlings were incubated for 1 d prior to transfer to hydroponics.

3.2.4 Growth in a hydroponic system

Seedlings were washed with sterile autoclaved water, transferred into hydroponic tubes (Figure 3.1), and incubated in a growth room at 20°C and 80% relative humidity, with a 12-h day/night cycle. For plants to be tested using the exudation method, each seedling was assigned to a random position in a hydroponic tank with control and inoculated seedlings in different tanks to prevent infection of the control plants. The hydroponic solution was a modified Johnson's solution (Johnson et al. 1957) as detailed by Melino et al. (2015) containing 0.5 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.25 mM Ca $(NO_3)_2$, 0.5 mM KNO_3; and the micronutrients, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM Na_2MoO_4, and 50 µM Fe-EDTA. Stock solutions of each nutrient component were prepared and diluted to the final

solution in the hydroponic tank on the day of transfer of seedling into hydroponics. The nutrient solution in the hydroponics tanks was changed twice a week.

For plants to be tested using a suction technique, seedlings were carefully inserted into open-ended Eppendorf tubes. Those tubes were then inserted into holes that had been cut within polystyrene disks, with four plants per disk before they were transferred into the hydroponic tanks.

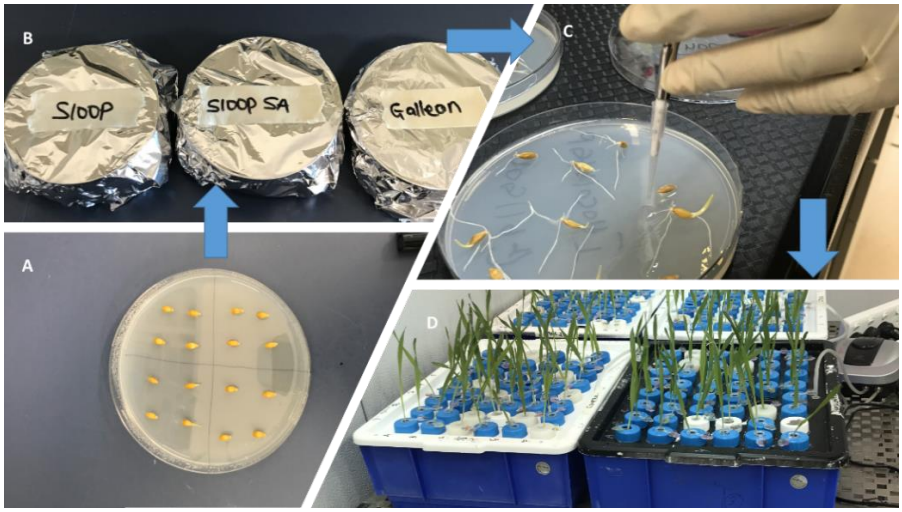

Figure 3.1 Plated seeds on 2% agar (A), seed germination in the dark (B), inoculation of seedlings root tips (C), and plants growing in hydroponics (D).

3.2.5 Exudation method for measurement of hydraulic conductivity

Root hydraulic conductivity measurements were performed using a root exudation method based on a protocol obtained from Dr. Wieland Fricke (personal communication).

A piece of silicon tubing that was narrow enough (inner diameter: 2.5 mm) to fit the shoot base of the plant to be tested was cut to about 10 mm long and a piece of thinner silicon tubing (inner diameter: 1.5 mm) was cut to about 5 mm long and inserted into one end of the larger tubing at about 2-3 mm deep. A glass capillary tube (inner diameter: 0.58 mm) was then fixed into the thinner tube such that its end was level with the larger tubing. The shoot of a plant was cut off

at an angle (20-30°) at about 1 cm above its base. The remaining base of the shoot was immediately inserted into the open end of the large silicon tubing until it almost touched the glass capillary. Vacuum grease was used to seal the shoot/ tubing junction to prevent leakage. The root system was placed in a tray filled with the plant growth medium (Figure 3.2) and the glass capillary was placed on and attached to an adjacent horizontal surface (Figure 3.2). Every 10 min, the distance travelled by the exudate through the glass capillary was marked with a marker pen. Once the capillary was full, exudate sap was collected. Distances travelled between marks on the capillary were measured with a ruler (precision 0.5 mm). Osmotic pressures of the xylem sap ($-\pi_{xylem}$) and the root medium ($-\pi_{medium}$) were measured on 20 µL samples of exudate sap and growth medium, respectively using a Fiske® Micro-Osmometer model 210. Each root system was scanned with an Epson Expression 10000XL scanner and analysed using the WinRHIZO software (Stranjnar et al. 2012). The cut shoot and roots were then weighed, oven-dried at 65°C for 4 d and weighed again.

Root hydraulic conductance (Lo, m^3s^{-1} MPa^{-1}) was calculated using the formula $Q/\Delta\psi$, in which Q is the exudation flow rate (m^3s^{-1}), $\Delta\psi$ is the driving force (MPa) for root water uptake during exudation. The driving force $\Delta\psi$ is calculated as the difference in water potential between the root medium ($-\pi_{medium}$) and xylem ($-\pi_{xylem}$, assuming a root reflection coefficient for solutes of 1.0). Using the Vaul Hoff equation, $\Delta\psi = R \times T \times \Delta C$, in which R is the Universal gas constant, T is temperature in Kelvin and ΔC is the difference in osmolality between xylem and nutrient medium. Root hydraulic conductivity (Lp_r, $ms^{-1}MPa^{-1}$) was calculated by dividing the root hydraulic conductance (Lo) by the root surface area (RSA).

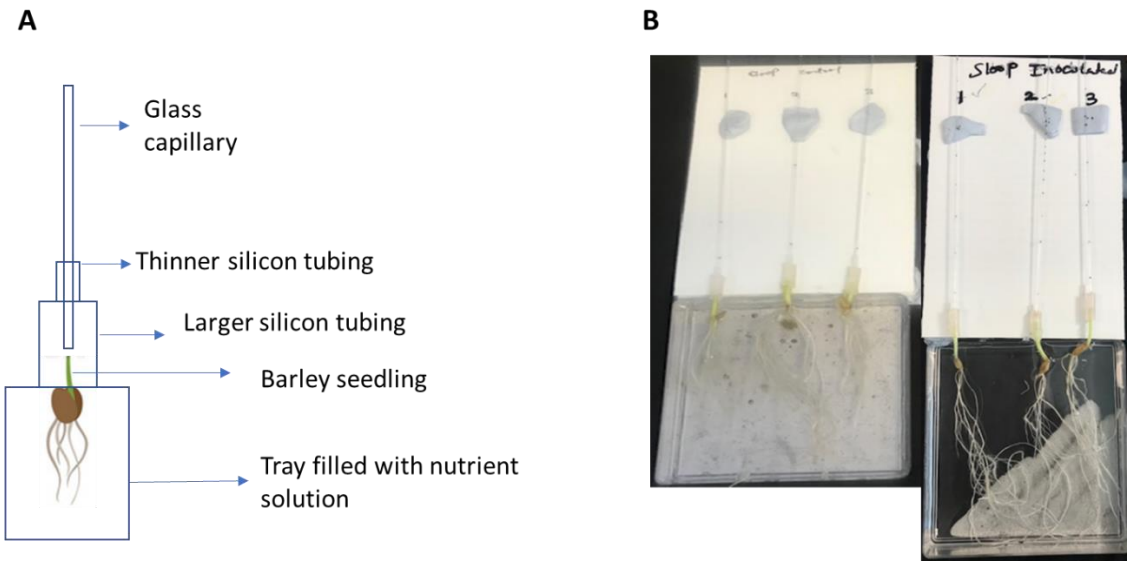

Figure 3.2 Diagram (A) and photograph (B) showing barley seedlings in the apparatus used for assessment of xylem sap exudation.

3.2.6 Suction method for measurement of hydraulic conductivity

Measurement of hydraulic conductivity using the suction method was based on methods described by Meng et al. (2016) with slight modification in the plant material used and the vacuum applied.

On the day of testing, buckets were filled with 1.25 L of nutrient solution and the solution was kept aerated throughout the measurement process. One polystyrene disk containing four plants was floated in each bucket. Pipette tips (100-1000 µL) were stuffed with cotton wool, weighed, and each attached to a thinner piece of silicon tubing that was narrow enough (inner diameter: 1.5-2.5 mm) to fit on the shoot base of a particular plant. Each pipette tip was then also attached to a piece of larger silicon tubing connected to a manometer and a vacuum pump. The shoot of each plant was cut off at about 1 cm above the shoot base and the shoot base was inserted into the thinner silicon tubing. Vacuum grease was applied around the junction of the insertion to prevent leakage of the sap out of the tubes and penetration of air into the tube. A vacuum of 100 kPa was applied for 20 min. This created a tension which caused xylem sap to be collected into the cotton wool. After 20 min, the pipette tip stacked with cotton wool was re-weighed and

the roots were scanned as described for the exudation method. Biomass data were taken as described for the exudation method.

Vacuum hydraulic conductance (Lo) was calculated using the formula Q × P, in which Q is the flow rate (m^3s^{-1}) and P is the gradient for water uptake (vacuum pressure of 100 kPa). Flow rate was calculated by dividing exudate volume (m^3) by the experiment duration (1200 s). Exudate volume was calculated from the difference between the initial and final weight of the pipette tips. Vacuum hydraulic conductivity (Lpr) was calculated by dividing vacuum hydraulic conductance (Lo) by root surface area (RSA).

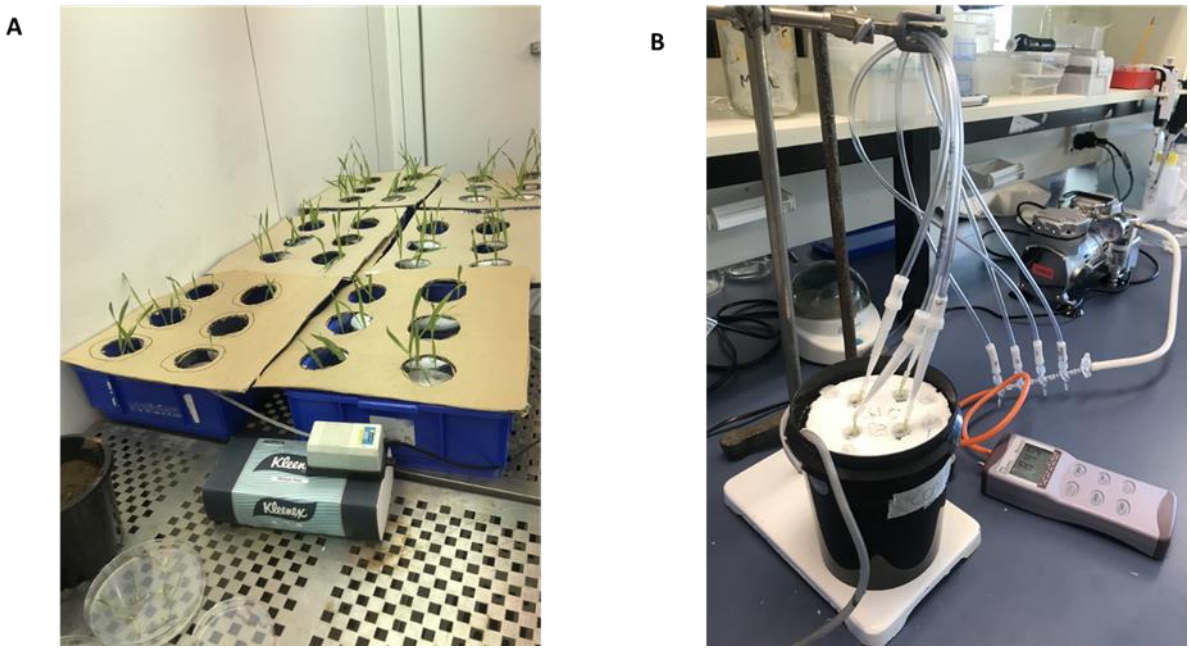

Figure 3.3 Seedlings arranged in polystyrene and randomised in hydroponics tanks. Cardboard was used to cover tanks to keep roots in the dark and to prevent algae growth (A). Experimental set-up of suction technique to measure hydraulic properties (B).

3.2.7 Experiment 1- measurement of hydraulic conductivity in Sloop barley using the exudation method to study the effect of CCN infection on hydraulic conductivity

This experiment was conducted to assess exudate flow rate, exudate osmolarity, hydraulic conductance, and hydraulic conductivity using the root exudation method.

Seed germination was staggered over three days, with 20 seeds per day surface sterilised, plated on agar, and kept for 3 d in the dark at room temperature (3.2.2). Seedlings were inoculated and transferred into the hydroponic system (3.2.3-3.2.4). After 9 d in hydroponics, four seedlings were sampled per treatment, hydraulic conductivity was measured using the exudation technique (3.2.5), and root length, root volume, root surface area, root diameter, and biomass data were taken. Plant parts (root and shoot) were dried at 65°C for 4 d.

3.2.8 Experiment 2- Measurement of the effect of CCN on root hydraulic conductivity in resistant and susceptible barley using the exudation method

This experiment was conducted to assess the effect of CCN infection on water transport in resistant and susceptible barley cultivars. The cultivars used in this study were Sloop (susceptible), Sloop SA (*Rha2* resistant), and Galleon (*Rha4* resistant). The root exudation method was used to measure exudate flow rate, driving force, exudate osmolarity, hydraulic conductance, and conductivity.

Seed germination was staggered over 6 d, with a total of 216 seeds, surface sterilised and plated on agar and kept for 3 d in the dark at room temperature. Twelve seeds were plated per cultivar per day. After 3 d, six seedlings of each cultivar were inoculated with J2 nematodes. The remaining six seedlings were used as control plants. All seedlings were incubated for 24 h in a growth room at 20°C, 12/12 h, and relative humidity 70%. Five plants of each cultivar-treatment combination were transferred to the hydroponic system. After 9 d in hydroponics, four seedlings per cultivar-treatment combination were sampled and assessed for root hydraulic properties (3.2.5), root length, root volume, root surface area, and biomass. Collected data was subjected to two-way ANOVA using GraphPad Prism version 8.

3.2.9 Experiment 3- Measurement of the effect of CCN on root hydraulic conductivity in resistant and susceptible barley using the suction method

This experiment was conducted to compare the effect of CCN infection on water transport between resistant and susceptible barley plants. The cultivars used in this study were Sloop (susceptible), Sloop SA (*Rha2* resistant), and Galleon (*Rha4* resistant).

Seed germination was staggered over 6 d with a total of 60 seeds, surface sterilised, plated on agar, and kept for 3 d in the dark at room temperature. Ten seeds of each cultivar were plated each day. After 3 d, five seedlings of each cultivar were inoculated with J2 nematodes and the remaining five were used as controls. They were then incubated in the growth room for 24 h at 20℃, 12/12 h, and relative humidity 70%. Four plants of each cultivar-treatment combination were inserted into open-ended Eppendorf tubes which were inserted into holes that had been cut within polystyrene disks. The polystyrene disks were randomly positioned in the hydroponic tanks with control and inoculated seedlings in different tanks. After 9 d in hydroponics, the suction protocol outlined in 3.2.6 in this thesis was followed to assess the effect of the infection on hydraulic properties (Figure 3.3). After each experiment, the roots were scanned for root surface area, root length, and root volume, and plant biomass. All data collected was subjected to two-way ANOVA using GraphPad Prism version 8.

3.3 Results

3.3.1 Experiment 1- measurement of hydraulic conductivity in Sloop barley using the exudation method to study the effect of CCN infection on hydraulic conductivity

By the time seedlings were transferred to hydroponics, their mean root length was 41.2 mm and their coleoptile had emerged, making them easy to handle and transfer into hydroponics. After 9 d in hydroponics, the roots of control plants were well developed with advanced lateral roots, while inoculated plants showed symptoms of *H. avenae* infection. Their overall root growth was impaired and infected regions were swollen and bent, with numerous lateral roots. (Figure 3.4).

For individual plants, xylem exudate volume increased linearly over time (Figure 3.5). For four control plants (C3, C4, C14, and C18) and three inoculated plants (I5, I21, and I22) exudates rose considerably more slowly than other plants in the same treatment. For two inoculated plants (I8 and I19), the exudate started to rise quite late. After exclusion of data for the seven plants with atypically slow flow rates (C3, C4, C14, C18, I5, I21, and I22), significant differences were detected between control and inoculated plants for some root, shoot and hydraulic variables (Table 3.1). In each case, the mean value was lower for inoculated plants than for control plants.

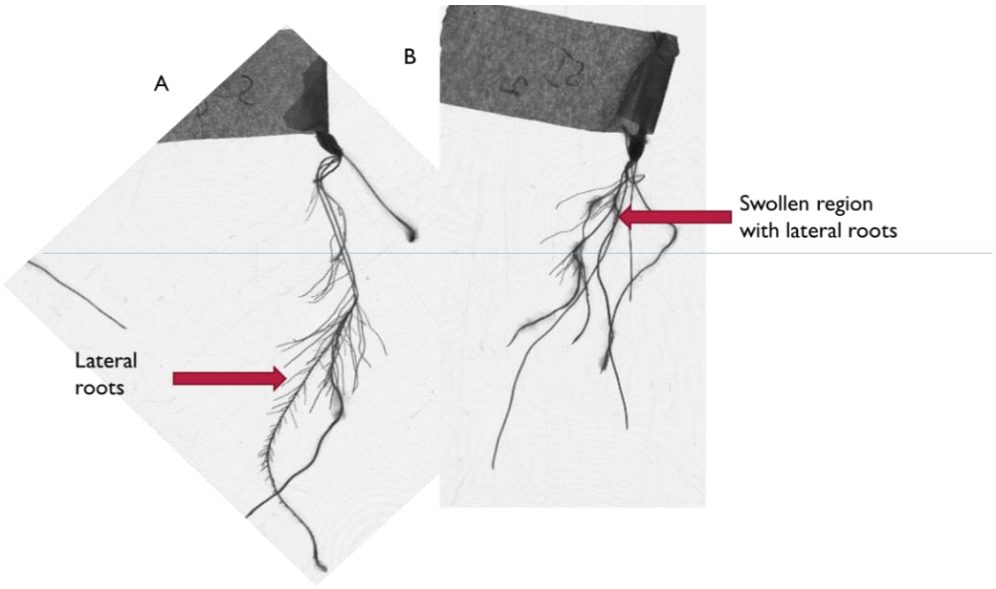

Figure 3.4 Scanned images of control (A) and CCN-inoculated (B) roots of Sloop barley grown in a hydroponic system and sampled at 9 d showing swollen regions with lateral roots.

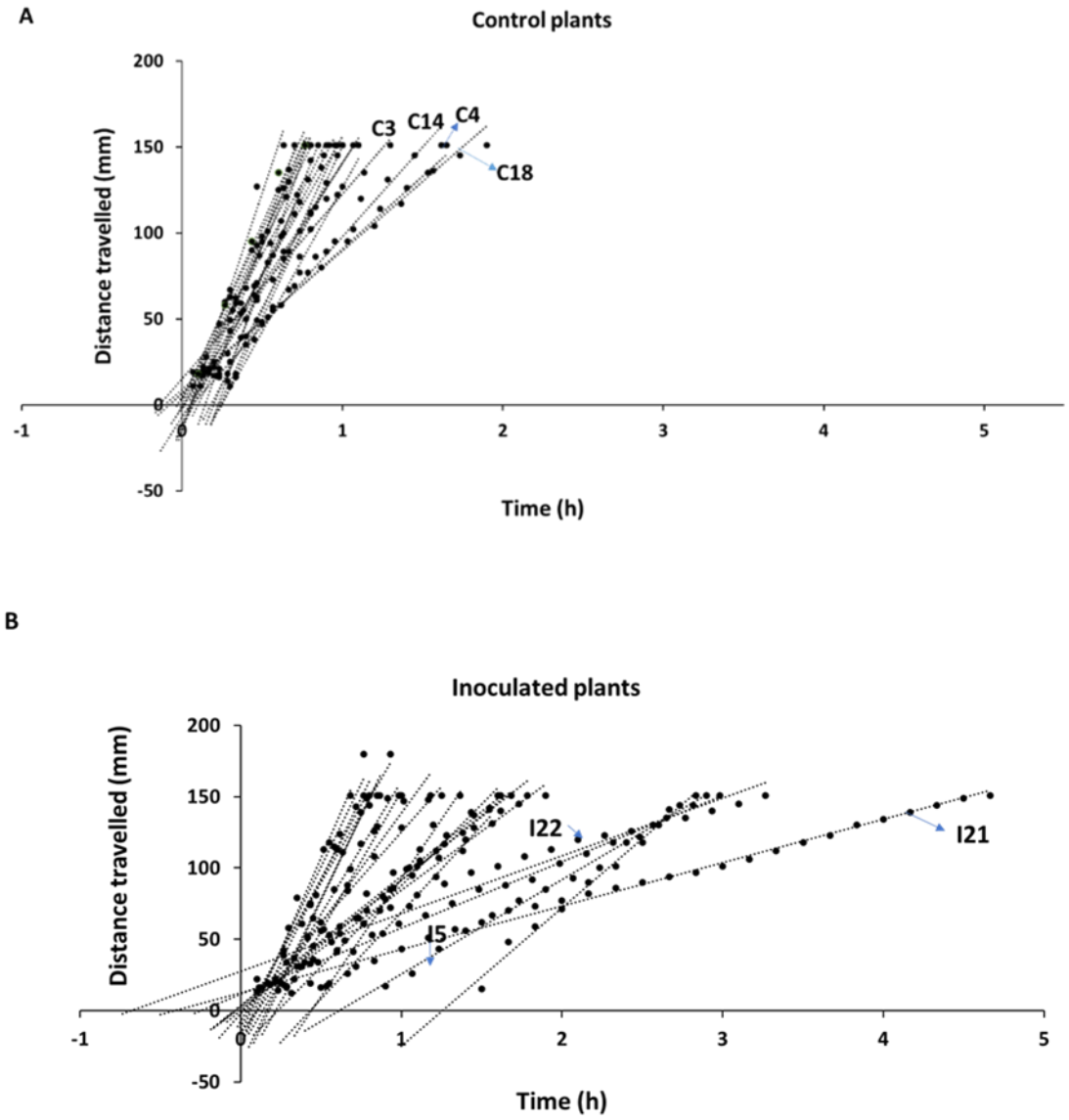

Figure 3.5 Distances travelled by xylem exudate through capillary tubes attached to the stem bases of seedlings of Sloop barley that had been grown in a hydroponic system for 9 d for (A) non-inoculated control plants and (B) plants that had been inoculated with juvenile cereal cyst nematodes 1 d prior to transfer into the hydroponic systems. Each black dot represents a measurement taken for an individual plant at a specific time (horizontal axis) after the capillary tube was attached to the seedling. Broken lines are linear regression lines for individual plants. Regression lines for which the slope differs substantially from other regression lines within the same treatment (control or inoculated) are labelled with their plant numbers (C3, C4, C14, C18, I5, I21 and I22).

Table 3.1 Mean values of traits for which significant ($p \leq 0.05$) differences were detected between control and CCN-inoculated plants of Sloop using unpaired t-tests to study the effect of CCN on root hydraulic conductivity.

Trait	**Control mean**	**Inoculated mean**	**p-value**
Root length (cm)	110.1	82.39	0.0084
Root surface area (cm^2)	15.13	12.11	0.0172
Leaf surface area (cm^2)	30.63	26.27	0.0177
Shoot dry weight (mg)	20.58	15.50	0.0006
Hydraulic conductance [$(m^3s^{-1}MPa^{-1}) \times 10^{-10}$]	4.405	3.062	0.0056
Hydraulic conductivity [$(ms^{-1}MPa^{-1}) \times 10^{-7}$]	2.985	2.343	0.0389

3.3.2 Experiment 2- Measurement of the effect of CCN on root hydraulic conductivity in resistant and susceptible sister lines of barley using the exudation method

Both resistant and susceptible cultivars were affected by infection. The roots of both resistant and susceptible cultivars were malformed (Figure 3.6). Among the five root and shoot variables measured in Experiment 2, only root dry weight exhibited a significant cultivar × treatment interaction (Table 3.2), with CCN infection reducing root dry weight more severely in Sloop SA than in Sloop or Galleon (Figure 3.7). For three of the other four root and shoot variables, both cultivar and treatment were significant sources of variation (Table 3.2). The exception was root surface area, for which there was a significant treatment effect but no significant cultivar effect.

Galleon had significantly longer roots than Sloop SA and significantly greater shoot dry weight than Sloop and Sloop SA. Sloop had significantly greater root volume than Sloop SA (Figure 3.8).

For each of the four variables with significant treatment effect, the value for CCN-inoculated plants was lower than the value for control plants, indicating that nematode infection had suppressed both root and shoot growth (Figure 3.9).

Among the five variables analysed for hydraulic properties, hydraulic conductivity was the only one for which cultivar was a significant source of variation (Table 3.3), with Galleon having significantly higher hydraulic conductivity than Sloop and Sloop SA (Figure 3.10). The inoculation treatment had significant effects for all five hydraulic variables (Table 3.3), with CCN-infection reducing exudation flow rate, xylem osmolality, hydraulic conductance, and hydraulic conductivity but increasing the driving force (Figure 3.11).

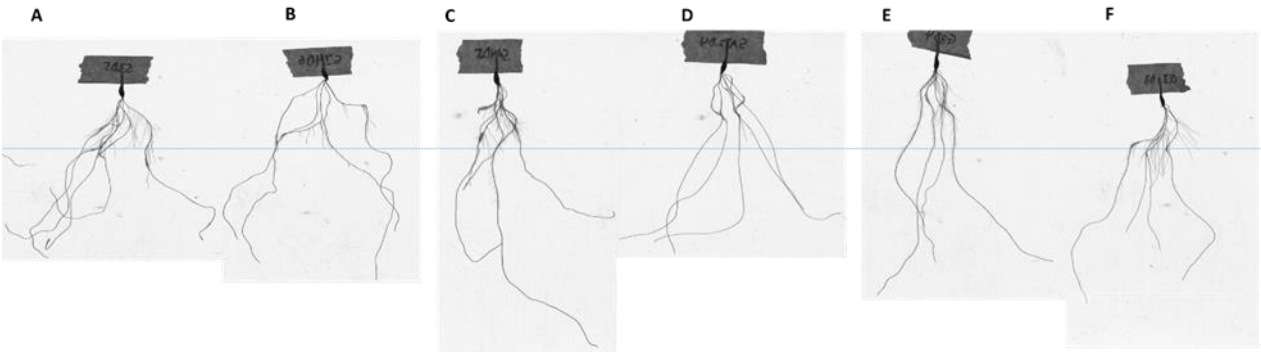

Figure 3.6 Scanned images of control (A, C, E) and CCN-inoculated (B, D, F) plants of Sloop SA (A, B), Sloop (C, D), and Galleon (E, F), barley plants grown in a hydroponic system and sampled at 10 d after inoculation.

Table 3.2 P-values from analysis of variance of root surface area, root length, root volume, shoot dry weights, and root fresh weight of control and CCN-inoculated plants of Sloop, Sloop SA, and Galleon barley plants to study the effect of CCN infection on morphological traits and how it could affect root hydraulic properties using the root exudation technique.

Trait	Cultivar p-value	Treatment p-value	Interaction p-value
Root surface area (cm^2)	0.3403	0.0139	0.4984
Root length (cm)	0.0125	0.0355	0.3578
Root volume (cm^3)	0.0071	0.0114	0.5640
Shoot dry weight (mg)	0.0001	0.0001	0.4275
Root dry weight (mg)	0.1619	0.2758	0.0001

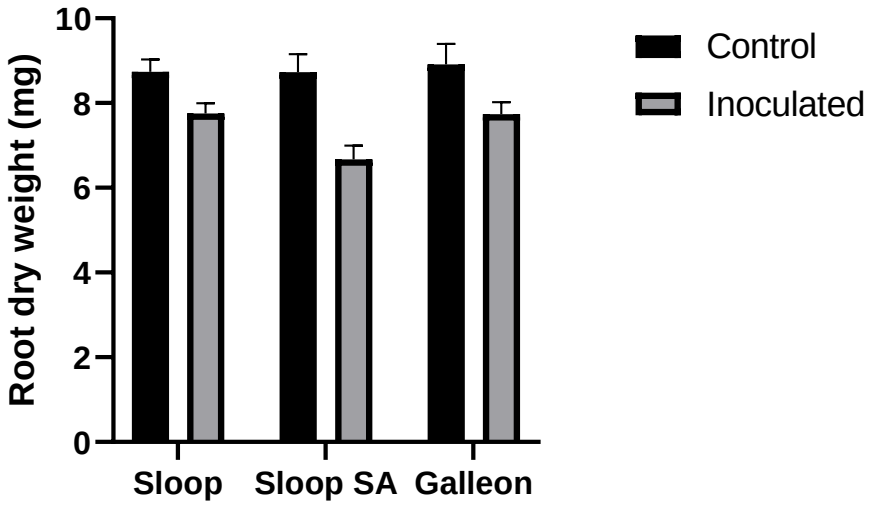

Figure 3.7 Means and standard errors for root dry weight of Sloop, Sloop SA, and Galleon plants inoculated with J2-stage cereal cyst nematodes, grown in a hydroponic system and sampled at 10 d after inoculation.

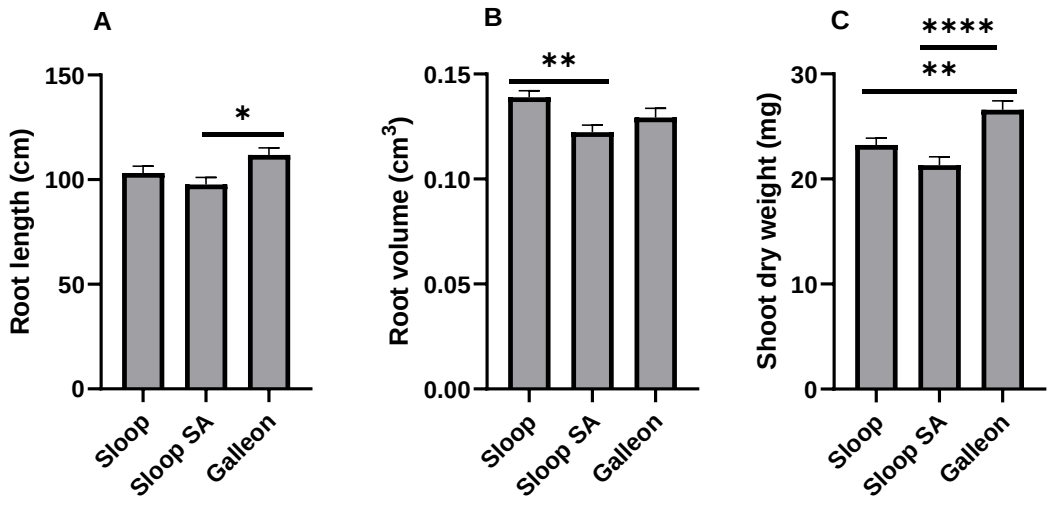

Figure 3.8 Cultivar means and standard errors of root length (A), root volume (B), and shoot dry weight (C) of Sloop, Sloop SA, and Galleon plants inoculated with J2-stage cereal cyst nematodes and transferred into a hydroponic system and sampled at 10 d after inoculation. *, **, **** Significance at the 0.05, 0.01, and 0.0001 levels of probability, respectively.

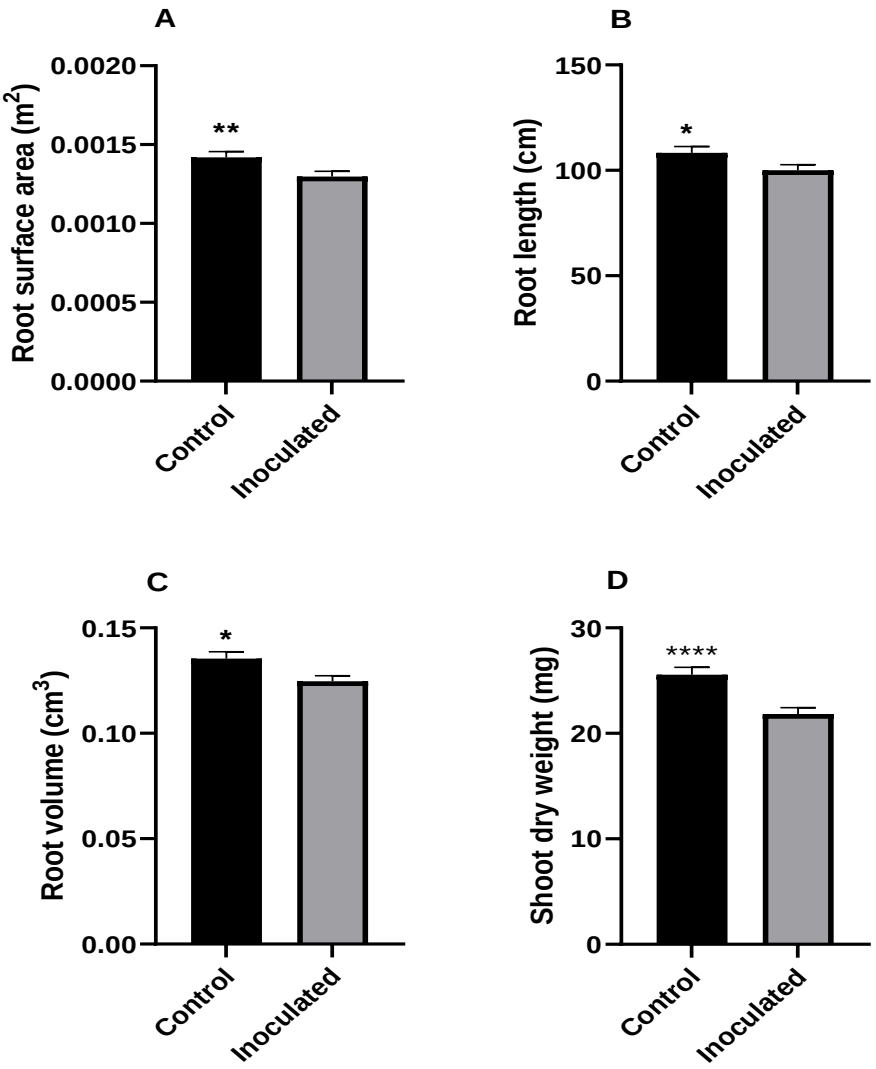

Figure 3.9 Treatment means and standard errors of root surface area (A), root length (B), root volume (C), and shoot dry weight (D) of control and CCN-inoculated barley plants transfer into a hydroponic system and sampled at 10 d after inoculation. *, **, **** Significance at the 0.05, 0.01 and 0.0001 levels of probability, respectively.

Table 3.3 P-values of analysis of variance of driving force, exudation flow rate, xylem osmolality, conductance, and hydraulic conductivity of control and CCN-inoculated plants of Sloop, Sloop SA, and Galleon barley to study the effect of CCN infection on root hydraulic properties using the root exudation technique.

Trait	**Cultivar p-value**	**Treatment p-value**	**Interaction p-value**
Driving force (MPa)	0.0847	0.0048	0.9256
Exudation flow rate (m^3s^{-1})	0.0903	0.0001	0.2325
Xylem osmolality ($mOsm\ kg^{-1}$)	0.0957	0.0044	0.9383
Hydraulic conductance $[(m^3s^{-1}\ MPa^{-1}) \times 10^{-10}]$	0.0536	0.0001	0.2774
Hydraulic conductivity $[(ms^{-1}\ MPa^{-1}) \times 10^{-7}]$	0.0114	0.0059	0.2396

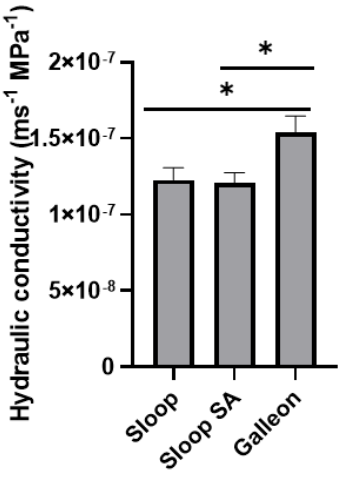

Figure 3.10 Cultivar means and standard errors of the effect of CCN infection on root hydraulic properties of Sloop, Sloop SA, and Galleon plants transferred into a hydroponic system and sampled at 10 d after inoculation. * Significance at the 0.05 levels of probability.

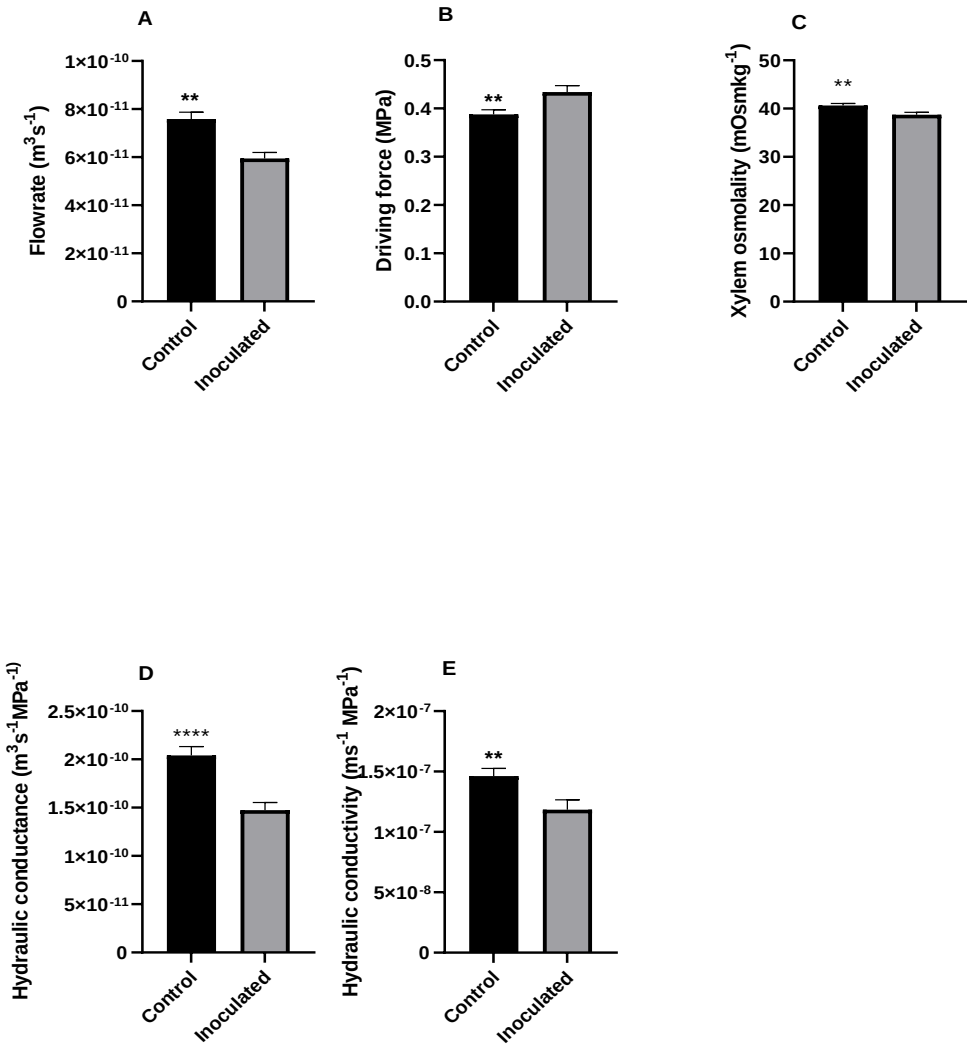

Figure 3.11 Treatment means and standard errors of flow rate (A), driving force (B), xylem osmolarity (C), hydraulic conductance (D), and hydraulic conductivity (E) of control and CCN-inoculated plants transferred into a hydroponic system and sampled at 10 d after inoculation **, **** Significance at the 0.01 and 0.0001 levels of probability, respectively.

3.3.3 Experiment 3- Measurement of the effect of CCN on root hydraulic conductivity in resistant and susceptible barley using suction method

For the five root and shoot variables measured, there were no significant cultivar × treatment interactions (Table 3.4). Treatment was a significant source of variation for all five variables, with lower values for the inoculated plants than for controls, showing that the infection had reduced root and shoot growth (Figure 3.12). Root length and shoot dry weights also had

cultivar as a significant source of variation. Galleon had significantly longer roots and greater shoot dry weight than Sloop (Figure 3.13).

For the three hydraulic variables, there were no significant cultivar × treatment interaction (Table 3.5). The inoculation treatment significantly reduced all three of these variables (exudation flow rate, hydraulic conductance, and hydraulic conductivity) (Figure 3.14).

Table 3.4 P-values from analysis of variance of root surface area, root length, root volume, shoot dry weights, root dry weight of control and CCN-inoculated plants of Sloop, Sloop SA, and Galleon barley to study the effects of CCN infection on morphological traits and how it could affect root hydraulic properties using the suction technique.

Trait	**Cultivar p-value**	**Treatment p-value**	**Interaction p-value**
Root surface area (cm^2)	0.2041	0.0413	0.3696
Root length (cm)	0.0017	0.0220	0.5523
Root volume (cm^3)	0.6421	0.0150	0.4209
Shoot dry weight (mg)	0.0188	0.0078	0.9485
Root dry weight (mg)	0.0983	0.0027	0.8774

Table 3.5 P-values from analysis of variance of exudation flow rate, conductance, and hydraulic conductivity of control and CCN-inoculated plants of Sloop, Sloop SA, and Galleon barley to study the effect of CCN infection on root hydraulic properties using the root suction technique.

Trait	**Cultivar p-value**	**Treatment p-value**	**Interaction p-value**
Exudation flow rate (m^3s^{-1})	0.2008	0.0001	0.9364
Hydraulic conductance [$(m^3s^{-1}\ MPa^{-1}) \times 10^{-10}$]	0.2122	0.0001	0.9716
Hydraulic conductivity [$(ms^{-1}\ MPa^{-1}) \times 10^{-7}$]	0.2067	0.0001	0.9767

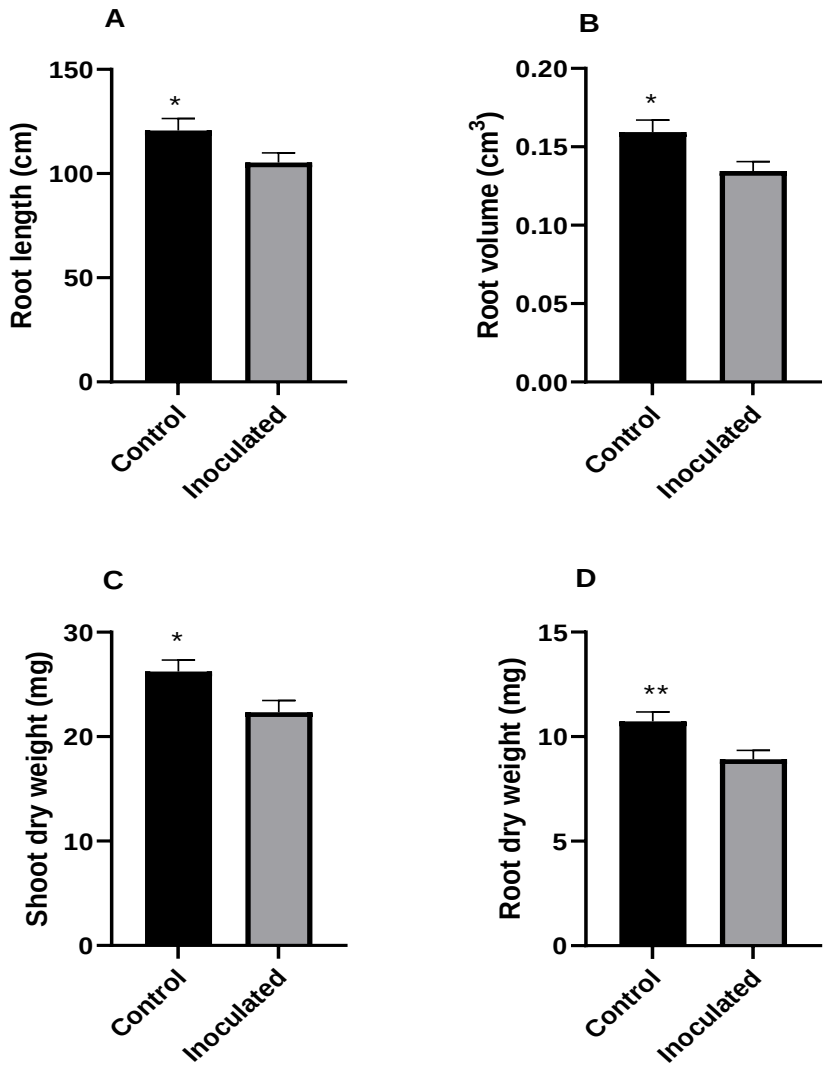

Figure 3.12 Treatment means, and standard errors of root length (A) root volume (B) shoot dry weight (C), and root dry weight (D) of control and CCN-inoculated barley plants grown in a hydroponic system and sampled at 10 d after inoculation. *, ** Significance at the 0.05 and 0.01 levels of probability, respectively.

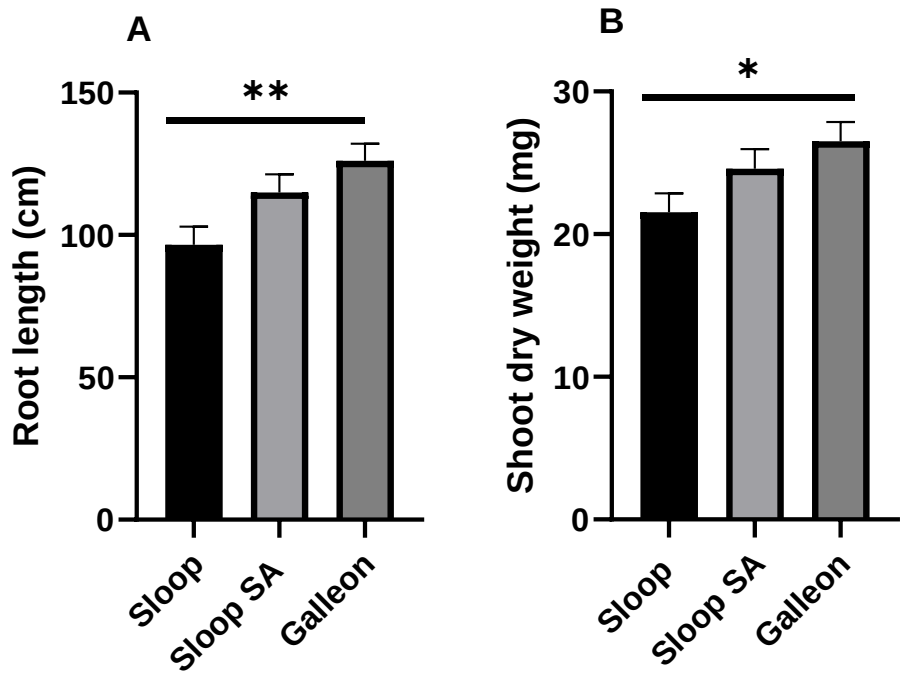

Figure 3.13 Cultivar means and standard errors of root length (A) and shoot dry weigh (B) of Sloop, Sloop SA, and Galleon plants grown in a hydroponic system and sampled at 10 d after inoculation. *, ** Significance at the 0.05 and 0.01 levels of probability, respectively.

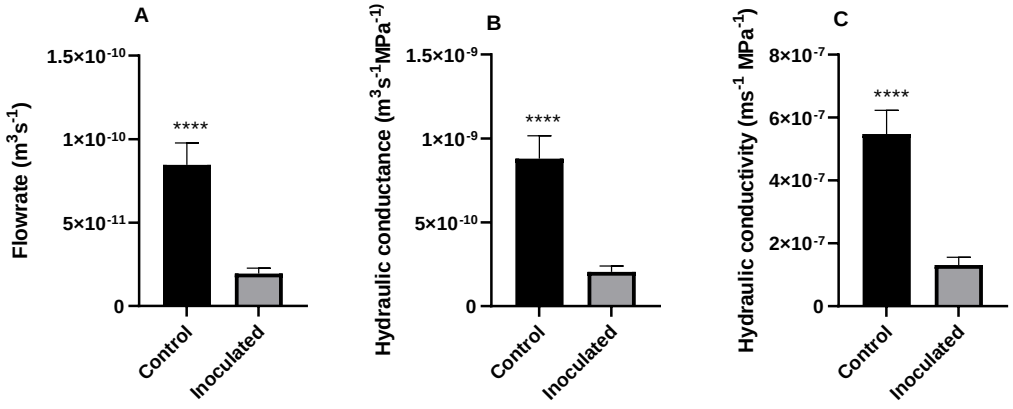

Figure 3.14 Treatment means and standard errors of flow rate (A) hydraulic conductance (B) Hydraulic conductivity, and (C) of control and CCN-inoculated barley plants grown in a hydroponic system and sampled at 10 d after inoculation. **** Significance at the 0.0001 level of probability.

3.4 Discussion/ conclusion

Because root hydraulic properties can be affected by the magnitude of water flow-induced through roots (Passioura and Munns 1984), it can be useful to use more than one technique for an analysis of hydraulic conductivity. In the present study, both exudation and suction methods were used to investigate the effects of *H. avenae* infection on barley seedlings. The two techniques used in this study differed in their experimental set-up, driving force, and flow rates across the roots. This resulted in different hydraulic conductivity values with control plants exhibiting higher hydraulic conductivity values (mean 5.47 ± 0.8 $\times 10^{-7}$ $ms^{-1}MPa^{-1}$) in the suction experiments than in the exudation experiments (mean 2.99 ± 0.2 $\times 10^{-7}$ $ms^{-1}MPa^{-1}$ and 1.46 ± 0.06 $\times 10^{-7}$ $ms^{-1}MPa^{-1}$).

The suction method imposed a hydrostatic pressure gradient across the roots of transpiring barley plants. This could affect both the apoplastic and cell-to-cell pathways, reducing hydraulic resistance in the roots and driving greater water flow through the roots (Steudle 2000a). Application of a vacuum to a sealed excised root system also creates xylem tension, which could cause the hydrostatic pressure of nutrient medium to force water transport through the apoplast and any intercellular air spaces, by-passing the hydraulic resistance of the endodermis. Accordingly, there could be both increased radial water flow across the root cylinder and increased axial water flow along the cortex (Suku et al. 2014; Meng et al. 2016).

In contrast, the exudation method relied on an osmotic pressure gradient (representing the absence of transpiration water demands from the shoot, that is in the night-time). Under this condition, the contribution of the apoplast would be negligible, resulting in a lower root hydraulic conductivity and water flow (Steudle 2000b).

In the present study, the osmotic hydraulic conductivity for control barley plants (2.99 ± 0.2 × 10^{-7} $ms^{-1}MPa^{-1}$ and 1.5 ± 0.06 × 10^{-7} $ms^{-1}MPa^{-1}$) was about one order of magnitude higher than those obtained in previous studies: 4.2 × 10^{-8} $ms^{-1}MPa^{-1}$ (Suku et al. 2014) and 3.2 ± 0.5 × 10^{-8}

and $11 \pm 1.7 \times 10^{-8}$ $ms^{-1}MPa^{-1}$ (Knipfer et al. 2010). Such differences could be due to minor differences in experimental conditions. For example, in the present study, the nutrient medium used for plant growth was a modified Johnson's solution and plants were grown at 20°C and 80% relative humidity, with a 12-h day/night cycle. In the published studies, the nutrient medium used was half-strength Hoagland solution, and plants were grown at of 21/15°C and 70 % relative humidity, with 16/8 h day/night cycle.

CCN infection caused root morphological changes including reduced root length, root volume, and RSA, and affected water relations and growth. Considerable plant-to-plant variation was observed for all measured and calculated variables making it necessary to use a large sample size to detect differences between treatments and among cultivars. The use of a large sample size made it possible to identify a few individual plants with atypically slow flow rates. These plants may have been damaged during the delicate process of attaching the silicon tubing and were considered to be outliers. Their data were excluded from the analysis and significant differences were identified in some measured and calculated variables.

Water transport from the root to the shoot of transpiring plants depends on morphological and hydraulic properties. The reduced RSA, total root length, and root volume of inoculated plants would have decreased the water absorption surface of the roots, which could affect water uptake by the roots. The flow rate of water transported through the root was decreased by the inoculation treatment, reducing the hydraulic conductivity. These reductions could indicate a strong diversion of water to syncytia and nematodes. The turgor pressure is higher in syncytia than the surrounding tissues (Böckenhoff and Grundler 1994), and it is thought that a large amount of water is transported into the syncytia at the expense of the host plant (Jones and Northcote 1972; Golinowski et al. 1996; Seah et al. 2000).

Comparing the effects of the infection between resistant and susceptible barley cultivars, the study showed that the nematodes were successful in causing physiological changes and reducing hydraulic conductivity in early vegetative stages. These effects were seen in both susceptible and resistant plants. However, Galleon (*Rha4+*) could exhibit a higher growth over Sloop SA (*Rha2+*) and Sloop. Resistance mediated by CCN R-genes is by the degradation of the syncytia. This commences by 10 d after inoculation (DAI) in *Rha4* plants (Galleon) and 13 DAI in *Rha2* plants (Sloop SA) (Seah et al. 2000). This difference could explain why Galleon had more robust growth than Sloop SA. The exudation and suction methods used in this study could not be used to assess how the resistance mediated by the CCN R-genes affected hydraulic conductivity in older plants, because older barley plants form tillers, and their roots are difficult to handle.

The inoculation treatment reduced hydraulic conductivity by 76.1% in the suction experiment and by only 21.5% and 19.0% in the exudation experiments. The hydraulic conductivity of inoculated plants in the suction experiment was about 1.84×10^{-7} $ms^{-1}MPa^{-1}$, which was not very different from what was observed in the exudation experiments 2.3×10^{-7} $ms^{-1}MPa^{-1}$ and 1.18×10^{-7} $ms^{-1}MPa^{-1}$. Yet there was a large difference in the control plants: about 5.5×10^{-7} $ms^{-1}MPa^{-1}$ compared to 3.0×10^{-7} $ms^{-1}MPa^{-1}$ and about 1.4×10^{-7} $ms^{-1}MPa^{-1}$. The high reduction between control plants and inoculated plants in the suction experiment could be because the vacuum is able to pull a lot of extra water through the control roots but not much through the infected roots due to their impaired root systems and/or to diversion of water out of the xylem into the feeding sites and could have affected the apoplastic pathway more than the symplastic pathway.

In conclusion, infection by CCN impedes plant growth and hydraulic properties in resistant and susceptible barley plants. There was considerable plant-to-plant variation for the traits measured here, requiring the use of large sample sizes. There were some significant cultivar

differences for plant growth traits, but only root dry weight exhibited a significant treatment × cultivar interaction. Effects of CCN infection on hydraulic traits could be detected using both exudation and suction methods but these effects were more evident with the suction method.

4 Chapter 4: Determination of water and solute transport pathways in CCN resistant and susceptible barley using 8-hydroxypyrene-1, 3, 6-trisulfonic acid trisodium salt (HPTS)

Summary

The apoplastic fluorescent tracer dye 8-hydroxypyrene-1, 3, 6-trissulfonic acid trisodium salt (HPTS) was used to investigate how far and quickly water travels in seminal roots of CCN-infected and control Sloop (susceptible) and Sloop SA (*Rha2* resistant) cultivars. In control plants the dye reached the base of the shoot within 60 min. Dye uptake was slower in infected plants and was blocked at the feeding site. The duration of the blockage on average was longer in Sloop than in Sloop SA. UV analysis showed that dye was concentrated at the feeding site, but there was some beyond the feeding site.

4.1 Introduction

The results presented in Chapter 3 demonstrate that CCN infection impairs the root system and affects water transport. The reduction in water transport from the root to the shoot could partly be due to the diversion of water to the feeding site and the nematode. For successful parasitism (completion of the nematode's life cycle) there needs to be a balance between the amount of water that is diverted to meet the needs of the nematode and the amount that is retained for use by the host plant. Little is known about how much water and solute are partitioned between the host and the nematode nor about pathways of water transport in infected roots.

Fluorescent dyes can be useful for tracing the pathway of water transport in plants. For example, Moon et al. (1986), Wright and Oparka (1996), and Faiyue et al. (2010) used fluorescent tracer dyes to trace the pathway of water and solute transport in various plant species. Dorhout et al. (1988) also used a fluorescent tracer, Tinopal CBS, to investigate a plant-nematode pathosystem. The study indicated water flow within the vascular cylinder from compartments of the xylem during feeding of the nematode (*Meloidogyne incognita*).

To understand the disruption in water transport caused by CCN infection, Levin (2020) applied drops of a fluorescent tracer dye, 8-hydroxypyrene-1, 3, 6-trissulfonic acid trisodium salt (HPTS-acetate) at the tips of seminal roots of wheat seedlings and observed a steady rise of the dye from the tip to the base of the root. In contrast, in CCN-infected plants, dye transport was blocked or delayed in the infected regions of the root. Nevertheless, there is still much to understand about the distribution of water and solute between nematodes and their host.

The work outlined here used the apoplastic fluorescent tracer dye 8-hydroxyprene-1, 3, 6-trisulfonic acid trisodium salt (HPTS) in accordance with methods by Levin (2020) to determine how far and quickly HPTS travels in seminal roots of CCN-infected susceptible and resistant barley cultivars. HPTS is a three sulphonate molecule that is highly stable in water and not membrane permeable due to its high charge at physiological pH (Wright and Oparka 1996).

Hence it has been used as an apoplastic dye to trace by-pass flow (apoplastic pathway in lateral roots) in plant species (Peterson et al. 1981; Moon et al. 1986; North and Nobel 1996, Faiyue et al. 2010).

4.2 Materials and methods

4.2.1 Seed germination, inoculation, and hydroponic growth

Plant materials used for this study were Sloop and Sloop SA cultivars of barley. Seed germination was staggered with five seeds of each of the two barley cultivars surfaced-sterilised, plated on agar, and placed in the dark at room temperature on each of six consecutive days. After 3 d, seedlings were inoculated and transferred to hydroponics using the methods detailed in 3.2.3 and 3.2.4. After 7 d in hydroponics, four control and four inoculated plants per cultivar were sampled to be tested for dye transport.

4.2.2 Fluorescent probe application and visualisation

The apparatus for the application of the fluorescent probe was set up as follows in the same growth room that was used for hydroponics. A plastic tray was set at an angle of approximately 45° and a reservoir was created at the bottom of the tray by gently pouring the dye solution at the base of the tray. Plants to be tested were arranged on the plastic tray such that root tips were in the reservoir. HPTS in aqueous solution at a concentration of 0.4% was added to the reservoir making sure all the root tips of each plant were submerged in the solution. Petri dishes of dimension 90 mm × 15 mm were used to cover the root system to prevent them from drying out. At 10 min intervals, the position of the dye front in each root was marked, and the distance travelled was measured with a tape measure. After 1 h roots were washed with autoclaved distilled water to remove excess dye and blotted dry with a paper towel. The stained roots were visualised on a UV transilluminator to confirm the position of the dye.

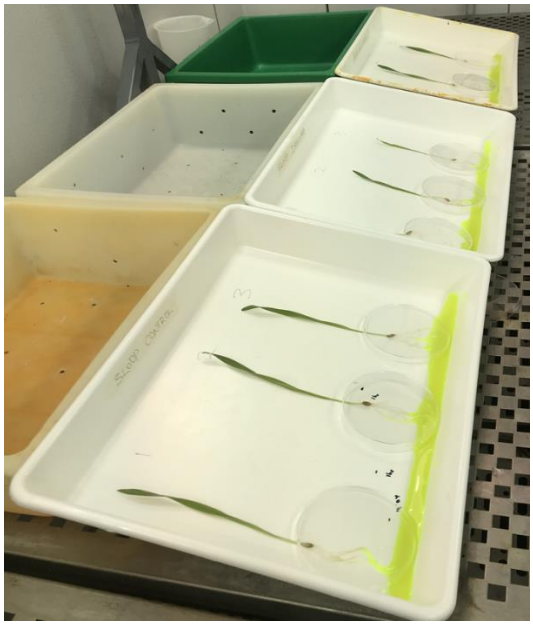

Figure 4.1 Experimental setup to trace HPTS in CCN- inoculated and control roots of Sloop (susceptible) and Sloop SA (*Rha2+*) barley cultivars. The test apparatus was inclined at an angle of about 45°C and plants were arranged such that their root tips were in the reservoir created at the bottom of the tray.

4.3 Results

Dye that was taken up by roots of both control and inoculated plants was visible as a bright yellow colour. Dye entry was mostly through the lateral roots into the main roots. In adjacent roots, the dye pick-up was sometimes through contact of their lateral roots with laterals of roots that have already picked up the dye (Figure 4.2).

In most control plants, the dye reached the base shoot within 60 min for control plants (Figure 4.3). In inoculated plants, dye transport was slow. In most cases, the transport of the dye was blocked at the swollen regions (Figure 4.4). In most but not all inoculated plants, the dye later began to rise again (Figure 4.5).

Figure 4.2 Tracer dye 8-hydroxyprene-1, 3, 6-trissulfonic acid trisodium salt (HPTS) pick-up by adjacent lateral roots from laterals of roots transporting the dye.

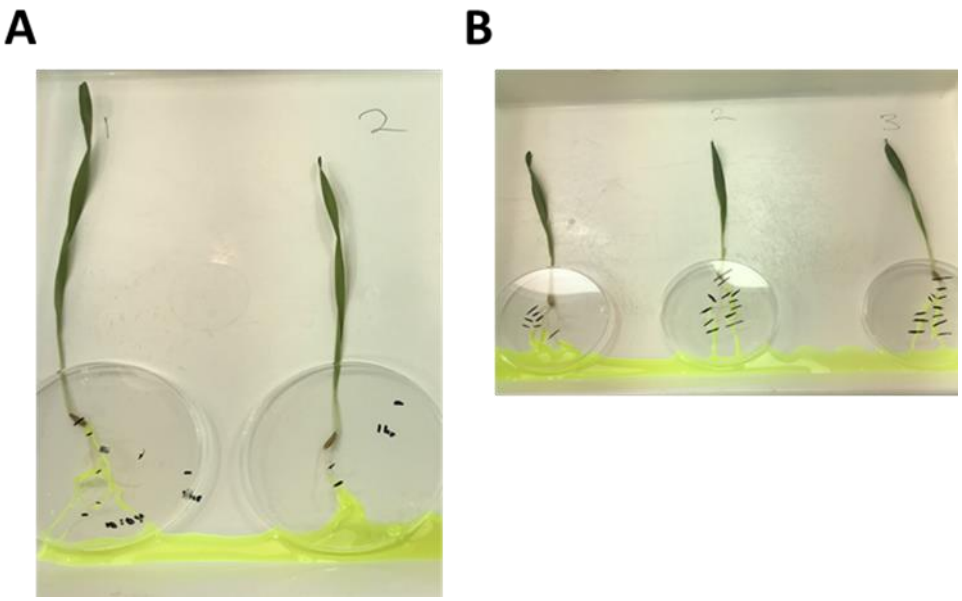

Figure 4.3 Uptake of fluorescent tracer dye, 8-hydroxyprene-1, 3, 6-trissulfonic acid trisodium salt (HPTS) in the roots of Sloop (A) and Sloop SA (B) control plants in 60 min.

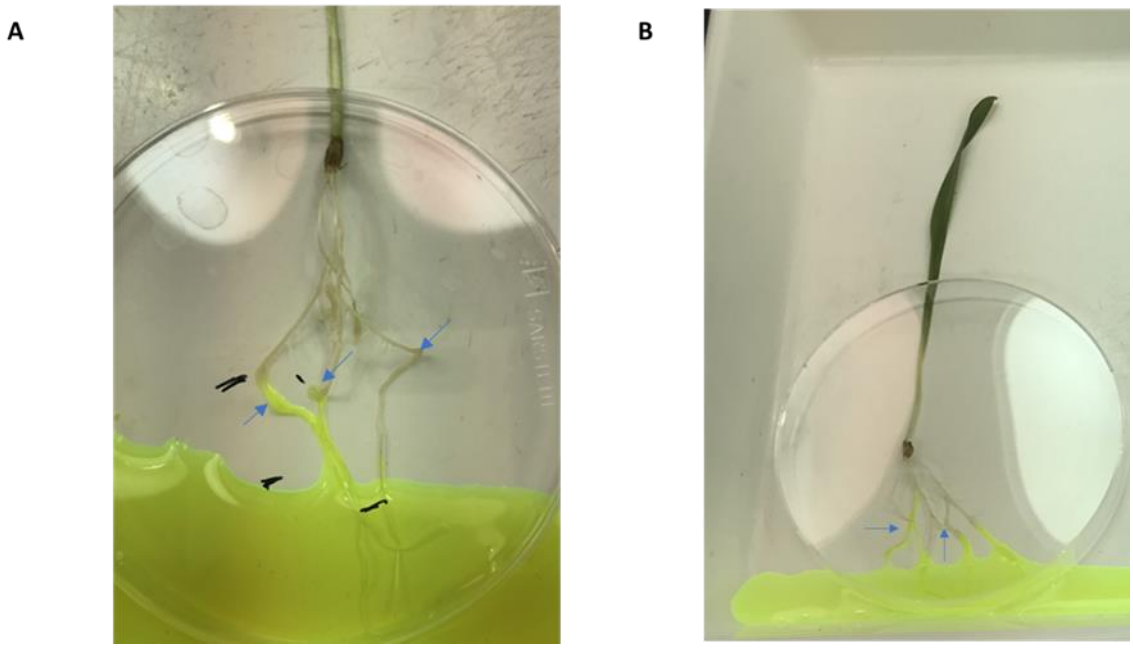

Figure 4.4 Pulse of fluorescent tracer dye, 8-hydroxyprene-1, 3, 6-trissulfonic acid trisodium salt (HPTS) at the swollen region in the roots of Sloop (A) and Sloop SA (B) inoculated plants. Arrows pointing to feeding site.

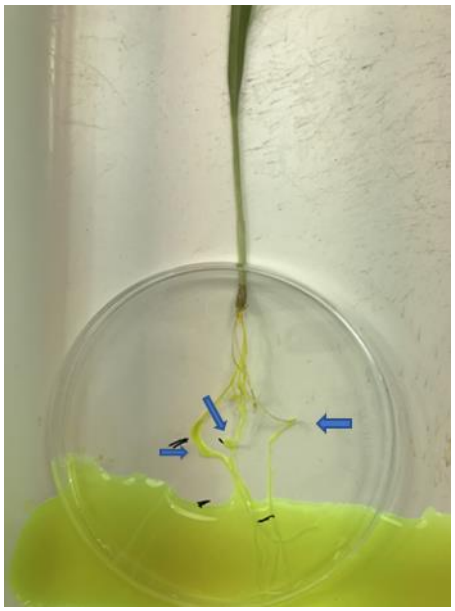

Figure 4.5 Continued rise of HPTS dye to the base of the shoot after pause at the swollen region. Arrows pointing to swollen regions.

Under the UV transilluminator, the dye was visible as a bright blue colour (Figure 4.6). It was concentrated at the swollen regions in the roots of inoculated plants, but some dye was present beyond the swollen regions.

Figure 4.6 HPTS dye visualisation under UV light 30 min after dye application at the tips of seminal roots with arrows pointing to dye concentration at the swollen regions in roots of Sloop inoculated (A), Sloop SA inoculated (B), Sloop (control with dye), and Sloop plant without dye (D).

In control plants, HPTS rose through the roots in an approximately linear manner. It rose more steeply in Sloop SA (mean slope = 1.32 mm min^{-1}) than in Sloop (mean slope = 1.04 mm min^{-1}) (Figure 4.6). In inoculated plants, the dye initially rose somewhat more slowly than in the control plants (Figure 4.7). When it resumed rising after being interrupted in the infected region, it rose slowly and rarely reached the base of the root. Because distances from root tip to infected

region varied among plants, these patterns were more evident when the position of the dye front was expressed relative to the position of the feeding site (Figure 4.8). In most cases the interruption of dye movement started at about 20 min, but the duration of the interruption varied considerably among plants. Once the dye started to rise again, it tended to rise more quickly in Sloop SA than in Sloop.

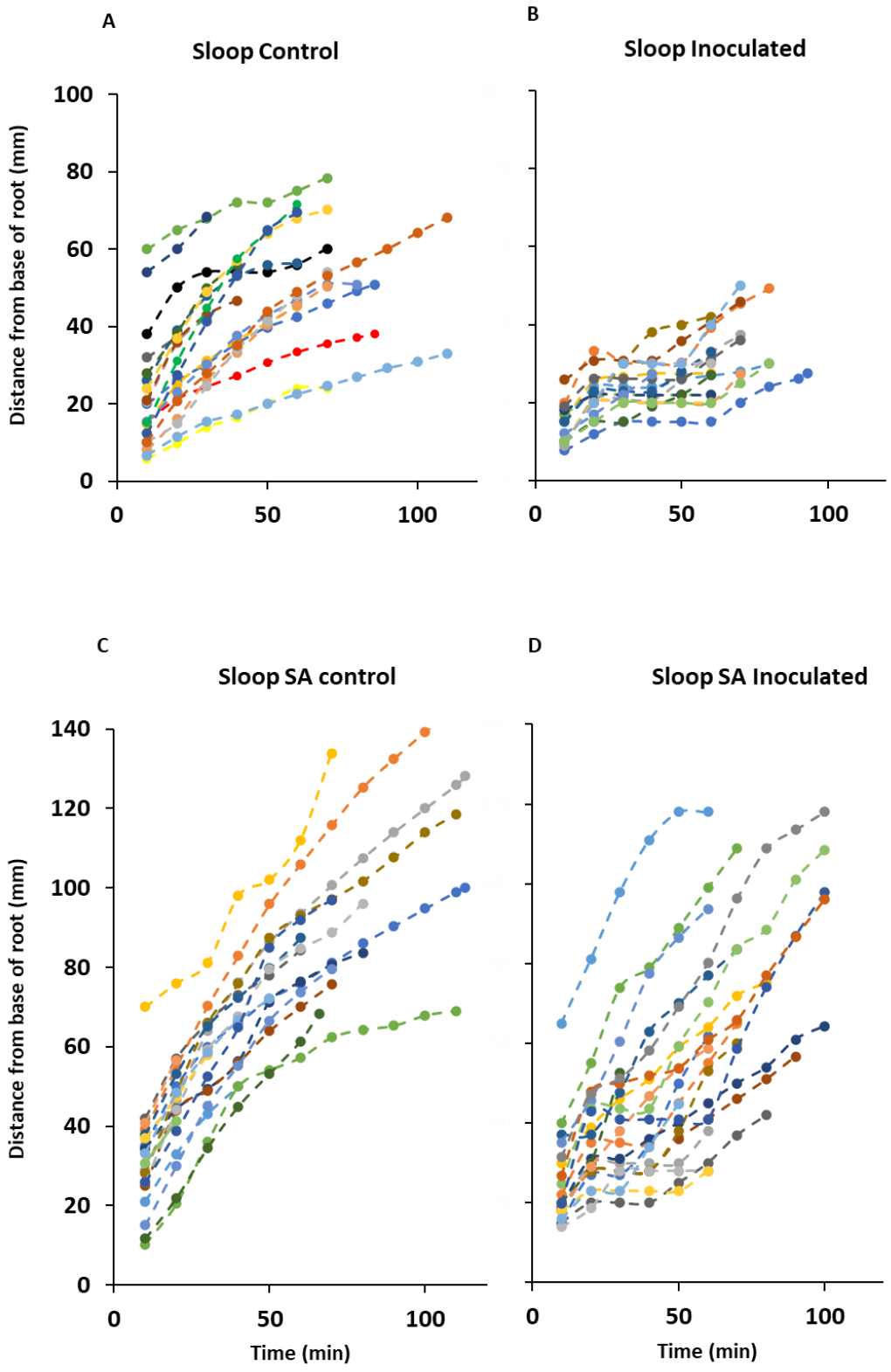

Figure 4.7 Dye movement from the root tip to the shoot base of individual plants of Sloop control (A), Sloop inoculated (B), Sloop SA control (C), and Sloop SA inoculated (D).

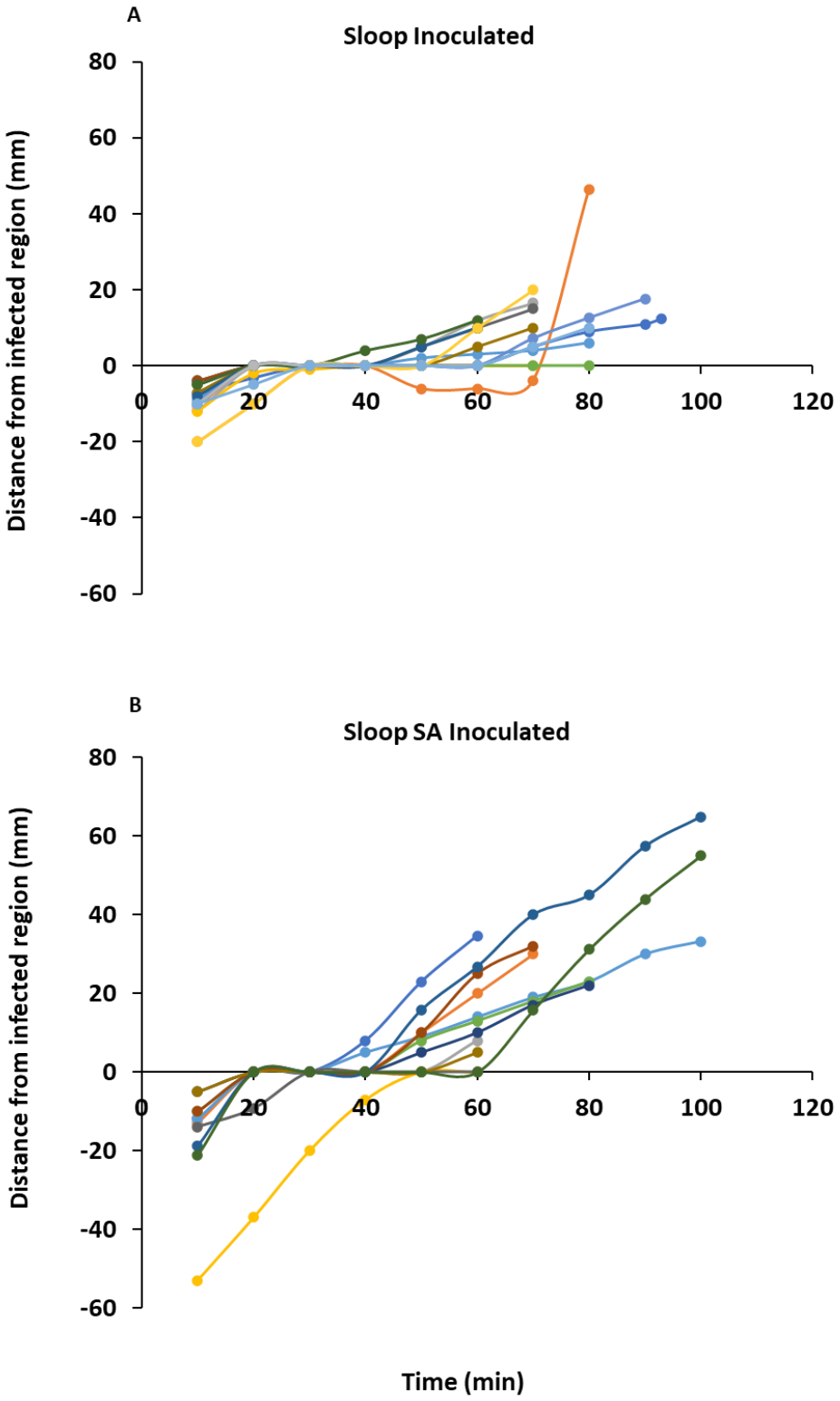

Figure 4.8 Movement of dye from the root tip to the shoot base of individual inoculated plants of Sloop (A) and Sloop SA (B), with the position of the dye front expressed as distance from the infected region of the root.

4.4 Discussion/ conclusion

In the experiments that were reported in Chapter 3, water transport was impeded by CCN infection in both susceptible and resistant plants. This study, therefore, aimed to investigate how far and quickly water travelled in the roots of resistant and susceptible barley infected with CCN.

In this study, dye applied to root tips reached the base of the shoot of Sloop and Sloop SA control plants in 60 min but was slower in inoculated plants. Dye was blocked at feeding sites of inoculated plant. The slower travel of the dye in inoculated roots could be due the impaired root system which reduced the absorption surface area and made the roots less conductive to water flow.

The blockage observed at the feeding site in inoculated plants could partly be due to the high demand for water and nutrients by the nematode creating a sink in the syncytium, thus reducing the amount of water transported through the roots. Alternatively, it could be related to infection-related damage to xylem vessels, such as that reported by Levin et al. (2020; 2021).

It is estimated that the daily uptake of solute by female nematodes is four times and the juveniles about 0.4-0.6 times the syncytial volume (Sijmons et al. 1991). Wyss (1991) indicated that about 60-70% of the activity of the juvenile nematode is feeding and defecating a minute after the start of feeding. With the syncytia having a high turgor pressure of 1 MPa (Böckenhoff and Grundler 1994), a high amount of water and solute could be transferred into the syncytia. Since nutrients are transported in solution, it is worthwhile for a larger amount of water to be transported into the feeding site, then after the nematode takes out the required nutrient, the water is defecated out. Results from the UV visualisation showed concentrated dye at the swollen region, and some dye beyond the feeding site towards the base of the shoot. This indicates that a little water makes it though intermittently and more water makes it through once the feeding site is full or the nematode is not feeding (Dorhout et al 1988).

The duration of the dye blockage varied in inoculated plants. However, the duration was on average shorter in inoculated Sloop SA compared to inoculated Sloop making the dye transport in inoculated Sloop SA faster. This could indicate that the effects of the interruption were less severe in Sloop SA than in Sloop, allowing better recovery in the resistant cultivar. The short duration of dye blockage in Sloop SA could be due to the syncytia not been well developed or was more active in susceptible plants.

Transport of the dye from the root to the base of the shoot could be reached in 60 min in control plants and was slower in inoculated plants. Although dye transport was blocked at the feeding site in both Sloop and Sloop SA inoculated plants, the delay was shorter in Sloop SA inoculated plants than Sloop inoculated plants.

5 Chapter 5: Assessment of whether the *Rha4* region on barley chromosome 5H of barley affects seedling vigour

Summary

In this Chapter, seedling vigour was assessed in Galleon (*Rha4*+), Sloop (*Rha4*-), and two families of an early-backcross generation of Galleon × Sloop (BC_2F_4) and two families of a later-backcross (Galleon × Sloop BC_6F_3) generation, to determine if increased growth was conferred by the *Rha4* region on chromosome 5H of barley. To study this, early biomass accumulation, shoot height, root length, root diameter, and volume were assessed in the parents and progenies. KASP primer sets flanking and within the *Rha4* region were used to genotype the progenies into genotypic classes for comparison within families. Assessing the growth of the parents and the progenies, Galleon had greater seedling vigour than Sloop, but this did not reflect in the progenies (BC_6F_3), showing that growth is not conferred by the *Rha4* resistance locus.

5.1 Introduction

Galleon is an Australian feed barley variety with resistance to CCN that is determined by the *Rha4* locus on chromosome 5H (Barr et al. 1998). In working with hydroponically grown barley seedlings, Dr. Pradeepa Bandaranayake (a visitor in our lab from the University of Peredeniya in Sri Lanka) noticed that Galleon seedings were considerably more vigorous than those of the CCN-susceptible cultivar Sloop. It therefore seemed possible that *Rha4* confers CCN resistance via an effect on seedling vigour. This motivated this study, in which experiments were conducted to compare seedling vigour between Sloop and Galleon and between *Rha4*+ and *Rha4*- plants from backcross-derived families.

5.2 Materials and methods

5.2.1 Experiment 1- Comparison of Galleon with Sloop for seedling vigour and examination of temperature effect on seed germination

This experiment was conducted as a follow up on Dr. Bandaranayake's observations and to also compare germination temperatures to choose experimental conditions for future work. The experiment was conducted with Sloop and Galleon seeds.

5.2.1.1 Method

For each of the germination temperatures (10℃, 15℃, 20℃) 50 seeds of each cultivar were surface sterilised and germinated as described in section 3.2.2 of this thesis. In all, 300 seeds were germinated for this experiment. Once 90% seed germination was achieved (after 3 d for seeds germinated at 15℃ and 20℃ and after 4 d for seeds germinated at 10℃), the seedlings were exposed to light in a growth room for 24 h at 15℃, 12/12 h and relative humidity of 70%. Root length was then measured on 20 seedlings of each cultivar-treatment combination, and these seedlings were transferred to hydroponics (3.2.4) or soil in a completely randomised arrangements. At 20 d after transfer into hydroponics or soil, biomass data were taken. For plants grown in soil, the soil was carefully washed off the roots before biomass data was taken.

Fresh shoot and root tissues were oven-dried at a temperature of 65°C for 4 d before dry weight measurements were taken.

5.2.2 Experiment 2- Comparison of seeding vigour between Rha4+ and Rha4- BC2F4 families

This experiment was designed to study the difference in root growth using materials derived by backcrossing *Rha4* from Galleon (the resistant donor parent) into Sloop (the susceptible recurrent parent). The seeds used here were BC_2F_4 seeds harvested from two BC_2F_3 plants (KK3-34-10-70-1 and KK3-34-10-70-19) that had been confirmed to be homozygous for the Galleon-derived allele at a marker (wri786) that is closely linked with *Rha4* (Kelvin Khoo, unpublished data) and from two BC_2F_3plants (KK3-20-2-6-39 and KK3-30-2-6-45) that had been confirmed to be homozygous for the Sloop-derived allele at that marker (Table 5.1).

Table 5. 1 Genotypes of Galleon, Sloop, and four BC_2F_3 progeny plants at four SNPs linked with the *Rha4* locus. In previous research, *Rha4* co-segregated with wri785 and wri787 and co-segregated with wri786.

DNA\Assay	*Rha4* status	wri785	wri786	wri787	wri647
Galleon	*Rha4+*	T:T	A:A	G:G	A:A
Sloop	*Rha4-*	C:C	G:G		G:G
KK3-30-2-6-39	*Rha4-*	C:C	G:G	G:G	A:A
KK3-30-2-6-45		C:C	G:G	G:G	A:A
KK3-34-10-70-1	*Rha4+*	T:T	A:A	G:G	G:G
KK3-34-10-70-19		T:T	A:A	G:G	G:G

5.2.2.1 Methods

Fifteen seeds from each family were surface sterilised, plated on agar, and kept in the dark for 3 d at room temperature (3.2.2). Seedlings were then exposed to light for 24 h in a growth room at 15°C, 12/12 h, and relative humidity of 70%. Ten seedlings were then randomly selected from each family. Their root length was taken, and they were transferred into hydroponics as detailed in 3.4.2 in this thesis. Plants were sampled at 7 d and 11 d, and data was collected on the number of leaves, shoot height and chlorophyll content (using chlorophyll meter SPAD-502). At 11 d leaf surface area and biomass data were also taken. Roots were scanned with an

Epson expression 10000XL scanner using WinRHIZO software on each sampling day. Root images obtained were analysed for the root length, surface root area, root volume, and the average root diameter. To compare trait values between resistant and susceptible lines, data was subjected to T-tests at a significance level of 0.05 using GraphPad version 8.

5.2.3 Experiment 3- Assessing seedling vigour in progeny of a heterozygous (Rha4+/-) BC6F3 plant

This experiment was conducted using seeds from a more advanced generation of the same backcrossing program. These seeds were harvested from one BC_6F_3 plant (KK7-11-8) that had been confirmed to be heterozygous for *Rha4*-linked markers.

5.2.3.1 Method

Eighty seeds were surface sterilised, plated on agar, and kept in the dark for 3 d at room temperature (3.2.2). Seedlings were then exposed to light for 24 h in a growth room at 15℃ 12/12 h and relative humidity of 70%. The seedlings were then transferred to hydroponics (3.2.4) at the same growth room conditions. Seedlings were arranged in a completely randomised design within the hydroponic tanks.

5.2.3.1.1 Genotyping using KASP assays

After 6 d in hydroponics leaf samples about 1 cm long were harvested, placed in tubes, and freeze-dried for 24 h. DNA was extracted from the freeze-dried samples using an SDS-method as detailed by Van Gansbeke et al. (2019). DNA quantity was checked with a Nanodrop 8000 spectrophotometer. Four KASP primer sets (wri777, wri647, wri776, and wri189) (Appendices 5 and 6), a SNPline platform and Kraken™ software (LGC Genomics Limited, Hoddesdon, UK) were used to genotype single nucleotide polymorphisms (SNPs) in the *Rha4* region.

5.2.3.1.2 Measurements and dimensions of root morphology and growth characteristics

Plants were sectioned into roots and shoots to study the growth characteristics and root morphology to determine similarities and differences between the plants. Growth variables including shoot length, the number of leaves, and the biomass of roots and shoots were taken.

Chlorophyll content was taken using chlorophyll meter SPAD-502. Roots were scanned with an Epson expression 10000XL scanner and analysed using the WinRHIZO software (Stranjnar et al. 2012). Root images obtained were analysed for the total root length, root surface area, root volume, and the average root diameter. Data was subjected to ANOVA using GraphPad Prism version 8.

5.2.4 Experiment 4- Assessing seedling vigour in Galleon, Sloop, and progeny of a heterozygous BC6F3 plant

The parent cultivars, Sloop and Galleon were grown along with segregating progenies of the BC_6F_3 plant KK7-11-12. Like KK7-11-8, KK7-11-12 had been confirmed to be heterozygous for SNPs linked with *Rha4*.

5.2.5 Methods

Fifty seeds of Sloop and Galleon, and 160 KK7-11-12 BC_6F_4 seeds were surface sterilised, plated on agar, and kept in the dark for 3 d at room temperature (3.2.2). Seeds were then exposed to light for 24 h on agar in the growth room at 20℃, 12/12 h, and relative humidity of 70%. The root lengths of all seedlings were taken and seedlings were transferred into hydroponics or soil. Completely randomised designs were used to arrange seedlings in hydroponics or soil.

After 6 d in hydroponics or soil, a piece of leaf tissue was excised from each plant and freeze-dried. DNA extraction and genotyping analysis were as detailed in 5.2.3.1.1 in this thesis. KASP assays used to interrogate 12 SNPs that are linked to *Rha4 (*Appendices 5 and 6). Seven of the markers investigated SNPs flank the *Rha4* region (wri777, wri189, wri192, wri197, and wri784 proximal to *Rha4*; wri647 and wri776 distal to *Rha4*), and six are within the *Rha4* region (wri780, wri781, wri782, wri783, wri779 and wri778) (Table 5.2).

Table 5. 2 Genotypes of Galleon, Sloop, and two BC_6F_3 plants at 12 SNPs linked with the *Rha4* locus and their positions on the Morex V2 genome assembly. Previous research had shown that *Rha4* is between markers wri784 and wri647. Black dotted lines indicates that markers wri777 was not assayed on BC_6F_3 plants.

	Position (bp) on the 7H pseudomolecule of Version 2 of the Morex barley genome sequence assembly												
	598120741	620514593	620558798	622168585	622192610	622267988	622268194	622297486	622368992	622833626	622818257	623756604	625423052
	wri 777	wri 189	wri 192	wri 197	wri 784	wri 780	wri 781	wri 782	wri 783	wri 779	wri 778	wri 647	wri 776
Galleon	A:A	C:C	T:T	G:G	A:A	A:A	T:T	T:T	G:G	G:G	G:G	A:A	A:A
Sloop	G:G	T:T	G:G	C:C	C:C	G:G	C:C	C:C	A:A	C:C	T:T	G:G	T:T
KK7-11-8	.....	T:C	T:G	C:G	A:C	G:A	C:T	T:C	A:G	C:G	T:G	G:A	T:T
KK7-11-12	.....	T:C	T:G	C:G	A:C	G:A	C:T	T:C	A:G	C:G	T:G	G:A	T:T

After 20 d in soil, biomass data was taken. Roots of plants grown in soil were carefully washed under running water to remove soil particles before measurements were taken. Plants were sectioned into shoot and root. Fresh weights were measured, then the roots and shoots were oven-dried at 65^{o}C for 4 d before dry weight measurements were taken.

5.3 Results

5.3.1 Experiment 1- Comparison of Galleon with Sloop for seedling vigour and examination of temperature effect on seed germination

For plants grown in hydroponics, there was no significant cultivar × treatment interaction, but germination temperature affected growth variables (Table 5.3). Initially, roots from the 20^{o}C treatment were longer than roots from the 15^{o}C, which were in turn longer than roots from the 10^{o}C treatment (Figure 5.1).

After growth in hydroponics, plants from seeds germinated at 15^{o}C or 20^{o}C had significantly higher root dry weight than plants from seeds germinated at 10^{o}C (Figure 5.2). For shoot dry weights, cultivar and temperature were significant sources of variation (Table 5.3). Shoot dry

weight was significantly greater for Galleon than for Sloop and for plants from seeds germinated at 15ºC than for plants from seeds germinated at 10ºC (Figure 5.3).

For seedlings grown in soil, the cultivar × treatment interaction was significant for root dry weight and shoot dry weight (Table 5.3). After germination at 15°C or 20°C, Galleon had higher root and shoot biomass than Sloop. After germination at 10°C, Sloop and Galleon had similar root biomass and Sloop had slightly higher shoot biomass than Galleon (Figure 5.4).

Table 5. 3 P-values from an analysis of variance for Sloop and Galleon plants from seeds germinated at three temperatures (10°C, 15°C, 20°C) after 20 d of growth in a hydroponics system (H) or soil (S).

Trait	**Cultivar p-value**	**Treatment p-value**	**Interaction p-value**
Initial root length (mm)	0.0572	< 0.0001	0.1584
Root dry weight (H) (mg)	0.6179	< 0.0001	0.1266
Shoot dry weight (H) (mg)	< 0.0001	< 0.0001	0.0715
Root dry weight (S) (mg)	0.0858	< 0.0001	0.0068
Shoot dry weight (S) (mg)	<0.0001	0.0009	0.0015

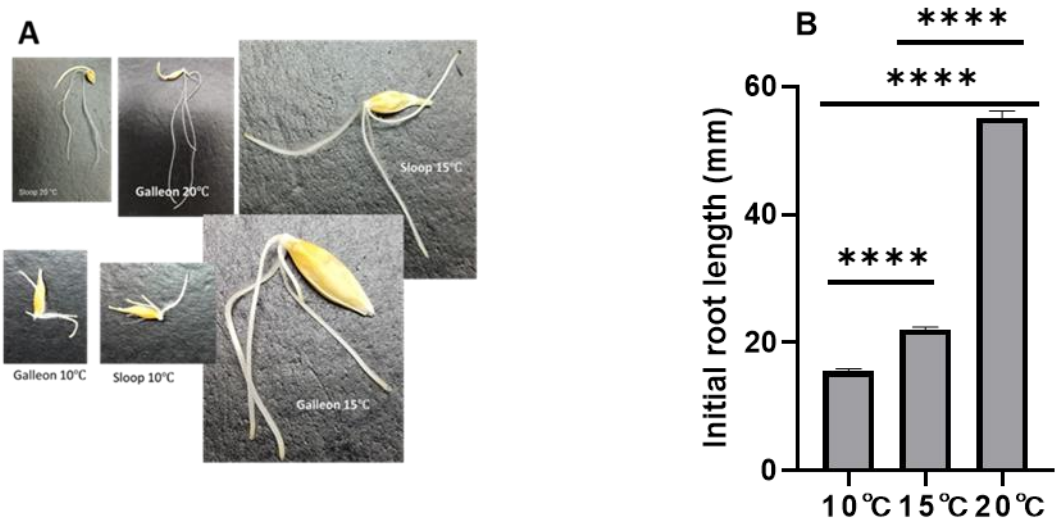

Figure 5.1 Initial root length of Sloop and Galleon barley seedlings after 4 d of germination at 20ºC,15ºC and 10ºC: photographs of typical seedlings (A) and means and standard errors (B). **** Significance at the 0.0001 level of probability.

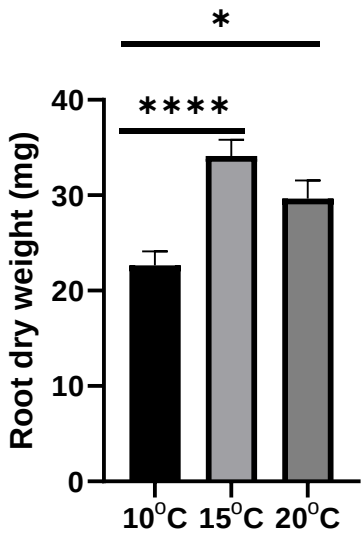

Figure 5.2 Means, and standard errors of root dry weight of Sloop and Galleon germinated at three temperatures (20ºC,15ºC and 10ºC), grown in a hydroponic system and sampled after 20 d. *, **** Significance at the 0.05 and 0.0001 levels of probability, respectively.

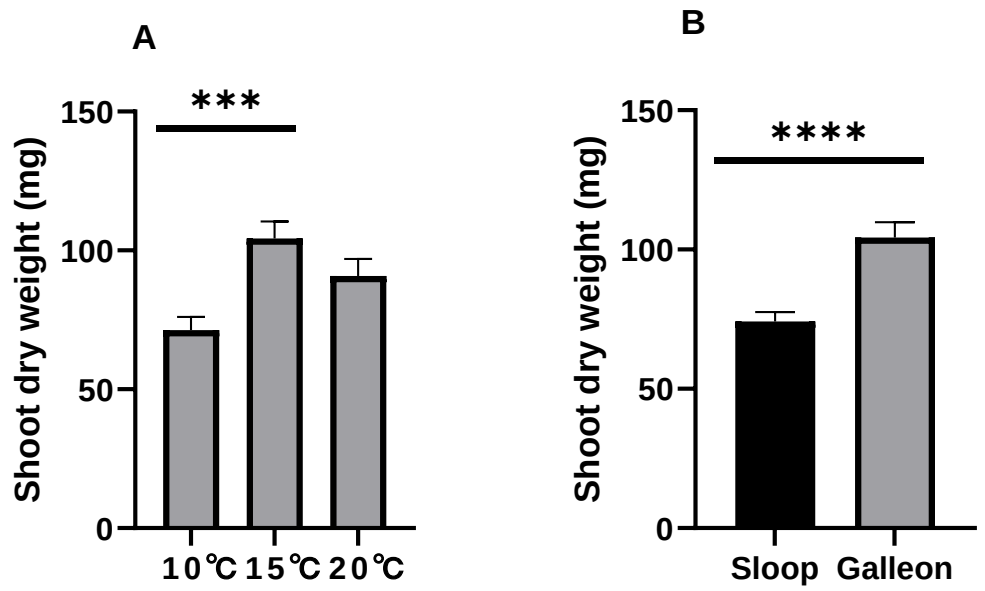

Figure 5.3 Means, and standard errors of treatment effect of shoot dry weight (A) and cultivar effect of shoot dry weight (B) of Sloop and Galleon germinated at three temperatures (20^{o}C,15^{o}C and 10^{o}C), grown in a hydroponic system and sampled after 20 d. ***, **** Significance at the 0.001, and 0.0001 levels of probability, respectively.

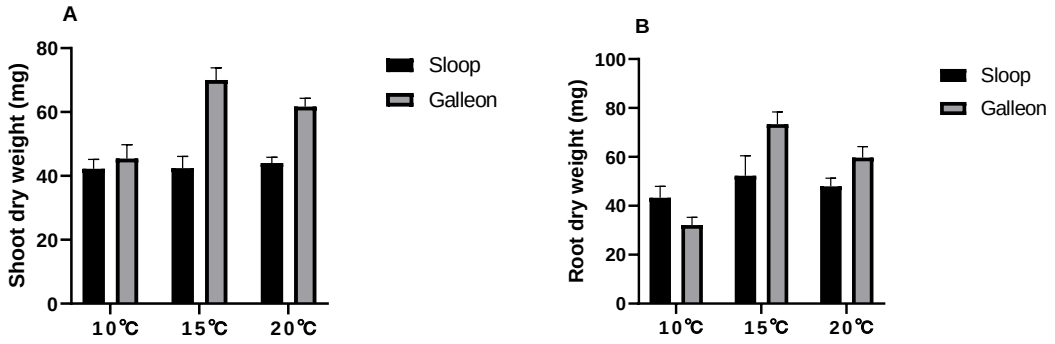

Figure 5.4 Means, and standard errors of shoot dry weight (A) and root dry weight (B) of Sloop and Galleon germinated at three temperatures (20^{o}C,15^{o}C and 10^{o}C), grown in soil and sampled after 20 d.

5.3.2 Experiment 2- Comparison of seeding vigour between Rha4+ and Rha4- BC_2F_4 families

After 6 d in hydroponics, plants from the two *Rha4+* families had larger root diameter and volume than those from the two *Rha4-* families (Figure 5.5) (Appendix 1). After 10 d in

hydroponics, plants from the *Rha4*+ families had larger root surface area, root diameter, root volume, root dry weight, and shoot dry weight than those from the two *Rha4*- families, indicating greater seedling vigour in *Rha4*+ lines (Figure 5.6) (Appendix 2).

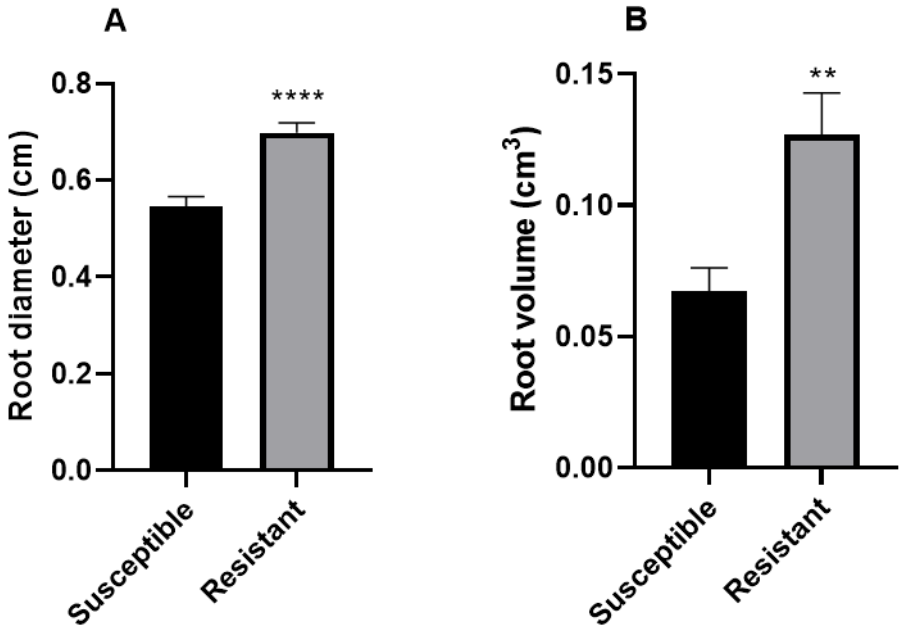

Figure 5.5 Means, and standard errors of root diameter (A) and root volume (B) of susceptible and resistant Sloop × Galleon $BC_2F_{3:4}$ families grown in a hydroponic system and sampled after 7 d. **, **** Significance at the 0.01, and 0.0001 levels of probability, respectively.

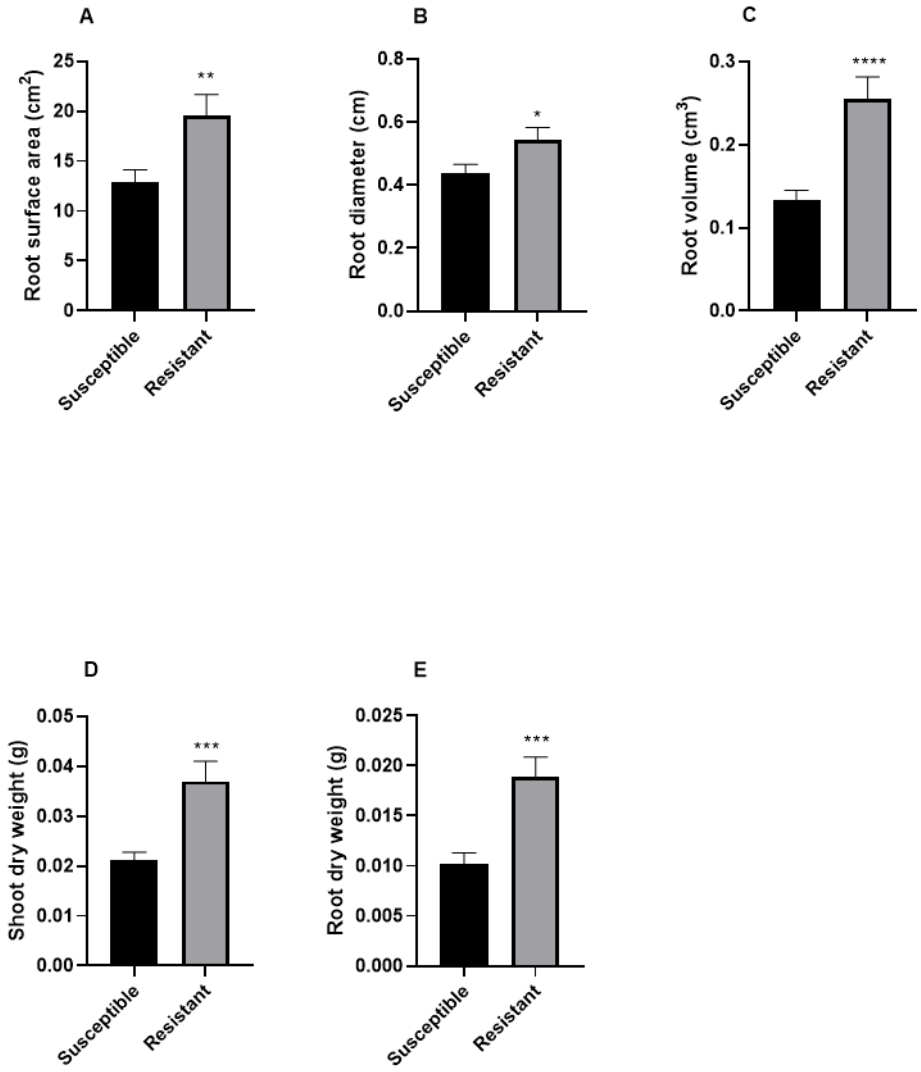

Figure 5.6 Means, and standard errors of root surface area (A), root diameter (B), root volume (C), shoot dry weight (D), and root dry weight (E) of susceptible and resistant Sloop × Galleon $BC_2F_{3:4}$ families grown in a hydroponic system and sampled after 11 d. *, **, ***,**** Significance at the 0.05, 0.01, 0.001, and 0.0001 levels of probability, respectively.

5.3.3 Experiment 3- Assessing seedling vigour in progeny of a heterozygous (Rha4+/-) BC_6F_3 plant

Genotyping of KK7-11-8 BC_6F_4 plants for four *Rha4*-linked molecular markers indicated that 20 plants were homozygous for Galleon alleles, 16 were homozygous for Sloop alleles, 35 were heterozygous, and two were possible recombinants (Table 5.5). The genotypic ratios observed for the two segregating markers did not deviate significantly from the expected ratio of 1:2:1(p = 0.793 for wri 189; p = .0.793 for wri 647) (Apeendix 3). The two possible recombinants were

removed from the experiment and transplanted to soil to be grown to maturity to obtain progeny for use in future fine-mapping studies. For the 11 growth variables measured, there were no significant differences among the three genotype classes (Table 5.5).

Table 5. 4 Genotyping results for Sloop × Galleon KK7-11-8 BC_6F_4 plants

Assay			
wri777 wri189 wri647 wri776	wri777 wri189 wri647 wri776	wri777 wri189 wri647 wri776	wri777 wri189 wri647 wri776
Rha4+/+ homozygote	*Rha4*-/- homozygote	*Rha4*+/- heterozygote	Possible recombinant
G:G C:C A:A T:T	G:G T:T G:G T:T	G:G T:C G:A T:T	G:G T:C G:G T:T KK 7-11-8-16
KK 7-11-8- 02 KK 7-11-8- 04 KK 7-11-8-21 KK 7-11-8-23 KK 7-11-8-28 KK 7-11-8-30 KK 7-11-8-31 KK 7-11-8-44 KK 7-11-8-48 KK 7-11-8-50 KK 7-11-8-53 KK 7-11-8-54 KK 7-11-8-55 KK 7-11-8-63 KK 7-11-8-66 KK 7-11-8-68 KK 7-11-8-72 KK 7-11-8-73 KK 7-11-8-74 KK 7-11-8-76	KK 7-11-8-08 KK 7-11-8-11 KK 7-11-8- 12 KK 7-11-8-18 KK 7-11-8-19 KK 7-11-8-22 KK 7-11-8-24 KK 7-11-8-29 KK 7-11-8-36 KK 7-11-8-43 KK 7-11-8-51 KK 7-11-8-59 KK 7-11-8-60 KK 7-11-8-61 KK 7-11-8-69 KK 7-11-8-79	KK 7-11-8-01 KK 7-11-8-03 KK 7-11-8-05 KK 7-11-8-06 KK 7-11-8-07 KK 7-11-8-09 KK 7-11-8-10 KK 7-11-8-13 KK 7-11-8-14 KK 7-11-8-15 KK 7-11-8-17 KK 7-11-8-20 KK 7-11-8-25 KK 7-11-8-26 KK 7-11-8-27 KK 7-11-8-32 KK 7-11-8-33 KK 7-11-8-35 KK 7-11-8-37 KK 7-11-8-38 KK 7-11-8-39 KK 7-11-8-40 KK 7-11-8-41 KK 7-11-8-42 KK 7-11-8-46 KK 7-11-8-49 KK 7-11-8-52 KK 7-11-8-56 KK 7-11-8-57 KK 7-11-8-62 KK 7-11-8-65 KK 7-11-8-67 KK 7-11-8-70 KK 7-11-8-71 KK 7-11-8-75 KK 7-11-8-77 KK 7-11-8-78	G:G C:C G:A T:T KK 7-11-8-582

Table 5. 5 Means and p-values of initial root length, number of leaves, chlorophyll content, leaf area, shoot height, root surface area, root length, root volume, root diameter, root fresh and dry weights, and shoot fresh and dry weights of Sloop × Galleon KK7-11-8 BC_6F_4 plants grown in a hydroponic system and sampled after 16 d.

Trait	*Rha4*+/+ homozygous	*Rha4*-/- homozygous	Rha4+/- heterozygous	p-value
Initial root length (mm)	40.29	41.21	39.99	0.843
Number of leaves	2.16	2.13	2.30	0.323
Chlorophyll content	29.87	28.81	29.88	0.437
Leaf area (cm^2)	42.06	48.38	45.55	0.363
Shoot height (cm^3)	19.61	20.17	20.01	0.650
Root surface area (cm^2)	20.66	20.91	22.44	0.334
Root length (cm^2)	108.50	113.819	124.84	0.358
Root diameter (cm)	0.65	0.60	0.60	0.160
Root volume (cm^3)	0.35	0.31	0.33	0.738
Root dry weight (mg)	16.51	17.12	17.23	0.630
Shoot dry weight (mg)	39.76	38.86	39.65	0.928

5.3.4 Experiment 4- Assessing seedling vigour in Galleon, Sloop, and progeny of a heterozygous BC_6F_3 plant

Genotyping of KK7-11-8 BC_6F_4 plants grown in hydroponics for 12 *Rha4*-linked molecular markers, indicated that 18 plants were homozygous for Galleon alleles, 27 were homozygous for Sloop alleles, and 33 were heterozygous, and two were possible recombinants (Table 5.6). The genotypic ratios observed for the 11 segregating markers did not deviate significantly from the expected 1:2:1 ratio ($p > 0.05$) (Appendix 3). The two possible recombinants were removed

from the experiment and transplanted to soil to be grown to maturity to obtain progeny for use in future fine-mapping studies.

Table 5. 6 Genotyping results for Sloop × Galleon KK7-11-12 BC_6F_4 plants

Assay																							
wri189	wri192	wri784	wri782	wri783	wri197	wri781	wri780	wri779	wri778	wri647	wri776	wri189	wri192	wri784	wri782	wri783	wri197	wri781	wri780	wri779	wri778	wri647	wri776
Rha4+/+ homozygous												*Rha4*-/- homozygous											
C:C	T:T	A:A	T:T	G:G	G:G	T:T	A:A	G:G	G:G	A:A	T:T	T:T	G:G	C:C	C:C	A:A	C:C	C:C	G:G	C:C	T:T	G:G	T:T
KK 7-11-12-13												KK 7-11-12-1											
KK 7-11-12-14												KK7-11-12-10											
KK7-11-12-16												KK 7-11-12-11											
KK 7-11-12-22												KK 7-11-12-12											
KK 7-11-12-23												KK 7-11-12-15											
KK 7-11-12-24												KK 7-11-12-17											
KK 7-11-12-25												KK 7-11-12-20											
KK 7-11-12-28												KK 7-11-12-31											
KK 7-11-12-3												KK 7-11-12-34											
KK 7-11-12-32												KK 7-11-12-35											
KK 7-11-12-39												KK 7-11-12-37											
KK 7-11-12-41												KK 7-11-12-40											
KK 7-11-12-43												KK 7-11-12-46											
KK 7-11-12-47												KK 7-11-12-48											
KK 7-11-12-58												KK 7-11-12-49											
KK 7-11-12-60												KK 7-11-12-5											
KK 7-11-12-7												KK 7-11-12-50											
KK 7-11-12-74												KK 7-11-12-53											
												KK 7-11-12-54											
												KK 7-11-12-59											
												KK 7-11-12-63											
												KK 7-11-12-65											
												KK 7-11-12-68											
												KK 7-11-12-69											
												KK 7-11-12-71											
												KK 7-11-12-75											
												KK 7-11-12-76											
Rha4+/- heterozygous												Possible recombinant											
T:C	T:G	A:C	T:C	A:G	C:G	C:T	G:A	C:G	T:G	G:A	T:T	T:C	T:G	A:C	C:C	A:A	-	C:C	G:G	C:C	T:T	G:G	T:T
KK 7-11-12-18												KK 7-11-12-79											
KK 7-11-12-19																							
KK 7-11-12-2												-	T:G	-	T:C	A:G	C:G	C:T	G:G	-	-	G:G	T:T
KK 7-11-12-21												KK 7-11-12-57											
KK 7-11-12-26																							
KK 7-11-12-27																							
KK 7-11-12-29																							
KK 7-11-12-30																							
KK 7-11-12-33																							
KK 7-11-12-36																							

KK 7-11-12-38 KK 7-11-12-4 KK 7-11-12-42 KK 7-11-12-44 KK 7-11-12-45 KK 7-11-12-51 KK 7-11-12-52 KK 7-11-12-55 KK 7-11-12-56 KK 7-11-12-6 KK 7-11-12-61 KK 7-11-12-62 KK 7-11-12-64 KK 7-11-12-66 KK 7-11-12-67 KK 7-11-12-70 KK 7-11-12-72 KK 7-11-12-73 KK 7-11-12-77 KK 7-11-12-78 KK 7-11-12-8 KK 7-11-12-80 KK 7-11-12-9	

Initially, Galleon had significantly longer roots than Sloop and the progeny plants (Figure 5.7). After growth in hydroponics, Galleon was more vigorous than Sloop. There were no significant differences among the genotypic classes of progeny plants (Figure 5.8) (Appendix 4).

Genotypes of KK7-11-8 BC_6F_4 plants grown in soil for 12 *Rha4*-linked molecular markers, indicated that 15 plants were homozygous for Galleon alleles, 20 were homozygous for Sloop alleles, and 30 were heterozygous (Table 5.7). The genotypic ratios observed for the 11 segregating markers did not deviate significantly from the expected 1:2:1 ratio ($p > 0.05$) (Appendix 3). For the two growth variables measured, there was no significant difference among the parents and the progenies (Appendix 4). Plants transferred into hydroponics had more robust root growth than those grown in soil (Figures 5.9 and 5.10).

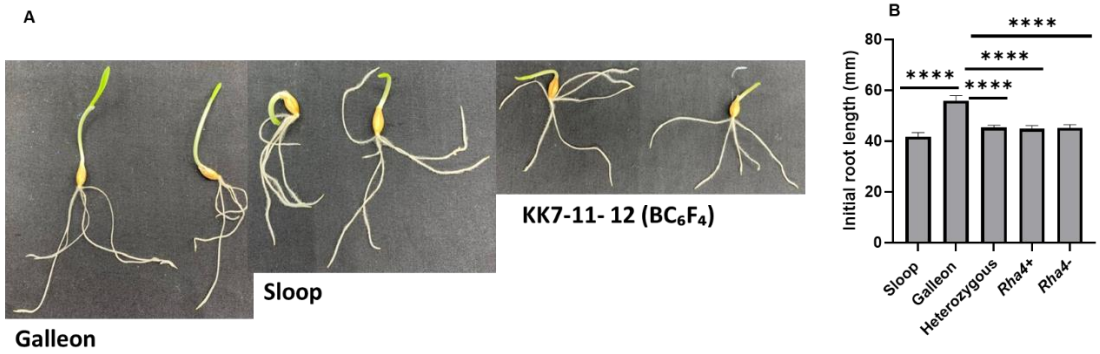

Figure 5.7 Initial root length of Sloop, Galleon, and progeny (BC_6F_4) barley seedlings after 3 d of germination: photographs of typical seedlings (A) and means and standard errors (B). **** Significance at the 0.0001 level of probability.

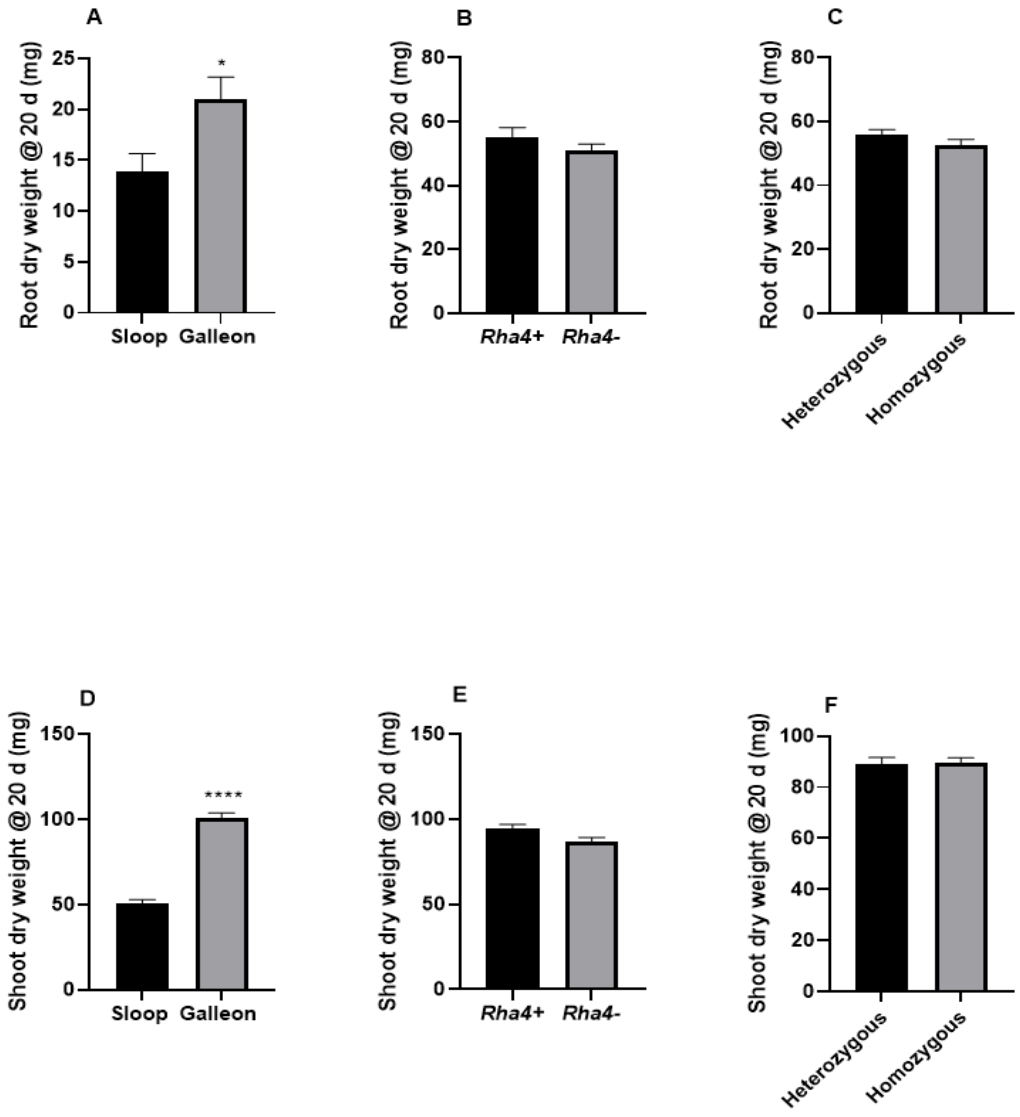

Figure 5.8 Means and standard errors of root dry weight (A, B, C) and shoot dry weight (D, E, F) of Sloop and Galleon (A, D), *Rha4+* and *Rha4-* homozygous and KK7- 11-12 BC_6F_4 progeny (B, E) and *Rha4* heterozygous KK7-11-12 BC_6F_4 progeny compared to the means of the two homozygous classes (F,G) after d in grown in a hydroponic system. *, **** Significance at the 0.05 and 0.0001 levels of probability, respectively.

Table 5. 7 Genotyping results for Sloop × Galleon KK7-11-12 BC_6F_4 plants.

Assay		
wri189 wri192 wri784 wri782 wri783 wri197 wri781 wri780 wri779 wri778 wrf647 wri776	wri189 wri192 wri784 wri782 wri783 wri197 wri783 wri780 wri779 wri778 wrf647 wri776	wri189 wri192 wri784 wri782 wri783 wri197 wri783 wri780 wri779 wri778 wrf647 wri776
Rha4+/+ homozygous	*Rha4*-/- homozygous	*Rha4*+/- heterozygous
C T A T G G T A G G A T : : : : : : : : : : : : C T A T G G T A G G A T	T G C C A C C G C T G T : : : : : : : : : : : : T G C C A C C G C T G T	T T A T A C C G C T G T : : : : : : : : : : : : C G C C G G T A G G A T
KK 7-11-12-7	KK 7-11-12-3	KK 7-11-12-1
KK 7-11-12-10	KK 7-11-12-5	KK 7-11-12-12
KK 7-11-12-11	KK 7-11-12-8	KK 7-11-12-13
KK 7-11-12-25	KK 7-11-12-14	KK 7-11-12-19
KK 7-11-12-32	KK 7-11-12-17	KK 7-11-12-2
KK 7-11-12-42	KK 7-11-12-27	KK 7-11-12-20
KK 7-11-12-45	KK 7-11-12-31	KK 7-11-12-21
KK 7-11-12-48	KK 7-11-12-33	KK 7-11-12-23
KK 7-11-12-58	KK 7-11-12-35	KK 7-11-12-24
KK 7-11-12-66	KK 7-11-12-38	KK 7-11-12-26
KK 7-11-12-68	KK 7-11-12-46	KK 7-11-12-28
KK 7-11-12-39	KK 7-11-12-50	KK 7-11-12-34
KK 7-11-12-47	KK 7-11-12-54	KK 7-11-12-36
KK 7-11-12-65	KK 7-11-12-55	KK 7-11-12-4
KK 7-11-12-60	KK 7-11-12-59	KK 7-11-12-41
	KK 7-11-12-64	KK 7-11-12-43
	KK 7-11-12-69	KK 7-11-12-44
	KK 7-11-12-70	KK 7-11-12-52
	KK 7-11-12-74	KK 7-11-12-56
	KK 7-11-12-75	KK 7-11-12-57
		KK 7-11-12-6
		KK 7-11-12-67
		KK 7-11-12-72
		KK 7-11-12-77
		KK 7-11-12-62
		KK 7-11-12-40
		KK 7-11-12-29
		KK 7-11-12-9
		KK 7-11-12-22
		KK 7-11-12-73

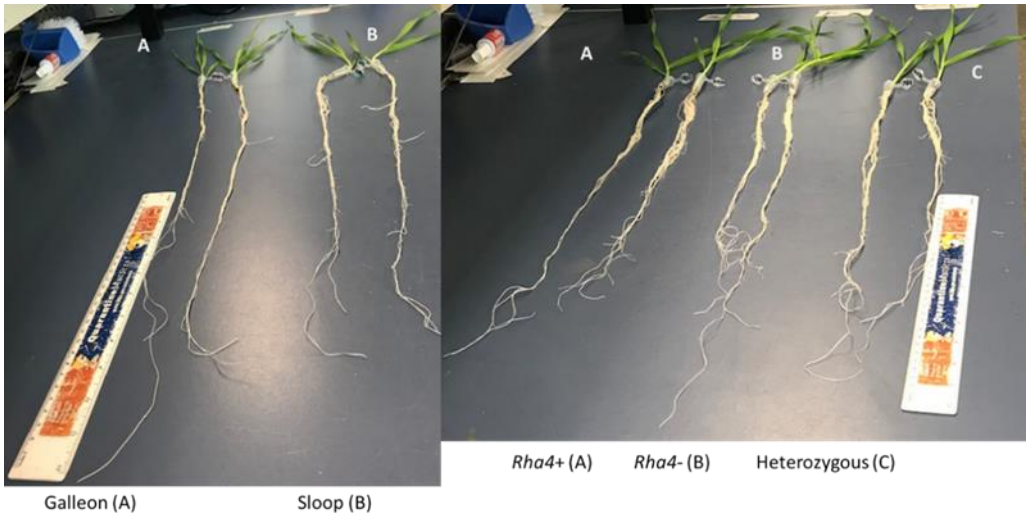

Figure 5.9 Galleon, Sloop, *Rha4+*, *Rha4-*, and Heterozygous (KK7-11-12 BC_6F_4) plants grown in a hydroponic system and sampled after 20 d.

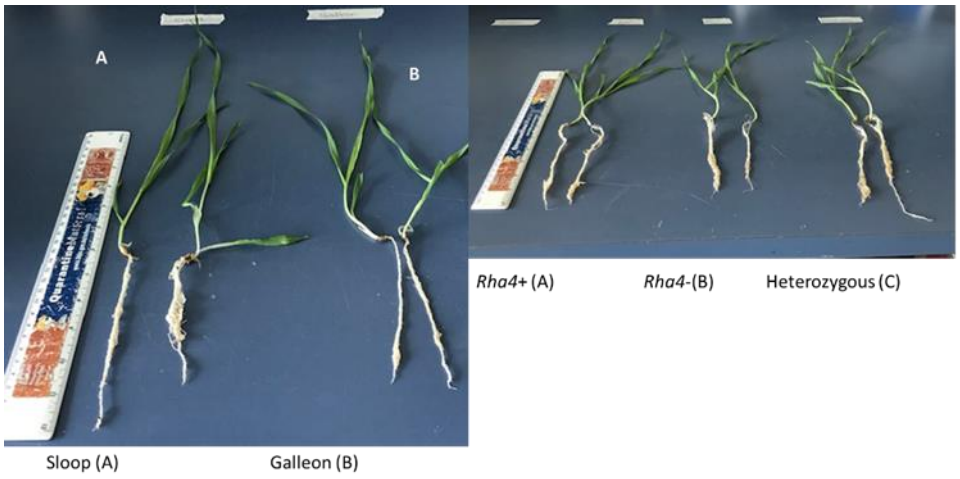

Figure 5.10 Sloop, Galleon, *Rha4+*, *Rha4-*, and Heterozygous (KK7-11-12 BC_6F_4) plants grown in soil and sampled after 20 d.

5.4 Discussion/ conclusion

Motivated by the robust root growth observed in Galleon, experiments were conducted to determine if the *Rha4* region on chromosome 5H of barley affected growth. Galleon, Sloop, and their progenies in the BC_2F_4 and BC_6F_3 generations were assessed for seedling vigour.

For most of the experiments described in this thesis, seedlings were grown in a hydroponic system at 15°C, a temperature that is suitable for CCN infection of plant roots (Van Gansbeke

et al. 2019; Levin et al. 2020) but may not be the best temperature for investigating seedling vigour. Accordingly, the first experiment described in this chapter used three germination temperatures (10°C, 15°C, and 20°C).

Temperature significantly affected seed germination and growth. Of the three germination temperatures that were tested here, 20°C provided the highest growth, but 15°C was the most useful for detecting differences in seedling vigour among the cultivars. Galleon seedlings were more vigorous at 15°C than Sloop seedlings regardless of the growth medium. The switch in temperature from 20°C to 15°C did not cause a significant change in the growth of the two cultivars. The vigorous growth of Galleon confirmed observations made by Dr. Bandaranayake.

In the early-generation backcross progeny plants ($BC_2F_{3:4}$), *Rha4+* families showed more robust growth than *Rha4-* families. However, the differences among these families cannot be attributed specifically to *Rha4.* They could be due to other genetic differences among the families. This led to investigating seedling vigour in more advanced families.

Progenies of the later-generation backcross families (KK7-11-8 and KK7-11-12 BC_6F_3) would have more of their genome fixed for Sloop alleles, making the families more homogeneous than the BC2-F3:4 families. These families were still segregating for *Rha4.* With genotyping of *Rha4*-linked markers, it was possible to classify progeny plants into three genotypic groups and make comparisons within families. The lack of significant differences among the genotypic groups seemed to indicate that the greater vigour of Galleon seedlings compared to Sloop seedlings could not be due to the *Rha4* locus.

Up to this point, experiments conducted did not include both the parents and the progeny, leaving open the possibility that the lack of difference among genotypic groups might be due to some unidentified difference in environmental conditions between the first and third experiments. Thus, in the final experiment, seedling vigour was assessed in both parents and

advanced-generation backcross progeny. In hydroponics, Galleon was again found to be more vigorous than Sloop, but this was not due to *Rha4* as there was no significant difference among the progeny groups. These experiments confirmed that the *Rha4* resistance cannot be attributed to greater seedling vigour. Roots of plants grown in a hydroponic system were about 3 times longer than roots of plants grown in soil. This was likely due to the ready availability of both nutrients and oxygen in the aerated nutrient medium. The more robust seedling vigour of Galleon was only observed in hydroponics and not in soil.

In conclusion, the study has shown that Galleon is more robust than Sloop when grown in hydroponics. There were no significant growth differences among the progenies. From this, it can be concluded that the *Rha4* region does not affect seedling vigour and thus seedling vigour does explain the CCN resistance conferred by *Rha4*. Although no differences were observed when Galleon and Sloop were grown in soil, it might be worth investigating a wider range of conditions, including field environments. If the observed effect in this study is expressed in the field, then it could be worthwhile to genetically map the cause.

6 Chapter 6: General discussion, conclusions, and Future work

6.1 General discussion

Cereal cyst nematode (CCN) *Heterodera avenae* is an endoparasite of cereals and can cause yield loss of 20-50% in cultivated cereals in temperate regions (Nicol 2002). The activities of this pathogen affect the physiological function of their host plant and reduce their yield. The works presented in this thesis investigated the effects of CCN on water transport and how this differs between resistant and susceptible barley.

Experiments reported in Chapter 3, assessed the effects of CCN infection on hydraulic conductivity on hydroponically grown resistant and susceptible barley plants using the exudation and suction techniques. CCN infection damaged root systems, reducing their root length, RSA, root volume in both susceptible and resistant cultivars used in the study. The infection also decreased the efficiency of bulk flow of water from the root to shoot and hydraulic conductivity. The reduction in hydraulic conductivity was (76.1%) in the suction experiment and (21.5% and 19.0%) in the exudation experiments.

Even though both Sloop SA and Galleon are known to be much more resistant to CCN than Sloop, resistance did not reduce the effect on hydraulic conductivity. Differences between resistant and susceptible plants may not have fully developed at the time (days after inoculation) that hydraulic conductivity was assessed. CCN feeding sites form in both resistant and susceptible plants. Initially, they can appear similar, but they later diverge, with the feeding sites in susceptible plants remaining active while those in resistant plants deteriorate (Seah et al. 2000). While it would be interesting to investigate hydraulic conductivity at a later stage (e.g., at 33 days after inoculation, based on results presented by Williams and Fisher (1993)), this was not feasible with the techniques used here. This is because the plants would have tillered, resulting in multiple shoot bases to be sealed within tubes, and because elongated roots might become tangled in the hydroponic tanks, making it difficult to separate individual plants.

A more recent and non-destructive technique such as magnetic resonance imaging (MRI) (Schneider et al. 2020) could be used to study the effect of CCN resistance on water transport in older plants. This technique can also be used to quantify the contribution of water transport in individual roots (seminal, lateral, and adventitious) when parasitised by the nematode.

In a recent study on hydroponically grown wheat seedlings inoculated with CCN, secondary thickening (maturity) of the central metaxylem vessel (cMX) was delayed with longer retention of end walls appearing to block the cMX (Levin et al. 2020). Immature cMX increases axial resistance (Steudle and Peterson 1998; Frensch and Steudle 1989) thus increasing the total hydraulic resistance (radial and axial resistance) to water flow. The reduced flow rate and hydraulic conductivity might in part be due to an increase in total hydraulic resistance. However, this could not be studied due to the time constraints in this study. In future experiments, the effect of CCN on axial flow could be studied to evaluate the consequence of altering the ratio of radial and axial resistance on water transport.

The reduced hydraulic conductivity of inoculated plants demonstrated in Chapter 3, motivated the interest to use an apoplastic tracer dye HPTS to determine how far and quickly the dye (coupled to water flow) could travel from the root to the shoot of CCN infected roots. Dye entry was at the base of the lateral roots into the main roots. The transport of the dye was interrupted in inoculated roots of both susceptible and resistant plants. In CCN inoculated roots, there was profuse formation of lateral roots at the region of the infection. According to Magnusson and Golinowski (1991), these lateral roots were formed at the head region of *H. schachtii* in mustard and could be supplying additional nutrients and water through by-pass flow to the syncytia. Since apoplastic dyes are transported into the stele at the point of emergence of lateral roots (Peterson et al. 1980; Faiyue et al. 2010), these tracers could be used to study the role of lateral roots in CCN-inoculated roots.

Nematodes require nutrients for their growth and development, and since nutrients are transported in solution form, a higher concentration of solute could be transported into the syncytia due to the high turgor pressure of the syncytia than the surrounding cells (Böckenhoff and Grundler 1994). Previous studies on PCN and SCN have shown a decrease in plant nutrients such as potassium, phosphorus, magnesium, calcium, sodium, and nitrogen (Trudgill et al. 1975; Blevins et al. 1995). However, little is known about the nutrients CCN deprives the host of. The multielement plant tissue analysis using inductively coupled plasma mass spectroscopy (ICP-MS) or inductively coupled plasma optical emission spectrometry (ICP-OES) (Hansen et al. 2013) could be used to study the regions below and above the feeding site, feeding site, and the isolated cyst to determine the nutrient composition and concentrations at those levels.

Investigating seedling vigour in Galleon, Sloop and the progenies in the early and advanced generations, Galleon had more robust root growth than Sloop. There was no significant difference among the genotypic groups in the BC_6F_3 families indicating that the *Rha4* region on chromosome 5H did not affect seedling vigour. The genetic cause of the difference in seedling vigour between Galleon and Sloop could be explored in the field environment and the difference could be genetically mapped.

6.2 Conclusions

There are three main conclusions from this thesis. Firstly, CCN infection reduced biomass, exudation flow rate, hydraulic conductance, and conductivity in resistant and susceptible barley. The techniques used to study hydraulic conductivity in inoculated plants were successful and revealed effects of infection on water transport.

Secondly, apoplastic tracer dye HPTS could be used to determine how quickly and far water travelled in the CCN infected roots of barley. UV analysis showed dye was concentrated at the feeding site and light colour of the dye beyond the feeding site. This indicated that water could

travel to the shoot when the nematode is not feeding or when the syncytia had reached its full capacity.

Thirdly, despite confirmation of greater seedling vigour in Galleon compared to Sloop and observed differences between early generation *Rha4+* and *Rha4-* backcross families, comparison between *Rha4+* and *Rha4-* segregants from advanced backcross families demonstrated that the *Rha4* region on chromosome 5H does not contribute to seedling vigour.

6.3 Future work

1. The study showed that CCN infection affected water transport at the early vegetative stages in resistant barley. Follow-up work might be carried out using magnetic resonance imaging (MRI) which is a non-destructive method that could be used to study hydraulic conductivity in older barley plants. Also, radial, and axial resistance in CCN infected roots could be studied to assess the influence of the infection on their ratio (Dorhout et al 1991, Knipfer and Frike 2011).

2. The fluorescent dye study could be followed up using a different apoplastic dye, disodium 4, 4'- bis (2- sulfostyryl) biphenyl (Tinopal CBS) (Peterson et al. 1981) to determine how water and solute are partitioned between the nematode and the host plant under a microscope. This dye is an aploplastic tracer and binds to cellulose and the dye position can be detected in sectioned tissues under a microscope without the dye washing out into the water during sectioning which is the case of HPTS. The point of entry and functions of Tinopal CBS are similar to those of HPTS (8-hydroxypyrene-1, 3, 6-trissulfonic acid trisodium salt).

3. Knowing the effect of CCN on water transport, further studies could be carried out to determine the nutrients the host plant is deprived of by CCN. This could be studied using multielement plant tissue analysis using inductively coupled plasma mass

spectroscopy (ICP-MS) or inductively coupled plasma optical emission spectrometry (ICP-OES).

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Appendices

Appendix 1: Means and p-values of traits for which significant ($p \leq 0.05$) differences were detected between susceptible and resistant Sloop × Galleon $BC_2F_{3:4}$ families grown in hydroponics and sampled after 6 d.

Trait	*Rha4-*	*Rha4+*	P-value
Root diameter (cm)	0.55	0.69	<0.0001
Root volume (cm^3)	0.07	0.13	0.0015

Appendix 2: Means and p-values of traits for which significant ($p \leq 0.05$) differences were detected between susceptible and resistant Sloop × Galleon $BC_2F_{3:4}$ families grown in hydroponics and sampled after 11 d.

Trait	*Rha4-*	*Rha4+*	P-value
Root surface area (cm)	12.85	19.51	0.0083
Root diameter (cm)	0.44	0.54	0.0332
Root volume (cm^3)	0.13	0.26	0.0001
Shoot dry weight (g)	0.02	0.04	0.0003
Root dry weight (g)	0.01	0.02	0.0003

Appendix 3: Chi-squares and p-values of KASP primer sets used for genotyping KK7-11-8 and KK7-11-12 BC_6F_4 plants grown in hydroponics or soil.

	KK7-11-8 (hydroponics)		*KK7-11-12 (hydroponics)*		*KK7-11-12 (soil)*	
Primer	Chi square	p-value	Chi-square	p-value	Chi-square	p-value
HvEPG001197	0.699	0.71	4.169	0.12	1.966	0.3743
HvEPG000044	0.444	0.80	6.09	0.05	1.839	0.40
HvEPG001200			6.507	0.04	2.898	0.24
HvEPG003925			4.671	0.10	1.571	0.46

HvEPG001969			4.595	0.10	1.839	0.40
HvEPG001971			4.949	0.08	1.600	0.45
HvEPG000040			3.582	0.17	1.097	0.58
HvEPG001986			4.30	0.12	2.500	0.29
HvEPG001967			3.923	0.14	1.839	0.40
HvEPG001956			6.227	0.05	1.433	0.49
HvEPG001955			5.077	0.08	1.571	0.46

Appendix 4: Means and p-values of initial root length, root dry weight, and shoot dry weight of Sloop × Galleon KK7-11-12 BC_6F_4 plants grown in hydroponics (H) and soil (S) sampled after 20 d.

Trait	Sloop	Galleon	Heterozygous	*Rha4+*	*Rha4-*	P-value
Initial root length (mm)	41.78	55.87	45.29	44.86	45.20	<0.0001
Root dry weight (H) (g)	49.52	57.61	55.95	55.26	50.96	0.0249
Shoot dry weight (H) (g)	80.88	101.06	89.03	94.24	86.74	<0.0001
Root dry weight (S) (g)	13.89	20.94	20.29	19.06	18.53	0.0892
Shoot dry weight (S) (g)	47.65	60.5	55.89	57.33	53.66	0.1856

Appendix 5: Sequences for which KASP Assays were developed

Sequence	KASP assay	Internal code[a]
TGCAGCTGGCGCCCTTCGCTGG[C/G]CACGCGGCCAACCATCTGGTCTTGAACATCACAAGTTTAATCTTCA	wri197	HvEPG000040
TGCAGCACACGATCGATGAAGCAATAGTGCAATACAGACACGAGTCTA[G/A]CTAGCTCACTTGCATTCATG	wri647	HvEPG000044
TGCAGTGCTTGGTTTGGTATGTCTCTGA[A/T]TATTTAGATACCAAACATTTTACGGAGTTTCTCTTTGACC	wri776	HvEPG000050
TCCCCTATATATATATAAGCAGCAGCAGCTCTTCCTGCAGCCATCCCCATCCATTCTTCACAGCGTCCATCCA CAAGAAGAACAAGATGAATGCG[C/T]TGTCCGCTCGCAAGTGCTCAGAGTTTTTCTGTGGGTTTGTTGTCGCTGCCTTTGCCGTTTATGCGCTAGT	wri189	HvEPG001197
AAGAGCCGATGCCAAAAGGGCTGCCCAGTTTTTGTGGCCGAATACAACTGCAGTTCACATTTGCCCT GTTTCTTTTTAATAAGCCGTT[G/T]CCATGGCCAGAACATGCACCGTTGCTTGTGGACAGCAACCACA CATAAGGAGGAAGAAGATCTAGAGAGGAATGATAGAAGTACAGAGGAAGGACACAGAGA	wri192	HvEPG001200
TGCAGCGAATCGAAGTGCGAGGT[A/G]AGAAGGAAGGACGCGAGGCTTACATCGAGGCACGTAGTTGGAGTC	wri777	HvEPG001320
TGTCCTAGAACGGAAAAGCTCTGCAGAGTTCAGTAGATAGTTTCCGGGACAATGTTCACATTCTACGTACTT GCTTGATCATCCTCT[G/T]CTTCCATACTAGGGATGTGCCATGCTGGTCTCCATGCTCCATACTTGATGGGCGATTTTGGAGCTAGCACGGCTGACA	wri778	HvEPG001955
TTGTTGCTTCCCTTCTTCCATACGCTCAGGAAAATGGCCGCCCCCACCATGACCGCCGCCAAGGCGTTCGCCAC CACCAGGTACCTGCACGCACCCACGAATCCGCCTTGTCA[G/C]CGTACGCACAACCCAGCATGGATACCGTCT CCAAGGGAGAAAAGCCTAGTAGACATACACAAAGGGGCCGAAGTCGCTGTAGGTGACGGTGTGTGCCTCGGCGCCGTTC	wri779	HvEPG001956
TTGTGACGTTGGAGATAATAATCCAGCAAACACTGCTTTCTTCTCAGATCAAGCTGGGACAATAATGCATCCAT CAACCCTGGCCTCCTCCTCAGAGAAT[A/G]TTGAGGTGGCCCAAGATGAGTACCGCGATCTGCTGCTCCTGCGCT CAAGCGGGCAGGTGGGCGACGTGGGGGAGTGCGGCGGATGGGAGGAAGTGCGCGA	wri780	HvEPG001967
GGCGGATGGGAGGAAGTGCGCGACAAGGAGAATGAGGGTGATGAGGAGAAAGGGGAACAGATGGAGAAAGA TGTGCAGGAGCTTTGCACGTTTGGGTTCAAGGCCATTGAGAATAAGGACTTCTCSGG[T/C]GCTGTAGACT GCCAACGCAGGATCCTCAAGACCAGGTTAGGGTTCTTCCTTTCCACCTGGCACCTGCCACTCTTATTTCAAA GCCTAGCCTGGCGTTGGCGTTTCTCTTATCTCAGATTGTTGAA	wri781	HvEPG001968
TGTCTCAACTTTGTATAGCTCTAGTATAAAGTTGGAGTAAG[C/T]TTGAGACACTCATTTTAAGACAGAAGAATATATTTTTAAAAATTCATTT TTTCTCATGGTAGTAAATTTCTTGCGTGATAGCCGTTCCAATTTTGAGAT	wri782	HvEPG001969
TCGACTCCCCAGTAAAATGGGACGCCGCCATCAGCATCCTGCAAGAAAATGGAGAGCCC[G/A]TATGAATTTATTTCGATATGACA TGGCATGGTTTTGTCTGTCTGTCAGACTGTCTTTGCCACATTAATGTGAAATGCGGAAATGCAGAATACTAGACAGG	wri783	HvEPG001971
TTGTTTAGTCGTTCCAATGAGGACTAAGAATCAATGCAACTTTTTATTCCACTATAATAGTTTCGTCGGTAAGGT[C/A]ATTGCTC ACGATCGTGATTATATATCGGGCGTCTTTTTTAAGAAATACTTTGCTTTTGCATTCAACATAATGTAC	wri784	HvEPG003925
TGCAGATGATTTCCATTCATTAAGATGTAATGAATGTACGTCCAAATCAAT[C/T]GCCATCATACAACTGTCCATTCATTCAAG CAACCCGAGTATATATGGTTCAACTGTTCGACGGTACTGTCTCCGT	wri785	HvEPG003926
CTTGCCATTGATTTGGTGCAAATAATGTGGTTAGTTAATTAGTTGGGGACACGTAAGAGCGTGTTAACCAGTTAC[G/A]TTAGA CCTCATAGAGCATGGTACCTTAATGGTATAGCAACAACGGCTATATGAGACTGTCACGTCGTACAAGGTT	wri786	HvEPG003953
TTGAAGAGCCTATTTTACCACGTTCGACTGTAGATGCTCCTATGAAATTCTCAAATCTATCCAAATGATATCCAT[A/G]ACACACCA CTCTTCTCCCGAACATACCCGCCGATGATCCCATGAAAC	wri787	HvEPG003968

[a]Internal codes are the unique identifiers for these markers in the University of Adelaide Kraken database.

Appendix 6: List of KASP assays

KASP Assay	Allele-specific primer 1	Allele- specific primer 2	Common primers
wri197	GAAGGTGACCAAGTTCATGCTAGCT GGCGCCCTTCGCTGGG	GAAGGTCGGAGTCAACGGATT AGCTGGCGCCCTTCGCTGGC	GTTCAAGACCAGAT GGTTGGCCGCGTG
wri647	GAAGGTGACCAAGTTCATGCTG CAATAGTGCAATACAGACACGAGTCTAA	GAAGGTCGGAGTCAACGGATTGCA ATAGTGCAATACAGACACGAGTCTAG	TGCACCATGAATG CAAGTGAGCTAG
wri776	GAAGGTGACCAAGTTCATGCT CAGTGCTTGGTTTGGTATGTCTCTGAA	GAAGGTCGGAGTCAACGGATTCAGTGC TTGGTTTGGTATGTCTCTGAT	GAAACTCCGTAAA ATGTTTGGTATCTAAAT
wri189	GAAGGTGACCAAGTTCATGCT GAGCACTTGCGAGCGGACAG	GAAGGTCGGAGTCAACGGATTGAGC ACTTGCGAGCGGACAA	GTCCATCCACAA GAAGAACAAGATGAAT
wri192	GAAGGTGACCAAGTTCATGCT CCCTGTTTCTTTTTAATAAGCCGTTG	GAAGGTCGGAGTCAACGGATTGCCC TGTTTCTTTTTAATAAGCCGTTT	GCAACGGTGCAT GTTCTGGCCAT
wri777	GAAGGTGACCAAGTTCATGCTCA GCGAATCGAAGTGCGAGGTA	GAAGGTCGGAGTCAACGGATTAGC GAATCGAAGTGCGAGGTG	CTCGATGTAAGCC TCGCGTCCTT
wri778	GAAGGTGACCAAGTTCATGCTACGTA CTTGCTTGATCATCCTCTG	GAAGGTCGGAGTCAACGGATTCTA CGTACTTGCTTGATCATCCTCTT	AGCATGGCACATCC CTAGTATGGAA
wri779	GAAGGTGACCAAGTTCATGCTCCCA CGAATCCGCCTTGTCAG	GAAGGTCGGAGTCAACGGATTCC CACGAATCCGCCTTGTCAC	TTTCTCCCTT GGAGACGGTATCCAT
wri780	GAAGGTGACCAAGTTCATGCTGG CCTCCTCCTCAGAGAATA	GAAGGTCGGAGTCAACGGATTG GCCTCCTCCTCAGAGAATG	GGTACTCATCTT GGGCCACCTCAA
wri781	GAAGGTGACCAAGTTCATGCTG CGTTGGCAGTCTACAGCA	GAAGGTCGGAGTCAACGGATTCT GCGTTGGCAGTCTACAGCG	GTTCAAGGCCATT GAGAATAAGGACTT
wri782	GAAGGTGACCAAGTTCATGCTTTC TGTCTTAAAATGAGTGTCTCAAG	GAAGGTCGGAGTCAACGGATTCTT TCTGTCTTAAAATGAGTGTCTCAAA	TTGTATAGCTCTAG TATAAAGTTGGAGTAA
wri783	GAAGGTGACCAAGTTCATGCTGCA AGAAAATGGAGAGCCCG	GAAGGTCGGAGTCAACGGATTCCTGCA AGAAAATGGAGAGCCCA	CCATGCCATGTCATA TCGAAATAAATTCAT
wri784	GAAGGTGACCAAGTTCATGCTACT ATAATAGTTTCGTCGGTAAGGTC	GAAGGTCGGAGTCAACGGATTCACTATA ATAGTTTCGTCGGTAAGGTA	CCGATATATAATCA CGATCGTGAGCAAT
wri785	GAAGGTGACCAAGTTCATGCTGATGTA ATGAATGTACGTCCAAATCAATC	GAAGGTCGGAGTCAACGGATTAGATGT AATGAATGTACGTCCAAATCAATT	GGTTGCTTGAATGA ATGGACAGTTGTAT
wri786	GAAGGTGACCAAGTTCATGCTGTACC ATGCTCTATGAGGTCTAAC	GAAGGTCGGAGTCAACGGATTGGTAC CATGCTCTATGAGGTCTAAT	GACACGTAAGAGCG TGTTAACCAGTT
wri787	GAAGGTGACCAAGTTCATGCTAA ATTCTCAAATCTATCCAAATGATATCCATA	GAAGGTCGGAGTCAACGGATTCTCAAATCTATCCAAATGATATCCATG	TCGGCGGGTATGTTCGGGAGAA