Factors stimulating germination of *Plasmodiophora brassicae* resting spores

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The important thing is not to stop questioning.

Curiosity has its own reason for existing.

- Albert Einstein physicist (1879 - 1955)

Table of content

Abbreviations

ANOVA	Analysis of variance
ASVs	amplicon sequence variants
CCD	Canadian clubroot differential set
CFW	Calcofluor white
CLSM	Confocal laser scanning microscope
CTAB	Cetyltrimethylammonium bromide
cv.	Cultivar
dai	Days post incubation
DAPI	4,6-Diamidine-2-phenylindole dihydrochloride
DI	Disease index
DIC	Differential interference contrast
dpi	Days post inoculation
EA-IRMS	Elemental analyser-isotope-ratio mass spectrometry
ECD	European Clubroot Differential set
EM	Emission wavelength
ESI	Electrospray ionization source
EX	Excitation wavelength
GLM	General linear model
GWASs	Genome-wide association studies
HTS	Hydroponic trapping system
LB	Luria-Bertani
LC	Liquid chromatography
LC-QTOF-MS	Liquid Chromatography Time-of-flight Mass Spectrometer
MS	Murashige and Skoog
NTCs	Non-template controls
PDC	Petri dish cultivation
PI	Propidium iodide
PMA	Propidium monoazide
qPCR reaction	Quantitative real-time polymerase chain
sdH_2O	Sterilized deionized water
TE	Tris-EDTA
var.	Variety
wpi	Weeks post inoculation
SynComs	Synthetic communities

1 Introduction

1.1 Cruciferous crops

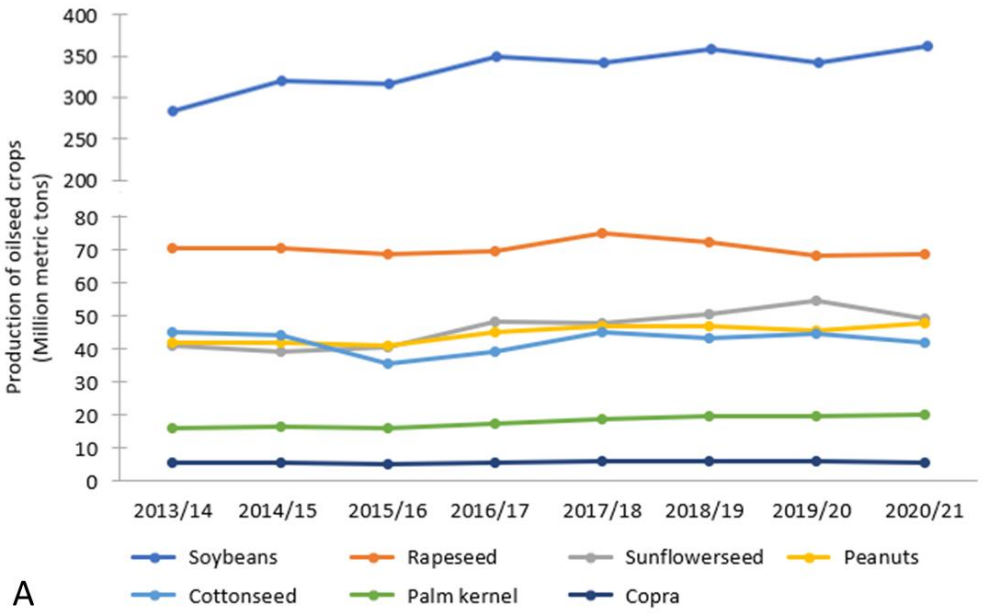
The Cruciferae, named for the cross shape of their four petaled flowers, include a number of genera and species that are economically important as crops, such as cabbage, broccoli, cauliflower, rapeseed, kale, turnip, as well as ornamentals and weeds. They are of considerable importance as vegetables, sources of edible and industrial oils, animal feeds, green manure and condiments (Vaughan et al. 1976; Wittkop et al. 2009). Cruciferous vegetables are rich in nutrients, including several carotenoids (beta-carotene, lutein, zeaxanthin), vitamins (ascorbic acid, folic acid, tocopherols and provitamin-A), folate and minerals (Singh et al. 2001). In medicine, cruciferous vegetables, which contain some chemoprotective substances like glucosinolates, isothiocyanates and indole-3-carbinol have shown a reduction in risk of carcinogenesis during initiation and promotion phases of cancer development (Murillo and Mehta 2001; Traka and Mithen 2009) and have proven to enhance cardiac health (Joshipura et al. 1999; Liu et al. 2000). Additionally, these substances in cruciferous vegetables play an essential role in plant defense against insects and pathogens. (Halkier and Gershenzon 2006).

Brassica oleracea, *B. rapa* and *B. napus* of the Brassicaceae (Cruciferae) family comprise the most important crops that are cultivated and consumed worldwide. There are indications that cruciferous vegetables were widely grown as early as 10,000 years ago. Natural populations of *B. oleracea* and associated types, which are native to the Mediterranean and European regions, are considered to be the potential progenitors of many European cole vegetables (Maggioni et al. 2018). *B. oleracea* comprises a wide array of vegetable crops showing extreme morphological diversity and crop forms that are cultivated for their leaves, inflorescences and stems. Brussels sprouts may have emerged during the Middle Ages and risen to be prominent in the 18th century around the city of Brussels. A remarkable diversity of cauliflower and broccoli-like vegetables developed in Europe, probably emanating from original highly localized crops in Italy and possibly evolved from germplasm introduced from the eastern Mediterranean region in Roman times (Dixon 2007).

B. rapa is considered the putative ancestor of many oriental *Brassica* vegetables. It has been suggested that the center of origin of *B. rapa* is the Mediterranean region, with its diversity center in East Asia and along ancient trade routes in Asia (Guo et al. 2014). *B. rapa* is an annual to biennial herb and is mostly distributed in temperate climate areas. This species contains high levels of phenotypic diversity that possesses many economically important traits resulting in a role as good potential gene donor for other Brassicaceae

(Mithila and Hall 2013). Products of various cultivars are consumed in markets around the world. The root vegetable of turnip and the leafy vegetables of Chinese cabbage and Pak Choi are used for cooking and some cultivars are grown for seed oil and as fodder.

B. napus (AACC genome, 2n = 38) is a digenomic amphidiploid species that originated through spontaneous interspecific hybridization between *B. rapa* (AA genome, 2n = 20) and *B. oleracea* (CC genome, 2n = 18) (Cai et al. 2014). Oilseed rape (*B. napus* L.) is the second most important oilseed crop, accounting for 11.6% of the global oilseed production after soybean (60.8%) in 2020 (USDA 2020) (Fig. 1.1A). Canada and the European Union are the main producers of rapeseed oil and meal followed by China and India (USDA 2021) (Fig. 1.1B). Oilseed rape includes spring and winter types, fodder and vegetable rape forms. Winter oilseed rape is predominantly cultivated in Europe and Asia due to the requirement of vernalization to initiate flowering. Spring rapeseed is mainly cultivated in Canada, northern Europe and Australia as it is sown in spring with stem development occurring immediately after germination that does not require vernalization.



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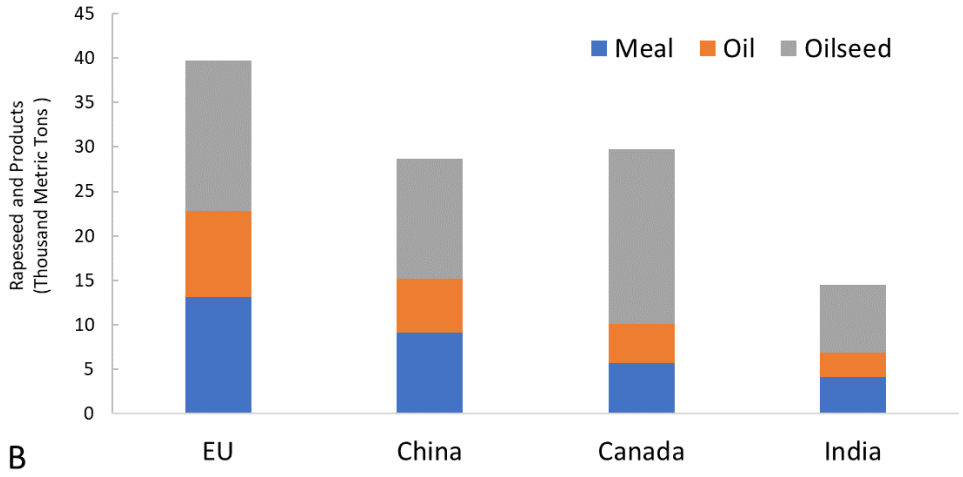

Figure 1.1 A) Global oilseed crop production from 2013/2014 to 2020/2021 and B) Rapeseed oil, meal and seeds production in the top producing countries in 2019/20 (USDA 2021).

Crucifers not only enrich the diet to meet people's nutritional needs, but also have a critical role in developing our understanding of plant genome mapping, as demonstrated by the extensive research on the model plant *Arabidopsis thaliana* (Provart et al. 2016). The rapid development of *Brassica* genomes research has provided comprehensive information for breeders to improve germplasm and cultivars. Considerable genetic diversity in the *Brassica* gene pool offers many valuable genetic resources for resistance to pathogens, pests and other agronomic traits. In response to increasing market demands of yield and quality, the need for efficient and sustainable cultivation of cruciferous crops is growing. However, pests and diseases are major threats to cruciferous crops. The common pests consist of aphids, beetles, butterflies and moths and the diseases include blackleg (*Leptosphaeria maculans*), powdery mildew (*Erysiphe cruciferarum*), clubroot (*Plasmodiophora brassicae*), stem rot (*Sclerotinia sclerotiorum*) and black rot (*Xanthomonas campestris*) (Zheng et al. 2020). In order to maintain or further increase productivity, a better understanding of the interaction between crops, other organisms and the environment is required for developing more effective crop protection systems. Integrated control strategies for pests and pathogens have been employed using various tools, such as cultivar resistance, manipulation of cropping systems, biological control and chemical control.

1.2 *Plasmodiophora brassicae*

Clubroot disease is one of the most serious soil-borne recurrent diseases in cruciferous crops worldwide. The causal agent is *Plasmodiophora brassicae* (Woronin), and the major

symptom of clubroot is single or multiple root galls that take on a finger or irregular shape leading to restriction of water and nutrients uptake. Infected plants display wilting in hot weather due to lack of water, while wilted plants may temporarily revive under cooler conditions. Plants become stunted and flowering is accelerated (Fig. 1.2A). Seed number and oil quality are depressed in oilseed and turnip rape crops, whereas the roots of swede and turnip become distorted and swollen. This disease leads to considerable losses in yield, quality, palatability and storability, as well as a reduction in land capital value (Dixon 2009b) thus seriously restricting the production of cruciferous vegetables (Tewari et al. 2005; Wang et al. 2008) (Fig. 1.2B).

Figure 1.2 Symptoms of *Plasmodiophora brassicae* on Brassica crops. A) Infected plants (*Brassica napus*. cv. Westar) show apparent stunting (left) and typical symptoms on roots of *B. rapa*. cv. Granaat (right). B) *P. brassicae* infected oilseed rape, field view. (Own photos)

The life cycle of *P. brassicae* comprises three stages: survival in the soil as resting spores, root hair infection, and cortical infection (Fig. 1.3). The resting spores can survive for a long period in the soil without stimulating factors. Each resting spore germinates to release one primary biflagellate zoospore. The released primary zoospores are short-lived and also are the only source of inoculum in natural infection. The zoospores attach to the root surface and become encysted, allowing them to penetrate the cell wall and form primary plasmodia in root hairs and epidermal cells (Aist and Williams 1971). The uninucleate primary plasmodium develops into multinucleate and then cleaves to form uninucleate zoosporangia (Liu et al. 2020b). Subsequently, multinucleate zoosporangia form and release secondary zoospores that are able to penetrate the cortex of the root. Once penetrated, they form a secondary plasmodium, which disturbs plant hormones and causes hypertrophy and hyperplasia of root cells to form galls (Jahn et al. 2013). Each secondary plasmodium will eventually be cleaved into large numbers of resting spores within the roots. When the root tissues decay, the resting spores are released into the soil to complete the

disease cycle. In infected roots, different life cycle stages of *P. brassicae* can occur simultaneously.

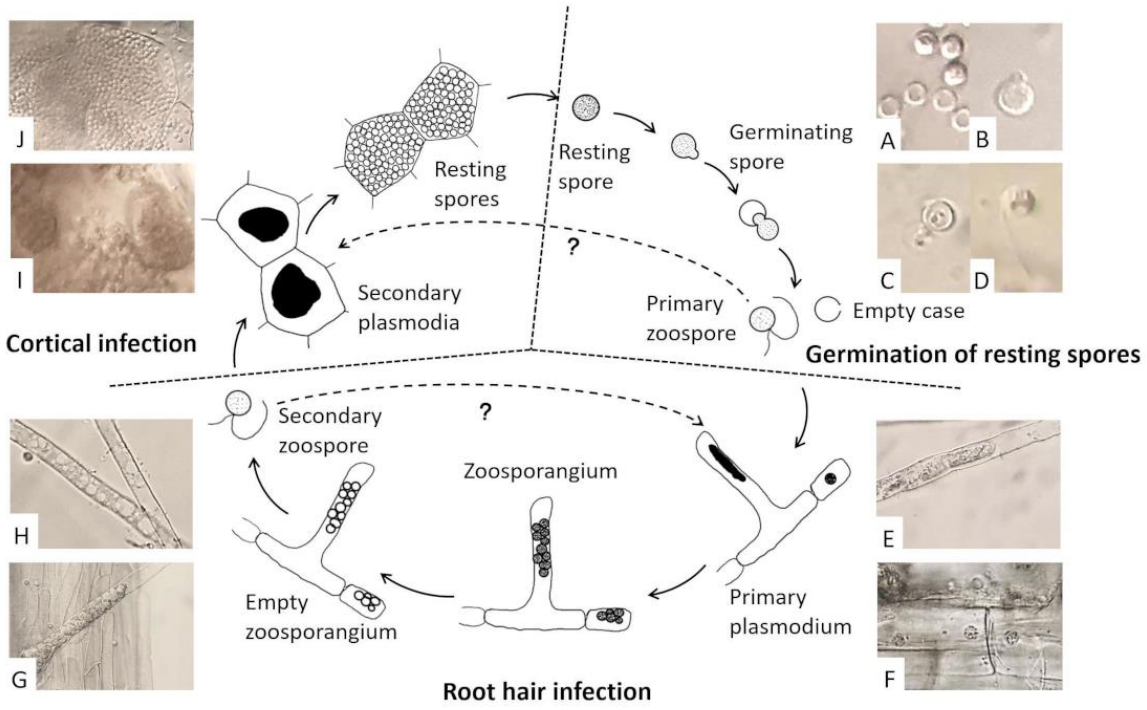

Figure 1.3 Life cycle of *Plasmodiophora brassicae*. A) Resting spores (full) and germinated spores (empty); B) emerged 'papilla'; C) germinating spore; D) primary zoospore; E) primary plasmodium in root hair; F) primary plasmodium in epidermal cells; G) zoosporangial cluster in root hair; H) empty zoosporangium; I) secondary plasmodia in cortical cells; J) resting spores in cortical cells. (Own photos and drawing)

Due to physiologic specialization in *P. brassicae*, several differential sets have been established in order to understand the pathogenic diversity in populations. The Williams differential series (Wallenhammar 1996), the European clubroot differential set (ECD; Buczacki et al. 1975) and the differential set of Somé et al. (Somé et al. 1996) have been broadly employed for the classification of different pathotypes of *P. brassicae* (Tab. 1.1). Recently, the Canadian clubroot differential (CCD) set (Strelkov et al. 2018) was developed to better fit the national conditions and have a greater differentiating capacity. The main prevalent physiological races differ in different countries and regions. In Germany, according to the differential set of Somé, pathotypes 1 and 3 were predominant and pathotypes 2 and 5 were in the minority. The Mendel (first clubroot-resistant oilseed rape cultivar, *B. rapa* ECD-04 x *B. oleracea* ECD-15) resistance-breaking pathotype is also expanding (Zamani-Noor 2017). Pathotype A3 classified by the CCD set, which is able to overcome the resistance in clubroot-resistant cultivars of *B. napus*, is predominant in Canada. (Fredua‐Agyeman et al. 2019). These improved differential clubroot sets will

facilitate the understanding of the classification, distribution, variation, and prevalence of *P. brassicae* populations, which in turn is supposed to greatly support the breeding of clubroot-resistant cultivars. However, the comparison of pathotypes in different countries can be difficult due to the use of various differential sets.

Table 1.1 The host cultivars used in various differential sets. ECD, European clubroot differential set; CCD, Canadian clubroot differential set; Somé, differential set of Somé et al.; Williams, Williams differential series.

Differential cultivar/line	Differential set
Brassica rapa	
subsp. *rapifea* line aaBBCC	ECD
subsp. *rapifea* line AAbbcc	ECD/CCD
subsp. *rapifea* line AABBcc	ECD
subsp. *rapifea* line AABBCC	ECD
var. *pekinensis* cv. Granaat	ECD/CCD
Brassica napus	
var. *napus* cv. Nevin	ECD/ Somé/CCD
var. *napus* cv. Giant Rape	ECD
var. *napus* selection ex.'Giant rape	ECD/CCD
var. *napus* New Zealand clubroot resistant rape	ECD/CCD
var. *napobrassica* cv. Wilhemsburger	Williams/ ECD/ Somé/CCD
var. *napobrassica* cv. Laurentian	Williams/CCD
var. *napus* cv. Brutor	Somé/CCD
var. *napus* cv. Mendel	CCD
cv. Westar	CCD
cv. 45H29	CCD
Brassica oleracea	
var. *capitate* cv. Badger Shipper	Williams/ ECD/CCD
var. *capitate* cv. Bindsachsener	ECD
var. *capitate* cv. Jersey Queen	Williams/ ECD/CCD

var. *capitate* cv. Septa	ECD
var. *acephala subvar. laciniata* cv. Verheul	ECD

Considering of the serious damage caused by *P. brassicae*, as well as its wide geographic distribution worldwide, various control methods have been developed, such as crop rotation, chemical treatments, soil liming, biocontrol and resistant cultivars. Long rotations with non-host plants (Peng et al. 2015) or the cultivation of bait crops (Hwang et al. 2015) are applied to reduce soil inoculum and relieve disease stress. Although a few pesticides, such as fluazinam (Suzuki et al. 1995), flusulfamide (Tanaka et al. 1999) and cyazofamid (Mitani et al. 2003), are effective against clubroot, they are often not accessible due to registration issues or are too expensive to utilize except where disease pressure is high. In EU countries, there are currently no pesticides approved for use against clubroot. In addition, increasing the soil pH via application of lime can reduce disease severity (Tremblay et al. 2005), but excessive use of agrochemicals may affect the soil ecosystem. Biofungicides like Serenade® (*Bacillus subtilis*) (Lahlali et al. 2013) and Prestop® (*Clonostachys rosea* syn. *Gliocladium catenulatum*) (Lahlali and Peng 2014), biofumigation, fungal endophytes and other bacteria have also been investigated for biocontrol of clubroot, but their efficacy in the field is limited (Zhou et al. 2014; Tremblay et al. 2005; Yu et al. 2015). Clubroot resistant cultivars of Brassica crops have been available for many years, but resistance in these crops has not been very sustainable, with several reports of virulent clubroot isolates overcoming the race-specific clubroot resistance (Rahman et al. 2014; Diederichsen et al. 2009).

All these factors result in a big challenge for effective clubroot control. With growing awareness of the requirements for better soil management and sustainable production practices, the advance of integrated approaches to the management of clubroot in Brassica crops is imperative. The development of cultivar resistance combined with the reduction of inoculum in various ways will propel integrated control systems forward. Meanwhile, it is indispensable to gain a better understanding of the interactions between plant roots, rhizosphere soil, and pathogens. With the evolution of technology and the application of omics, new perspectives have emerged to improve integrated control systems, which rely on the modification of physico-chemical and biological soil conditions to reduce the inoculum of *P. brassicae* or prevent infection.

1.3 Plant-soil microbe-pathogen interactions

Plants harbor a variety of microorganisms (the plant microbiota) and supply a multitude of niches for their growth and proliferation. These microorganisms including bacteria, fungi, protists, viruses and nematodes can form complex co-associations with plants and play important roles in promoting plant fitness and productivity in natural environments. A concept of 'holobiont' has been proposed (Vandenkoornhuyse et al. 2015) by which plants and their associated microbiota are considered as a single entity, whereby evolutionary selection between plants and microorganisms contributes to the overall stability of the system. In recent years, the knowledge of the repertoire of microorganisms in complex plant microbial communities residing in various plant compartments has been greatly expanded with the help of high-throughput sequencing (Lundberg et al. 2012; Peiffer et al. 2013; Ofek-Lalzar et al. 2014).

Several studies have noted the presence of 'core microbiota', a subset of microbial lineages which systematically associate with a particular host in almost all the communities (Fitzpatrick et al. 2018; Hamonts et al. 2018; Roman-Reyna et al. 2019). Within the core microbiota, the 'hub microorganisms' which are substantially more connected within a co-occurrence network may represent keystone species that can influence the community structure by strong biotic interactions with the host or with other microbial species, rather than simply through their own high abundance (Agler et al. 2016; Hamonts et al. 2018). These hub species possess powerful direct and indirect impacts on microbiome assembly and perform a mediating role between the plant and its associated microbiome. Moreover, genome-wide association studies (GWASs) and metagenome-wide association studies have shown that individual microbial taxa and genes are linked to plant colonization, physiology and fitness (Wagner et al. 2016; Bergelson et al. 2019). Genomics and multi-'omics' based analyses have allowed us to identify and characterize genes that govern plant interactions with the associated microbiomes (Cole et al. 2017; Levy et al. 2017), thereby increasing our understanding of how microorganisms adapt to the plant environment.

A plant microbiota comprises beneficial, neutral and pathogenic microorganisms. The integration of surrounding plant microbiota and pathogenic agents has contributed to the concept of 'pathobiome', which represents the interaction between the pathogen, the host-plant and the associated biotic microbial community, affecting pathogenesis (Vayssier-Taussat et al. 2014; Bass et al. 2019). It is essential to understand the impact of the microorganism community on the persistence, transmission and evolution of pathogens and the knowledge of abiotic and biotic factors that may interrupt the pathobiome and lead to the onset of pathogenesis. An increasing number of studies have described the structure, composition and assembly of various plant-associated microbial communities comparing healthy and infected plants (Zhao et al. 2017; Da Saraiva et al. 2020). It is crucial to shift

from descriptive to functional studies of the interactions in order to gain a mechanistic understanding of how microbes act on plant growth and defense, and/or on pathogen development and pathogenicity. Some studies have attempted to reveal the impacts of soil microbial environments on the functions of a pathogen and its pathogenesis, as well as the molecular response of the plant to infection (Daval et al. 2020). Microbe-microbe interactions also play an important role in shaping microbial communities, which should not be overlooked (Niu et al. 2017).

The various microbial communities in different plant compartments including the soil microbiota, the rhizosphere, the phyllosphere and the endosphere may have a role in the modulation of disease development and plant responses. Soil microbiota represents a great reservoir of biological diversity that acts as the seed bank for root microbiome assembly (Armalytė et al. 2019). Rhizosphere, corresponding to the thin layer surrounding the plant roots, consists of a gradient in chemical, biological and physical properties that vary both radially and longitudinally along with the roots (Mendes et al. 2013). The endosphere is the environment inside plant tissues that possesses less microbial diversity than that outside the roots. It shows a rapid loss of diversity and complexity of microbial network from soil to endosphere compartments (Bulgarelli et al. 2012). The rhizosphere is a hot spot of microbial interactions as root exudates are the main food source for microorganisms and also provide a basis for communication and recognition that directs their population density and activities (Raaijmakers et al. 2009; Zhalnina et al. 2018). The composition of root exudates is dynamic and varies depending on plant species, growth stages, stress conditions, nutrition, soil types, and root traits, among other factors (Zhalnina et al. 2018). Rhizosphere microbiome composition is primarily modulated by root exudates, which is not only beneficial for plant growth promotion (Vannier et al. 2019), but can also serve as an additional layer of defense against biotic and abiotic stress (Berendsen et al. 2012). Recruited microorganisms constitute different types of defense barriers such as stimulating the production of defense-related compounds by plants, direct antagonism against pathogens (production of antibiotics or antifungal compounds) and competition with pathogens for resources (Raaijmakers et al. 2009). The invasion by a soil-borne pathogen may also lead to changes in indigenous plant-associated microbial communities (Lebreton et al. 2019).

Recently, the importance of eco-evolutionary processes in the assembly of plant-associated microbiomes has been emphasized. The main ecological processes include selection (biotic and abiotic effects causing fitness differences), dispersal (movement of microorganisms between local communities), ecological drift (stochastic changes in population size) and diversification (generation of new genetic variation in a population) (Cordovez et al. 2019). These ecological processes relate to the stochastic-deterministic framework governing

microbial community structure. Likewise, selection is solely a deterministic process, whereas ecological drift is exclusively stochastic. Dispersal and diversification are often considered stochastic processes but could be deterministic in some cases. The order and timing of species arrival and dispersal can influence how species interact with one another affecting the local communities, which is referred to as priority effects. Priority effects can operate through early colonizing species depleting resources (niche preemption) and/or early colonizing species physically/chemically modifying the local niche (niche modification) to facilitate the inhibition, resulting in long-lasting consequences for plant performance (Fukami 2015). This provides new lines of research strategies aiming at optimizing microbiome functions in agroecosystems, like the application of synthetic communities (SynComs). Determining the principles governing the assembly, dynamics, stability and vulnerability to disturbance of the microbiome, including the roles of biotic and abiotic factors, will contribute to a better mechanistic understanding of the plant-soil microbe-pathogen interaction to improve the fitness and function of plant hosts.

1.4 Aims of the study

Clubroot is a severe disease that threatens the production of cruciferous crops worldwide. A deeper understanding of the pathobiome on *P. brassicae* will help to pave the way for improving innovative integrated control systems. Resting spores of *P. brassicae* can survive in the soil for many years. The percentage of resting spore germination is a crucial variable in determining disease severity. Spore dormancy is influenced by the surrounding external stimulants, which forms the initial relationship between *P. brassicae* and the environment. Germination of dormant *P. brassicae* resting spores may occur spontaneously at low rates but can be stimulated by various biotic and abiotic factors in the environment. It has been reported that root exudates of host and non-host plants can stimulate resting spore germination under experimental conditions (Suzuki et al. 1992; Rashid et al. 2013). However, further research is needed to accurately determine the role of root exudates from host and non-host plants on the rate of germination of clubroot resting spores. It is unclear why root exudates from non-host plants can also stimulate the germination of resting spores and whether and how this stimulation differs from a host-derived trigger.

Root exudates are considered the key regulators in rhizosphere communication and can modify the biological and physical interactions between roots and soil microorganisms. Research on the interactions of soil microbes and *P. brassicae* mediated by root exudates has important implications for elucidating the functions of rhizosphere microbes and providing practical guidelines in crop management to reduce disease incidence. Currently,

only a few studies focus on plant-soil microbe-pathogen interactions and how ecological factors affect this complex interaction network.

This study aims to illustrate the interactions of plant roots with *P. brassicae* and associated microbes in the soil. Specifically, the primary goal is dedicated to clarifying the direct germination stimulating factors on resting spores and their underlying mechanisms.

In order to achieve this overall goal, several specific sub-objectives are addressed:

1 To optimize the approaches to assess resting spore viability and germination rates contributing to a good basis for the determination of germination stimulants.

2 To compare the metabolic profiles of root exudates from host (oilseed rape) and non-host plants (tomato, ryegrass) and to investigate their role in stimulating resting spore germination under laboratory conditions.

3 To explore the effect of soil microbes and nutrients on resting spore germination focusing on soil microbial community diversity and abundance modulated by biotic and abiotic factors with regard to their impact on the pathogen.

4 To establish a model to elucidate plant-microbiome-pathogen interactions associated with breaking spore dormancy.

To this end, a series of experiments was conducted to deeply investigate the influence on resting spore germination in relation to abiotic and biotic factors. Based on the findings from this research, a functional model is presented to illustrate the plant-microbiome-pathogen relationship as a foundation to develop novel tools for the integrated sustainable control of this notorious pathogen. This study sheds light on the interaction of soil microbiota-mediated modulation of clubroot disease.

2 Materials and methods

2.1 Propagation of single spore isolate H1

The single spore isolate H1 provided by Prof. Elke Diederichsen (Institute of Biology/Applied Genetics, Freie Universität Berlin) was propagated on Chinese cabbage in the greenhouse. The dehydrated root galls were macerated in sterile water and ground into slurry. Susceptible Chinese cabbage, *B. rapa*. cv. Granaat (N.L. Chrestensen, Germany) was used as host to propagate the single spore isolate H1. The seeds were surface sterilized with 70% ethanol for 10 min, followed by 1% NaClO for 10 min with constantly shaking, and subsequently rinsed three times with sdH_2O. One surface-sterilized seed was sown in each new plastic pot (11×11×12cm) filled with autoclaved substrate of soil-sand mixture (3:1, v/v) individually and kept at 21±3°C with 14 h daytime in the greenhouse. The seven-day-old seedlings were inoculated with 1 ml 1×10^8 spores/ml spore suspension. The root galls were harvested at 8 weeks post inoculation (wpi) and stored at -20°C after washing with tap water.

2.2 Preparation of spore suspension

Resting spores were extracted from Chinese cabbage (cv. Granaat) root galls stored at -20°C. The galls were surface-sterilized in 70% ethanol for 10 min, and then with 1% sodium hypochlorite (NaClO) for 10 min. The surface-sterilized galls were rinsed three times with sdH_2O and cut into small pieces. The root pieces were homogenized with sdH_2O by an autoclaved homogenizer (Ultra-Turrax T25, IKA®-Werke GmbH & Co. KG, Germany). The homogenate was filtered through eight layers of sterile 50 µm nylon sheet, and the filtrate was centrifuged at 500 rpm for 10 min. The supernatant was transferred and centrifuged at 3500 rpm for 10 min. After discarding the supernatant, the pellet was rinsed twice with sdH_2O. The concentration of the spore suspension was determined using a haemocytometer. This spore suspension was considered as crude extraction containing bacteria contamination, which was the non-sterile spore suspension.

2.3 Surface-disinfection of resting spores

The resting spore pellet was resuspended with freshly prepared 2% chloramine-T (Sigma-Aldrich, Germany) (w/v) for 20 min. After centrifugation and rinsing with sdH_2O twice, the spores were incubated in the dark overnight with antibiotics consisting of 2 µg/ml colistin sulfate (Sigma-Aldrich, Germany), 2 µg/ml vancomycin hydrochloride (Sigma-Aldrich, Germany) and 12 µg/ml cefotaxime sodium (Fluka, Germany). After incubation, the spore suspension was centrifuged and rinsed twice with sdH_2O. The surface-disinfected spores

were suspended in sdH_2O to obtain 1×10^8 spores/ml. The surface-disinfected spore suspension was considered as sterile spore suspension.

2.4 Assessment of resting spore viability

The performance of methods for determining the spore viability was assessed using suspensions with known ratios of dead and viable spores. The autoclaved spore suspension was considered as 100% mortality and the freshly prepared spore suspension was 100% viability. Two spore suspensions were mixed at different ratios to obtain the assumed percentage of viable spores (0%, 25%, 50%, 75%, 100%). To figure out the optimal staining time for each stain, the resting spores were evaluated after incubation in the staining solution for 0, 0.25, 0.5, 2, 4, 24, 48 and 72 hours at room temperature.

2.4.1 Evans blue staining

The azo dye Evans blue was used to discriminate viable from non-viable resting spores. A volume of 500 µl spore suspension was transferred to a 1.5 ml microcentrifuge tube containing an equal volume of freshly prepared Evans blue stain solution (20 mg/ml) and incubated at room temperature. About 10 µl of each sample was placed on the slide and observed under light microscopy (Standard 20, Zeiss, Germany).

2.4.2 Calcofluor white - propidium iodide dual staining

The calcofluor white solution (CFW, Merck KGaA, Germany) consisting of 1 g/l Calcofluor White M2R and 0.5 g/l Evans blue was used for the staining of resting spore cell walls. Propidium iodide (PI, Carl Roth GmbH & C o. KG, Karlsruhe, Germany) is a cell-membrane impermeable dye that intercalates with nucleic acids. The stock solution was prepared by dissolving 1 mg of PI in 1 ml sdH_2O and stored at -20 °C. An aliquot of 500 µl spore suspension was mixed with 50 µl CFW solution and 1 µl PI stock solution and incubated at room temperature in the dark. Individual dye was performed and imaged with detection configured for both to ensure that there was no overlap in fluorescence detection between CFW and PI and obtain the optimal excitation and emission wavelength (EX, EM). CFW was excited using a 405 nm laser and was recorded at EM of 480-510 nm. The fluorescence of PI was excited at 514 and 561 nm and collected at 600-610 nm. Confocal laser scanning microscopy (CLSM) was carried out on a Leica TCS SP2 Laser Scanning Microscope 510 (Leica Microsystems CMSGmbH, Germany). Images were processed using the Leica Application Suite Advanced Fluorescence (LAS AF) software. The mortality was calculated as the ratio of red spores over blue spores.

2.4.3 Propidium monoazide treatment and qPCR

To distinguish between DNA of viable and dead spores, the effect of spores treated with propidium monoazide (PMA), which inhibits amplification of DNA from dead spores, was examined.

2.4.3.1 Lethal treatment

As heating treatments can cause DNA degradation, alternative methods for inactivating the resting spore were investigated. The resting spores were treated with different methods including chemical and heat treatments. One milliliter of spore suspension was incubated with an equal volume of methanol (HPLC grade) and acetone (HPLC grade) for 30 min, respectively. Sterile deionized water treatment served as control. For heat treatments, each 2 ml Eppendorf tube containing 1 ml spore suspension was subjected to different temperatures, including 80°C for 1h in a water bath, 95°C, 10 min in the digital dry bath heat block (NIPPON Genetics Europe, Germany) and 121°C, 20 min in the autoclave. The viability of treated spores was examined using CFW-PI dual staining immediately and Evans blue for overnight incubation, respectively.

2.4.3.2 Propidium monoazide treatment

A 300 µl spore suspension (1.5×10^8 spores/ml) was prepared for each treatment. After treatment (1.4.3.1), the suspension was divided into three parts equally. One part was mixed with 10 µl CFW and 0.2 µl PI as used for viability assessment. The other two parts were treated with 2.56 µl of 2 mM cell membrane-impermeable dye propidium monoazide (PMA, Biotium, USA) or an equal volume sdH_2O. The tubes were incubated at room temperature for 30 min on an orbital shaker at 300 rpm in the dark. Subsequently, the tubes were placed in a transparent box wrapped with a layer of aluminum foil and filled with ice. The samples were exposed to 500W halogen light for 15 min at a distance of 20 cm and kept at continuous mixing on a shaker. After treatment, the samples were spun at 10,000 rpm to pellet the cells and sniped in liquid nitrogen.

2.4.3.3 DNA extraction

The samples in each 2 ml tube were ground with stainless beads (4mm) into fine powder. Total DNA was extracted by cetyltrimethylammonium bromide (CTAB) method (Brandfass and Karlovsky 2008). Briefly, the sample powder was suspended in 1 ml CTAB solution with 1 µl proteinase K (20 mg/ml) and 2 µl β-mercaptoethnol and thoroughly mixed. The samples were then incubated at 42°C for 10 min, followed by 65°C for 10 min. Subsequently, the samples were purified by chloroform/isoamyl alcohol (24:1, v/v) extraction. DNA was precipitated by the mixture of 30% PEG (polyethylene glycol) and 5 M NaCl. Pelleted DNA

was rinsed twice with cold 70% ethanol, allowed to dry in speed vacuum, and resuspended in 200 µl of sterile Tris-EDTA (TE) buffer.

2.4.3.4 Quantitative PCR detection

P. brassicae DNA was amplified and quantified by a CFX384 real-time PCR instrument (Bio-Rad, Germany) using primer pair of CRqF2 (CTAGCGCTGCATCCCATATC) and CRqR2 (TGTTTCGGCTAGGATGGTTC) (Harding et al. 2019). A 10 µl PCR reaction consisted of 5 µl premix (qPCRBIO SyGreen Mix Lo-Rox, Nippon Genetics Europe GmbH), 0.4 µM of each primer and 1 µl DNA template and filled up with ddH_2O. The qPCR program was one cycle of 2 min initial denaturation at 95° C followed by 40 cycles at 94° C for 15 s and 60° C for 30 s, and a cycle of 7 min at 72°C for final elongation.

To verify the accuracy of the PMA qPCR, spore suspension at a concentration of 1.5×10^8 spores/ml was treated with acetone for 30 min. After centrifugation and rinsing twice with sdH_2O, the treated spores were mixed with non-treated spores in different ratios in order to obtain the assumed viability of 0%, 25%, 50%, 75% and 100%. Half portion was treated with PMA, another portion was left untreated and the DNA was extracted by CTAB method. Plasmid pPK2II of *P. brassicae* constructed by Dr. Birger Koopmann (Göttingen University) with a length of 10,505 bp was used. The DNA amount of the plasmid was quantified using the Qubit Fluorimeter (Invitrogen, Carlsbad, USA). Plasmid DNA was serially diluted five times by 10 fold and used for generating a standard curve. The copy number was calculated using the following equation (Whelan et al. 2003):

$$\text{copy number} = \frac{6.02 \times 10^{23}(\text{copy/mol}) \times \text{DNA amount(g)}}{\text{DNA length(bp)} \times 660((\text{g/mol})/\text{bp})}$$

A linear regression was generated by plotting the logarithm of the template copy number against the corresponding CT value. The number of resting spores in each DNA sample could be calculated from the qPCR CT value according to the standard curve to obtain viability.

2.5 Determination of resting spore germination rate

2.5.1 DAPI staining

A blue fluorescent nucleic acid stain of 4,6-Diamidine-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich, Germany) was used in a working solution of 1 µg/ml. After 5 min incubation, the nuclei in non-germinated spores was observed with an EX/EM of 405/430-550 nm.

2.5.2 Calcofluor white - Nile red dual staining

Nile red (9-diethylamino-5H-benzo (alpha) phenoxazine-5-one, Sigma–Aldrich, Germany) was used for detecting intracellular lipid droplets. It was dissolved in methanol to make a 1 mg/ml stock solution. The resting spores were incubated with CFW (100 µl/ml) and Nile red (1 µg/ml) for 10min in the dark. An EX/EM of 405 nm/480-510 nm was used for detecting the signal of CFW. Lipid droplets stained by Nile red was detected with an EX of 488 nm and an EM of 590-620 nm.

2.5.3 Differential interference contrast microscope

A Zeiss light microscope (Standard 20) with a differential interference contrast (DIC) prism was used to differentiate germinated spores from non-germinated spores according to the description by Suzuki et al. (1992). Images produced by a DIC microscope were relief-like and seem to have a shadow cast.

2.6 Collection of root exudates

Seeds from each of host plant (rapid cycling rape, *B. napus*), non-host plant (perennial ryegrass, *Lolium perenne* and tomato, *Solanum lycopersicum*) were surface disinfected, separately, with 70% ethanol for 10 min, followed by 1% NaClO for 10 min and then rinsed with sdH_2O three times. The seeds were placed on Murashige and Skoog (MS) basal medium with a layer of autoclaved cellophane to pregerminate in a growth chamber at 25°C with 14 h photoperiod. The contaminated seeds were discarded. Two root exudates collection systems were used in this study.

2.6.1 Petri dish cultivation (PDC)

Forty 7-day-old seedlings of each plant species were transferred to a new Petri dish with 20 ml sterilized 1/10 strength Hoagland solution (Sigma-Aldrich, Germany) or sdH_2O. The Petri dishes containing seedlings were placed in a sterile plastic container and incubated at 25°C on 16h/8h day/night cycle, 70% humidity, irradiance at 180µE m^{-2} s^{-1} in the growth chamber. After 7 days of incubation, the root exudates of each plate were collected with a syringe and passed through a 0.2 µm sterilization filter. The collected root exudates were stored at 4°C until use.

2.6.2 Hydroponic trapping system (HTS)

A hydrophobic root exudate trapping system modified from Tang and Young (1982) was used. Based on perlite substrate, a hydroponic system was established for trapping exudates from undisturbed living roots under sterile condition. 1/5 strength Hoagland solution was continuously circulated through the root system and a XAD8 resin (Amberlite® Sigma-Aldrich, Germany) column, followed by a XAD4 resin (Amberlite® Sigma-Aldrich,

Germany) column. Extracellular hydrophobic metabolites were selectively adsorbed by the resin, while inorganic nutrients were recycled to sustain plant growth (Fig. 2.1).

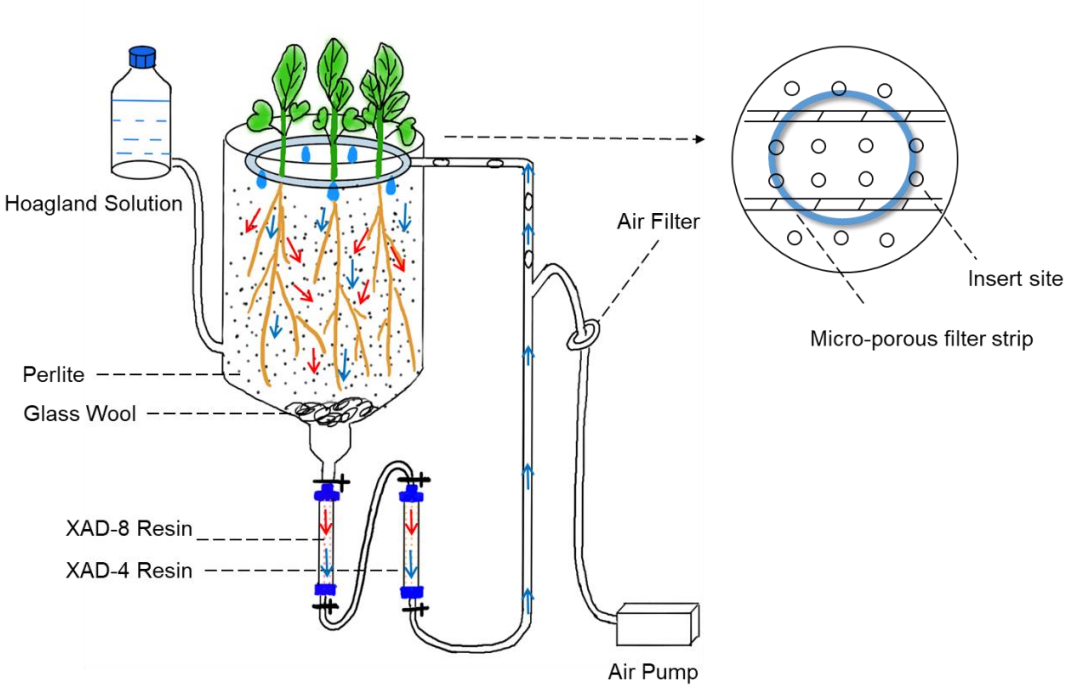

Figure 2.1 A hydroponic root exudate trapping system based on perlite substrate to collect root exudates under sterile conditions. The root exudates (red arrows) released from undisturbed living roots are selectively captured by the columns containing XAD8 and XAD4 resin. 1/5 strength Hoagland solution (blue arrows) is continuously circulated through the entire system to sustain plant growth. The container is covered with a plastic membrane with micro-porous filter strips (right).

2.6.2.1 Cultivation of plants

The experiments were carried out in a closed cabinet placed in a climate chamber to obtain sterile cultivation conditions. Before assembling the trapping systems, the cabinet was disinfected by fumigating with 10 ppm ozone for at least 5 hours. The containers filled with perlite and all the other parts were wrapped with aluminum foil and autoclaved twice at 121°C for 20 min. One milliliter tips cut at the bottom were autoclaved and filled with 800 µl 1% water agar. Surface-sterilized seeds (see 1.6) with radicles on MS plate were placed on the surface of water agar in 1 ml tips. Twenty-two tips of each plant species were inserted into a plastic membrane with micro-porous filter strips covered on the top of the container. The whole system was assembled in the ozone chamber. 1/5 strength Hoagland solution was circulated through the entire system by an air pump (SCHEGO® Schemel & Goetz, Germany). Additional Hoagland solution was supplied as needed during the whole cultivation period. The cultural condition was 23/18°C (day/night), 70% humidity, and 14h daytime. One complete similar system without plants served as blank control.

2.6.2.2 Collection of root exudates

The root exudates were collected separately at three growth stages, BBCH14, 52 and 64. The liquid chromatography columns (Luer Lock, Non-jacketed, bed volume 8 ml, I.D. × L 1.0 cm × 10 cm) (Sigma-Aldrich, Germany) and the stopcocks were washed with sdH_2O and soaked in 75% ethanol until using. Amberlite XAD4 resin and XAD8 resin were rinsed with sdH_2O, followed by HPLC grade methanol. The cleaned resin was stored in methanol at 4°C in the dark until using. Columns were packed with 6 ml XAD4 resin or 8 ml XAD8 resin, and then rinsed with sdH_2O to remove methanol. At different growth stages, one XAD8 resin column followed by one XAD4 resin column was connected to the system to absorb the substances. After two days absorption, the columns were taken off and washed with 40 ml of gradient (5%-75%) methanol to pre-wash the columns. Subsequently, the columns were eluted with 80 ml HPLC grade methanol and the eluate was stored at -20°C until use.

2.7 Non-target profiling analysis of root exudates by LC-QTOF-MS

2.7.1 Sample preparation

A 10-ml volume of root exudates eluted from the resin columns in the hydroponic trapping system was concentrated to dryness in a speed vacuum at 30°C. The residue was thoroughly dissolved in 500 µl 10% methanol (first 50 µl of HPLC grade methanol, then 450 µl of ddH_2O), sonicated for 5 min at room temperature and centrifuged at 4,500 rpm for 5 min. 10% methanol were used as blank. The supernatant was transferred into a glass vial and subjected to the Liquid Chromatography Time-of-flight Mass Spectrometer (LC-QTOF-MS) analysis.

2.7.2 LC-QTOF-MS analysis

The root exudates from the hydroponic trapping system were analyzed via HPLC-QTOF-MS using an Agilent 6545 QTOF-MS coupled with an Agilent 1290 Infinity II liquid chromatography (LC) system (Darmstadt, Germany) consisting of a LC system, an electrospray ionization source (ESI), a quadrupole mass filter, a time-of-flight analyzer and a mass detector. The samples were separated on a Nucleodur C18 Pyramid column (particle size 3 µm, 125 x 2 mm, Macherey-Nagel, Germany) with the following gradient mobile phases of solvent A (distilled H_2O /formic acid, 99.9/0.1 (v/v)) and solvent B (Methanol /formic acid, 99.9/0.1 (v/v)) at a flow rate of 0.2 ml/min: a gradient of solvent B from 5% to 98% in 22 min, holding at 98% solvent B from 22 min to 26 min and then the column was re-equilibrated to 5% solvent B in 30 s, followed by 5% of solvent B 3.5 min for

cleaning. 10 µl of each sample was injected and the column temperature was set to 35°C. Eluted compounds were monitored on the Agilent 6545 QTOF with positive and negative ionization mode of ESI. The flow rate of sheath gas was 11 l/min and the temperature was set to 350 °C. Two TOF spectra per second were captured in MS1 mode ranging from 100 to 1,700 m/z. The nitrogen temperature was set to 320°C with a flow of 8 l/min and a nebulizer pressure of 35 psig. The voltages were set as below: Nozzle voltage 1000 V, capillary voltage 3500 V, fragmentor 175 V and skimmer 65 V. The data was processed using Agilent MassHunter Workstation software (B05.00).

2.8 Root exudates bioassay

The root exudates from Petri dish cultivation were tested directly after collection. The eluted root exudates from XAD4 and XAD8 resin were dried using a speed vacuum concentrator at 30°C. The residue was dissolved with 1/10 strength Hoagland solution or sdH_2O. Germination of resting spores treated with root exudates from perennial ryegrass, rapid cycling rape and tomato was evaluated by incubating 100 µl crude extracted (non-sterile) spore suspension or surface-disinfected (sterile) spore suspension with 1 ml of each root exudate preparation in 2 ml Eppendorf at 25°C in the dark. As control, 1 ml Hoagland solution or sterile deionized water mixed with spores were used. At least 100 resting spores of each sample were examined every 24 h under the DIC microscope. Resting spores were considered to have germinated if they appeared empty.

2.9 Soil suspension bioassay

The soil was collected from different locations. The field sample was collected from an oilseed rape (cv. Bender) field in Weende near Goettingen (51°33'48.6"N 9°56'47.7"E). The grassland soil was collected from a place covered with grass (51°33'32.17"N, 9°57'9.43"E). The greenhouse soil was harvested from the pots used to propagate *P. brassicae* in the greenhouse, including soil with inoculated oilseed rape (cv. Westar, 6 wpi), soil with healthy oilseed rape (7-week-old), and soil without plants. All the soil samples were stored at 4°C. Thirty grams of soil were thoroughly soaked with 40 ml sdH_2O for 1 h. The slurry was passed through sterile filter paper. The filtrate was considered as non-filtered soil suspension. A portion of the filtrate was subjected to a 0.2 µm sterilization filter (Sartorius Minisart $^{@}$ non-pyrogenic) and considered as the filtered soil suspension. The resting spores were extracted from the frozen root galls of Chinese cabbage into a 1.3×10^8 spores/ml suspension (see 2.2) and then a half portion of the spore suspension was surface-disinfected (see 2.3). An aliquot of 100 µl spore suspension was incubated with 1 ml non-filtered or filtered soil suspensions at 25 °C. Hoagland solution or sdH_2O mixed with spores served as control. The germination rate of resting spores was checked daily.

2.10 Bacteria isolation and bioassay

Thirty grams of different soils (see 1.9) was soaked with 30 ml sdH_2O and mixed well. The slurry was filtered through sterile filter paper and diluted to 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} with sdH_2O. A volume of 100 µl of each diluted suspension was plated on Luria-Bertani (LB) plates and incubated at 25°C. Single colony was isolated based on morphology on LB plate and stored at 4°C. The bacterial isolates were mixed with 25% glycerol and stored at -80°C as stock.

One of the isolated bacterial strains, Iso4, and a known bacterial strain of *Escherichia coli* GSPB48 (Göttingen Collection of Phytopathogenic Bacteria, Germany) as control were respectively cultured in LB medium at 37°C overnight and then the bacterial suspensions were centrifuged and resuspended with sdH_2O. The concentration was determined using a spectrophotometer at 600 nm (BioTek µQuant Monochromatic Microplate Spectrophotometer, BioTek Inc., USA). The bacterial suspensions of Iso4 and *E. coli* at a concentration of 10^8 CFU/ml were diluted 5 times by 10 fold. Then 100 µl of each diluted bacterial suspension was mixed with an equal volume of 1×10^8 spores/ml non-sterile spore suspension in 1 ml 1/10 strength Hoagland solution or sdH_2O, respectively. The samples were incubated at 25°C and checked daily. Twelve additional isolated bacterial strains were randomly selected (OD_{600} = 0.2) and incubated with non-sterile spores to further check the effect of triggering resting spore germination. *E. coli* was used as the positive control. Hoagland solution and sdH_2O without bacteria isolates were used as control.

2.11 Bacteria classification

The bacterial isolates were classified on the basis of several methods, including morphological observation, nitrate reduction test, oxidase test, fermentation test and differential medium culture. *E. coli* GSPB48 was used as positive control.

The morphological characteristics were observed by naked eyes or microscopy.

2.11.1 Nitrate reduction test

The isolates were cultured in the differential medium of nitrate broth (Merck KGaA, Germany) at 37°C overnight. After cultivation, reagent A (sulfanilic acid solution) and reagent B (α-naphthylamine solution) were added into the culture tube and shaken to mix well with the medium. A red or pink color appeared within a few minutes indicating nitrate reduction. When the culture was colorless after the addition of reagent A and B, a tiny amount of zinc powder (Sigma-Aldrich, Germany) was added into the tube to check the color changes. If the color turned pink that indicated a negative result, colorless indicated a positive result.

2.11.2 Oxidase test

Oxidase test was performed by spreading the isolated colony on an oxidase disc (Merck KGaA, Germany) containing N,N-dimethyl-p-phenylenediamine oxalate and α-naphthol. The appearance of dark-blue color indicated the presence of the enzyme cytochrome oxidase (gram-negative bacteria).

2.11.3 Fermentation test

Fermentation test was conducted by glucose broth with a pH indicator of bromothymol blue. The glucose broth consisted of 5 g NaCl, 10 g peptone from casein, 5 g glucose for 1 liter medium. A Durham tube was placed in each tube containing glucose broth. The medium was inoculated with the isolates and incubated at 37°C. The color changes and gas production were checked daily.

2.11.4 Differential medium

MacConkey agar, a selective and differential medium is used for the isolation of gram-negative bacteria and the differentiation of lactose fermenting ability. The isolates were spread out on MacConkey agar plates and incubated at 37°C. The bacterial growth was observed daily.

2.12 Hoagland based components test

Several components of Hoagland solution were tested based on the compositions in the product formulation (Tab. 2.1) and some other inorganic components were also tested at the concentration listed in Tab. 2.2. The spore suspension was prepared from the single spore isolate H1 root galls stored at -20°C. A portion of spore suspension was sterilized by freshly prepared 2% chloramine-T for 20 min and antibiotics overnight. The isolated bacterial strain Iso4 from glycerol stock was scraped with a sterile loop and cultured in LB broth at 37°C overnight. 100 µl sterile/non-sterile spores of 1.2×10^8 spores/ml were incubated in 1 ml of different inorganic solutions with 100 µl of Iso4 suspension (OD_{600} = 0.326) at 25°C in the dark. The spores mixed with inorganic solution alone were also compared. Hoagland solution diluted to 1/10 strength and sdH_2O were used as control. The germination rate was checked at 7 days post incubation (dai) under the optical microscope. A series concentration (0.3 mM - 9 mM) of calcium nitrate, potassium nitrate and potassium nitrite were also tested.

Table 2.1 Type and concentrations of used inorganic components

Chemical	Manufacturer	Original Conc.[1] (mg/l)	Working Conc.[2]
$Ca(NO_3)_2$	Merck KGaA, GER	656.4	4/0.4 mM
$MgSO_4$	Carl Roth, Germany	240.76	2/0.2 mM
KNO_3	Carl Roth GmbH & Co. KG, GER	606.6	6/0.6 mM
NH_4H_2PO4	Sigma-Aldrich, Germany	115.03	1/0.1 mM
H_3BO_3	Carl Roth, Germany	2.86	46/4.6 µM
$CuSO_4 \cdot 5H_2O$	Carl Roth GmbH & Co. KG, GER	0.08	0.32/0.032 µM
$ZnSO_4 \cdot 7H_2O$	Merck KGaA, GER	0.22	0.77/0.077 µM

1. The concentration of inorganic components originated from Hoagland product information
2. The concentration of inorganic components used for bioassays that calculated based on the full strength or 1/10 strength Hoagland solution.

Table 2.2 Inorganic components used for bioassays

Chemical	Manufacturer	Working Conc.[2]
KNO_2	Merck KGaA, Germany	6/0.6 mM
NH_4Cl	Merck KGaA, Germany	6/0.6 mM
$(NH_4)_2CO_3$	Merck KGaA, Germany	3/0.3 mM
NH_4NO_3	Sigma-Aldrich, Germany	6/0.6 mM
KCl	Merck KGaA, Germany	6/0.6 mM
$CaCl_2$	Merck KGaA, Germany	6/0.6 mM

2.13 ^{15}N isotope analysis

2.13.1 Bacterial strains and cultivation

Escherichia coli GSPB48 strain was cultured in LB medium at 37°C overnight. The bacterial suspension was centrifuged at 5000 rpm for 10 min to collect the pellet. The pellet was suspended with sdH_2O to a density of OD_{600} = 0.28 (ca. 1.5×10^8 cells/ml). Surface-disinfected resting spores were prepared from the Chinese cabbage root galls of the single spore isolate H1. The spore suspension was adjusted to 1.2×10^8 spores/ml. 0.6 mM potassium nitrate in ^{15}N-enriched form (10 atom% $K^{15}NO_3$, Sigma-Aldrich) was used in this experiment. Two milliliters of spore suspension with or without 3 ml of *E. coli* suspension

was incubated in 20 ml $K^{15}NO_3$ at 25°C. Resting spores with or without *E. coli* were cultured in sdH_2O without labelling as control.

2.13.2 Sample preparation and isotope analysis

The samples of each treatment were harvested after 0, 3 and 7days of incubation. The resting spores and *E. coli* were separated by 16% Ficoll ® 400 (w/v) (Carl Roth GmbH & Co. KG, Germany) at 1,850 rpm for 15 min twice. The separated resting spores were rinsed with sdH_2O four times and incubated in the oven at 50°C overnight. The samples were weighed into tin cups and then formed into small balls. The total N and total ^{15}N abundances of samples were measured using elemental analyser-isotope-ratio mass spectrometry (EA-IRMS) at the Centre for Stable Isotope Research and Analysis (University of Göttingen, Germany).

2.14 *Bacillus subtilis* mutants

The *B. subtilis* wild-type strain JH642 (*trpC2 pheA1*), *narG* mutant strain THB1 (*trpC2 pheA1 narGH::tet*, tet 0.5 µg/ml) and *nasD* mutant strain 1972 (*trpC2 pheA1* Δ *nasD::phleo*, phleo 0.4 µg/ml) were obtained from Prof. Dr. Dieter Jahn, Institute of Microbiology, TU Braunschweig. The *narG* mutant strain is no longer able to perform nitrate respiration and the *nasD* mutant strain is unable to convert nitrite into ammonia. The strains were grown aerobically at 37°C in Luria broth medium. Each cell culture was centrifuged at 5,000 rpm for 10 min to get the cell pellet which then was suspended with sdH_2O (OD_{600} = 0.4). 0.4/4 mM $Ca(NO_3)_2$, 0.6/6 mM KNO_3 and KNO_2 were used as nutrient sources in bioassay. 1/10 strength Hoagland solution and sterile deionized water were used as control. The resting spore suspension of 0.8×10^8 spores/ml was freshly prepared from frozen Chinese cabbage root galls and sterilized by antibiotics (see 2.2 & 2.3). One hundred microliters of sterile/non-sterile spores and 100 µl *B. subtilis* suspension were mixed with 1 ml inorganic solution or their 10 fold dilution, respectively. Hoagland solution at 1/10 strength and sterile deionized water were used as control. The germination rate was determined at 7 days post incubation.

2.15 Bacterial product bioassays

2.15.1 Living and dead bacteria

The bacterial strains of Iso4, *B. subtilis* JH642 and *E. coli* GSPB48 were cultured in LB medium at 37°C overnight. The bacterial culture was centrifuged at 5,000 rpm for 10 min and then discarded the supernatant. The bacterial cells were suspended with sdH_2O to get an OD value at 600 nm of 0.2. Half of the bacterial suspension was autoclaved at 121°C for 20 min, which is considered as dead bacterial suspension. 0.4/4 mM $Ca(NO_3)_2$, 0.6/6 mM

KNO_3 and KNO_2 were used for the bioassay. A volume of 100 µl of 0.9×10^8 spores/ml non-sterile spores and 100 µl dead bacterial suspension were incubated in 1 ml Hoagland solution, inorganic solutions and sdH_2O, separately. Resting spores with live bacteria in Hoagland solution were used as positive control.

2.15.2 Bacterial filtrate

Four bacterial strains A4, Iso4, *B. subtilis* JH642 and *E. coli* GSPB48 were cultured in LB medium at 37°C overnight. The bacterial suspension was centrifuged at 5000 rpm for 10 min and the supernatant was discarded. The bacterial cells were suspended with sdH_2O to the OD_{600} = 0.2. Each cell suspension was passed through a 0.2 µm sterilization filter to get the filtrate. For bioassay, 100 µl sterile/non-sterile spores and 100 µl bacterial filtrate were cultured with 1 ml 1/10 strength Hoagland or sdH_2O at 25°C.

2.16 Bacterial community analysis

2.16.1 Sample preparation

B. subtilis wild-type strain JH642 bacterial suspension and isolate A4 filtrate were used as carbon sources. Hoagland solution (1/10 strength) and 6 mM KNO_3 were used as nitrogen sources. The resting spores were extracted from Chinese cabbage root galls inoculated with the single spore isolate H1. A portion of spore suspension was surface-disinfected by 2% chloramine-T for 20 min and antibiotics overnight. For the treatments (Tab. 2.3), 2 ml of spore suspension and 2 ml of carbon source were incubated in 20 ml nitrogen source at 25°C for 7days. The germination rate in each sample was examined. The sterile spores (SS) and non-sterile spores (NS) were collected at 0 dai for DNA extraction as the initial microbial community.

Table 2.3 Treatments used for bacterial community analysis

Treatment	Spores	Carbon source	Nitrogen source	Incubation time
HB	Non-sterile	*B. subtilis* suspension	Hoagland	7days
KB	Non-sterile	*B. subtilis* suspension	KNO_3	7days
A4f	Non-sterile	A4 filtrate	Hoagland	7days
H	Non-sterile	None	Hoagland	7days
sd	Non-sterile	None	sdH_2O	7days
NS	Non-sterile	None	sdH_2O	0days
SS	Sterile	None	sdH_2O	0days

2.16.2 DNA extraction

The samples were centrifgued at 10,000 rpm to pellet the cells and sniped in liquid nitrogen. The samples were lyophilized and crushed with 4 mm beads into powder for DNA extraction. Total DNA was extracted by the sterile CTAB method (see 1.4.3.3 DNA extraction) that the reagents were autoclaved. Pelleted DNA was washed twice with cold 70% ethanol, allowed to dry in a speed vacuum, and resuspended in 50 µl of sterile TE buffer. The quality and quantity of total DNA were examined by staining with Midori green (Nippon Genetics Europe GmbH, Germany) and running 1% agarose gel electrophoresis. DNA of *E. coli* strain GSPB48 was used as positive template. Empty tubes were extracted in parallel as non-template controls (NTCs) to assess the presence of contaminants.

2.16.3 16S rRNA gene amplification and sequencing

The V3 and V4 regions of the 16S ribosomal RNA gene were amplified using a pair of primers D-Bact-0341-b-S-17: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and D-Bact-0785-a-A-21: 5'-GTCTCGTGGGCTCGGAGATGTTATAAGAGACAGGACTACHVGGGTATCTAAKKCC-3' that included Illumina adapter overhang nucleotide sequences and the gene-specific sequences. The amplification reaction was performed in 50 µl volumes that consisted of 10 µl 5×Phusion GC Buffer, 10 µM primer of each, 0.2 µl of 50 mM $MgCl_2$, 2.5 µl DMSO, 1 µl of 10mM dNTPs (Thermo Fisher Scientific, USA), 1 Unit Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific, USA) and 25 ng DNA template. PCR program in a thermal cycler was conducted as following: initial denaturation at 98°C for 1 min, followed by 25 cycles consisting of denaturation at 98°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 30 s and a final extension step at 72°C for 5 min. Amplified PCR products of each sample were examined by running the samples on 1% agarose gel (Roth, Germany) with FastGene®100 bp DNA ladder. The PCR products with the appropriate size (~550bp) were individually purified using bead-cleanup (SPRIselect, Beckman Coulter, USA). The amplicons were quantified using the Qubit Fluorimeter (Invitrogen, Carlsbad, USA) twice and placed in 96 well plate. A second PCR was carried out to add barcodes to the amplicons. The amplicons were pooled and sequenced with MiSeq instrument and v3 chemistry (Illumina, San Diego, USA) using the dual index paired-end approach (2×300 bp) at Göttingen Genomics Laboratory.

2.16.4 Bioinformatic processing of 16S rRNA gene amplicons

Paired-end sequencing data from Illumina MiSeq were quality filtered with fastp38 (version 0.20.0) using default settings with additional modifications, a minimal per-base phred score of 20, a sliding window base clipping (4 bp, phred score 20), minimal length of 50 bp, base pair correction by overlap and automatic adapter detection for paired-end. After quality filtering, paired-end reads were merged using PEAR (version 0.9.11) and forward and reverse primers were clipped by cutadapt (version 2.5) with default settings. The sequences were continued to be processed with VSEARCH (version 2.15.0), including sorting and size filtering of paired reads to ≥ 300 bp and dereplication. Dereplicated amplicon sequence variants (ASVs) were denoised using UNOISE3 and chimeras were removed by UCHIME3 *de novo* and reference-based against the SILVA SSU NR database (version 138.1) resulting in a total of 452 ASVs. Raw reads were mapped to ASVs with VSEARCH to create abundance table. The taxonomy was assigned using BLAST 2.9.0+ against the SILVA SSU 138.1 NR database with an identity of at least 90% to the query sequence.

2.17 Carbon source bioassays

2.17.1 Sugars

Different concentrations of glucose, sucrose, trehalose and soluble starch were tested as carbon sources. Stock solutions of 0.2% sugars were prepared. The spore suspension was extracted from the fresh 8 wpi root galls of oilseed rape cv. Westar grown in the greenhouse. Part of the spores was surface-disinfected with 2% chloramine-T for 20 min and antibiotics overnight. The spore suspension was adjusted to 1×10^8 spores/ml with sdH_2O. Each stock solution was mixed with sdH_2O or 50 mM potassium nitrate in different ratios to get a working concentration of 0.1% and 0.01%, respectively. A volume of 100 µl of sterile/non-sterile spores was incubated in 1 ml sugar solution with different working concentrations at 25°C in the dark. The number of germinated spores was counted every week with Zeiss DIC microscope.

2.17.2 Amino acids

A 50 mM concentration of the L-amino acids alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), and valine (Val) were used to examine the effects on *P. brassicae* resting spores germination. For the bioassay, 100 µl sterile/non-sterile spores were incubated in 500 µl 50 mM L-amino acids mixed with 50 mM KNO_3 at 25°C in the dark. The treatments without KNO_3 was also included to

evaluate the effect of organic nitrogen alone. The germination rate of resting spores was examined after 7 and 14 days incubation under the microscope.

2.18 Auto/non-autoclaved soil bioassay

The soil collected from a field associated with oilseed rape cv. Bender (see 1.9) was used. Part of field soil was autoclaved at 121°C for 20 min to exclude the effect of live microbes.

2.18.1 Assessment of germination rate

The autoclaved and non-autoclaved soil was put into 2 ml tubes separately and mixed with 1 ml of spore suspension. The mixture was incubated at 25°C for three weeks. The germinated resting spores were distinguished from the dormant spores and soil particles to calculate the germination rate in each treatment at different time points.

2.18.2 Soil moisture bioassay

Twenty-two grams of autoclaved and non-autoclaved soil were filled in multiple trays. 5 ml 7×10^7 spores/ml of *P. brassicae* spore suspension was inoculated into the soil and thoroughly mixed. The soil was supplied with different amounts of sdH_2O to get different levels of humidity as 6%, 50% and 90%, respectively. The samples were incubated in a climate chamber at 25°C for 3 weeks. The germination rate of resting spores was examined under the microscope.

2.18.3 Disease assessment of *P. brassicae*

The autoclaved and non-autoclaved soil were inoculated with *P. brassicae* resting spores to obtain infested soil with 1×10^6 spores/g. A weight of 35 g of infested soil was filled in multiple trays. The soil without inoculum was used as control. Seeds of oilseed rape cv. Westar were surface-disinfected by 70% ethanol and 1% NaClO and then sown into the soil. The plants were cultivated in the climate chamber at 25°C with 75% humidity. Hoagland solution and sdH_2O were supplied daily. Disease severity in each treatment was assessed at 5 weeks post inoculation followed the assessment key: 0) healthy root without any galls, 1) roots with a few tiny galls, 2) roots with moderate galls, 3) obvious swelling of tap root. Disease index (DI) was calculated using the below formula (Strelkov et al. 2006):

$$\mathrm{DI}\ (\%) = \frac{\sum(n\times 0 + n\times 1 + n\times 2 + n\times 3)}{N\times 3}\times 100\%$$

where Σ is the sum; *n* is the plant number of each category; *N* is the total assessed number of plants; 0,1,2 and 3 are severity assessment categories.

2.19 Nutrients bioassay in sand culture

2.19.1 Sample preparation

Oilseed rape cv. Westar seeds were surface sterilized with 70% ethanol for 10 min and then 1% NaClO for 10 min with constantly shaking, and subsequently rinsed three times with sdH_2O. Quartz sand was pre-washed with 2 M HCl and autoclaved at 121°C for 20min in 5×5 cm heat-stable plastic pots. Resting spores were extracted from 8 weeks post inoculation Chinese cabbage root galls stored at -20°C. A portion of crude spore suspension was disinfected by 2% chloramine-T for 20 min and antibiotics overnight. The concentration of crude spore suspension and disinfected spore suspension were determined using hemocytometer. The various inorganic solutions of $Ca(NO_3)_2$, KNO_3, NH_4Cl, NH_4NO_3 were prepared to have equal molarity (6 mM) of NO_3^- or NH_4^+ and autoclaved to avoid additional contaminations. Autoclaved quartz sand treated with 10 ml inorganic solution containing 6 mM/0.6 mM of NO_3^- or NH_4^+ were inoculated with 5 ml of 0.8×10^8 spores/ml crude spore suspension or disinfected spore suspension respectively and mixed well. Subsequently, disinfected seeds were sown in the inoculated sand and grown in a sterile container placed in a climate chamber with a 16 h photoperiod and 90% humidity at 25˚C. Root hair infection was observed by staining with trypan blue. The roots of seedlings were collected at 4 and 7 dpi and lyophilized to dry. After lyophilization, the samples were stored at -20°C until DNA extraction.

2.19.2 DNA extraction and quantification in root tissues

The roots from three seedlings were merged in one tube and ground by 4 mm stainless steel beads at 28Hz 1'×3. Total DNA of root samples was extracted using CTAB method (see 1.4.3.3 DNA extraction). Pelleted DNA was dissolved in 50 µl TE buffer. Total DNA was checked on 1% agarose gel to assess the quality and quantity. A CFX384 real-time PCR instrument was used for the amplification and quantification of *P. brassicae* DNA using CqR forward primer (5'-CTAGCGCTGCATCCCATATC-3') and reverse primer (5'-TGTTTCG GCTAGGATGGTTC-3'). The plant root DNA was amplified using the GAPDH primer pair forward (5'- CGCTTCCTTCAACATCATTCCCA-3') /reverse (5'-TCAGATTCCTCCTTGA TAGCCTT-3'). Total volume of 10 µl amplification reaction consisted of 5 µl premix (qPCRBIO SyGreen Mix Lo-Rox, Nippon Genetics Europe GmbH), 0.4 µM of each primer and 1 µl DNA template and filled up with ddH_2O. The PCR conditions consisted of 2 min initial denaturation at 95°C, 40 cycles of 15 s at 94°C, 30 s at 60°C, and 72°C for 25 s, followed by a final extension of 5 min at 72°C. Plasmid pPK2II was used to generate the standard curve of *P. brassicae* (see 1.4.3.4). All treatments were performed

with three biological replicates and data was analyzed using CFX Manager Software (Bio-Rad laboratories, Inc.).

2.20 Statistical analysis

Statistical analyses were conducted using SPSS (version 26, IBM) and R (version 4.0.2). A Mixed-model Analyses of Variance (ANOVA) was performed using a General Linear Model (GLM) to determine significant differences for main factors and interactions between factors. Subsequently, Tukey's Multiple Comparisons Tests were applied to determine statistically significant differences of means within each main factor.

3 Results

3.1 Viability assessment by staining

Proper assessment of spore viability is the foundation for the determination of factors affecting resting spore germination, thus different methods for assessing spore viability were first examined. The staining methods with Evans blue and CFW-PI were evaluated, showing good performance to distinguish viable from non-viable spores. For Evans blue, viable spores with intact cell wall could not be stained and appeared colorless, while non-viable resting spores appeared in blue color due to the uptake of stain into the cytoplasm (Fig. 3.1). For dual staining with CFW-PI, CFW staining alone only produced detectable blue fluorescence, while PI staining alone exhibited red fluorescence, indicating that each fluorescence was specific to each dye without the interference of autofluorescence. After configuration optimizations, the images of CFW-PI staining showed a clear differentiation between viable and dead spores (Fig. 3.2). The cell wall of both dead and viable spores was stained by CFW showing blue color. The nuclei of dead spores were intercalated with PI resulting in red color.

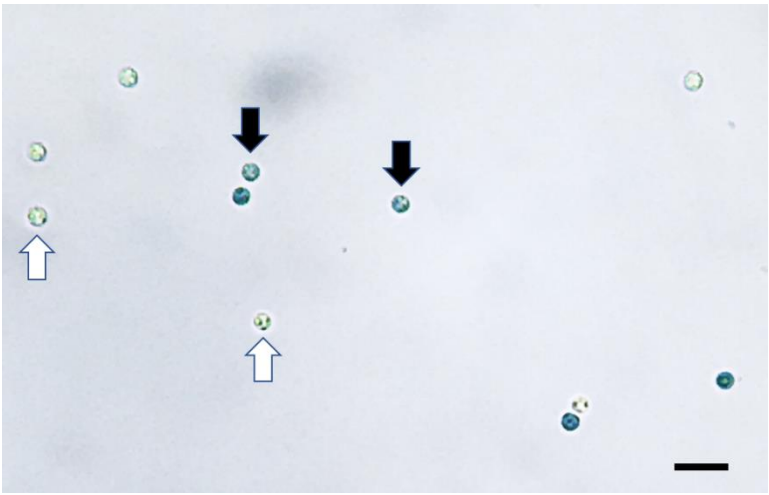

Figure 3.1 *P. brassicae* resting spores stained with Evans blue visualized under optical microscope. Black arrows indicate dead spores (blue), white arrows indicate viable spores (colorless), bar=10 µm.

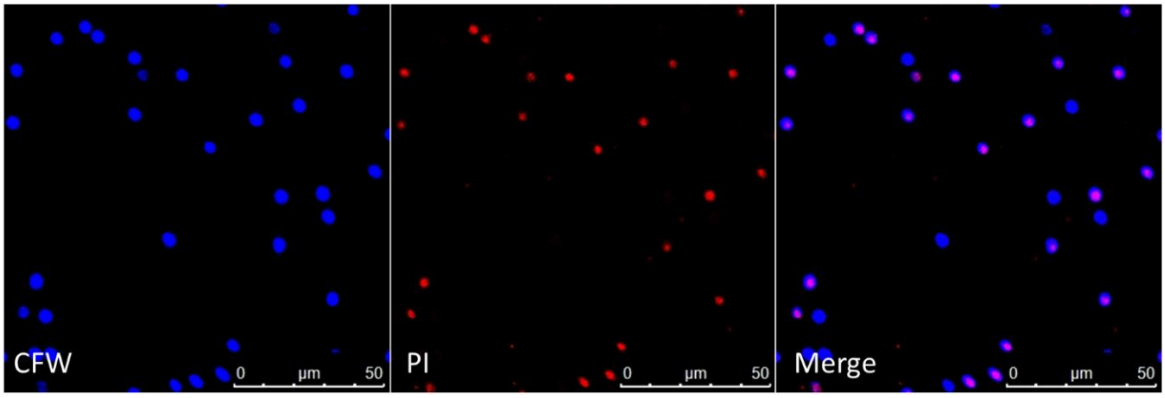

Figure 3.2 Confocal laser scanning microscopy (CLSM) images of *Plasmodiophora brassicae* resting spores double stained with calcofluor white (CFW) - propidium iodide (PI). Both, dead and viable spores, appear blue after CFW staining (left) while the nuclei of dead spores appear in red after PI staining (middle). After merging both images (right), the blue spores indicate viability and the pink spores indicate to be dead.

To determine the optimal incubation time, viable spores and heat-killed spores were mixed with Evans blue and CFW-PI, respectively. After 0, 0.25, 0.5, 2, 4, 24, 48 and 72 hours incubation, Evans blue and CFW-PI showed different performances as the incubation time varied (Fig. 3.3). The autoclaved samples assessed by CFW-PI showed the viability was 0% and remained stable without any changes over time. However, when assessed with Evans blue, it showed dramatic decreases and then stabilized after 24 hours of incubation. For the non-autoclaved samples, the viability detected by Evans blue was slightly higher than when estimated by CFW-PI staining after incubation of 4 hours. The viability remained stable at about 84% over 72 hours of incubation.

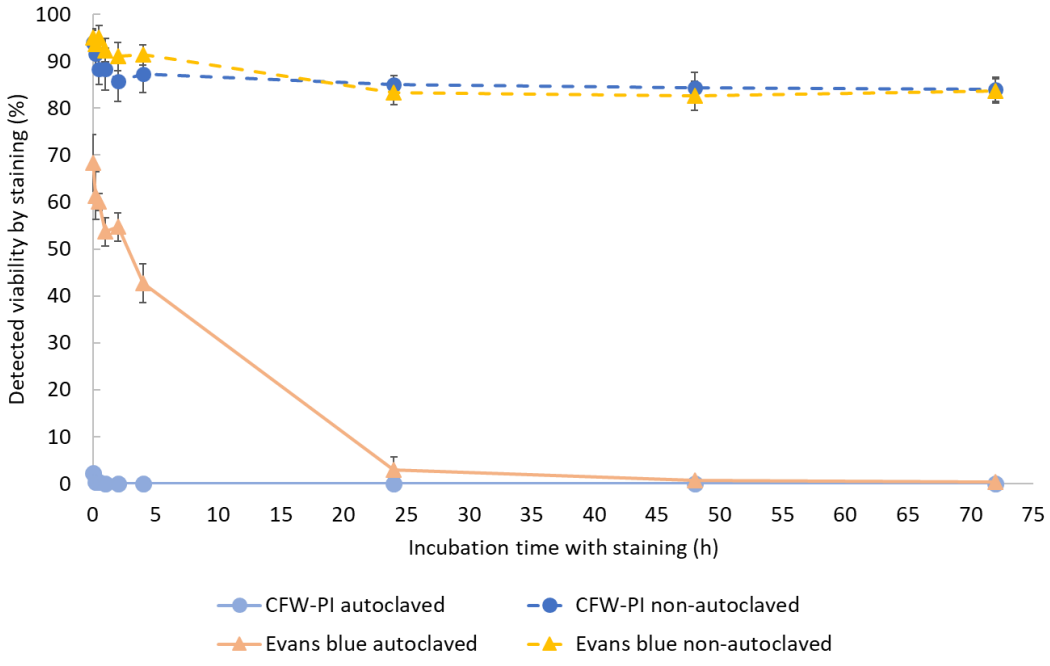

Figure 3.3 Viability of autoclaved and non-autoclaved spores of *Plasmodiophora brassicae* determined by different staining methods during 72 hours of incubation. Error bars indicate standard deviations.

The accuracy of the two staining methods was evaluated using known ratios of spore suspensions by incubating with Evans blue overnight or CFW-PI for 10 min to get predictable viability. Figure 3.4 shows a strong positive linear correlation between predictable and assumed viability with coefficients of determination of R^2 = 0.9981 (Evans blue) and R^2 = 0.9941 (CFW-PI). This indicates a high accuracy of predictable viability by staining.

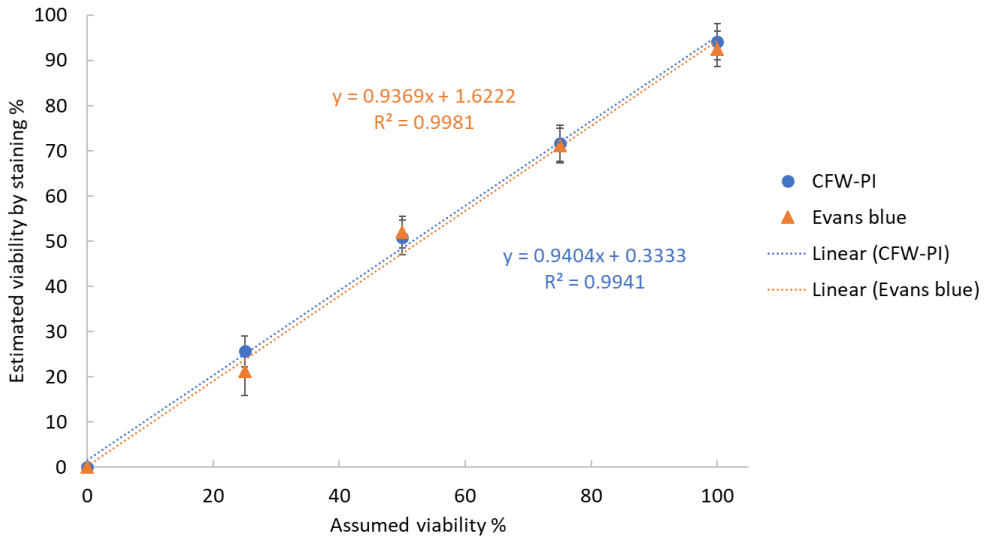

Figure 3.4 Correlation between assumed viability and estimated viability by staining was calculated using known ratios of dead to viable resting spores. Error bars represent standard deviations.

3.2 PMA qPCR detection of resting spore viability

Resting spores quantified by qPCR cannot distinguish between DNA of viable and dead spores. In order to quantify viable spores, the impact of propidium monoazide on inhibiting the amplification of DNA from dead spores was investigated.

3.2.1 Effect of lethal treatments on resting spores

The mortality of resting spores was determined by Evans blue and CFW-PI, which showed significantly a higher mortality in the heat and chemical treatments compared to untreated spores (Fig. 3.5). The untreated spores had a mortality of 5.75% by assessed both staining. Estimating resting spore mortality by incubation with CFW-PI for 10 min showed that the autoclave treatment had the highest mortality of 100%, followed by acetone of 95%. The

mortality assessed by overnight incubation with Evans blue showed the highest mortality rate of 100% for autoclave, acetone and methanol treatments, followed by 95°C for 10 min heat treatment of 95.5% mortality. The mortality after 1h treatment at 80°C was assessed significantly lower with CFW-PI (60.25%) than the one estimated by Evans blue (78.25%). For 95°C 10 min treatment, the mortality predicted by CFW-PI was 35% less than the estimates of Evans blue. For methanol treatment, the spore mortality was 79% given by CFW-PI, whereas it was 100% by Evans blue. In all lethal treatments, the mortality assessed by CFW-PI was lower than Evans blue except for the autoclave treatment.

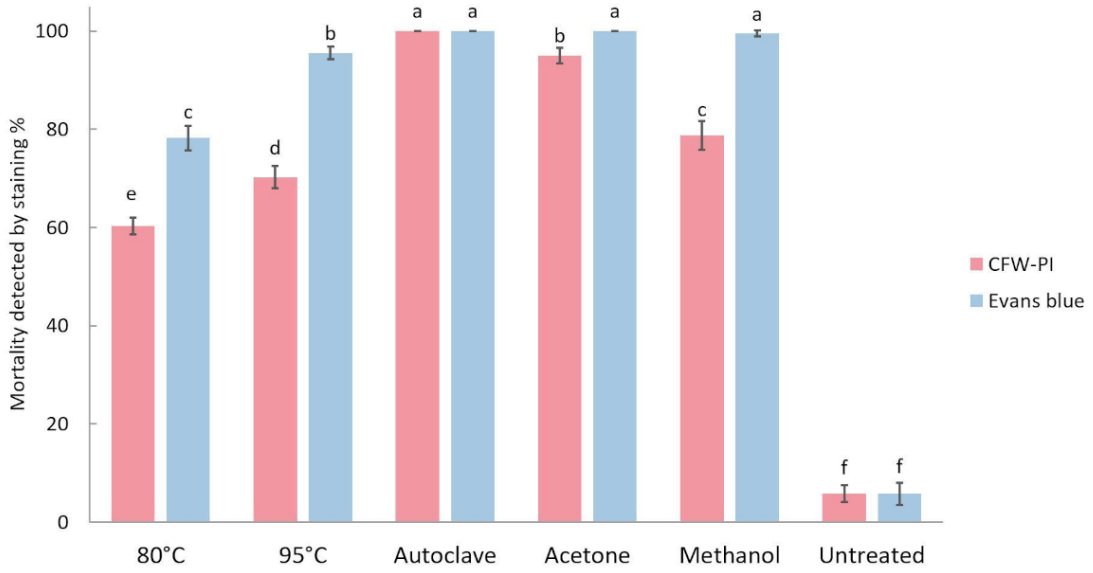

Figure 3.5 Mortality of resting spores of *P. brassicae* assessed by two staining methods after different lethal treatments. Different letters indicate significant differences among the treatments (Multi-factor ANVOA, Tukey test, $P<0.05$). Error bars indicate standard deviations.

3.2.2 DNA degradation of lethal treatments

The amount of DNA in *P. brassicae* resting spores was quantified in the presence or absence of PMA and showed significant differences between various heat and chemical treatments. The DNA amount in the control with and without PMA was similar indicating that the PMA treatment alone did not affect the quantity of DNA. For the samples without PMA treatment, which was the total DNA amount in each treatment, there was no significant difference in DNA amount between acetone, methanol and control treatments, while the DNA amount in heat treatments was significantly lower than in the control treatment. Especially after autoclave treatment, the DNA amount of *P. brassicae* was about 100-fold lower compared to the control. Relative to the total DNA amount, DNA quantified in

presence of PMA was considerably lower. Acetone treatment had the largest decrease when comparing the amount of DNA in the same lethal treatment with and without PMA.

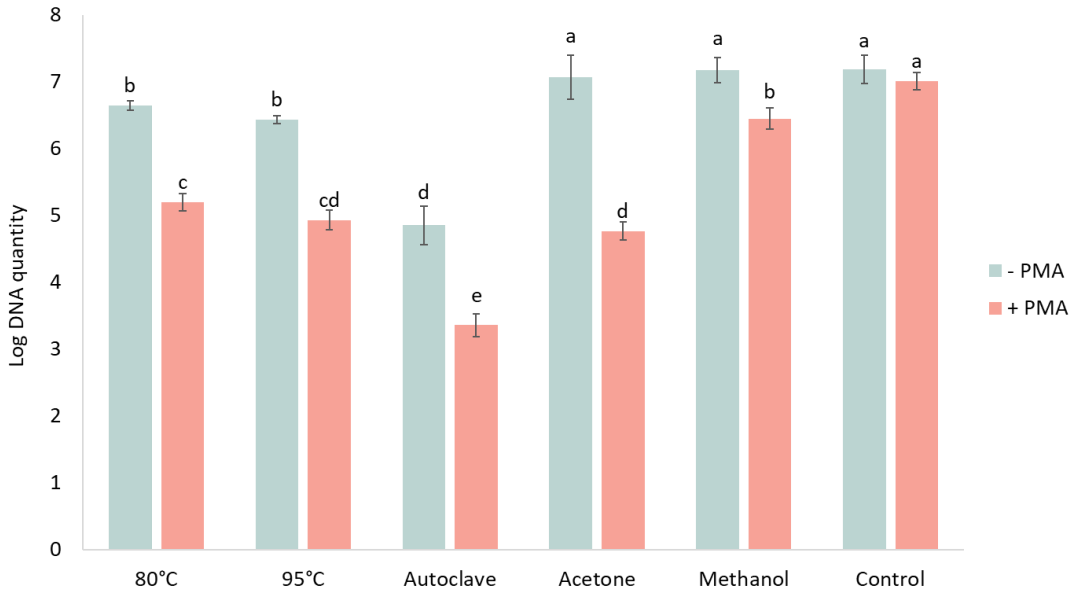

Figure 3.6 Log DNA amount of *P. brassicae* resting spores quantified by qPCR with (+PMA) or without (-PMA) propidium monoazide after different heat and chemical treatments. – PMA: total DNA amount; + PMA: DNA of viable spores. Different letters indicate significant differences among the treatments (Tukey test, $P<0.05$). Error bars indicate standard deviations.

3.2.3 Accuracy of PMA qPCR for assessment of spore viability

The viable and total spore numbers were calculated using the CT value obtained in PMA qPCR to obtain the viability. The regression analysis of the relationship between the percentage of viable spores detected by PMA qPCR and the assumed percentage of viable spores in the spore suspension showed that these values were positively correlated. The correlation was significant with a *P*-value of 0.0005 and explained a large percentage of the variance with an R^2 value of 0.989 (Fig. 3.7).

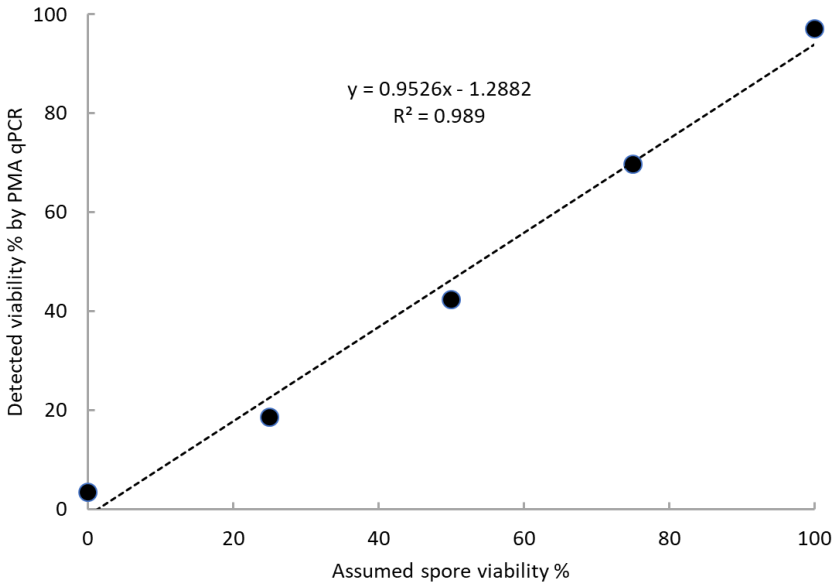

Figure 3.7 Accuracy of PMA qPCR to predict viability calculated by using known ratios of viable to acetone-killed resting spores of *P. brassicae*.

3.3 Germination process and determination of germination rate

The germination process of *P. brassicae* resting spores was observed using light microscopy. There were tiny particles observed in the resting spores that appeared to be in Brownian movement. Occasionally, conjugated spores were observed in spore suspension (Fig. 3.8A). The emergence of 'papilla' and the germinating spore that was releasing a primary zoospore were shown (Fig. 3.8B-D). The released zoospore was spherical and biflagellate and the size was similar to a resting spore (Fig. 3.8E). Zoospores swam vigorously at times and not so much at others. The observation of zoospores could be disturbed by contaminations, such as some protozoa having similar size and motility. After germination, an empty case with exit pore was left and this was considered as a germinated spore (Fig. 3.8F). The entire process from the emergence of papilla to release of a zoospore in one resting spore was not observed. Using differential interference contrast microscopy, it was suitable to differentiate germinated spores from non-germinated spores as germinated spores showed empty cases and non-germinated spores appeared filled (Fig. 3.8).

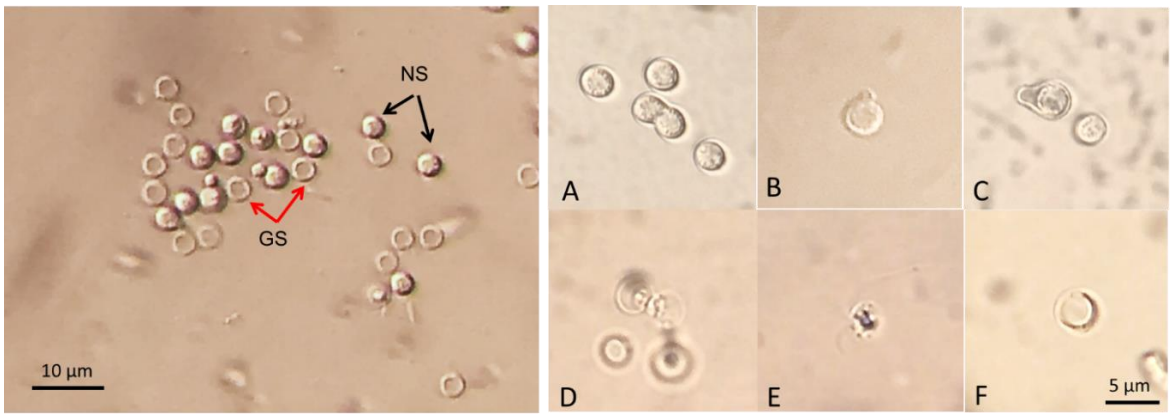

Figure 3.8 Resting spores of *P. brassicae* under the optical microscope with DIC prism (left) and the germination process of *P. brassicae* resting spores (right). Red arrows indicate germinated spores (GS), black arrows indicate non-germinated spores (NS). A) resting spores and conjugated spores; B &C) Emerged 'papilla'; D) Germinating spore; E) Primary zoospore; F) Empty case.

The germinated spores could be distinguished from non-germinated spores by DAPI and CFW-Nile red staining. The nuclei of non-germinated spores were stained by DAPI and appeared bright blue (Fig. 3.9). Similar to the dual stain of CFW-PI, configuration optimizations were conducted for CFW-Nile red that showed there was no overlap of detectable fluorescence between them. Figure 3.10 showed that only the lipid droplets in dead spores were stained by Nile red with an excitation wavelength of 488 nm and an emission wavelength of 590-620 nm. With a lethal pre-treatment, the non-germinated spores, which contained lipid droplets, stained by both CFW and Nile red, while germinated spores showed only blue staining of the cell wall (Fig. 3.10).

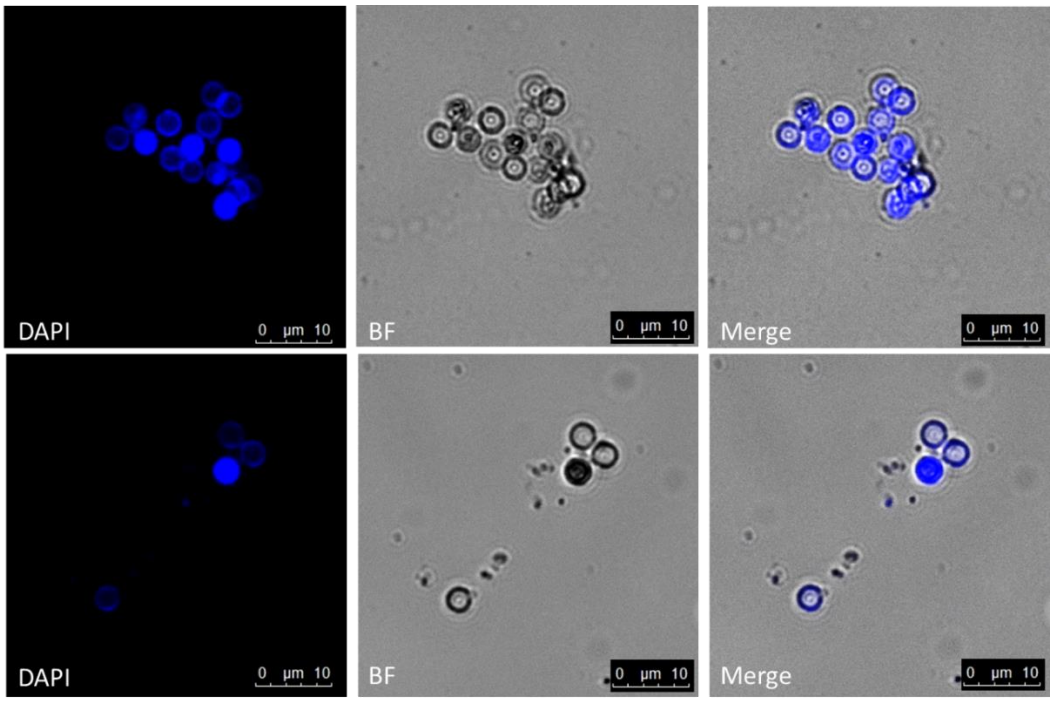

Figure 3.9 Resting spores stained with DAPI to distinguish the germinated from non-germinated spores with CLSM. The blue spores are non-germinated spores and the colorless spores are germinated spores.

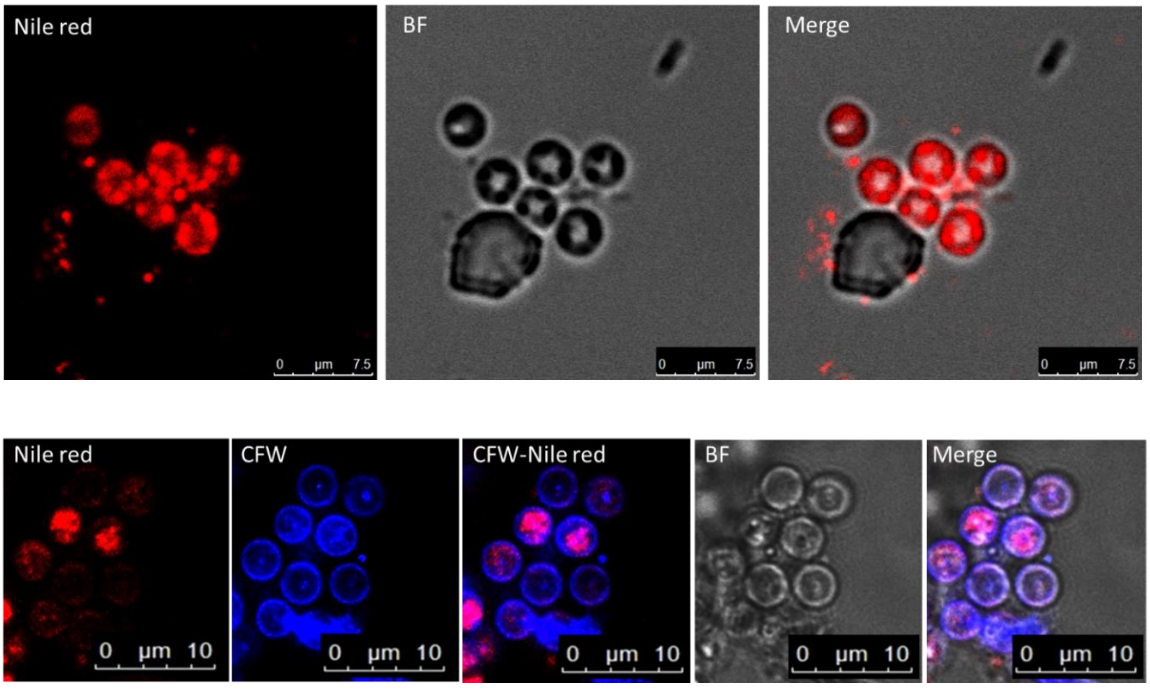

Figure 3.10 Resting spores of *P. brassicae* stained with Nile red alone (upper panel) or the dual staining of CFW-Nile red (lower panel) with confocal microscopy. BF indicates the view under bright field.

3.4 Non-target profiling analysis of root exudates

Non-target profiling analysis of root exudates collected from the hydroponic trapping system was conducted by LC-QTOF-MS. The compounds of root exudates collected at the four-leaf stage (BBCH 14) from different plant species were highly diverse. Different ion mode of LC-QTOF-MS was sensitive to different compounds resulting in different composition of root exudates (Fig. 3.11). In positive mode (Fig. 3.11A), the entities of root exudates from oilseed rape, ryegrass and tomato were 239, 375 and 106, respectively. There were only 13 compounds in common. In negative mode (Fig. 3.11B), root exudates of tomato had the most entities of 254, followed by ryegrass of 90 entities and rape of 79 entities. There was only one common compound among them. After filtering by abundance, 133 formulas and 204 formulas were calculated in positive and negative mode, respectively.

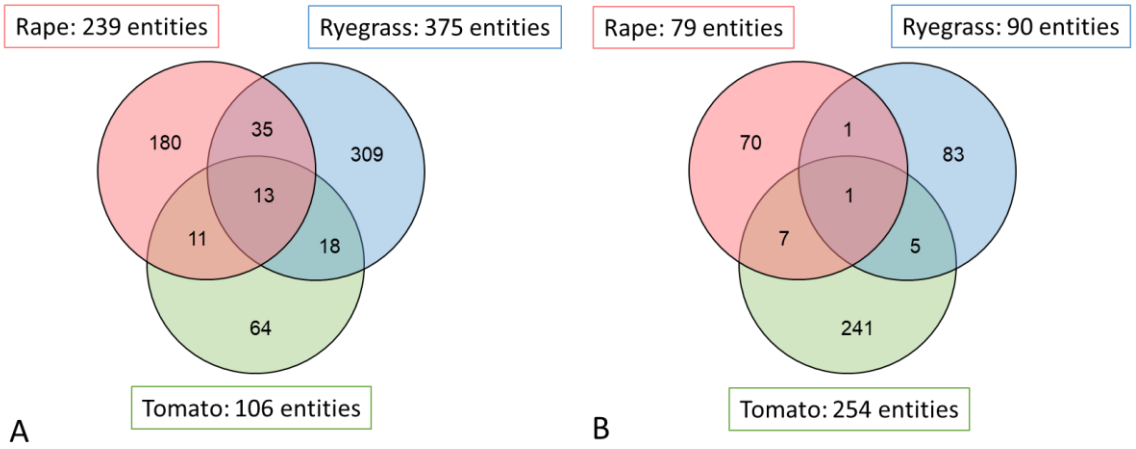

Figure 3.11 Venn diagrams of root exudates obtained in the hydroponic system and measured in positive mode (A) and negative mode (B) by LC-QTOF-MS. The total entities of root exudates from oilseed rape, ryegrass and tomato obtained in positive and negative mode are marked after subtraction of blanks.

3.5 Effect of root exudates on resting spores

The filtered root exudates collected from petri dish cultivation (PDC) and the hydroponic trapping system (HTS) were mixed with sterile and non-sterile spores to examine the effects on stimulating resting spore germination. The results showed that the germination rates of sterile and non-sterile spores incubated with root exudates from PDC were approximately zero percent. However, the root exudates collected from HTS showed different effects. The eluted root exudates from XAD4 and XAD8 resin were dried using speed vacuum and then dissolved with Hoagland solution and sdH_2O, respectively. Similar to the root exudates from PDC, when the root exudates of HTS were resuspended in sdH_2O, the germination was almost 0% indicating that root exudates did not induce germination of sterile and non-sterile spores. However, HTS root exudates resuspended in Hoagland solution had germination

stimulating effects on the non-sterile spores resulting in higher germination rates (Fig. 3.12). The root exudates from XAD8 resin had relatively higher germination rates than those from XAD4 resin. There were no significant differences in resting spore germination rates incubated with root exudates from different plant species and different growth stages.

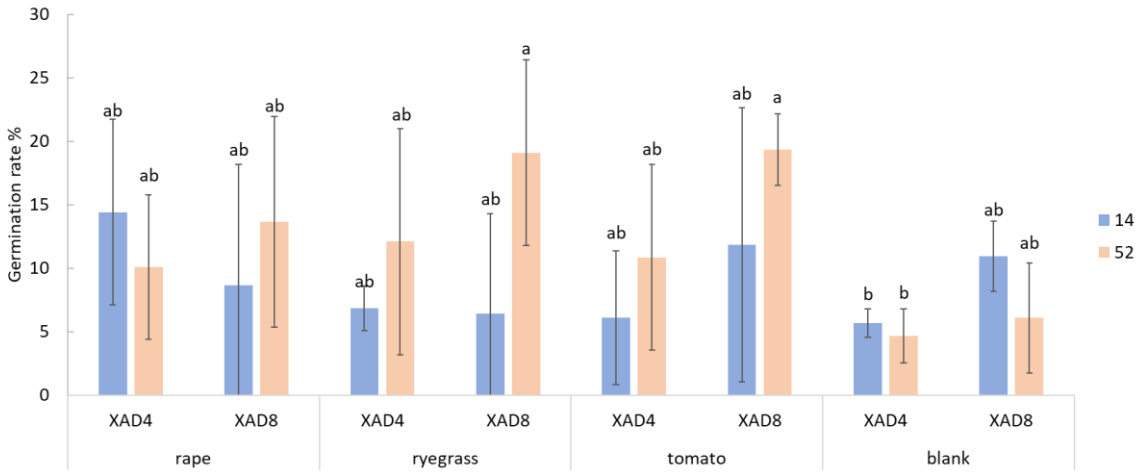

Figure 3.12 Effect of root exudates collected from the hydroponic trapping system at BBCH 14 (blue) and BBCH 52 (pink) and resuspended in Hoagland solution on the germination of non-sterile resting spores. Different letters represent significant differences among the treatments (Tukey test. $P<0.05$). Error bars indicate standard deviations.

3.6 Role of soil bacteria in spore germination

3.6.1 Germination of resting spores in soil suspensions

The non-sterile spores were incubated with filtered or non-filtered soil suspensions from grassland soil resulting in different germination rates. Figure 3.13 shows that there was no significant difference in germination rate of resting spores between filtered and non-filtered soil suspensions after 5 days of incubation. At 18 dai, the germination rate of resting spores in non-filtered soil suspension was dramatically higher than that in the filtered soil suspension. The percentage of germinated spores in filtered treatment was slightly increased after 18 days of incubation, while that in non-filtered treatment increased up to 32.75%. The biggest difference between filtered and non-filtered soil suspensions was the presence of soil microbes, obviously having some stimulatory effects on the germination of resting spores.

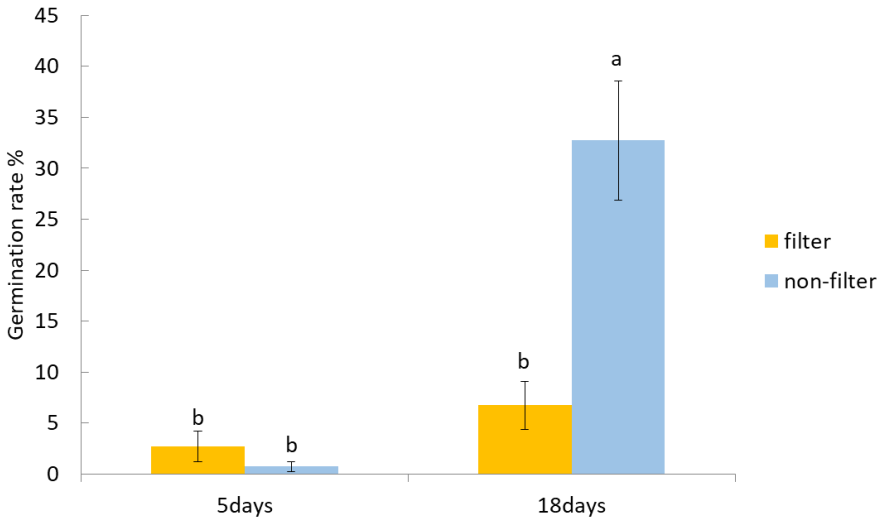

Figure 3.13 Germination rates of *P. brassicae* resting spores incubated with the filtered and non-filtered soil suspensions from grassland soil after 5 and 18 days of incubation. Error bars represent standard deviations. Different letters represent significant differences among the treatments (Tukey test. $P<0.05$, n=3).

Both rhizosphere and bulk soil suspensions from an oilseed rape field showed similar effects on the germination rate of non-sterile spores. The results (Fig. 3.14) showed that the resting spores incubated in the filtered soil suspensions of both rhizosphere soil and bulk soil barely germinated up to 7 days post incubation. Non-filtered soil suspensions had remarkable promoting effects on the germination of resting spores compared to the filtered ones. At 5 dai, the germination rate of resting spores in the non-filtered bulk soil suspensions was significantly higher than that in the non-filtered rhizosphere soil suspensions, while no difference in germination rates between them was found at 7 dai. These results indicated that although both non-filtered rhizosphere and bulk soil suspensions stimulate the germination of resting spores, the germination rate increased much faster in bulk than in rhizosphere soil suspensions.

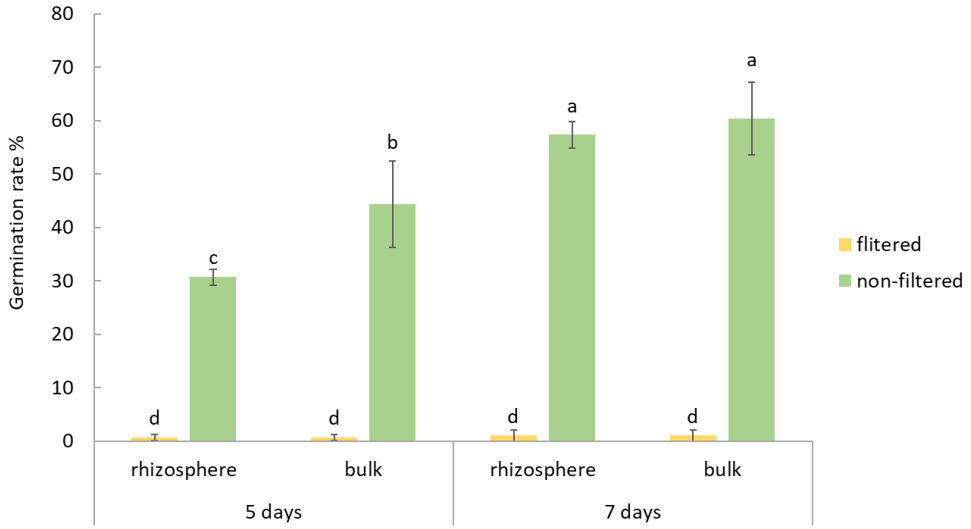

Figure 3.14 Germination rates of *P. brassicae* resting spores incubated in the filtered and non-filtered soil suspensions from an oilseed rape field after 5 and 7 days of incubation. Rhizosphere soil was collected from root of oilseed rape cv. Bender at BBCH 18 (eight-leaf stage). Bulk soil was collected from a neighboring field without plants. Error bars represent standard deviations. Different letters represent significant differences among the treatments (Tukey test. $P<0.05$, n=4).

Besides, soil suspensions from greenhouse soil (1:3 of sand and soil) with and without diseased and healthy plants had various effects on the germination rates after 10 days of incubation (Fig. 3.15). Both sterile and non-sterile resting spores cultured in sdH_2O or Hoagland solution had relatively low germination rates. Similar to the control groups, soil suspensions from soil with healthy plants did not show any positive effect on the germination rate of the sterile and non-sterile resting spores. For the soil suspensions of soil with inoculated plants, both sterile and non-sterile spores had much higher germination rates in the non-filtered soil suspensions than in the filtered soil suspensions. The highest germination rate of both sterile and non-sterile spores was observed in the non-filtered blank soil suspensions, whereas for the filtered blank soil suspensions (without plant), non-sterile spores had a dramatically higher germination rate than that of the sterile spores in the filtered blank soil suspensions.

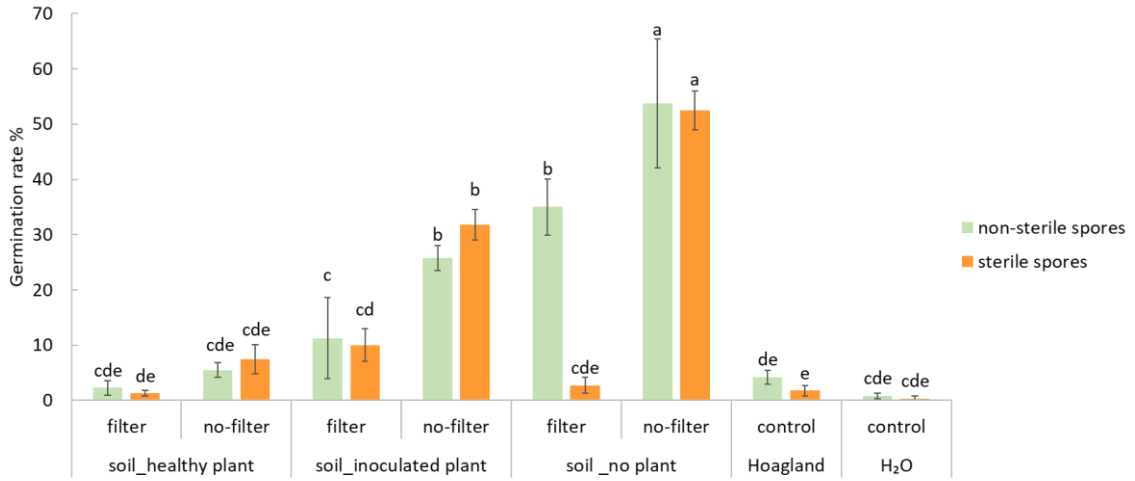

Figure 3.15 Germination rates of *P. brassicae* resting spores incubated with the filtered and non-filtered soil suspensions from greenhouse soil mixed by sand and potting soil after 10 days of incubation. Three soils were used: soil with healthy oilseed rape (cv. Westar, 7-week-old), soil with oilseed rape inoculated with *P. brassicae* (6 weeks post inoculation) and soil without plants. Hoagland solution (1/10 strength) and sdH_2O were control treatments. Error bars indicate standard deviations. Different letters represent significant differences among the treatments (Tukey test. $P<0.05$, n=4).

3.6.2 Bacteria isolation, bioassay and classification

In total, 78 bacterial strains were isolated from grassland, field and greenhouse soil suspensions. The bacterial isolates Iso4 and *E. coli* incubated in 1/10 strength Hoagland solution were able to stimulate the germination of non-sterile resting spores, when bacterial concentration was higher than 10^7 CFU/ml (Fig. 3.16). The germination rate of *P. brassicae* resting spores was rising with time and it was up to 60% at 10 dai (Fig. 3.16). The resting spores with bacteria in sdH_2O did not germinate which indicated that nutrients were essential for the stimulation of resting spore germination. The germination rate of resting spores in Hoagland solution without bacteria was 0%, suggesting that bacteria were involved in triggering the germination of resting spores.

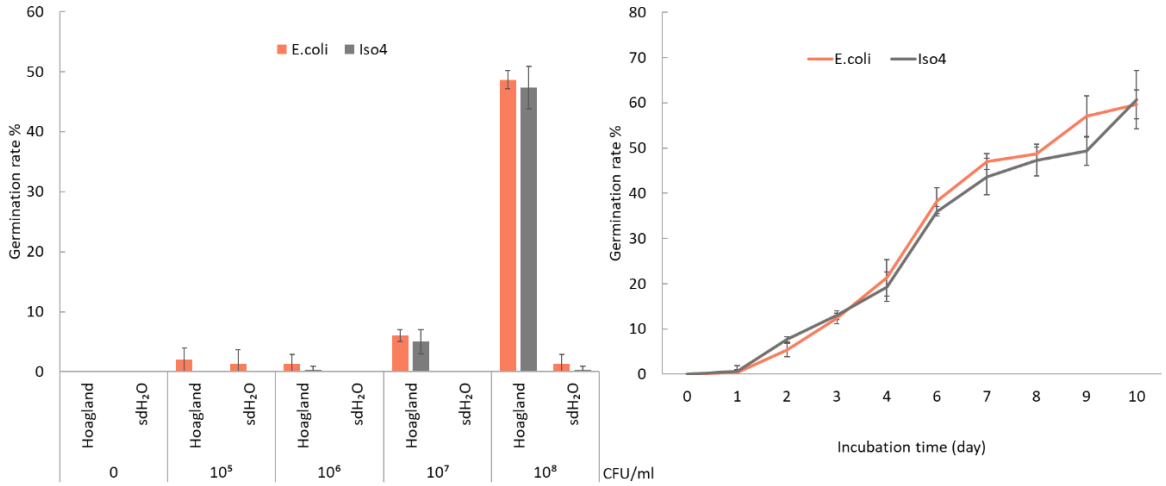

Figure 3.16 Germination rate of *P. brassicae* resting spores cultured with different concentrations of suspensions of two bacteria after 8 days of incubation (left). Changes in germination rates of *P. brassicae* resting spores over time when cultured with 10^8 CFU/ml bacteria in Hoagland solution (right). Error bars represent standard deviations.

Almost all the selected bacterial strains were able to stimulate the germination of non-sterile resting spores, when they were provided in Hoagland solution (Fig. 3.17). The germination rate of six strains (A1, A3, A5-1, A6-1, P1, P2) was significantly lower than that of the positive control of *E. coli* (58.3%), but they still induced germination rates of 9.6%-21.6%, which was higher than the negative control (Hoagland solution/sdH_2O without bacteria). The highest germination rate was 69.6% in the P3-3 treatment.

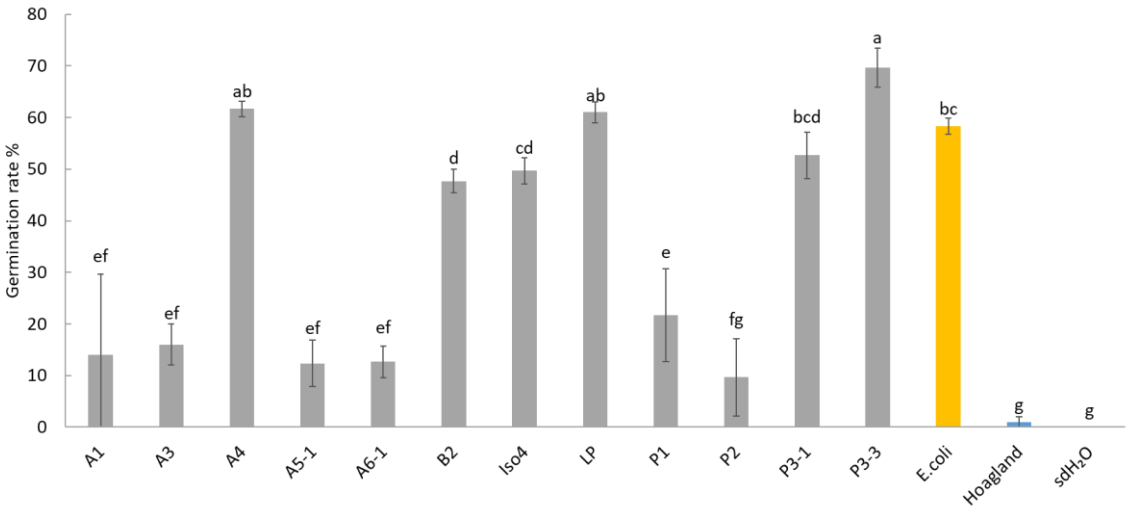

Figure 3.17 Germination rates of non-sterile resting spores of *P. brassicae* incubated with different bacterial strains in Hoagland solution. Positive control (yellow): *E. coli* with Hoagland solution; negative control (blue): Hoagland and sdH_2O without bacteria. Different letters represent significant differences among the treatments (Tukey test. $P<0.05$). Error bars represent standard deviations.

The results indicated that different bacterial strains had diverse impacts on the germination of resting spores. The selected bacterial strains were classified using several methods (Tab 3.1), showing that most bacterial strains that induced higher germination rates were able to reduce nitrate into nitrite, except for LP.

Table 3.1 Classification of bacterial isolates by various methods

Isolate	Motility	Gram	Fermentation			King's B	Nitrate reduction	Oxidase
			Lactose	Glucose	Gas			
A1	+	-	+	-	-	-	-	+
A3	+	-	+	-	-	-	-	+
A4	-	+	-	-	-	-	+	+
A5-1	-	+	-	-	-	-	-	-
A6-1	-	+	-	-	-	-	-	-
P1	-	-	+	+	+	-	+	+
P2	-	-	+	-	-	+	-	+
P3-1	-	+	-	-	-	-	+	(+)
P3-3	-	+	-	-	-	-	+	(+)
B2	+	-	+	+	+	-	+	+
Iso4	-	-	+	+	+	-	+	+
LP	-	+	-	-	-	-	-	+
E. coli	+	-	+	+	+	-	+	-

+ represent positive reaction

(+) represent weak positive reaction

- represent negative reaction

3.7 Role of nitrogen and carbon sources on resting spore germination

3.7.1 Effect of inorganic components on resting spore germination

Individual components of Hoagland solution mixed with sterile or non-sterile spores and bacterial strain Iso4 showed different effects on resting spore germination. Sterile spores barely germinated in all treatments and the germination rates of both sterile and non-sterile resting spores cultured in various inorganic solutions without bacteria was almost 0%.

Figure 3.18A shows the germination rates of non-sterile spores in different solution with bacterial strain Iso4. The germination rate of the negative control (sd) was close to 0%, and the positive control, 1/10 strength Hoagland solution had the highest germination rate of 34.6%. Calcium nitrate and potassium nitrate had considerably stronger effects in stimulating the germination of resting spores, compared to other components.

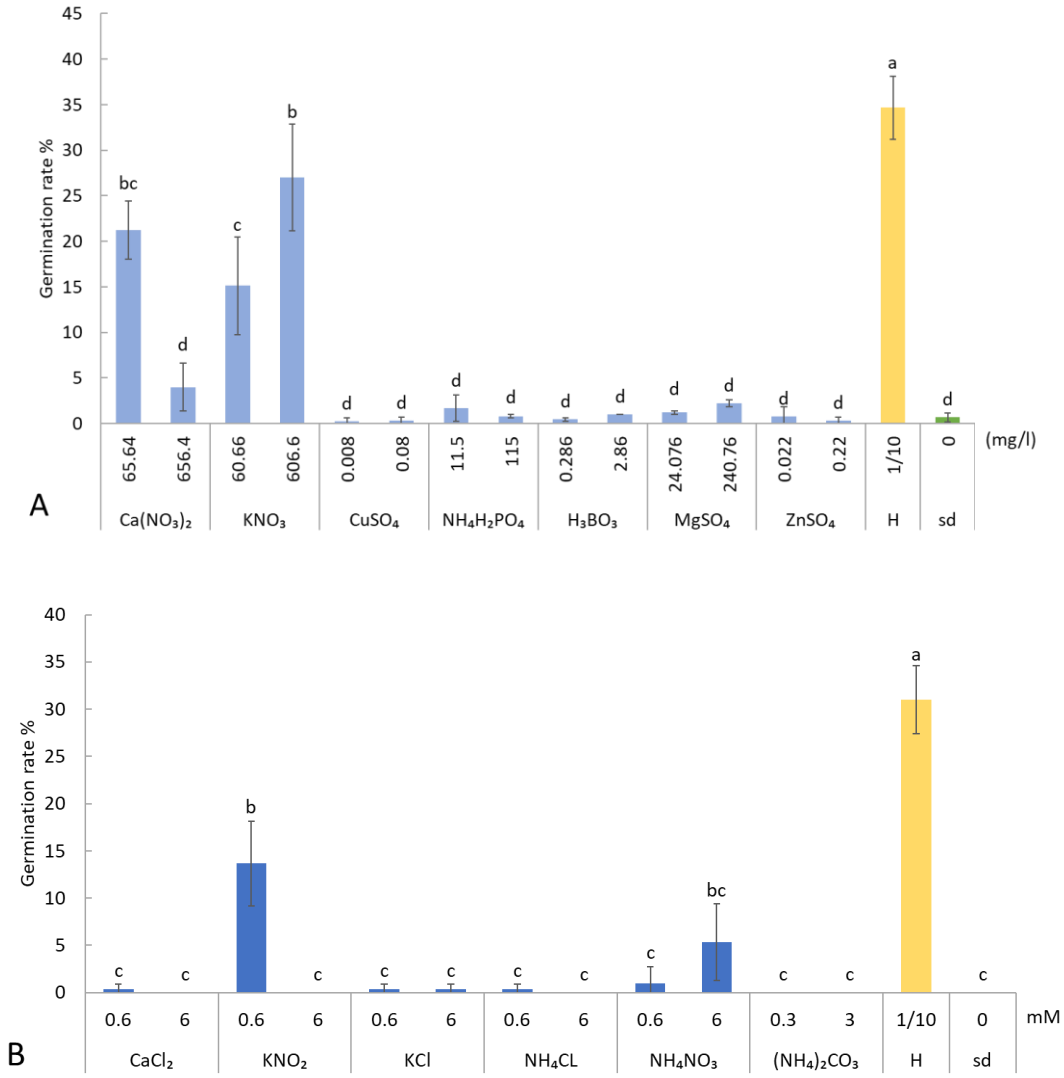

Figure 3.18 Germination rates of non-sterile resting spores of *P. brassicae* incubated with bacterial strain Iso4 in various solutions after 7 days. A) Individual components of Hoagland solution tested in the bioassay. B) Resting spore germination rates in solutions with different cations. The positive control of 1/10 strength Hoagland solution (H) is indicated in yellow. The negative control of sdH_2O (sd) is indicated in green. Error bars represent standard deviations. Different letters represent significant differences among the treatments (Tukey test. $P<0.05$).

A higher concentration of potassium nitrate (606.6 mg/l) resulted in a higher germination rate of 27%, and conversely, a higher concentration of calcium nitrate (656.4 mg/l) resulted

in lower germination rate of 4%. This indicates that nitrate may be essential for resting spore germination, but the cations were also capable of affecting germination rates. Moreover, 0.6 mM potassium nitrite had a stimulation effect on the germination of resting spores, but 6 mM KNO_2 not (Fig. 3.18B). The germination rate was slightly higher in 6 mM ammonium nitrate.

In the presence of bacteria, the germination of resting spores cultured in gradient concentrations of different inorganic solutions showed different patterns (Fig. 3.19). The germination rates decreased with increasing concentration of calcium nitrate, while the germination rates in different concentrations of potassium nitrate slightly increased with increasing concentration without significant differences. When equal amounts of nitrate were present, high concentrations of calcium ions inhibited the germination but not potassium ions. The germination rate decreased significantly with the increase of potassium nitrite concentration, and the germination rate was zero when the concentration was greater than 1.2 mM. This indicates that a high concentration of nitrite is not suitable for resting spore germination.

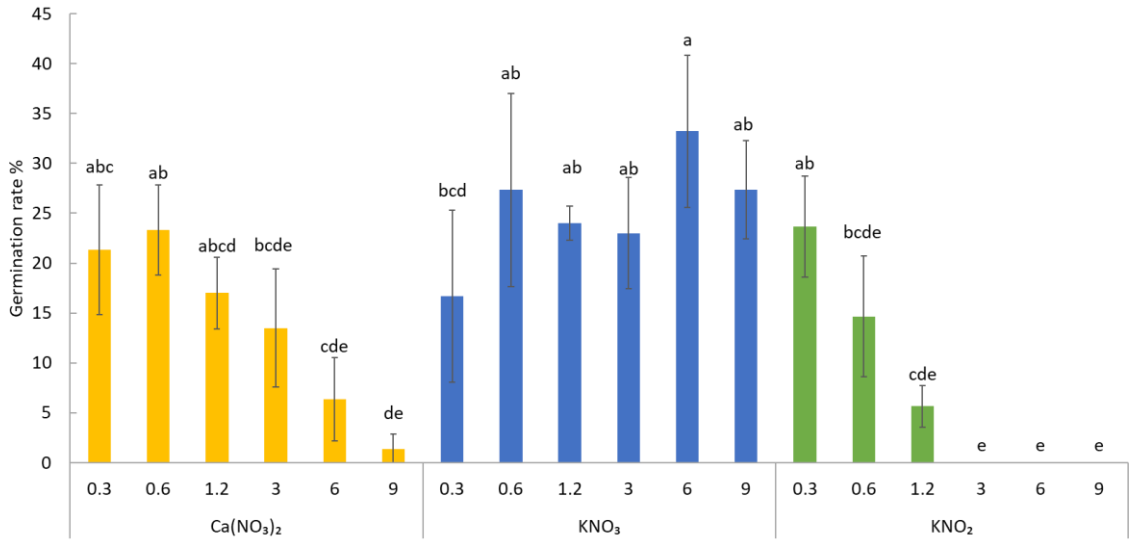

Figure 3.19 Germination rate of *P. brassicae* resting spores incubated with different concentrations of $Ca(NO_3)_2$, KNO_3 and KNO_2. Different letters represent significant differences among the treatments (Tukey test. $P<0.05$). Error bars represent standard deviations.

3.7.2 $K^{15}NO_3$ isotope analysis

The ^{15}N content in resting spores was measured after incubation with potassium nitrate in 10% ^{15}N-enriched form or natural abundance form in the presence of *E. coli* or not. The nitrogen content of resting spores was about 4.5%. The results showed that ^{15}N enrichment in resting spores increased with incubation time with or without the additional bacteria

compared to natural abundance (Fig. 3.20). There was no relationship between the enrichment of ^{15}N and the germination rate of resting spores, indicating that nitrate is not directly involved in stimulating resting spore germination.

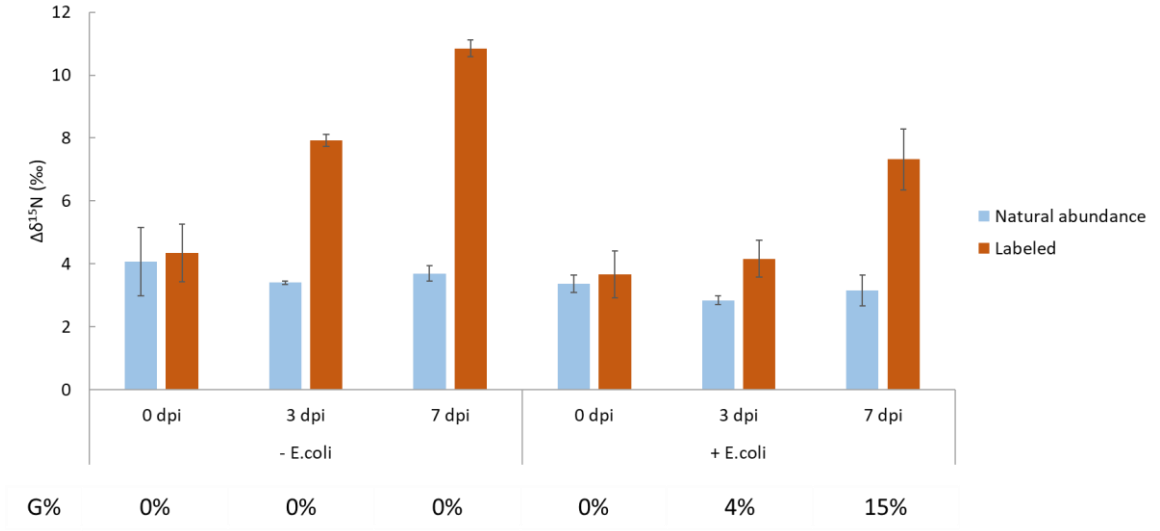

Figure 3.20 ^{15}N enrichment ($\Delta\delta^{15}N$) of *P. brassicae* resting spores incubated with potassium nitrate with (+ *E. coli*) or without (- *E. coli*) the presence of *E. coli* and the corresponding germination rates (G%) after different incubation time. Error bars indicate standard deviations.

3.7.3 Effect of *Bacillus subtilis* mutants

B. subtilis wild-type strain JH642, *narG* mutant strain THB1 (unable to convert nitrate to nitrite) and *nasD* mutant strain 1972 (unable to convert nitrite into ammonia) were used for the bioassay to examine the effect of nitrate reductase and nitrite reductase on the germination of resting spores. There was no major difference in the effect of different *B. subtilis* mutants on germination rate. The effect of *B. subtilis* mutants in different inorganic solutions on the germination rate showed the same trend as that of the isolated bacterial strain Iso4 (see 3.7.1). Neither the *narG* mutant strain nor the *nasD* mutant strain regulated the germination rate of resting spores different from the wild type strain.

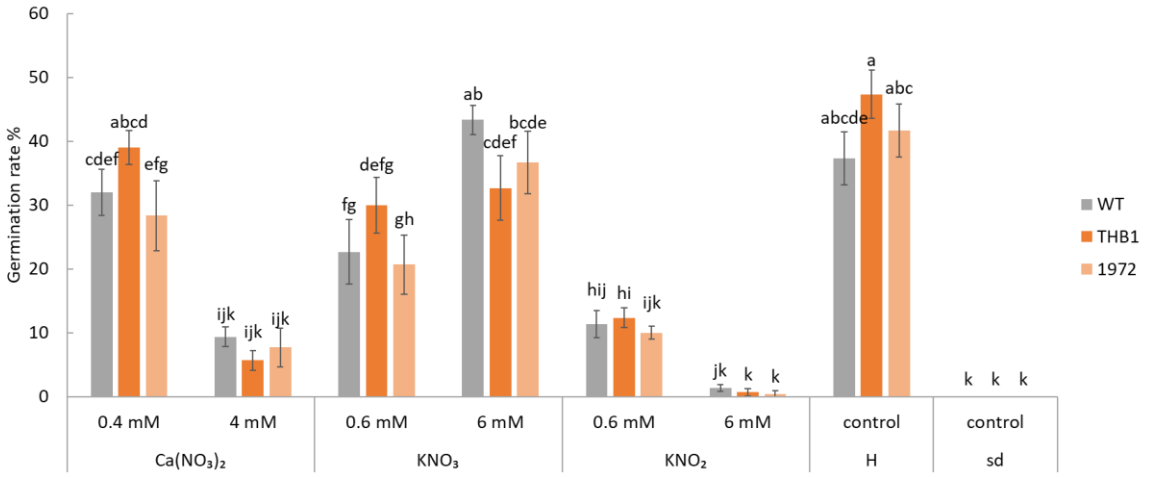

Figure 3.21 Effect of *B. subtilis* mutants on the germination of resting spores of *P. brassicae* incubated in different inorganic solutions. WT: *B. subtilis* wild-type strain JH642 (*trpC2 pheA1*), THB1: *narG* mutant strain (*trpC2 pheA1 narGH::tet*, tet 0.5 µg/ml) and *nasD* mutant strain 1972 (*trpC2 pheA1* Δ *nasD::phleo*, phleo 0.4 µg/ml). H indicates 1/10 strength Hoagland solution and sd indicates sdH_2O. Different letters represent significant differences among the treatments (Tukey test. $P<0.05$). Error bars represent standard deviations.

3.7.4 Effect of bacterial products

Although the bacterial strains were killed to offset their bioactivity, they were still able to stimulate the germination with addition of nitrogen source. Figure 3.22 shows that there was little difference in germination rates between living and dead bacterial treatments. The dead bacterial treatments had similar tendencies as living bacteria, when they were mixed with nitrate solution. This indicated that the activity of additional bacteria was not essential for the stimulation of germination.

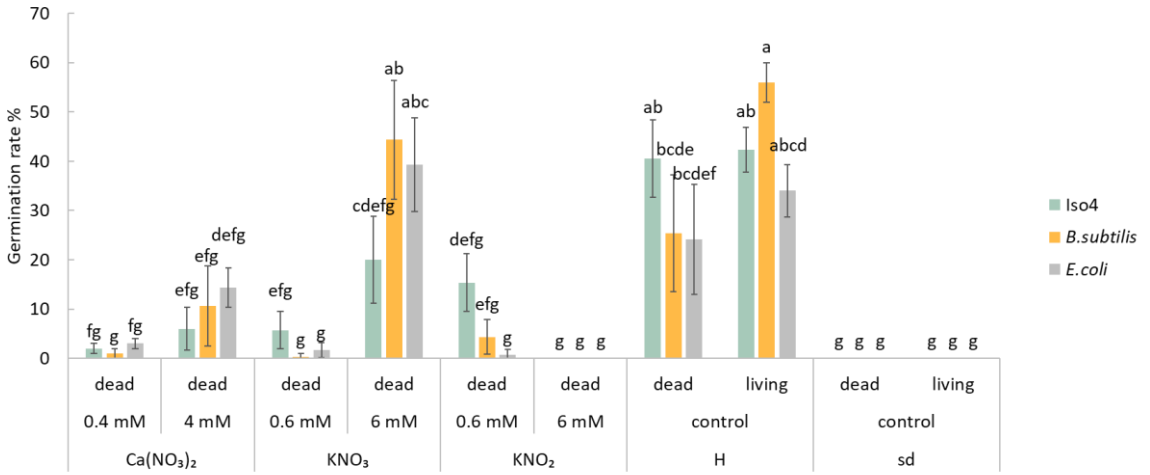

Figure 3.22 Effect of dead bacterial suspension on the germination rate of resting spores of *P. brassicae* in various inorganic solutions. Control: 1/10 strength Hoagland solution (H) and sterile deionized water (sd). Error bars represent standard deviations.

The filtrates of three bacterial cultures were tested to determine their effects on resting spore germination. Figure 3.23 shows that the combination of bacterial filtrates and Hoagland solution was capable of stimulating the germination of non-sterile resting spores, while this was not observed with sterile spores. The treatments of filtrates alone and the mixture of filtrates and sdH_2O had a poor stimulation effect on resting spores. This suggests that filtrates of isolated strains were not the direct factors of triggering germination.

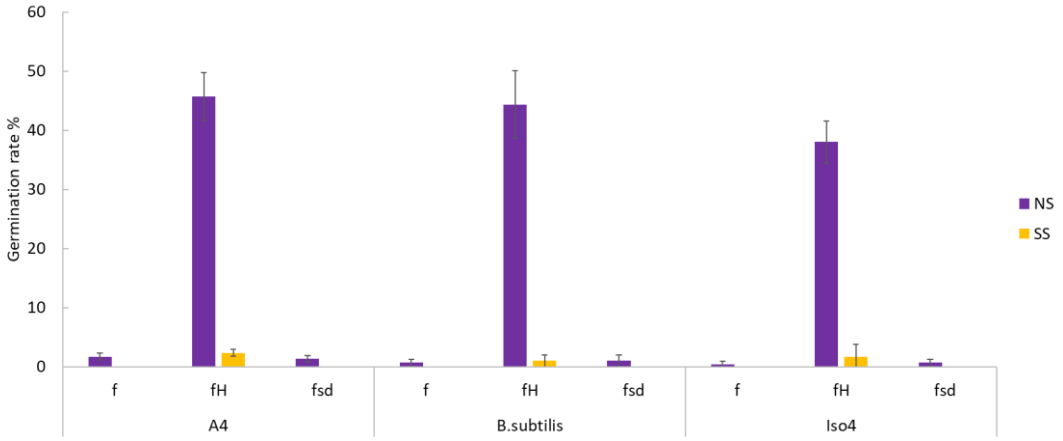

Figure 3.23 Effect of bacterial filtrate on the germination rate of resting spores of *P. brassicae* in Hoagland solution or sdH_2O. NS: non-sterile spores; SS: sterile spores; f: bacterial filtrate; fH: bacterial filtrate and Hoagland solution; fsd: bacterial filtrate and sdH_2O.

3.7.5 Bacterial community analysis

The germination rate of resting spores in each sample (Tab. 2.3) was examined under the microscope. The initial germination rate of non-sterile spores was 0%. The negative treatments of resting spores incubated in Hoagland solution (H1-H3) and sdH_2O (sd1-sd3) showed significantly lower germination rates than the other treatments (A4f, HB and KB). With the presence of bacterial filtrate/ bacterial suspension and Hoagland solution/potassium nitrate, the germination rate dramatically increased.

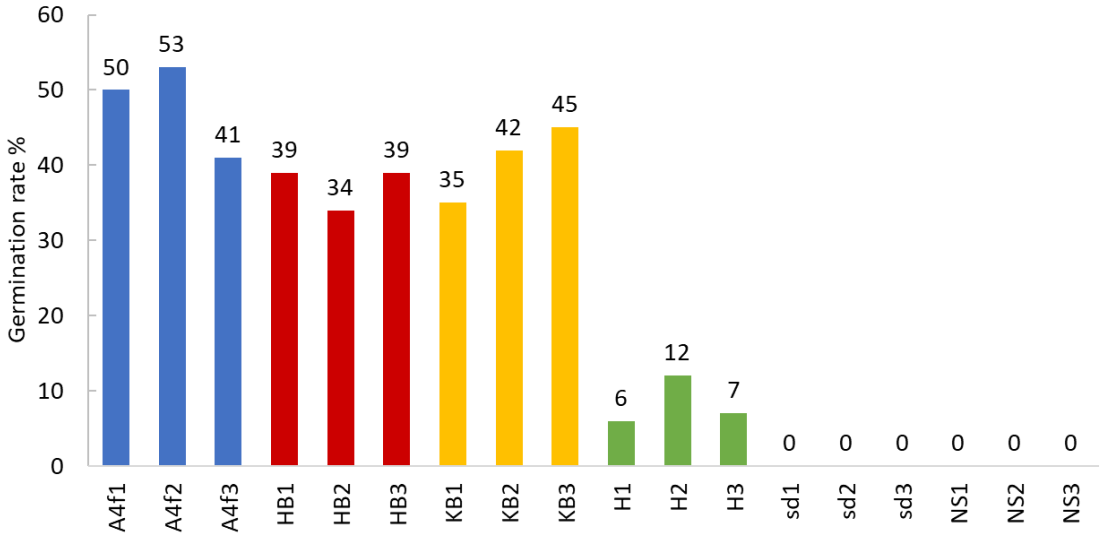

Figure 3.24 Germination rate of resting spores of *P. brassicae* in each treatment used for 16S rRNA gene amplicon sequencing. A4f (blue): filtrate of bacterial strain A4 with 1/10 strength Hoagland solution; HB (red): *B. subtilis* cell suspension with 1/10 strength Hoagland solution; KB (yellow): *B. subtilis* cell suspension with 6 mM potassium nitrate; H (green): 1/10 strength Hoagland solution; sd: sterilized deionized water; NS: non-sterile resting spore suspension (initial bacterial community).

The 16S ribosomal RNA gene of each sample (Tab. 2.3) was amplified with primers of D-Bact-0341-b-S-17 and D-Bact-0785-a-A-21. PCR products were examined on 1% agarose gel (Fig. 3.24) that showed a consistent size of the products of approximately 550 bp compared to the 100 bp ladder DNA. There was no amplification in the sterile spore samples (SS1-SS3) that indicated the bacterial amount was quite low after treatment with antibiotics, in contrast, there were clear bands in the non-sterile spore samples (NS1-NS3). The samples of sterilized spores were excluded from the sequencing experiment. The no template control (NTC) had no PCR product confirming that there was no contamination in the extraction materials.

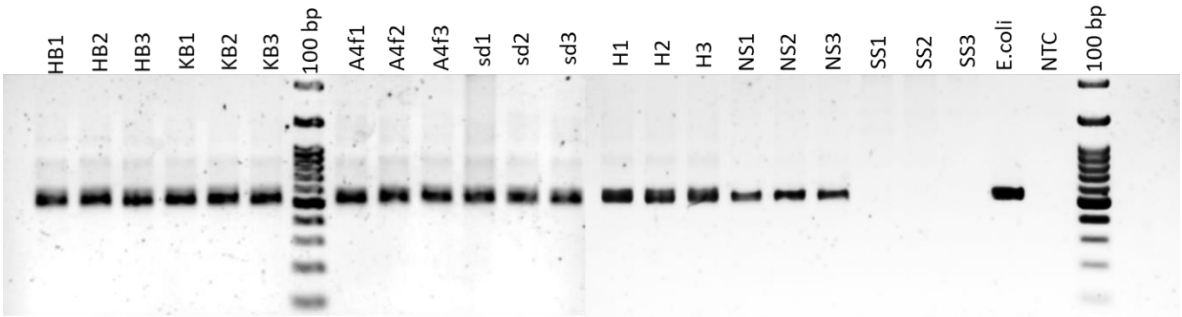

Figure 3.25 Image of amplified 16s ribosomal RNA of each sample on 1% agarose gel. HB: *B. subtilis* cell suspension with 1/10 strength Hoagland solution; KB: *B. subtilis* cell suspension with 6 mM

potassium nitrate; A4f: the filtrate of bacterial strain A4 with 1/10 strength Hoagland solution; sd: sterilized deionized water; H: 1/10 strength Hoagland solution; NS: non-sterile resting spore suspension (initial bacterial community); SS: sterile resting spore suspension; *E. coli*: positive control; NTC: no template control; 100 bp: ladder DNA.

There were 620,712 reads of 16S rRNA bacterial gene sequences, after cleaning and filtering. There were 452 operational taxonomic units (OTUs) obtained at 100% genetic identity level using the UNOISE3 algorithm. The composition of the bacterial community at the phylum and family level is shown in Fig. 3.26. In total, nine bacterial phyla were identified. The initial bacterial communities (NS) were dominated by the phyla Proteobacteria (91.7%) and Actinobacteriota (7.4%). After 7 days of incubation, the relative abundance of Proteobacteria in each sample decreased, while the portion of Bacteroidota increased. These changes of relative abundance in each community resulted in a shift in the ratio of Proteobacteria and Bacteroidota. Proteobacteria and Bacteroidota ratios were lower in A4f, HB and KB than that in H and sd. Actinobacteriota with average relative abundances of 3.9% in A4f, 0.99% in HB and 0.1% in KB were lower than that of 18% in H and 19.6% in sd. At family level, the diversity of bacterial communities significantly increased compared to the initial bacterial community (NS). The average relative abundance of families *Weeksellaceae* and *Flavobacteriaceae* was considerably higher in the treatment (A4f, HB, KB) with higher germination rate than those (H, sd) with lower germination rate.

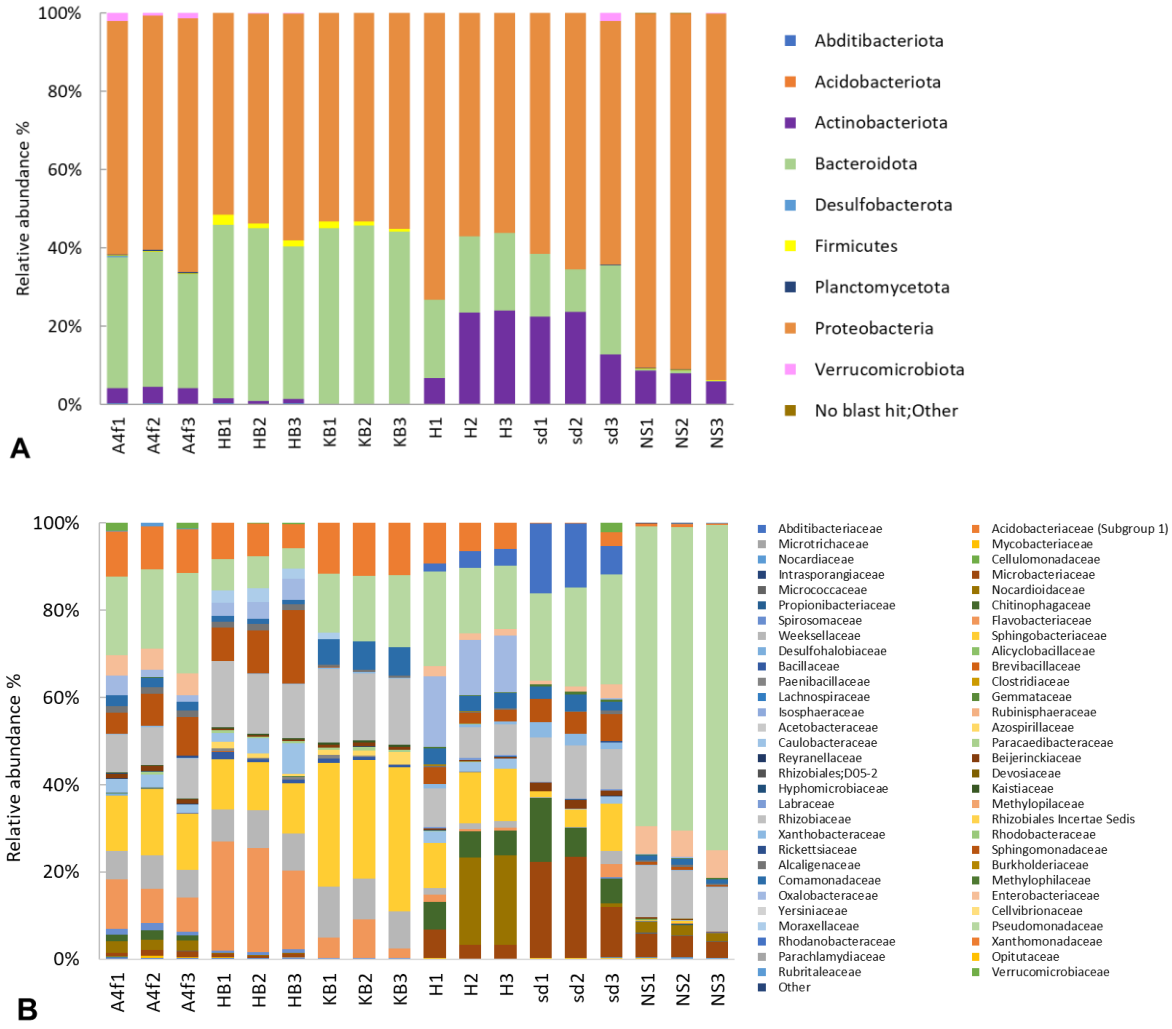

Figure 3.26 Relative abundance of bacterial communities in each treatment at phylum level (A) and family level (B). A4f: filtrate of bacterial strain A4 with 1/10 strength Hoagland solution; H: 1/10 strength Hoagland solution; HB: *B. subtilis* cell suspension with 1/10 strength Hoagland solution; KB: *B. subtilis* cell suspension with 6 mM potassium nitrate; NS: non-sterile resting spore suspension (initial bacterial community); sd: sterilized deionized water.

The samples were divided into two groups depending on the germination rate (Fig. 3.25), those with germination rates higher than 15% belonged to the high germination rate group and those lower than 15% were assigned to the low germination rate group. Alpha diversity indices including observed OTUs, Shannon index and phylogenetic diversity were used to reveal the bacterial community diversity in different samples (Fig. 3.27). Species richness was reflected by the observed OTUs. The richness of A4f was the highest among the treatments. The number of OTUs in the samples of low germination rate group were similar. Shannon index represented the number of species and the inequality between species abundances. It showed that the bacterial abundances of A4f were well balanced and there was a single dominant species in NS. Phylogenetic diversity (PD) was expressed as the

number of tree units which are found in a sample that was used to measure biodiversity at the level of features. The samples in the high germination group had similar feature diversity.

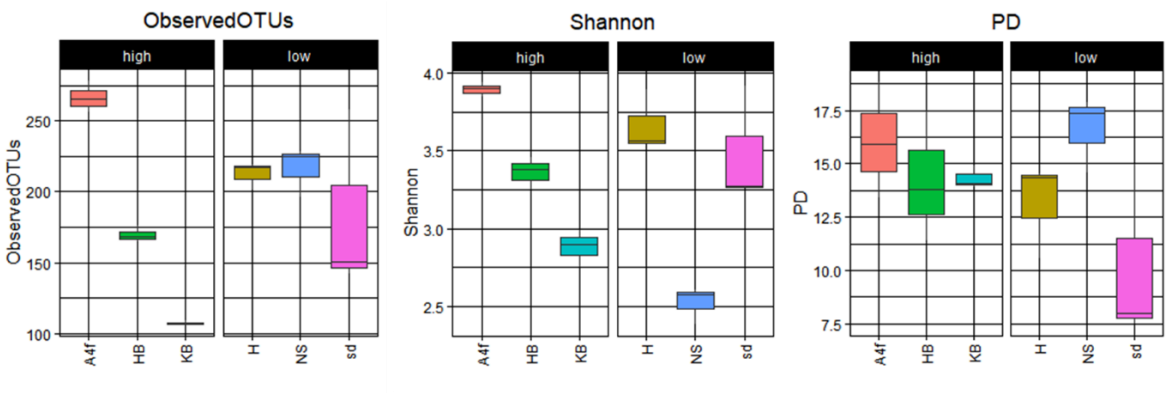

Figure 3.27 Richness and diversity of bacterial communities in different treatments grouped by germination rate. Richness was estimated by number of observed OTUs. Diversity was represented by Shannon's index and Faith's PD (phylogenetic diversity).

Regarding the beta-diversity, principal coordinate analysis (PCoA) was used to reveal the variability of bacterial community composition in different treatments (Fig. 3.28). The samples were grouped by germination rate. This explained 47.1% and 27.9% of the total variance, respectively. The results suggested that the treatments had strong effects on the bacterial community, which in turn had an impact on the germination rate. Some bacterial genera were the key drivers contributing to the difference, which is indicated in the graph (Fig. 3.28), such as *Chryseobacterium, Stenotrophomonas, Delftia* and *Sphingobacterium* which were the most important drivers for the high germination rate group.

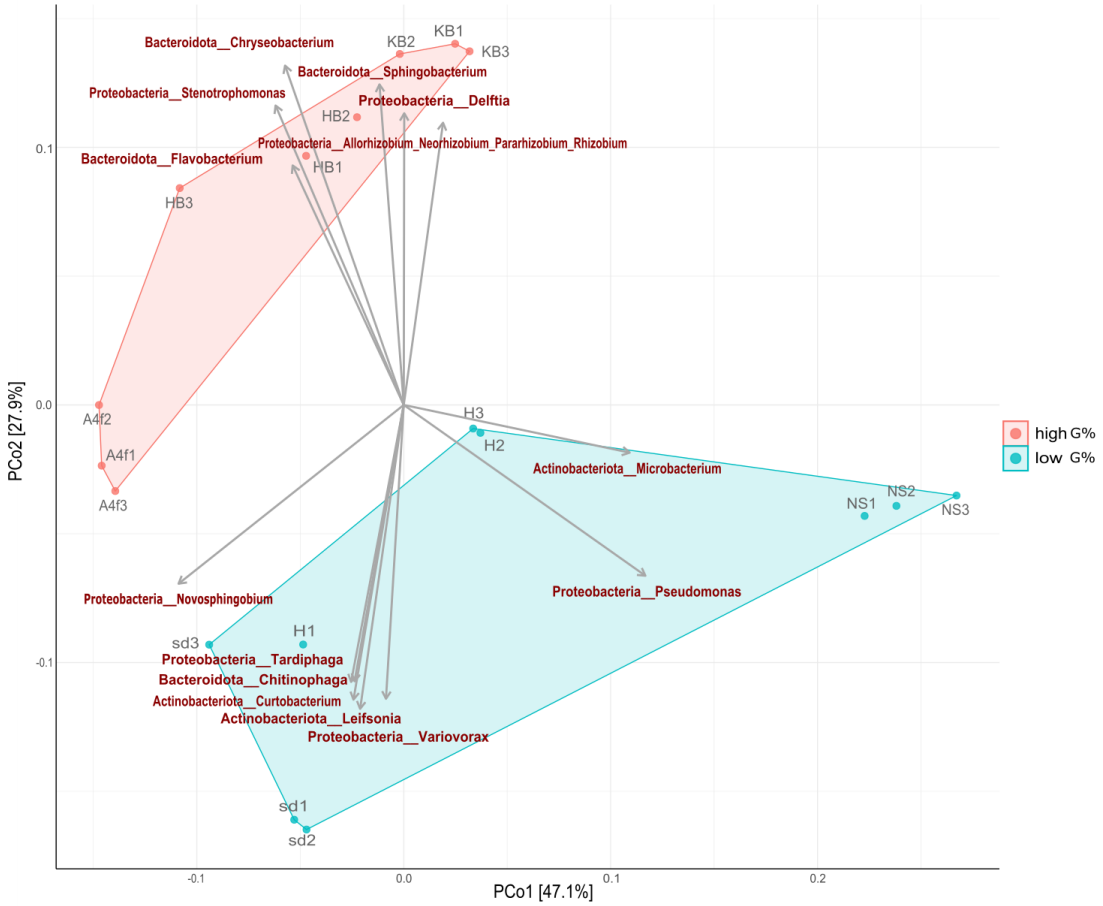

Figure 3.28 Principal coordinate analysis (PCoA) of bacterial community based on high germination rate group (red) and low germination rate group (blue). The percentages of variation explained by each ordination axis. The gray arrows indicate the correlation between bacterial species and the PCoA axes.

To further clarify the stimulation associated bacteria, correlation between germination rates and bacterial relative abundance was calculated. It is clear that the relative abundance of *Stenotrophomonas* and *Chryseobacterium* was positively correlated with spore germination rates (Fig 3.29). The correlation was extremely significant with an R-value of 0.89. The key bacteria, which is able to stimulate the spore germination could belong to these genera.

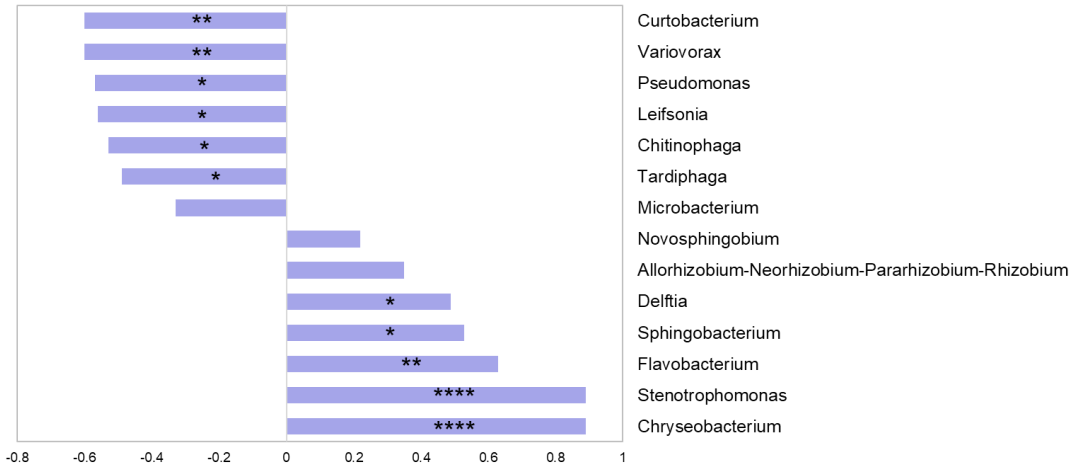

Figure 3.29 Correlation between germination rates and relative abundance of bacteria (Pearson's r, $P < 0.0001$ ****, $P < 0.001$ ***, $P < 0.01$ **, $P < 0.05$*).

3.7.6 Effect of amino acids and sugars

Nineteen L-amino acids (50 mM) were separately mixed with 50 mM KNO_3 to examine their effects on the germination of resting spores. The sterile spores basically did not germinate, while the germination of non-sterile spores could be triggered by some treatments (Fig. 3.30). The resting spore germination started earlier in Asn, Pro and Ser than in other treatments. There was no germination of resting spores in Asp, Cys, Glu and Met. Amino acids alone were not able to stimulate the germination, but the mixture of amino acid and potassium nitrate did. This indicates that the effect of organic nitrogen on the germination of resting spores is different from that of inorganic nitrogen.

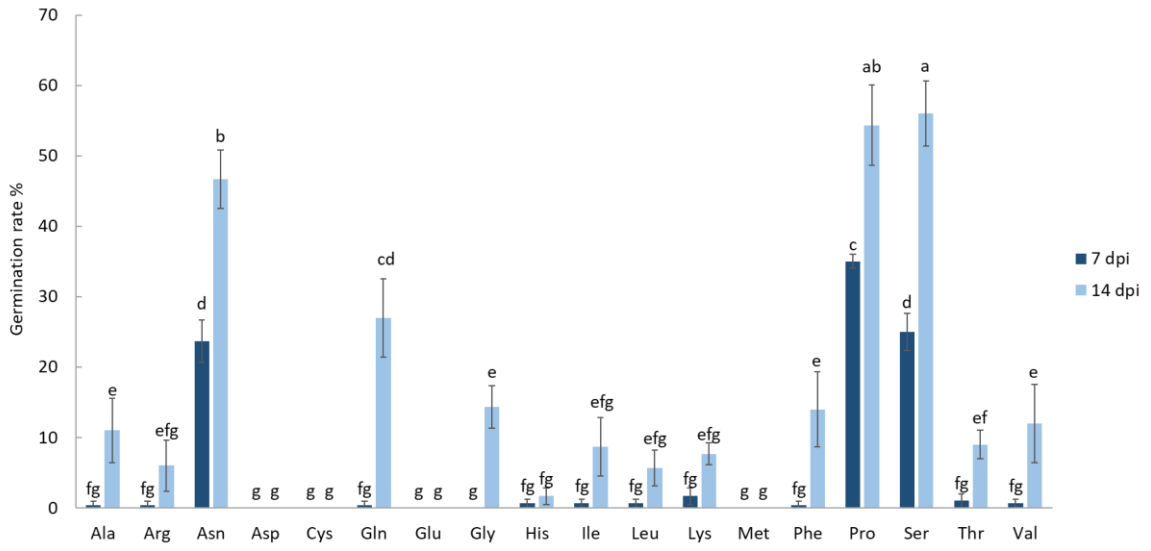

Figure 3.30 Effect of amino acids on *P. brassicae* resting spore germination at 7 and 14 dai. Error bars indicate standard deviations. Different letters indicate significant differences among samples (Tukey test, $P<0.05$)

The effects of different concentrations of various sugars on triggering sterile and non-sterile spore germination were examined with or without addition of potassium nitrate. The results showed that the resting spore germination was not induced without the presence of potassium nitrate. With potassium nitrate, the germination rate of non-sterile spores was much higher than of sterile spores (Fig. 3.31). The sterile spores cultured with 0.02% glucose (11.25%) and 0.2% trehalose (6%) had relatively higher germination rate than those incubated in other sugars. A lower concentration of glucose and soluble starch had bigger impacts on stimulating spore germination than higher concentration, in contrast the germination rate in lower concentration of trehalose was lower. Both concentrations of sucrose had a similar level of germination rate.

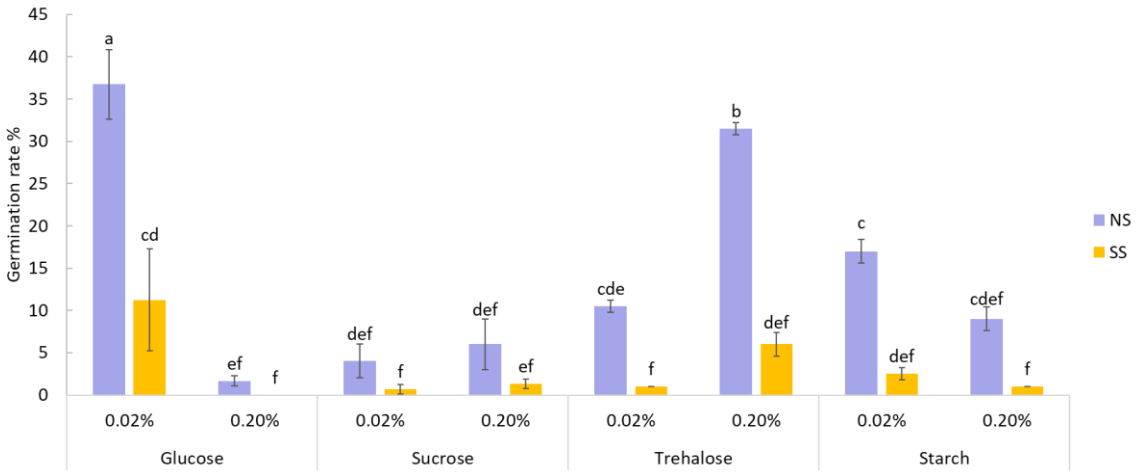

Figure 3.31 The effect of sugars as carbon sources on the germination of resting spores of *P. brassicae* with addition of potassium nitrate. NS: non-sterile spores; SS: sterile spores. Error bars represent standard deviations. Different letters indicate significant differences among samples (Tukey test, $P<0.05$)

3.8 Effect of soil moisture on resting spore germination

The germinated spores could be distinguished from non-germinated spores in soil using DIC microscopy (Fig. 3.32A). The samples had to be diluted to facilitate the observation. The results show that the resting spores incubated with non-autoclaved soil had a much higher percentage of germinated spores. The spores cultured with the autoclaved soil remained dormant even after 60 days of incubation.

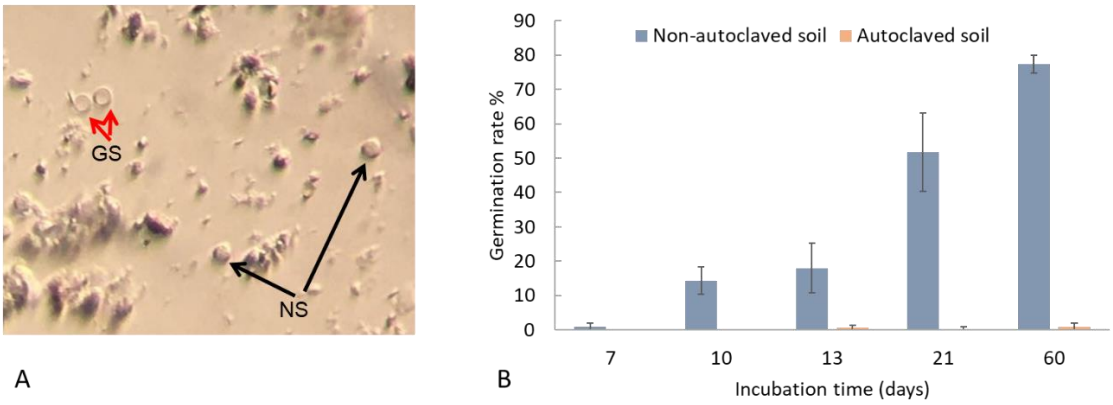

Figure 3.32 Germinated spores (GS) and non-germinated spores (NS) in soil samples under the microscope (A). The germination rate of resting spores cultured in autoclaved and non-autoclaved soil after different periods (B). Error bars represent standard deviations.

The resting spores were incubated in autoclaved and non-autoclaved soil with different levels of water holding capacity. The results (Tab. 3.2) showed that the germination rate was significantly higher when the soil moisture was higher. The resting spores had dramatically higher germination rate in non-autoclaved soil in comparison to that in autoclaved soil, when the soil moisture was greater than 50%. This indicates that soil moisture is one of the environmental factors required in stimulation of resting spores.

Table 3.2 Germination rate of *P. brassicae* resting spores incubated in autoclaved and non-autoclaved soil with different levels of moisture.

Soil moisture	Germination rate %	
	Autoclaved soil	Non-autoclaved soil
6%	0.0±0.0 a	0.0±0.0 a
50%	2.0±1.0 a	28.3±3.1 c
90%	12.3±5.0 b	70.6±2.1 d

Value=mean ± SD; Different letters indicate significant differences among samples (Tukey test, $P<0.05$)

3.9 Effect of nutrients and soil microbiome on disease severity

3.9.1 Plant disease severity

The seeds of *B. napus* cv. Westar were sown in the autoclaved and non-autoclaved soil with the supplement of Hoagland solution or sdH_2O and the development of *P. brassicae* root hair infection was examined. There was root hair infection in 5-day-old seedlings and

the infection was mainly on the oldest 1 cm of the tap root. Primary plasmodia and zoosporangia could be observed in the plant roots cultivated in the inoculated soil and empty zoosporangia were also observed occasionally (Fig. 3.33). After 5 weeks of cultivation, plant disease severity (Fig. 3.34A) was assessed using a 0-3 scale. The disease index (Fig. 3.34B) was highest in non-autoclaved soil with sdH_2O, followed by autoclaved soil with Hoagland solution supplement. The plants cultured in autoclaved soil had a minimum of symptoms. This indicates that soil microbes and nutrients have a crucial impact on the infection with clubroot.

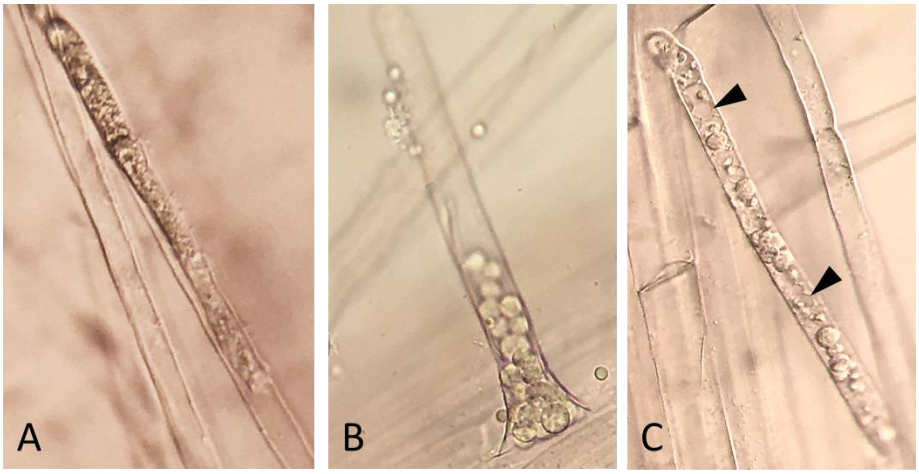

Figure 3.33 The development of *P. brassicae* root hair infection in the roots of *B. napus* cv. Westar. A) primary plasmodium; B) zoosporangium; c) empty zoosporangium indicated with black arrowheads.

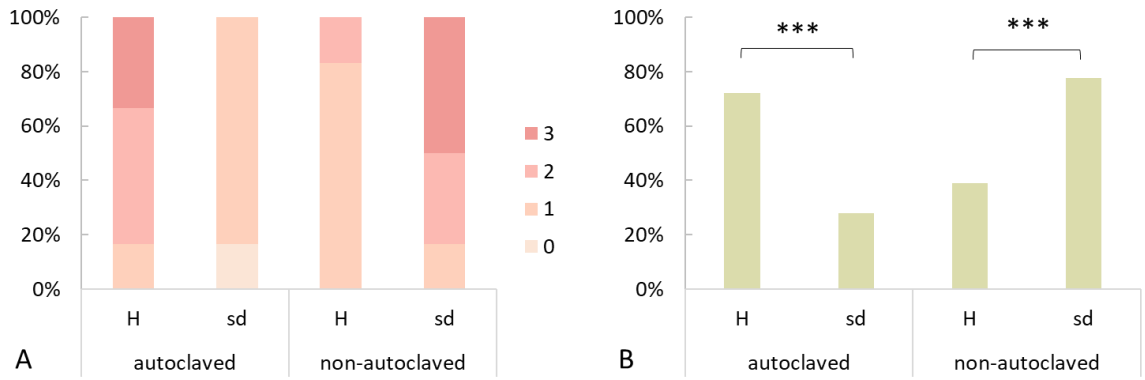

Figure 3.34 Disease severity (0-3; A) and disease index (B) of plants grown in autoclaved and non-autoclaved soil with the supplement of 1/10 strength Hoagland solution (H) or sterilized deionized water (sd). Asterisk indicates statistically significant difference.

3.9.2 Quantification of *P. brassicae* DNA in plants

The seeds were sown in sterilized sand inoculated with non-sterile spores and supplied with various nutrients containing 0.6/6 mM of NO_3^- or NH_4^+. The total amount of *P. brassicae* DNA in the roots in each treatment was quantified by qPCR at 4 and 7 dpi. The DNA amount in each treatment at 4 dpi was generally similar. The DNA amount at 7 dpi significantly increased than that at 4 dpi. The DNA amount of the treatment containing nitrate was higher than the treatment containing ammonium, especially 0.3 mM calcium nitrate and 6 mM potassium nitrate. Calcium nitrate and potassium nitrate had equal molar amounts of nitrate, but they showed different effects that high concentration of calcium resulted in lower amounts of pathogen DNA. The DNA amount in each treatment had similar tendency to the germination rate with various inorganic solutions (see 3.7.1). This indicates a positive correlation of the germination rate of resting spores with infection.

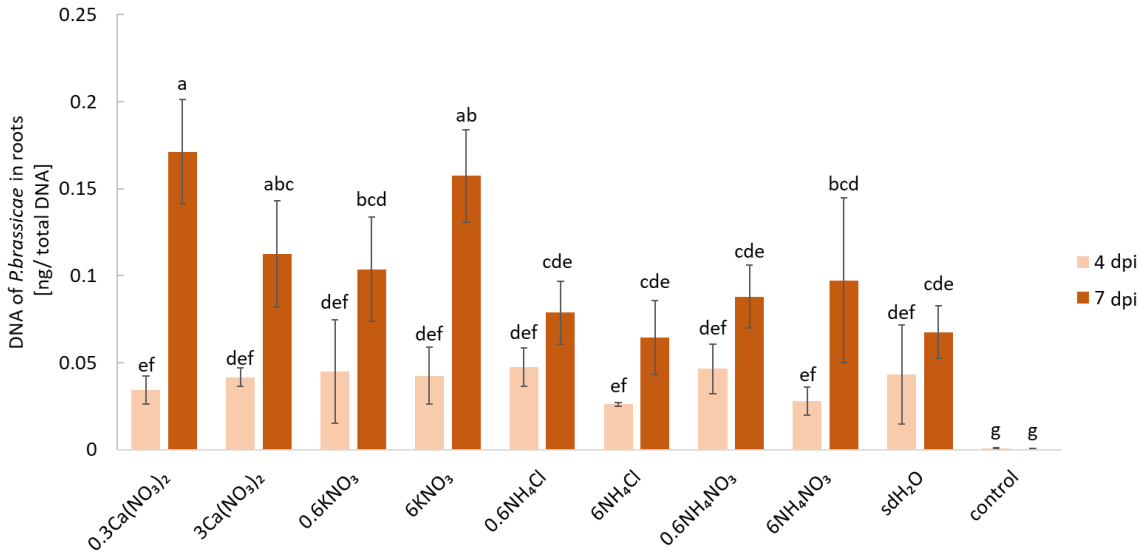

Figure 3.38 DNA amounts of *P. brassicae* in seedling roots of *B. napus* cv. Westar supplied with various nutrients at a concentration of 6 mM/0.6 mM of NO_3^- or NH_4^+ at 4 dpi (light red) and 7 dpi (dark red). Error bars represent standard deviations. Different letters indicate significant differences among samples (Tukey test, $P<0.05$)

The effect of sterile and non-sterile spores with addition of various nutrients on the infection was examined. Figure 3.39 shows that the treatment with 6 mM KNO_3 induced the highest amount of DNA, when it was inoculated with sterile spores. The other treatments inoculated with sterile spores caused a similar level of DNA content in roots. When the seedlings were inoculated with non-sterile spores, the treatments supplied with 0.3 mM $Ca(NO_3)_2$, 0.6 and 6 mM KNO_3 displayed a higher DNA content than the other treatments. This suggests that the bacteria originating from non-sterile spore suspension were also important for *P. brassicae* infection.

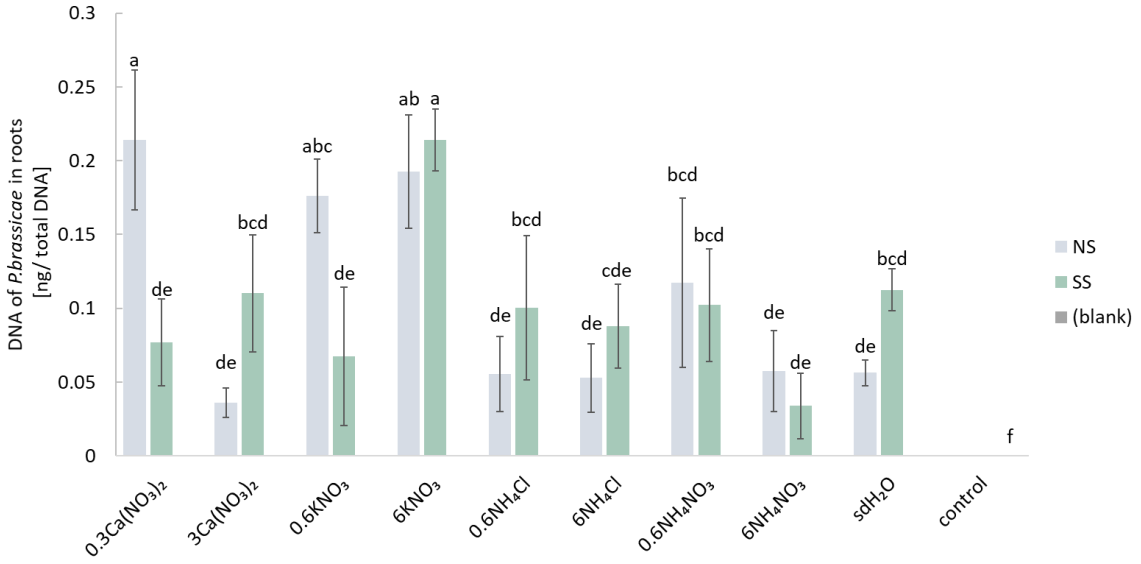

Figure 3.39 DNA amount of *P. brassicae* in seedling roots of *B. napus* cv. Westar inoculated with sterile spores (SS) and non-sterile spores (NS) and supplied with various nutrients at 7 dpi. Error bars represent standard deviations. Different letters indicate significant differences among samples (Tukey test, $P<0.05$)

4 Discussion

The obligate biotrophic parasite *Plasmodiophora brassicae* is the causal agent of clubroot disease that induces the enlargement of roots of Brassica crops. Infected plants become stunted and flowering is accelerated leading to the reduction of seed number and oil quality. Infections with this soil-borne pathogen seriously restrict the production of cruciferous crops and diminishes the land capital value, as currently there are no pesticides available in the EU against this notorious disease. The present study is dedicated to clarifying the biotic and abiotic factors affecting *P. brassicae* resting spore germination in order to facilitate the development of control strategies from novel perspectives. As a first step, the performance of methods for assessing the viability and germination rate of *P. brassicae* resting spore was evaluated, which provided a reliable foundation for the determination of germination stimulating factors. It is generally accepted that root exudates can stimulate the germination of resting spores of *P. brassicae*. However, our research showed certain soil bacteria are more likely to be the direct germination stimulants of resting spores than root exudates. In addition, various further factors were shown to play important roles in modulating the interactions of soil microorganisms and *P. brassicae*. Based on these findings, we established a model illustrating the interaction of *P. brassicae* and its soil environment and propose a novel integrated sustainable control strategy.

4.1 Assessment of *P. brassicae* resting spore viability and germination rate

4.1.1 Viability assessment for resting spores

To provide a good foundation for determining the germination stimulating factors, several methods were evaluated for their performance to assess *P. brassicae* resting spore viability, including acridine orange, fluorescein diacetate, trypan blue, methylene blue, Evans blue and calcofluor white (CFW)- propidium iodide (PI) as well as propidium monoazide (PMA) qPCR. After comparisons, Evans blue and CFW-PI staining methods showed the most reliable results for determining resting spore viability. Evans blue has been widely applied for many studies to distinguish dead cells from living cells as it can penetrate ruptured or destabilized membranes leading to a blue-stained cytoplasm in dead cells, while living cells that maintain membrane integrity appear colorless (Jacyn Baker and Mock 1994; Crutchfield et al. 1999; Lu and Higgins 1999). Evans blue has been used to assess *P. brassicae* resting spore viability by Tanaka et al. (1999). However, Al-Daoud et al. (2017) proposed that the estimated viability obtained by incubating with Evans blue for 30 min was inaccurate. Our results showed that the viability of *P. brassicae* resting spores estimated by Evans blue became stable after 24 hours of incubation (Fig. 3.3), which was consistent with

Harding et al (2019) reporting that a longer incubation time (8 h or more) can enhance the accuracy and consistency of evaluation. Incubation time of less than 4 h resulted in an underestimation of mortality by 40%, introducing experimental error. When the exposure time exceeded 24 hours, there was no significant difference in the Evans Blue estimates, indicating that it had no toxic effect on the spores during the 72-hour incubation period. The intensity of staining was also not affected by incubation time. Therefore, when used to assess the viability of *P. brassicae* resting spores, overnight incubation is more appropriate and convenient for Evans Blue.

Moreover, the cellulose and chitin binding fluorochrome calcofluor white (CFW) has been used in combination with the nucleic acid stain ethidium bromide (EB) to examine the viability of *P. brassicae* resting spores (Takahashi and Yamaguchi 1988; Takahashi 1991; Donald et al. 2002). Due to the mutagenic risks of EB, the cell-membrane impermeable dye propidium iodide (PI), which binds to nucleic acids by intercalating between bases with little sequence preference was chosen as an alternative in our study. It has been reported that 30 µM of PI was cell permeable and cytotoxic to J774 cells after 1 day of incubation (Chiaraviglio and Kirby 2014). A concentration of 10 µM PI can promote partial PI penetration leading to potential false positive results (Krämer et al. 2016). In the present study, a final concentration of 3 µM (2 µg/ml) PI was applied. The dual staining of CFW-PI showed a clear differentiation between viable and non-viable spores (Fig. 3.2). CFW-PI provided stable viability over a long-term exposure of 72 h, suggesting that 3 µM PI was applicable as it did not cause cytotoxicity or excessive permeability to the spores. In addition, dual staining of CFW-PI was capable of determining the viability of resting spores within 15 min. The accuracy of both staining methods was evaluated by the correlation between assessed viability by stain and assumed viability based on ratios of living and autoclaved spores in the given suspensions. The results indicated that both staining methods are capable of reliably predicting the viability of resting spores.

After optimization, the staining methods of CFW-PI and Evans blue were employed for examining the lethal effects of heat and chemical treatments on resting spores. Among the treatments, autoclaved spores showed the highest mortality rate with both staining methods. For each heat and chemical treatment, there was a significant difference in viability estimated by CFW-PI and Evans blue, which could be explained by the difference in the staining time between the two methods (Fig. 3.5). The treated spores estimated with CFW-PI were assessed within 10 min, which was the immediate viability after treatment. However, the spores stained with Evans blue were examined after overnight incubation, which was the final mortality of each treatment. After an overnight incubation, the mortality rate increased significantly, implying that there was a delay in the death of the spores after

treatments. Perhaps, the speed (or accessibility) of staining also depended on the effect of the treatments on the degree of physical damage to the spores, e.g. mortality increased with increasing temperatures.

Furthermore, propidium monoazide (PMA) combined with qPCR has been employed for the quantification of viable cells (Nocker et al. 2006; Nocker et al. 2007a; Nocker et al. 2007b). PMA is a cell membrane impermeable dye that photoreactively binds with high affinity to double-stranded DNA (dsDNA). Upon photolysis, it selectively modifies DNA from dead cells with compromised membrane integrity, while DNA from living cells is unaffected. PMA is known to inhibit PCR amplification of modified DNA templates by removing the modified DNA during purification and by inhibiting amplification of template by DNA polymerase (Nocker et al. 2006).

Several studies have employed heat treated spores for determining the viability of *P. brassicae* resting spores (Al-Daoud et al. 2017; Harding et al. 2019). However, our results showed that heat treatments would affect DNA integrity, which could have an impact on the evaluation for the accuracy of PMA qPCR (Fig. 3.6). To verify the accuracy of PMA qPCR, the spores were treated with acetone to obtain spore suspension in different ratios of viability since acetone treatment does not interfere with DNA integrity. Resting spore viability was calculated using plasmid DNA and the results showed a strong linear correlation between detected viability and assumed viability. PMA qPCR can thus be used to detect the viability of resting spores, but the results were more variable than the staining methods.

In comparison, Evans blue and CFW-PI staining were more cost-effective and accessible approaches to determine *P. brassicae* resting spore viability. While Evans blue was more applicable for long period observation to determine the final viability, CFW-PI was more suitable for tests that require instant detection of viability and also can be applied for detecting the viability of spores in soil. PMA-qPCR is also an option that can be applied for the samples which cannot be determined by staining methods, such as soil samples. Our studies provide multiple options for the assessment of *P. brassicae* resting spores viability that can be applied for various demands.

4.1.2 Germination of resting spores and assessment of germination rates

Resilient *P. brassicae* resting spores containing chitin in cell walls can survive in soil for a long time, germinating and releasing zoospores when conditions are favorable. The emergency of papilla was the first sign of germination. Each spore released one primary zoospore that is capable of infecting plant tissue. Germinated spores showed empty cases

with exit pore. The observed germination process in the present study was consistent with the observation by Macfarlane (1970). However, both studies did not observe the entire process from the emergence of papilla to the release of a zoospore in one resting spore. Therefore, it is not clear how long the whole germination process takes and whether it is a slow and gradual process or an instantaneous one. The movement pattern of zoospores also remains unknown. The tiny size of resting spores (ca. 3 µm) and microbial contaminations in spore suspensions hinder competent and high-quality observation. Live cell imaging using high resolution time-lapse microscopy may be a good alternative for long term observation of the germination process.

Primary zoospores are believed as the main source of infection, but they are not suitable for direct measurement, instead, the presence of empty spores is a good evidence of germination for calculating the germination rates. Reliable methods showing clear distinction between germinated spores and non-germinated spores are essential to assess the impacts of germination stimulating substances that have the potential to control the disease. In previous studies, several methods have been applied for distinguishing germinated from non-germinated spores, such as orcein (Friberg et al. 2005; Rashid et al. 2013). However, orcein did not perform very well in our tests that only a part of the spores were stained. A blue fluorescent nucleic acid stain DAPI has been applied to examine the germination of resting spores by the absence of nuclei of spores (Niwa et al. 2008), which forms fluorescent complexes with AT clusters with one molecule of dye per 3 base pairs (Kapuściński and Szer 1979). *P. brassicae* nuclei stained with DAPI showed bright blue, while germinated spores without nuclei were colorless (Fig. 3.9). The dual staining of CFW combined with a lipophilic fluorescent dye Nile red has been used to detect the structures and development of *P. brassicae* in *B. napus* callus tissues (Tu et al. 2019). Nile red has also been employed to stain the abundant intracellular lipid droplets in *P. brassicae* resting spores (Bi et al. 2016). The absence of lipid droplets could be also considered as an indicator to discriminate between germinated spores and non-germinated spores. However, we found that when CFW-Nile red was used to assess the germination rate of resting spores. Nile red could only stain the lipid droplets of non-viable spores, instead of all the spores containing lipids,. This may be due to the fact that the excessive thickness of the cell wall of the mature spores interferes with the intrusion of the dye. CFW-Nile red method with a prerequisite to inactivate the spores could be used for successful distinguishing the germinated from non-germinated spores (Fig. 3.10). Differential interference contrast microscopy has been employed in several studies (Naiki et al. 1987; Suzuki et al. 1992; Asano et al. 2000), consistently with our results that non-germinated appeared full and germinated spores showing empty cases (Fig. 3.8). After examining the ability of different

methods to differentiate between germinated and non-germinated spores, we selected differential interference contrast microscopy to check the germination rate for further research since it has the advantage of being simple, fast and easy to operate.

4.2 Impact of biotic and abiotic factors on the germination of *P. brassicae* resting spores

4.2.1 Role of root exudates

It has been generally accepted that the resting spores are dormant in the soil and germinate spontaneously at low rates, but the germination of resting spores can be stimulated by various biotic and abiotic factors. It has been shown that root exudates of several host and non-host plants have stimulation effects on resting spore germination under experimental conditions (Suzuki et al. 1992; Rashid et al. 2013). It is interesting to note that the germination rates of resting spores cultured with root exudates from the non-host *Lolium perenne* was higher than those with root exudates from the host plant *B. rapa* var. *pekinensis* (Friberg et al. 2005). A complex hexasaccharide carbohydrate derived from *B. oleracea* root exudates was identified to be capable of stimulating the germination of resting spores (Mattey and Dixon 2015). Based on these findings, we conducted a series of experiments in an attempt to characterize the underlying mechanism of root exudates on stimulation of resting spore germination. A hydrophobic root exudate trapping system (HTS) was established for trapping exudates from undisturbed growing roots under sterile conditions. Similar systems have been applied to collect root exudates in several studies (Hao et al. 2007; Lu et al. 2014; Li et al. 2014). A work flow for non-target profiling analysis of root exudates from HTS using LC-QTOF-MS was established. Approximately 60% of compounds were consistently detected in two independent experiments. The data provided a valuable basis for the characterization and quantification of root exudates in subsequent studies. The profile of detected compounds revealed a high metabolic diversity of root exudates from different plant species (Fig. 3.11). It showed that this system could be applied as a model system for the investigation of root exudation.

The root exudates collected by HTS as well as PDC were used for bioassays. Crude spore suspension (with bacterial contamination) and surface-disinfected spore suspension (without bacterial contamination) were used for examining the stimulation effects of root exudates. The results showed that the pure root exudates collected from HTS and PDC under sterile condition cannot stimulate the germination of sterile *P. brassicae* spores. Only the root exudates from HTS resuspended with Hoagland solution somehow stimulated the germination (Fig. 3.12). The effects of XAD4 and XAD8 resin eluates on stimulation of spore germination were similar and there was no host specificity, despite the fact that the

composition of root exudates from different plant species was variable. The effect of root exudates collected by other methods were also tested, showing consistent results with root exudates from HTS and PDC that the sterile spores with pure root exudates did not germinate (data not shown). We therefore suggest that the root exudates has no stimulation effects on spore germination which was contrary to several previous studies. However, this could be a reasonable explanation for the low efficacy of bait crops or crop rotation with non-host plants on reduction of resting spores in the greenhouse and field trials (Friberg et al. 2006; Ahmed et al. 2011). *P. brassicae* is an obligate biotrophic parasite, host plants are essential for the completion of its life cycle. However, resting spores germination and primary zoospores infection are two distinct processes. The occurrence of resting spore germination may be not limited to the presence of host plants. It has been reported that the formation of primary plasmodium was observed in some non-host plants, but this does not allow the pathogen to complete its life cycle (Ludwig-Müller et al. 1999; Liu et al. 2020a). Our data indicate that host plants may be essential for pathogen propagation, but not for resting spores germination. Moreover, only the combination of root exudates and Hoagland solution can trigger the germination of non-sterile resting spores, which emphasizes the importance of nutrients and bacterial contamination in spore suspension on inducing spore germination.

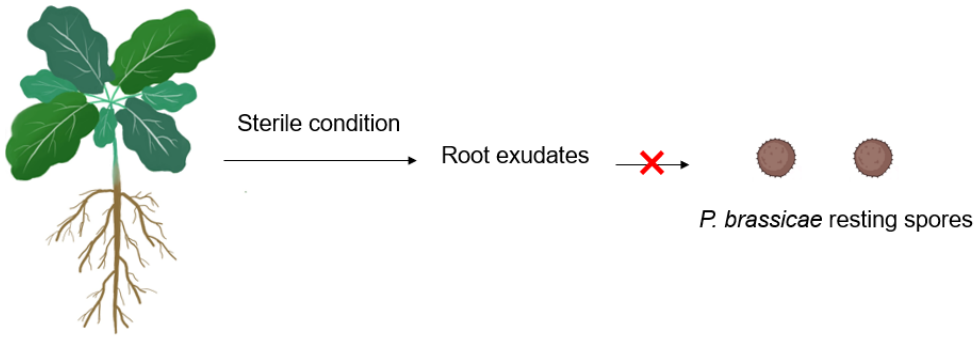

Figure 4.1 Effect of root exudates. Pure root exudates collected under sterile condition cannot stimulate the germination of *P. brassicae* resting spores.

4.2.2 Role of soil microbes and soil nutrition

In most soils, fungal germination and growth are inhibited to a certain extent, the phenomenon known as soil fungistasis (Lockwood 1977). It has received considerable attention because of its association with a widespread suppression of soil-borne fungal diseases. The intensity of fungistasis is dependent on the physical and chemical soil properties as well as soil microbial activity (Qian 1987; Alabouvette 1999). There are several hypotheses about the causal mechanisms of fungistasis caused by microorganisms such

as competition leading to carbon limitation (nutrient deprivation hypothesis) and production of antifungal compounds (antibiosis hypothesis) (Ho and Ko 1986; Liebman 1992; Zou et al. 2007). Although soil fungistasis has not been recognized in *P. brassicae* resting spores, Takahashi (1994a) mentioned that the long term survival of resting spores seemed to be associated with activity of soil microorganisms that may correspond to soil fungistasis. To clarify the impact of soil fungistasis on *P. brassicae* resting spore germination, the filtered and non-filtered soil suspensions from grassland, field and greenhouse soil samples were examined. Unexpectedly, we found that the germination rate of non-sterile resting spores incubated with non-filtered soil suspensions was significantly higher than that with filtered soil suspensions from grassland soil after 18 days of incubation (Fig. 3.13). The soil suspensions from an oilseed rape field soil showed a similar tendency that the resting spores in non-filtered soil suspensions had a higher germination rate (Fig. 3.14). This indicates that the presence of soil microbes is essential for the stimulation of resting spore germination, since the most significant difference between filtered and non-filtered soil suspensions is the presence or absence of soil microorganisms. In addition, the occurrence of germination was earlier in field soil suspensions than that in grassland soil suspensions. After 5 days of incubation, the germination rate of spores was 30% with rhizosphere soil suspensions, 44% with bulk soil suspensions and only 1% with grassland soil suspensions. After 7 days of cultivation, there was no significant difference in the germination rate between rhizosphere and bulk soil suspensions. This might be explained by the differences in initial microbial communities in different non-filtered soil suspensions.

From the results of non-filtered soil suspensions from greenhouse soil (Fig. 3.15), the presence of soil suspensions from soil with healthy plants lead to a strong suppression of the germination of both sterile and non-sterile resting spores. In contrast, suspension from soil without plants had apparently stimulating effects on resting spore germination. The germination rate in the soil with *P. brassicae* inoculated plants was moderate and there was no significant difference in germination rate between the sterile and non-sterile spores. Comparing the germination rates in different greenhouse soil suspensions was, the differences may be attributed to the diversity of microbial communities in soil samples. It is well known that plants are able to alter the soil microbial composition, structure and diversity. The soil microorganisms are recruited by the plants to facilitate nutrient acquisition, mitigate stress and enhance the defense to pathogen (Mendes et al. 2011; Naylor and Coleman-Derr 2017; Backer et al. 2018). Soil with healthy plants may contain certain microorganisms that are able to suppress *P. brassicae* germination. This implicates the presence of intense microbial competition. For the filtered soil suspensions from greenhouse soil, there was a similar tendency of germination rate as the non-filtered soil suspensions, except for the

samples of soil without plants where non-sterile spores showed dramatically higher germination rate than the sterile spores. Overall, the germination rate in the non-filtered soil suspensions was higher than that in the filtered soil suspensions, which was consistent with the other two soil suspensions tests. This again highlights the role of soil microorganisms (originated from spore suspension as well as from soil) on stimulating the germination of resting spores.

To further verify the role of soil bacteria, several bacterial strains were isolated and tested *in vitro* to examine their effects on resting spore germination. We found that high concentrations of bacterial suspension with addition of Hoagland solution was able to trigger the germination of resting spores and the germination rate increased with incubation time (Fig. 3.16). More bacterial isolates isolated from soil were tested that showed similar effects, the presence of bacterial suspension and Hoagland solution triggered the germination of resting spores (Fig. 3.17). Interestingly, all tested isolates were able to stimulate the germination to some extent and there were no common characteristics among them (Tab.3.1). In addition, the importance of Hoagland solution on spore germination was confirmed. After individual compounds of Hoagland solution have been examined, compounds containing nitrate were identified to induce a much higher germination rate than the other compounds and high concentrations of calcium suppressed the germination (Fig. 3.18). This indicates that nitrate plays an important role in triggering the germination and the concentration of calcium was related to the germination. In Palm Elmer Thurman's thesis (1958), the effects of mineral nutrition on invasiveness and development of *P. brassicae* were studied and the results are consistent with the present findings on the effects of calcium and potassium. Several former studies suggested that application of calcium may significantly reduce the germination rate of resting spores, as well as root-hair infections and therefore liming was capable of decreasing disease severity (Murakami et al. 2002; Tremblay et al. 2005; Niwa et al. 2008). Additionally, low concentrations of nitrite had similar stimulation effects as nitrate, whereas ammonium displayed low stimulatory potential on the germination of resting spores.

To clarify the role of nitrate, $K^{15}NO_3$ isotope analysis was performed. It showed that the resting spores slightly adsorbed nitrogen after 7 days of incubation, but this uptake did not correlate with germination rate (Fig. 3.20). This demonstrates that nitrate is not directly involved in the stimulation of germination, but plays another role. *P. brassicae* resting spores lack genes encoding for proteins involved in nitrate uptake and also lost the capacity to synthesize a number of amino acids (Schwelm et al. 2015; Rolfe et al. 2016), so it is reasonable that nitrate is not the direct stimulation factor.

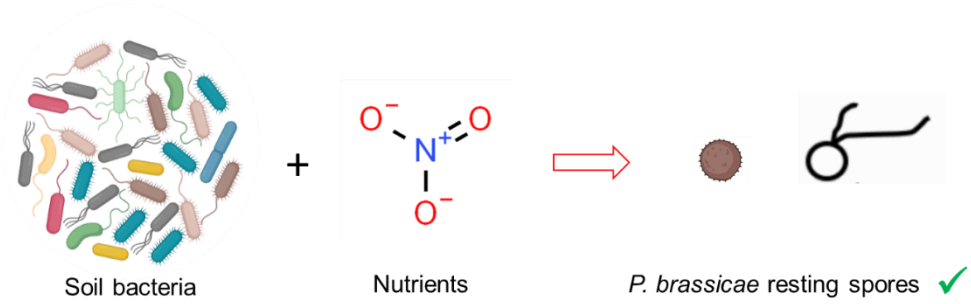

Figure 4.2 Effect of soil bacteria and nutrition. The presence of bacteria and nitrate is able to trigger the germination of *Plasmodiophora brassicae* resting spores.

Additionally, we noticed that most of tested bacterial isolates were able to reduce nitrate to nitrite and nitrate reduction was detected in the bioassay samples. Therefore, *Bacillus subtilis* mutants were used to check if nitrate reduction was related to the germination. The *narG* mutant strain THB1 is unable to perform nitrate respiration and the *nasD* mutant strain 1972 is unable to convert nitrite into ammonia. *B. subtilis* wild-type strain JH642 was used as control. However, there was no significant difference in the germination rate between the mutants and the wild type (Fig. 3.21). This suggests that the germination of resting spores was not affected by the different nitrate related properties of the mutants, as they induced similar germination rates as the wild type. To understand the role of added bacteria, three bacterial strains were autoclaved to exclude their bioactivity, however, their presence was still able to stimulate the germination as long as a nitrogen source was added, leading to similar effects as living bacteria (Fig. 3.22). This indicates that the activity of additional bacteria is not essential for stimulation of germination. Further experiments with bacterial filtrates showed that the combination of bacterial culture filtrate and Hoagland solution was able to stimulate the germination of non-sterile spores, while sterile spores remained dormant (Fig. 3.23). Actually, these data emphasized the importance of the relationship between nitrate and the added bacterial suspension as well as the non-sterile spore suspension. The bacteria originating from spore suspension displayed substantial impacts. Macfarlane (1970) also mentioned that something in the resting spore suspension was important for the germination which was not readily replaced by the presence of host plants.

To clarify the role of added bacterial suspension or bacterial culture filtrate and nitrate, 16S rRNA gene amplification and sequencing were conducted to explore the bacterial community in different treatments. There was no 16S rRNA gene amplification in the sterile spore samples that demonstrated the spore suspension after surface-disinfection was excluded from bacterial contamination. The germination rate of each sample was checked that the samples with addition of bacterial suspension or bacterial filtrate and nitrogen

source had much higher germination rates than the Hoagland and sdH_2O control (Fig. 3.24). After sequencing, the results of non-sterile spore suspension represented the initial bacterial community in it and the other treatments showed the altered bacterial community after 7 days of incubation (Fig. 3.26). The phyla Proteobacteria (91.7%) and Actinobacteriota (7.4%) dominated in the initial bacterial communities (NS). After 7 days incubation, the portion of Bacteroidota increased leading to substantial changes in the ratio of Proteobacteria and Bacteroidota in each treatment. Interestingly, Proteobacteria and Bacteroidota ratios were lower in the samples with higher germination rates (A4f, HB, KB) than that with lower germination rates (H, sd). At family level, the diversity of bacterial community was clearly higher than in the initial community after 7 days incubation. In particular, the average relative abundance of the families *Weeksellaceae* and *Flavobacteriaceae* was considerably higher in the treatments with higher germination rates. It has been reported that the microbial community composition is important in the development of fungistasis (Boer et al. 2003). Based on our results, we conjecture that there might be certain bacteria (stimulation associated bacteria) that are capable of stimulating the germination of resting spores. In addition, we assume the existence of some other bacteria (stimulation suppressing or non-stimulating bacteria) which are competing with the previous group, resulting in an intense arms race between them. In our experiments, the spore germination was only triggered, when the portion of stimulation associated bacteria reached certain relative abundance or absolute abundance within the total microbial community.

Alpha and beta diversity analysis revealed the variation of bacteria within a single sample and between samples, respectively. The bacterial community composition of different treatments can be classified into two groups based on the germination rates. The diversity analysis demonstrated a dissimilarity of the bacterial community between the high and low germination rate groups. In the high germination rate group, the genera *Chryseobacterium, Sphingobacterium, Stenotrophomonas* and *Delftia* were the most significant drivers for the dissimilarity of two groups. Interestingly, *Pseudomonas* is one of the crucial factors in the low germination group and *Pseudomonas* has been suggested to be related to the development of fungistasis (Boer et al. 2003; WU et al. 2008). The correlation between relative abundance of bacteria and germiantion rates indicated that the genera of *Stenotrophomonas* and *Chryseobacterium* may be essential for triggering resting spore germiantion. The germination could be activated through biological reactions or as a response to bacterial metabolites. The underlying mechanism of bacteria induced *P. brassicae* resting spore germiantion still needs to be further explored.

For developing novel control strategies, it is crucial to characterize the specific stimulation associated bacteria and the underlying interaction on the stimulation of *P. brassicae* resting spore germination. 16S rRNA gene amplicon sequencing only provides the relative abundance of bacteria in each sample. It is difficult to compare the difference between the samples without the information on quantitative changes. Absolute abundance quantification of microbial community can be achieved through combining of qPCR and high-throughput sequencing techniques (Lou et al. 2018; Tkacz et al. 2018; Jian et al. 2020; Zemb et al. 2020). The absolute abundance provides a more comprehensive understanding of the dynamics and interactions of the microbiome. The absolute amount of changes of certain bacteria groups could be one of the key factors in regulating resting spore germination. Not only the soil bacterial community composition, structure and diversity may contribute to the stimulation effect, but also the functional characteristics of bacteria might be relevant. It is intuitive and generally accepted that the functional traits assigned to the microbiomes are more informative and intriguing than taxonomic information. Phenotyping and functional potential of microbial communities can provide meaningful insights into the possible mechanisms driving pathogen-microbe interactions. It is beneficial to identify the associated genes that are responsible for the triggering of resting spore germination and such knowledge could provide a powerful approach to generate novel control strategies.

Through bacterial community analysis, we recognized that either bacterial suspension or filtrate as well as nitrate added in the bioassay samples were carbon and nitrogen sources, which affected the bacterial community. In other words, when certain carbon and nitrogen sources are applied, the initial bacterial community in the spore suspension will shift to a suitable condition for the resting spore germination (Fig. 4.3). Furthermore, the results from studies with various amino acids and sugar also support this hypothesis. Amino acids alone were not able to stimulate germination, but the mixture of some amino acids (carbon source) and potassium nitrate (nitrogen source) did (Fig. 3.30). Sugars probably played a role as C source in a similar way. When combined with nitrate, their presence likely changed the initial bacterial community leading to the stimulation of germination. The diversity of carbon sources and their concentrations or the carbon to nitrogen ratio might be the important factors in reshaping the bacterial community and it may also be related to the initial bacterial composition and diversity. The time required to reach a suitable bacterial community for germination depends on the interaction of carbon source, nitrogen source and the initial bacterial community. Ohi et al. (2003) found that caffeic acid, coumalic acid and corilagin stimulated the germination of resting spores, but we failed to reproduce these results (data not shown). In our study, 20 mM citric acid, succinic acid and lactic acid were examined that seemed to have a lethal effect on the spores rather than stimulating the germination due to

their low pH (data not shown). This still does not exclude an effect of organic acids on reshaping the bacterial community, since the composition of root exudates is diverse. It has been reported that different bacterial strains presented distinct preference for certain amino acids (Liu et al. 2020c). Bacteria possess two strategies of carbon source utilization, one after another (diauxie) and simultaneously consumed (co-utilization), and make choices dependent on the available carbon source (Wang et al. 2019). Microbes coordinate uptake and utilization of nutrients to adapt to complex nutritional ecosystems and this dynamic process affects their growth rate (Zampieri et al. 2019). Therefore, the preferences of microbes could be a good explanation for the different germination rates obtained in various nutrients treatments. We speculate that stimulation associated bacteria prefer certain carbon sources and nitrate, which help them take a dominant position in the soil microbial competition and thus affect the germination of *P. brassicae* resting spores.

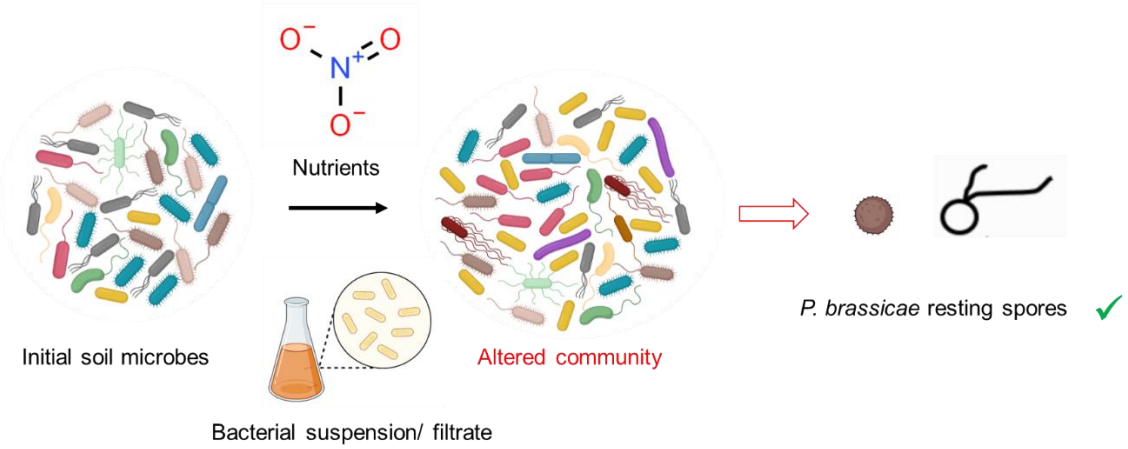

Figure 4.3 Role of carbon sources and nutrition. The initial soil microbial community is affected by various nutrients and carbon sources. When a certain bacterial community is shaped, in which the stimulation associated bacteria increase, the germination of *Plasmodiophora brassicae* resting spores is stimulated.

4.3 Ecological factors affecting *P. brassicae* in soil

It is generally accepted that *P. brassicae* spores remain persistent and dormant in the soil to withstand adverse conditions and the dormancy appears to be broken by the compatible host. However, our results revealed that certain stimulation associated bacteria were the key factors on triggering the germination of resting spores instead of the presence of host plants. Under natural conditions, highly diverse microbial communities are present in soil and the germination state of *P. brassicae* resting spore is still unclear. *P. brassicae* in soil was usually detected by bait plant bioassays, fluorescent microscopy and qPCR, which have been used to assess the viable spores and the total amount of spores(Takahashi and Yamaguchi 1989; Wallenhammar 1996; Wallenhammar et al. 2012). Few studies have

monitored the spore germination rate in soil, thereby the resting spores were mixed with autoclaved and non-autoclaved soil to verify the germination status in our study. The results showed that the germination rate could reach up to 80% after 60 days of incubation, while the spores in autoclaved soil remained dormant (Fig. 3.32). An interesting question that arose was whether the spores have such a high germination rate in natural soil, which they will gradually become extinct without the help of a host to propagate. However, regarding the consistent infection in the field, this may not be the case. Besides the higher germination rate in the non-autoclaved soil, we found that soil moisture was one of the environmental factors affecting the germination rate (Tab. 3.2). Soil moisture is a dominant environmental factor in interactions with *P. brassicae* (Dixon 2009a), which is important for the infection and development (Dobson et al. 1982; Gossen et al. 2014), but not affecting the spore viability (Takahashi 1994b). It is also known that soil moisture has an impact on the soil microbial community, with drought having a much stronger effect on bacterial than on fungal networks (Naylor and Coleman-Derr 2017; Vries et al. 2018).

B. napus seeds were sown in the inoculated autoclaved and non-autoclaved soil and supplied with Hoagland solution or water. During cultivation, different infection stages can be observed simultaneously in the same samples (Fig. 3.33). The disease severity assessment showed interesting results that plants grown in autoclaved soil were more affected when supplied with Hoagland solution, while the opposite was true in non-autoclaved soil (Fig. 3.34). Nutrients had different impacts on plant disease severity when the soil microorganisms were various. It seems that nutrients suppress the disease development in non-autoclaved soil, but assist the disease development in autoclaved soil. This is probably because nutrients contribute to the growth of stimulation associated bacteria in autoclaved soil, since there are much less competitors. On the contrary, nutrients are more beneficial for the growth of competitors in non-autoclaved soil rather than the stimulation associated bacteria. There is an intense microbial-microbial arms race. Moreover, plants also behave differently under different conditions, affecting their metabolism and root architecture. This may lead to different levels of resistance. The effects of nutrients and bacteria on *P. brassicae* infection were also examined. We found these inorganic compounds had similar impacts on the germination of resting spores and the infection of *P. brassicae*. The bacteria originating from spore suspension also influence the infection of *P. brassicae*. It seems that the amount of *P. brassicae* in seedlings is positively correlated with the germination rate and the relationship of *P. brassicae* and nutrients is merely disturbed by the presence of plants. It is evident that calcium has a great impact on the germination rate as well as on the infection and development of *P. brassicae,* which has been supported by several studies (Webster and Dixon 1991; Dixon and Page 1998). It has

been observed that calcium application was not fungicidal to the pathogen, the effects were more likely to create unfavorable conditions for the pathogen (Myers 1985; Webster and Dixon 1991). Calcium also plays a role on regulating plant defense (Zhang et al. 2014; Thor 2019). The relevant suppression mechanisms are still unclear and the role of calcium in affecting bacterial community need to be further studied.

In present study, a series of experiments has been conducted to investigate the influence on resting spore germination in the perspective of abiotic and biotic factors. We propose a model to elucidate plant-microbe-pathogen interactions associated with breaking spore dormancy in soil (Fig. 4.4). Firstly, the pure root exudates collected under sterile conditions cannot stimulate the germination of resting spores that have no direct stimulation effect on the resting spores. Secondly, root exudates are believed to be a kind of carbon source affecting the initial soil microbes. Thirdly, the initial soil microbes are influenced by various factors including carbon source, nutrients and soil moisture to shift to a favored condition leading to the germination of resting spores. From the perspective of *P. brassicae* resting spores, there is a progressive relationship. Resting spores have direct interactions with soil bacteria, which are regulated by plants and other environmental factors. We suppose that this could be a sophisticated strategy for the long-term survival of *P. brassicae*. Under natural conditions, bacteria display very rapid responses to environment changes to ensure growth and survival. They constantly sense and rapidly adapt to the metabolite and nutrients changes (Massalha et al. 2017; Zampieri et al. 2019). Therefore, the characteristics of bacterial communities reflect the surrounding conditions. If resting spores only react to a specific bacterial pattern, they can save a lot of energy to maintain longevity instead of sensing the environment and determining which conditions are favorable. The stimulation associated bacteria could be considered to have a role of a messenger to wake up the resting spores.

Wet soil provides a good condition for zoospore movement and sustains the growth of plants. The optimal temperature for *P. brassicae* is 20-25 °C, which is also suitable for the growth of cruciferous crops. It is interesting to note that nitrate is the most important nitrogen source for forming germination associated bacterial community rather than ammonium. Nitrate is the most prevalent reactive form of nitrogen required by plants, although ammonium and amino acids are also present (Filleur et al. 2005; Gent and Forde 2017). Plants regulate the growth of the lateral roots into NO_3^- rich patches (Drew 1975; Zhang and Forde 1998). Plant engagement with microorganisms can enhance nutrient acquisition (Wang et al. 2018; Oldroyd and Leyser 2020). Moreover, nitrate is always present in the soil solution and moving with runoff water, while ammonium ions attach on the soil cation exchange complex that limits its mobility. We suppose that nitrate or its associated bacteria may serve as an

indicator for *P. brassicae* for the presence of roots. Calcium may influence the function of bacteria involved in spore germination and also regulate the plant defense, leading to the suppression of disease.

Another main factor is the presence of host plants, which are essential for *P. brassicae* to complete the life cycle. A concept of 'holobiont' has been proposed (Vandenkoornhuyse et al. 2015) that plants and their associated microbiota are considered as a single entity, whereby evolutionary selection between plants and microorganisms contributes to the overall stability of the system. It has been noted the presence of 'core microbiota', a subset of microbial lineages which reproducibly associates with a particular host in almost all the communities (Fitzpatrick et al. 2018; Hamonts et al. 2018; Roman-Reyna et al. 2019). Within the core microbiota, the 'hub microorganisms' which are substantially more connected within a co-occurrence network may represent keystone species that can influence the community structure by strong biotic interactions with the host or with other microbial species, rather than simply through their own high abundance (Agler et al. 2016; Hamonts et al. 2018). These hub species possess powerful direct and indirect impacts on microbiome assembly and perform a mediating role between the plant and its associated microbiome. We speculate that these hub species may have the potential to be the lighthouse for *P. brassicae,* which help it to locate the position of host plants, although the relationship between hub species and stimulation associated bacteria is unclear.

Soil microbiota represent a great reservoir of biological diversity that act as the seed bank for root microbiome assembly (Armalytė et al. 2019). The rhizosphere is a hot spot of microbial interactions as root exudates are the primary food source for soil microorganisms and also provide a basis for communication and recognition that direct their population density and activities (Raaijmakers et al. 2009; Zhalnina et al. 2018). The composition of the root exudates is dynamic and varies depending on plant species, growth stages, stress conditions, nutrition and soil types, and root traits, among other factors (Zhalnina et al. 2018). Therefore, the rhizosphere microbiome composition primarily modulated by root exudates also represent the surrounding conditions. All these ecological factors are represented by specific bacterial community patterns that will increase the successful rate of infection of *P. brassicae*. Instead of constantly sensing and adapting the environmental changes by *P. brassicae* itself, directly utilizing bacteria may be a more cost-effective way to survive. The interaction of *P. brassicae* with its surrounding environment could be the result of an ecological evolution during the long-term natural selection. The underlying mechanisms for this complicated interaction are only just beginning to be understood.

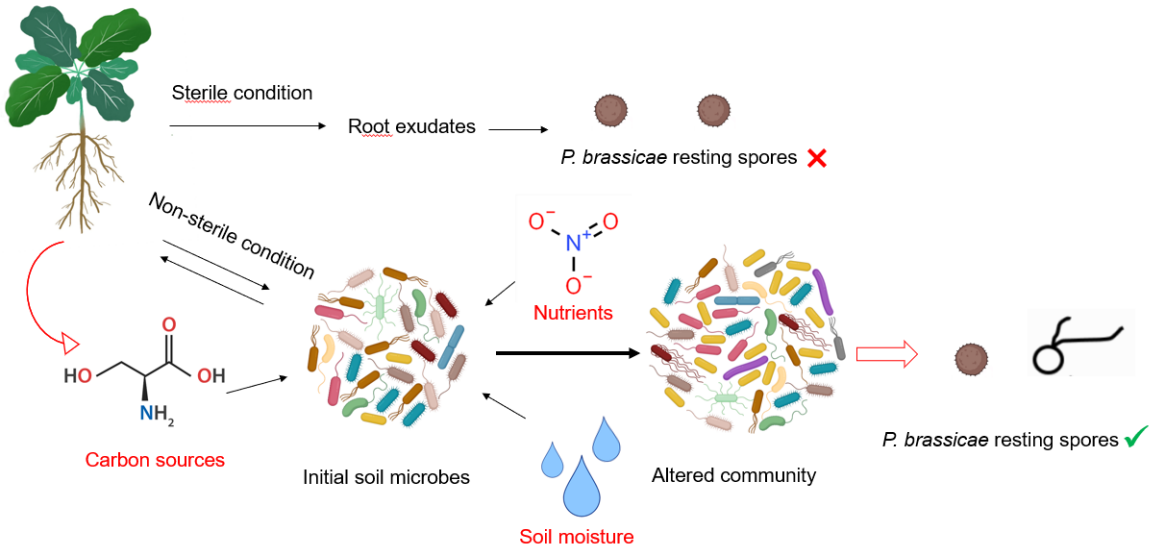

Figure 4.4 Factors affecting the germination of *Plasmodiophora brassicae* resting spores. Root exudates collected under sterile condition cannot stimulate the germination of resting spores, whereas they can serve as a carbon source to modulate the microbial community under non-sterile conditions. The initial microbial community is reshaped by various factors (e.g. carbon sources, nutrients and soil moisture) to a community favoring the germination of *Plasmodiophora brassicae* resting spores.

A better understanding of this complex interaction gives us more targeted directions for the effective control of clubroot disease. We are poised to use the knowledge generated in this model system to optimize integrated control strategies. The present results clarify the importance of bacterial communities in the soil on the germination of *P. brassicae* resting spores and the presence of strong microbe-microbe competition. Synthetic communities (SynComs), artificial assembly for certain purposes, could be introduced as a powerful tool to control clubroot disease. Microorganisms have been applied as inoculants for biocontrol for many years, but the field efficacy is limited to environmental factors (Lahlali et al. 2013; Zhou et al. 2014). Many biocontrol products are single isolates from different environments, thus the competitiveness is probably not sufficient to antagonize the indigenous microbiome. The highly inconsistent performance of microbial inoculants suggests that the functionality and persistence of microorganisms are dependent on interactions with the environment as well as with other microorganisms within a community (Trivedi et al. 2020).

Explicit consideration of the fundamental ecological processes underlying the relationship of complex microbial communities and *P. brassicae* is still in its infancy. However, it is essential for the rational design and manipulation of microbial communities in agricultural systems. The most significant advantage of SynComs is that changes can be implemented not only at the microorganism level, but also at the functional level. SynComs with different

complexities can be designed as practical needs. For example, application of Synthetic communities consisting of stimulation associated bacteria before sowing may be able to trigger the germination of resting spores in order to reduce the soil inoculum, and SynComs of microorganisms with broad, persistent and durable plant-growth-promoting traits have the potential to suppress stimulation associated bacteria and enhance plant resistance thereby alleviating disease severity. SynComs constructed on the basis of knowledge of the microorganisms in disease suppressive soils have been reported to control diseases in field condition (Mendes et al. 2011; Santhanam et al. 2015). Priority effects can also be utilized to engineer and manipulate the dynamics of soil and plant-associated microbiomes in order to facilitate the control efficacy (Toju et al. 2018).

To effectively harness the microbiome, other approaches to manage the system are required, such as disease prediction, fertilizer application, cultural practices and plant breeding. The disease prediction and expected yield loss are the foundation upon which integrated clubroot control strategies can be implemented. In recent years, molecular methods have been developed to detect *P. brassicae* in soil (Faggian et al. 1999; Wallenhammar and Arwidsson 2001; Cao et al. 2007; Faggian and Strelkov 2009; Gossen et al. 2019). The emergence of high-throughput sequencing technologies has provided unprecedented information about the microbial composition of diseased versus healthy soils (Liu et al. 2018; Lebreton et al. 2019; Daval et al. 2020; Da Saraiva et al. 2020). The development of a model for disease prediction can be a powerful decision support tool, when combined with key common features of clubroot associated soil microbiome and local information. Soil nutrition is important for plant growth. While nitrate has stimulatory effects on resting spore germination, ammonium is a better choice for inorganic nitrogen supply. However, the presence of nitrifying organisms can lead to conversion of ammonium to nitrate. SynComs may slow down the conversion. Potassium and calcium have different roles for the pathogen and plants, a balance of fertilizer application needs to be provided in order to maintain the plant growth and suppress the disease (Walker and Hooker 1945; Dixon and Page 1998). Long period crop rotation and continuous fallow are useful methods to reduce soil inoculum, as we recognize that the presence of plants is not essential for resting spore germination when the conditions are favorable. The absence of host plants impedes the accomplishment of the pathogen life cycle resulting in poor generation of new resting spores, while the germination of resting spores continually reduces soil inoculum. Crop rotation needs to be maintained for a long enough period due to the large population and slow germination rate of spores in soil. Crop rotation is also beneficial for establishing new soil microbiomes to compete with stimulation associated bacteria. Together with the varied SynComs, it can accelerate the reduction of *P. brassicae* or enhance the microbial

competition to suppress it. Many studies are dedicated to finding resistance genes and most resistance sources are race-specific (Diederichsen et al. 2009; Rahman et al. 2014). Since plants have the ability to form core microbiomes, crop breeding can be considered as a way to corporate the selection of beneficial plant–microorganism interactions in order to breed 'microorganism-optimized' plants. Multiple methods can be combined according to practical requirements. Our studies shed light on the interactions related to the soil microbiota-mediated modulation of clubroot diseases and provide novel insights into this complex system that may enable us to develop innovative tools for the integrated sustainable control of this notorious pathogen.

5 Summary

Cruciferous crops have significant economic value as vegetables, edible and industrial oil sources, animal feeds, manure and biofuel. Cruciferous vegetables are rich in nutrients and also have medical benefits for human health. With the growing market demands of yield and quality, the global needs for efficient and sustainable cultivation of cruciferous crops are increasing. However, clubroot caused by *Plasmodiophora brassicae*, a worldwide spread soil-borne disease seriously restricts the production of cruciferous crops. The major symptom of clubroot is single or multiple root galls that limit the uptake of water and nutrients leading to stunting and considerable losses in yield and quality. The resting spores of *P. brassicae* are able to survive in the soil for many years that also diminish the land capital value. So far, there are no pesticides approved to control clubroot in Europe. The efficacy of available control strategies including soil liming, crop rotation and biocontrol, is limited in the field. Clubroot resistant cultivars have been utilized for many years, but resistance in these crops have not been very sustainable and several cases of virulent clubroot isolates overcoming the race-specific clubroot resistance have appeared. With the growing awareness for better soil management and sustainable production practices, we face a big challenge for developing effective clubroot control methods. A better understanding of interactions between the plant roots, rhizosphere soil and the pathogen will provide us with new perspectives to improve integrated control systems.

P. brassicae resting spores can be persistent in the soil for many years. The biotic and abiotic stimulants for spore germination are crucial factors in clubroot management. Several studies have mentioned that root exudates of several host and non-host plants can stimulate the germination of resting spores. However, the specific drivers and the underlying mechanisms on triggering resting spore germination are still unclear. In the present study, a series of experiments has been conducted to deeply investigate the ecological factors on stimulating the germination of resting spores. A functional model is presented to illustrate the plant-microbiome-pathogen relationship as a foundation to develop novel tools for the integrated sustainable control of clubroot disease.

Firstly, several methods for determining the resting spore status were examined, including the viability assessment and germination rate estimation. After comparison, Evans blue and the dual staining of calcofluor white (CFW) and propidium iodide (PI) were the most effective staining methods for determining the viability of *P. brassicae* resting spores. Accurate assessment of viability by Evans blue requires at least 10 h incubation time, which was more applicable for long period observation. The dual staining of CFW-PI showed a clear differentiation between live and dead spores that provide the accurate viability within 10 min

and can also be applied for detecting spores in the soil. The accuracy of propidium monoazide (PMA) qPCR has been verified and used to detect the viability of resting spores in the present study. Because heat treatments can affect DNA integrity, the evaluation of the accuracy of PMA qPCR may be influenced. Instead of heat treatment, in order to establish a standard curve without DNA degradation for PMA qPCR, acetone can be used to treat the spores. For PMA qPCR, sufficient replicates are required to obtain stable results. After comparison, the staining method of CFW-PI was selected to assess the spore viability in the bioassays to warrant a good baseline for further studies.

Reliable identification of germinated and non-germinated resting spores is crucial to investigate the stimulants of germination. Therefore, three assays have been tested in the present study. A blue fluorescent nucleic acid stain, DAPI, can be used to distinguish the germinated spores from non-germinated spores. Besides, CFW-Nile red dual staining was also able to examine the germination rate, when a pre-treatment to inactivate the spores was conducted before staining. Among them, differential interference contrast microscopy was a fast and easy way to check the germination rate and was selected for further experiments in this study.

Our studies revealed that certain bacteria can be direct drivers for triggering the germination of *P. brassicae* resting spores. 16S rRNA gene amplification and sequencing showed clear shifts in the relative abundance and composition of taxa in the bacterial community in the samples with high germination rates compared to the initial community, indicating a functional relationship between certain bacteria and resting spore germination. Genera of *Chryseobacterium* and *Flavobacterium* were considerably more abundant in the high germination rate treatments.

Further research is required to identify the specific stimulation associated bacteria and the underlying mechanisms. Moreover, the initial microbial community around resting spores in the soil is influenced by many factors, like soil moisture, soil nutrition and carbon sources. Pure root exudates collected under sterile conditions were not the direct factors for stimulating the germination of *P. brassicae,* whereas they could play a role as carbon sources modulating the bacterial community under non-sterile conditions. Nitrate is a more favorable nitrogen source than ammonium to form favored conditions for resting spore germination. High concentration of calcium may suppress the growth of stimulation associated bacteria or benefit for the non-stimulation associated bacteria. Soil moisture also related to the microbial community. All these environmental factors not only influence the soil microbial community, but also affect plant growth. Conversely, plants continuously modulate the surrounding microbial community to maintain fitness. Plants and their

associated microbiota are considered as a single entity, holobiont, whereby evolutionary selection between plants and microorganisms contributes to the overall stability of the system. During the evolutionary selection, stimulation associated bacteria could act as a messaging mediator between plants and *P. brassicae*. It is a sophisticated way for *P. brassicae* to survive for long periods by utilizing bacteria to respond to environmental changes and stimuli. Our studies shed novel light on the interaction of soil microbiota-mediated modulation of clubroot diseases and provide a powerful insight into this complex system to develop novel tools for the integrated sustainable control of clubroot disease.

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7 Curriculum vitae

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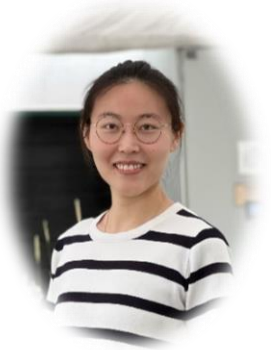

Research experience

- **The biotic and abiotic stimulation factors on the germination of *Plasmodiaphora brassicae* resting spores** Oct. 2016 - Dec. 2020
  Gene expression, Isotopic analysis, HPLC-MS, Soil microbial analysis

- **Assessment of *Plasmodiaphora brassicae* resting spore viability and germination rate** Oct. 2016 – Nov. 2019
  Fluorescent microscopy, Staining, qPCR

- **Plant activator & fungicides compound in controlling clubroot disease of cruciferous** Mar. 2015 - Nov. 2015
  Greenhouse and field bioassays, Physiological and biochemical tests
  (Commonweal Specialized Research Fund of China Agriculture)

- **ISSR analysis on genetic diversity of *Paecilomyces Farinosus*** Oct. 2012 - Oct. 2013
  Collection and purification of pathogens, Identification of *P.farinosus*
  (National Natural Science Foundation)

Education Background

- **University of Göttingen, Germany** Oct. 2016 - Present
  Ph.D. (Plant Pathology and Crop Protection)

- **Sichuan Agricultural University, China** Sep. 2014 - Jun.2016
  Master (Plant Protection)

- **Sichuan Agricultural University, China** Sep. 2010 - Jun. 2014
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Languages & Skills

Chinese: ■■■■■■■■■■
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9 Statutory declaration

Herewith I declare that this dissertation was prepared autonomously and without any unaccredited aid.

Date: 03.04.2021 Signature: