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Effects of the endophyte *Acremonium alternatum* on oilseed rape (*Brassica napus*) development and clubroot progression

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Abstract:

The clubroot pathogen *Plasmodiophora brassicae* infects economically important Brassica crops such as oilseed rape and vegetable brassicas. Clubroot results in abnormally growing roots and restricts the flow of water and nutrients to the upper plant parts, thereby inducing wilting. Yield loss affects about half the percentage of infected plants. Due to its complex and well-adapted life cycle the pathogen is difficult to control by chemical and cultural means and therefore continues to spread around the globe. Infested fields can no longer be used effectively for cultivation of crop plants for at least the next ten years. Despite costly breeding of resistant cultivars, recent research leans towards alternative, low-impact and environmentally friendly methods to control clubroot. To this end we have previously identified the endophyte *Acremonium alternatum*, a known biological control agent in several countries, to show promising antagonistic effects in clubroot-infected *A. thaliana* and Chinese cabbage (*Brassica rapa*). Here, we will describe its effect on the growth, development and clubroot control of oilseed rape (*Brassica napus*). While the clubroot symptoms were not clearly reduced after co-inoculation with *A. alternatum* and *P. brassicae* on oilseed rape roots, the aboveground plant parts were delayed in senescence and produced more seeds, which is indicative of an increase in yield after *A. alternatum* treatment. The long-term goal of this work is to contribute to a fundamental understanding of endophyte-plant interactions and an effective reduction of clubroot to be used in integrated pest management for oilseed rape and other cabbage varieties.

Keywords: *Acremonium alternatum*; *Brassica napus*; clubroot disease; endophytic fungus; *Plasmodiophora brassicae*; seed yield.

1. Introduction

The clubroot disease (*Plasmodiophora brassicae*) causes severe crop loss in Brassica cultivars worldwide, among them the important food plants Chinese cabbage (*Brassica rapa*) and oilseed rape (*Brassica napus*) [4]. The complex, two-stage life cycle of the obligate protist *P. brassicae* makes it hard to control this pathogen [7]. In addition, the protist forms durable resting spores that remain infectious in the soil for up to 18 years [22], therefore posing a threat to all subsequently farmed Brassicas on infested fields. Typical clubroot symptoms are a stunted growth and abnormally large root galls, called clubs. Agrochemicals are partially effective against clubroot, but with growing interest in environmental safety and human health concerns they become less available [7]. Recent attempts to control clubroot include the use of microbes that can act as biocontrol agents and reduce symptoms of infected plants at least to some extent [3, 13, 15].

Recently, we have investigated the potential of *Acremonium alternatum*, a known biocontrol agent [1, 12, 17] to control the clubroot disease. *Acremonium alternatum* is an endophytic Ascomycete that is widespread in all soil types [8]. So far, the endophytic fungus has not been observed to alter host growth [18]. We have applied a spore solution of the endophytic fungus on *Brassica rapa* [6] and *Arabidopsis thaliana* [11] to assess its potential to reduce clubroot. In Chinese cabbage co-inoculation with the endophyte resulted in smaller root galls and better performing plants [6]. Similarly, *A. thaliana* plants were more tolerant to the clubroot pathogen when co-inoculated with *A. alternatum* [11]. In this work it was also shown that the effect was a delay in clubroot development, but the endophyte did not inhibit resting spore germination of the pathogenic protist [11].

To test whether the same effect is true in other *Brassica* species we carried out similar experiments using *A. alternatum* on *Brassica napus* roots. Oilseed rape (canola in North America) is Europes most important oilseed crop and the second most important worldwide [16]. The clubroot disease causes huge losses on fields with this crop worldwide [5], so the control with an environmentally friendly alternative is of enormous interest.

2. Material and Methods

2.1 Plant cultivars and growing conditions

Commercially available oilseed rape seeds cv. Ability (summer oilseed rape) and cv. Visby (winter oilseed rape) were obtained from the Deutsche Saatveredlung GmbH, Germany and the Sächsisches Ministerium für Umwelt und Landwirtschaft, Germany. These *B. napus* varieties are adapted to the German climate and soils. To simulate growth conditions throughout the year two growth periods were chosen: one for summer rape cv. Ability (end of May to October) and another for the winter period cv. Visby (end of October to April). Seeds were washed from their coating of fungicides and pre-germinated for two days on wet paper in the light. The emerging seedlings were planted in 11 x 11 x 12 cm pots filled with a mixture of standard soil (Pikiererde Cl P Profisubstrat, Einheitserde Classic; steam sterilized for two hours) and sand (commercial playground sand, sterilized for 24 hours) at the ratio 4:1. All experiments were performed in a green house with temperatures ranging from 14 (night) to 27°C (day) and a relative humidity of 40 to 80 %.

B. napus cv. Ability was fertilized three times throughout the five months growth period with commercial fertilizer (Blumendünger mit Guano, COMPO GmbH, Germany), *B. napus* cv. Visby was fertilized weekly with a nutrient solution diluted 1/8 according to Smeets et al. [21].

2.2 Inoculation procedure

Spore suspensions of *Plasmodiophora brassicae* and *Acremonium alternatum* were prepared as described elsewhere [11].

For the inoculation of the summer rape cv. Ability a spore concentration of 10^7 spores ml^{-1} was used. Since all clubroot infected plants died during the summer before the flowering stage a slightly lower concentration (10^6) was used for winter rape cv. Visby to ensure flowering and seed production of the plants. There were four treatment groups: control, *A. alternatum* inoculated, *P. brassicae* inoculated and a combined inoculation with *A. alternatum* and *P. brassicae*. Spore suspensions were adjusted to the desired concentration of 10^6 or 10^7 spores ml^{-1} using potassium buffer (50 mM KH_2PO_4, pH 5.5). Each plant was inoculated by pipetting 3 ml of the spore suspension directly on the soil next to the plant. Control plants were treated with the same volume of buffer.

2.3 Disease rating

Per treatment 14 to 16 plants were harvested for disease classification of the roots six (summer oilseed rape cv. Ability) or nine (winter oilseed rape cv. Visby) weeks after inoculation. For this, the soil around the roots was carefully removed and the gall size categorized according to Yoshikawa et al. [23]: 0 – no visible swelling, 1 – very light swelling at lateral roots, 2 – moderate swelling, 3 – severe swelling, 4 – severe swelling or decay of lateral roots. The disease severity is shown as percentage of plants in individual disease classes or by calculating the disease index (DI) according to the following equation: $(n_0*0) + (n_1x10) + (n_2*30) + (n_3*60) + (n_4*100)/n_{total}$ [23].

2.4 Determination of fresh weight, stem lengths and growth stages

Fresh weight of the green parts was determined for harvested plants at the time point of the disease rating 6 to 9 weeks after treatment. Stems of the cv. Ability were measured five months and of cv. Visby nine weeks after treatment. Growth stages of cv. Ability were assessed at two time points according to the BBCH-scale for oilseed rape [14].

2.5 Root histology

Scanning microscopy was used to assess the disease progress inside the plant roots. For this, root samples of cv. Visby were harvested nine weeks after treatment and fixed on slides suitable for the scanning electron microscope (n = 2 per treatment). The samples were cryofixed for 30 minutes and broken to preserve the natural structures of cells and prevent artefacts that result from cutting with blades.

2.6 Statistics

All data sets were tested for normal distribution by graphical methods. Data showing a skewed distribution were log-transformed prior to further analyses. This applied to stem lengths and growth stages of five month old cv. Ability plants. With these a two-sided t-test was carried out with a probability value of 0.05. An ANOVA was carried out using the software PSPP with stem lengths and biomass for all data sets available.

3. Results and Discussion

3.1 Acremonium alternatum cannot control clubroot in oilseed rape

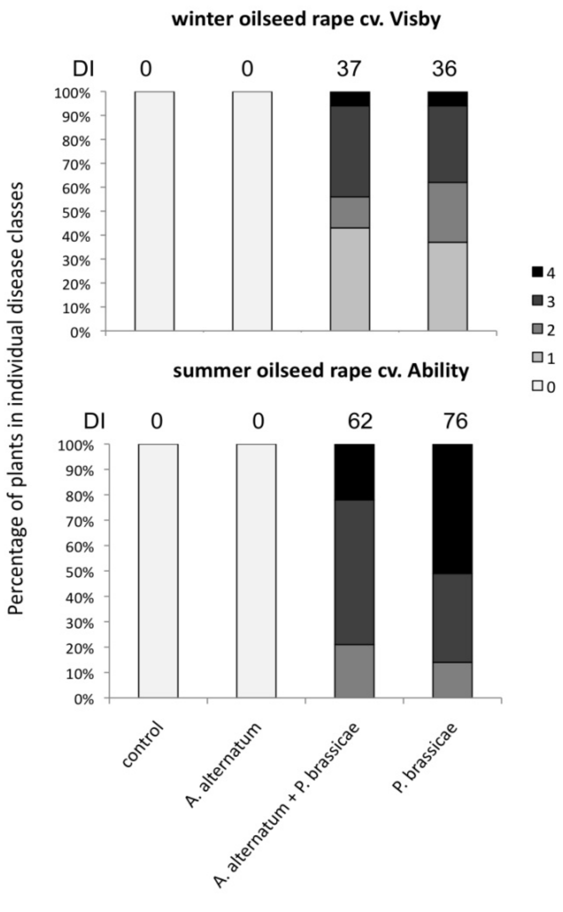
According to experiments with *Brassica rapa* (Chinese cabbage) and *Arabidopsis thaliana* the clubroot disease symptoms could be reduced by co-inoculation with the endophytic fungus *Acremonium alternatum* [6, 11]. These results encouraged us to investigate the effect of *A. alternatum* on a major energy and oil crop worldwide *Brassica napus* (oilseed rape). Two cultivars, one winter and one summer oilseed rape, were chosen for this investigation. In both cultivars the effect of *A. alternatum* on root symptoms caused by *P. brassicae* was barely visible. The disease index was only slightly decreased in summer oilseed rape (cv. Ability) six weeks after inoculation and not affected in winter oilseed rape (cv. Visby) nine weeks after treatment (Figure 1). Disease indices in the fertilized plants were generally lower. It is known that some plant nutrients reduce disease development of *P. brassicae* [9].

This was supported by microscopy at late stages of the infection (9 weeks after treatment of cv. Visby). No changes in disease progression were observed in co-inoculated samples (Figure 2). In contrast to Chinese cabbage [6] and Arabidopsis [11], no delay in *P. brassicae* development was observed in the two root samples of co-inoculated plants in comparison to the plants inoculated with *P. brassicae* only. This was also reflected in the general appearance of root galls and the disease index (Figure 1).

Another goal in this experiment was to assess the performance of the aboveground plant parts after treatments with the different microorganisms. For this stem length and weight, and different developmental stages (i.e. flower development) were recorded since the endophytic fungus was able to shift the onset of senescence (see 3.2). No changes could be detected between *P. brassicae* and combined treatments on any of the above mentioned parameters for the upper plant parts (data not shown). In summer rape with the higher spore concentration of 10^7 spores ml^{-1} all plants inoculated with clubroot died within the five months growth period at the stage of leaf development.

Oilseed rape plants inoculated with both organisms were doing better in terms of overall survival compared to *P. brassicae* infection alone. Nine-week-old cv. Ability plants showed a shift in senescence. While the controls and endophyte inoculated plants were already in the stage of flowering, *P. brassicae* inoculated plants did not produce flowers and some were dead already (Figure 3).

Figure 1. Percentage of plants in individual disease classes and the disease index (above the histograms) of two oilseed rape (*Brassica napus*) cultivars. In cv. Visby (n = 16 per treatment) there is not much difference between treatments, whereas in cv. Ability (n = 14 per treatment) more plants were found in lower disease classes, which is also reflected in a lower disease index, when the plants were co-treated with *Acremonium alternatum.*



3.2 A. alternatum promotes senescence of oilseed rape and increases seed production

Five months old plants treated with *A. alternatum* were further developed than the control group but the effect was not significant (p=0,134). There were 10% more plants already in the stage of flowering and ripening of the seeds (Figure 3).

Figure 2. Scanning Electron Microscopy images of control (A), *Acremonium alternatum* (B), *Plasmodiophora brassicae* (C) and co-inoculated with both organisms (D) *Brassica napus* cv. Visby roots. The scale bar always represents 100 µM. The typical enlarged cells contain resting spores (RS) of *P. brassicae*. Hyphae of *A. alternatum* are not visible in these pictures, but the fungus was detected by PCR (data not shown).

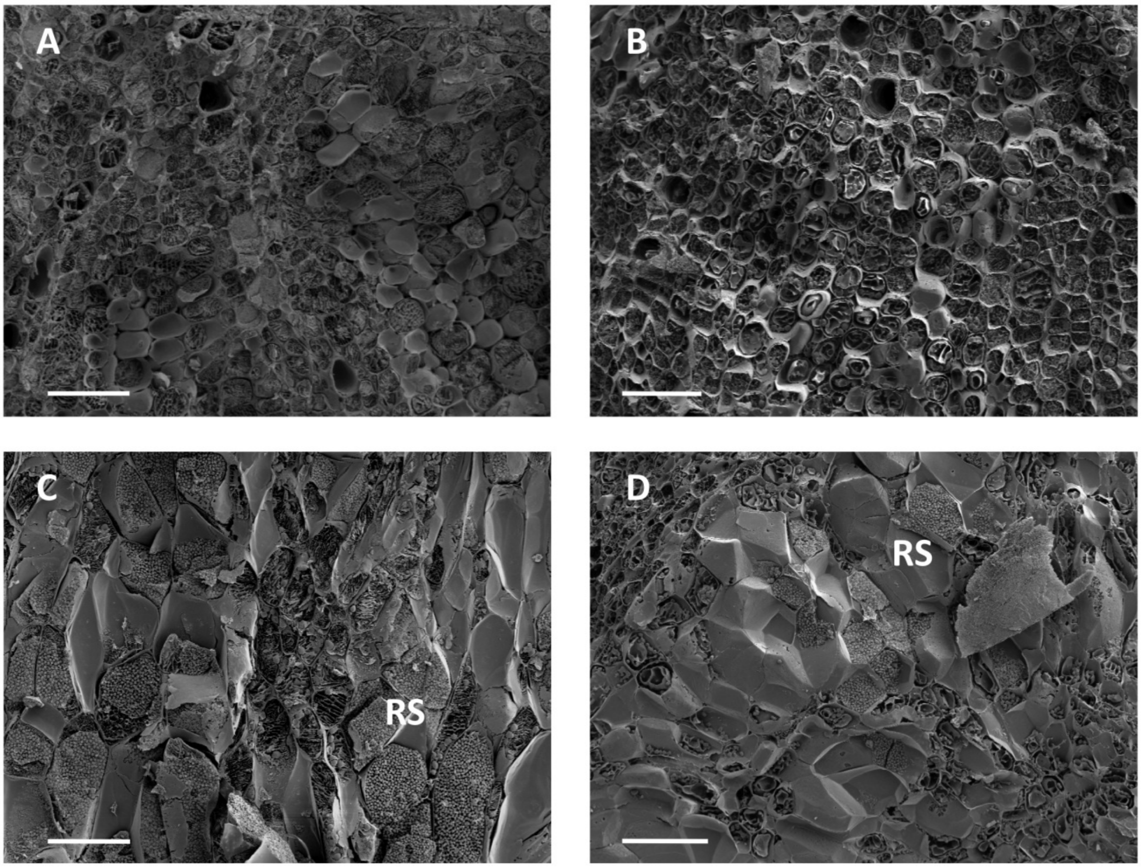
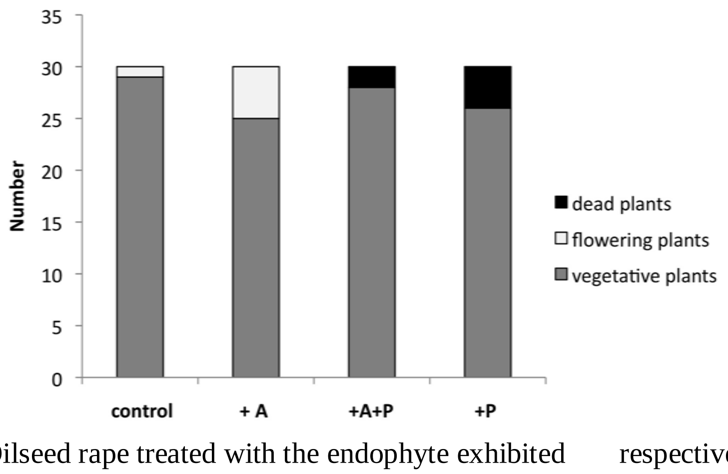


Figure 3. Growth stages of summer oilseed rape cv. Ability. For evaluation the plants were 5 months old. *Acremonium alternatum* (+A) increased the number of flowering plants compared to controls, while the plants only inoculated with *Plasmodiophora brassicae* (+P) showed the highest mortality rate compared to all other treatments. Co-inoculation with *A. alternatum* and *P. brassicae* (+A+P) did reduce the number of dead plants within the population, but could not delay senescence.



Oilseed rape treated with the endophyte exhibited more siliques per plant, resulting in more seeds respectively (Figure 4). However, seed weight was not increased in endophyte-infected plants. In grasses

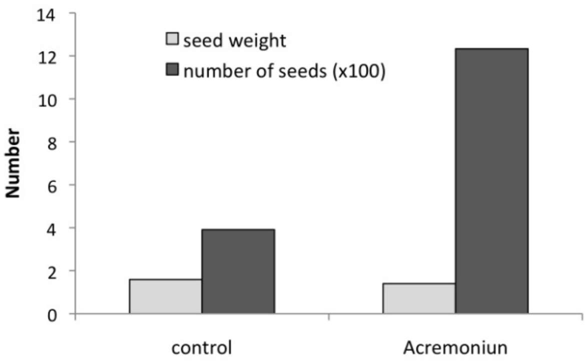
Acremonium species are known to grow within the seeds and therefore have a better chance for propagation [2].

The cultivar Visby did not produce flowers within the growth period of six months. In all experiments the fresh weight of treated plants was lower than that of the controls, but this was only statistically significant in the Ability cultivar (ANOVA; $p<0.05$).

It has been reported that due to biochemical changes in the plant upon pathogen challenge less resources are available for growth [10]. The process of inducing resistance is costly for the plant and detection mechanisms to distinguish hostile from non-hostile influences are switched on within minutes of an invasion. Hence plants which are "busy" to defend themselves are smaller in comparison to control plants.

Interestingly, *A. alternatum* treated five months old cv. Ability had significantly longer stems than the controls (t-test; p=0,007). This could indicate that the fungus is able to modify the hormonal balance of the plant, probably by inducing gibberellins, which are known inducers of internode length [19, 20].

Figure 4. Seed weight and yield of summer oilseed rape cv. Ability.



4. Conclusion

Viable spores of the endophyte *Acremonium alternatum* are not suitable for clubroot control in oilseed rape. A recent paper [13] used a formulation of an endophyte to suppress clubroot in oilseed rape successfully. Therefore, a suppressive effect might well be dependent on the microorganism used for biocontrol, but also maybe on the cultivars. Interestingly, most biocontrol agents used to suppress clubroot were root endophytes, whereas *A. alternatum* readily colonizes leaves [11]. This difference in colonization behavior of the endophyte could also influence the outcome of a disease suppression reaction, especially in a plant with large stems such as oilseed rape. In the summer cultivar Ability endophyte inoculated plants were more successful in terms of competition (longer stems) and reproduction (more seeds per plant). *Brassica napus* seeds are used to manufacture oils for the industry, biofuel and for food products. Therefore the impact on seed production is crucial for a broader application of this potential biocontrol agent.

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