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A rapid method to screen wild *Solanum* for resistance to early blight

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Abstract Early blight of potato and tomato is caused by *Alternaria* fungi and negatively impacts crop yields. Environmental factors and plant maturity influence disease development, which is usually kept under control by fungicide applications. Wild tuber-bearing *Solanum* section *Petota* species are a promising source of resistance to early blight that could be used to control the disease, for example by crossbreeding or modern breeding approaches. An efficient screening method is a first prerequisite for the identification of resistant genotypes in wild *Solanum* germplasm. Here, we describe a protocol that can be used to rapidly screen for resistance to early blight in wild *Solanum* collections. This protocol provides a good starting point for the identification of resistant genotypes and is a step towards breeding for resistance to early blight using wild *Solanum* species.

Keywords *Alternaria* · *A. grandis* · *A. protenta* · *A. solani* · Disease assay · Wild potato

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Alternaria fungi are associated with diseases in potato. Small-spored *Alternaria* species such as *A. alternata* and *A. arborescens* can cause brown leaf spot disease (van der Waals et al. 2011; Tymon et al. 2016a; Tymon et al. 2016b), while large-spored *A. solani*, *A. grandis* and *A. protenta* can cause early blight (Duarte et al. 2014; Woudenberg et al. 2014; Bessadat et al. 2015; Ayad et al. 2017). Early blight is characterised by the appearance of dark necrotic lesions on infected leaves, which are often restricted within the leaf veins and have a typical concentric ring pattern. Affected leaves have a reduced photosynthetic potential and severe infections lead to defoliation of the plant, which negatively influences crop yield (Johnson and Teng 1990; Rotem 1994; Shtienberg et al. 1996).

Paul Sorauer first described early blight in Europe and considered *A. solani* a weak pathogen with a mainly saprophytic lifestyle that only infects plants with the right predisposition under especially favourable conditions (Sorauer 1896). He recognised the importance of environmental and host factors in disease development at a time when the attention of most plant pathologists was focused on the pathogens and when almost all fungi that occur on plants were considered parasites (Westerdijk 1917).

Many studies have since confirmed that early blight development is greatly influenced by factors such as temperature, humidity, leaf wetness and abiotic stress, or by the occurrence of other plant diseases (Holley et al. 1985; Bussey and Stevenson 1991; Rotem 1994). Genetic host factors also contribute to resistance to early blight in potato (Herriott et al. 1990; Christ and Haynes

2001; Xue et al. 2019). It was found that early maturing potato cultivars are generally more susceptible to early blight than late maturing cultivars, but no fully resistant cultivars have been identified (Rodriguez et al. 2006; Duarte et al. 2014; Odilbekov 2015; Abuley et al. 2018; Xue et al. 2019).

Early blight control is currently based on the application of fungicides (Abuley and Nielsen 2017), but various studies have reported a reduced efficacy of the chemicals that are being used (Pasche et al. 2005; Rosenzweig et al. 2008; Fairchild et al. 2013). Moreover, the use of fungicides presents a cost for farmers and could lead to adverse environmental effects.

Some wild potato relatives show high levels of resistance and could possibly provide an alternative solution to control early blight (Jansky et al. 2008; Weber and Jansky 2012; Meier et al. 2015; Haynes and Qu 2016). Such genotypes could be used in classical breeding, but could also provide a starting point to identify genes that contribute to resistance for use in modern breeding approaches.

An efficient screening method is required for testing large numbers of genotypes from wild germplasm collections for resistance to early blight. Several protocols for testing early blight resistance have already been described by others (Bussey and Stevenson 1991; Jansky et al. 2008; Weber and Jansky 2012; Odilbekov et al. 2014), but not all methods are suitable for screening wild *Solanum* species. Such species are very diverse and may respond differently to the varying environmental conditions that are encountered in a field. We have therefore developed a greenhouse protocol, based on previously described methods (Rodrigues et al. 2010; Odilbekov et al. 2014), in which environmental conditions are controlled to favour early blight infection. This allows us to properly evaluate the role of genetic factors in resistance. The protocol is optimised for the fast characterisation of plants that are maintained in vitro.

Potato (*S. tuberosum* cv. Désirée) plants and wild *Solanum* genotypes (listed in Table 1) with various levels of resistance to early blight were propagated in vitro on MS20 medium (4.4 g/l Murashige and Skoog basal salt mixture including vitamins, 20 g/l sucrose, and 8 g/l micro agar in water) (Murashige and Skoog 1962). Two weeks after propagation, plants were transferred to 11x11x12 cm pots with sterilized soil and grown in a climate regulated greenhouse under long-day conditions (16 h light and 22 °C, 8 h dark and 18 °C) with sufficient space in between to allow adequate light exposure.

Five isolates belonging to three different species of *Alternaria* from different locations were used in this study (Table 2). We have previously described *A. solani* isolate CBS 143772 (Iftikhar et al. 2017; Wolters et al. 2018) and additional *Alternaria* isolates were ordered from the fungal culture collection (CBS) of the Westerdijk Institute (Utrecht, the Netherlands). The *Alternaria* isolates were maintained on potato dextrose agar (Beever and Bollard 1970) in Petri dishes.

To produce conidia, agar plugs containing mycelium of the *Alternaria* isolates were used to inoculate V8 medium (5x diluted V8 juice), followed by incubation on a shaking incubator at 28 °C until the colour of the culture changed from red to almost black after 4–5 days. The *Alternaria* culture was then poured directly into Petri dishes containing potato dextrose agar and incubated without lid in an incubator equipped with blacklight fluorescent tubes (Philips TL-D 18 W BLB) at 25 °C (12 h dark, 12 h blacklight) for 3 days. The plates were covered and stored at 25 °C in the dark until needed.

Five-week old plants were transferred to a climate cell (25 °C, relative humidity >70%, 16 h light and 8 h dark) one day before inoculation. The plants were placed inside a tent that was constructed by covering a frame with transparent plastic sheeting. An atomiser (Condair 505) was placed inside the tent together with the plants, to create a fine mist and increase humidity after inoculation of the plants (Fig. 1a).

On the day of the inoculation, conidia were harvested by flooding the *Alternaria* cultures with tap water and gently brushing the surface. Next, the conidial suspension was filtered through a tea strainer to remove pieces of mycelium and the conidia were left to sink so the bottom. Supernatant was poured off and the conidia were resuspended in 1/5 strength potato dextrose broth

Table 1 *Solanum* genotypes used in this study

Genotype	*Solanum* species
PLD 782–9	*S. polyadenium*
IMT 63–3	*S. immite*
PLD 207–1	*S. polyadenium*
Désirée	*S. tuberosum*
GIG 715–1	*S. microdontum* subsp. *gigantophyllum*

Table 2 *Alternaria* isolates used in this study

Accession	*Alternaria* species	Original host	Origin
CBS 143772	*A. solani*	*Solanum tuberosum*	Netherlands
CBS 109157	*A. solani*	*Solanum tuberosum*	USA
CBS 116695	*A. grandis*	*Solanum tuberosum*	USA
CBS 116437	*A. protenta*	*Hordeum vulgare*	New Zealand
CBS 116696	*A. protenta*	*Helianthus annuus*	Israel

(PDB) supplemented with 0.3% micro-agar to obtain a suspension of 1×10^5 conidia/ml. The agar was added to increase the density of the spore suspension, leading to firmer droplets that do not easily run off the inoculated leaves. Diluted PDB was used to make sure that the conidia germinate consistently (Fig. 1b).

The upper three fully expanded compound leaves (further sub-classified as 'upper', 'middle' and 'lower' leaves) of the plants were drop-inoculated with 10 μl droplets of conidial suspension. It should be noted that wild *Solanum* genotypes are morphologically very diverse. Therefore, it can be difficult to pick comparable leaves for inoculation. Depending on the size of the leaves of the genotype tested, 4–6 droplets can be used to inoculate each compound leaf. For each genotype, three replicates were included in the experiment and the experiment was repeated once. Both experiments were carried out at the Laboratory of Plant Breeding at Wageningen University (Wageningen, the Netherlands) in 2016.

Directly after inoculating the plants, lights were switched off and the atomiser was turned on. The normal light regime was resumed the next day and the atomiser was only switched on during subsequent nights. This mimics the alternating leaf wetness during the night and gradual drying of the leaves during the course of the day that can occur outdoors. Figure 1c shows an example of the lesions that are produced on the second fully expanded leaf from the top of potato cultivar Désirée, 5 days after inoculation with six droplets of spore suspension. In the experiment that is summarized in Figs. 2 and 3, we have used four droplets of spore suspension to inoculate each leaf (12 droplets per plant). Diameters of lesions produced on all the inoculated spots were recorded 5 days after inoculation using a digital calliper (Mitutoyo 500–161-30). The averages of the lesion sizes formed by each isolate on the different *Solanum* genotypes are summarized in Fig. 2.

The disease protocol that is described here differs in several aspects from the protocol described by Odilbekov et al. (2014). We start with in vitro maintained plants, which allows the testing of wild *Solanum* germplasm. By using an atomiser, leaf wetness at night is ensured. Combined with using an increased spore concentration of 1×10^5 spores/ml, consistent lesion development is achieved, allowing the measuring of lesions at 5 days post inoculation already.

The tested *Solanum* genotypes ranged from resistant to susceptible, with average lesion sizes of less than 1 mm in the most resistant genotype (*S. polyadenium* 207–1), to over 10 mm in susceptible genotypes (*S. tuberosum* cv. Désirée and *S. microdontum* subsp. *gigantophyllum* 715–1, Fig. 2). While the effects of maturity are likely to be less pronounced in young plants, they cannot be fully eliminated. When recording the lesion sizes, it is important to distinguish between the different positions of the leaves that are inoculated. A pronounced effect of leaf position on lesion size was seen in the susceptible genotypes, pointing to an effect of leaf maturity on early blight development (Fig. 3), as was also observed previously by others (Rodriguez et al. 2006; Odilbekov et al. 2014). Interestingly, this effect seems to be absent in the more resistant genotypes (*S. polyadenium* 207–1 and 782–9 and *S. immite* 63–3, Fig. 2).

The method for spore production that was used by us is based on the protocol described by Rodrigues et al. (2010). We omitted the grinding step, but did expose the mycelium to UV light and drought stress. Spores were produced within 10 days for all isolates. The tested isolates were all found to be pathogenic on potato cultivar Désirée, but were shown to differ in virulence. CBS 116695 (*A. grandis*) appeared to be the most virulent of the isolates that were tested, whereas CBS 116696 (*A. protenta*) showed the lowest virulence in the assay (Fig. 2). CBS 143772 (*A. solani*) displayed an

Fig. 1 Setup of the disease test (a), pictures of germinating spores (b) and lesions produced by *Alternaria solani* (c). a. Plants were incubated inside a transparent plastic tent and an atomiser was placed inside the tent to create a fine mist. **b.** Addition of potato dextrose to the inoculation medium results in high spore germination efficiency, as evidenced by abundant mycelial growth 6 h after resuspending the spores. **c**. The adopted inoculation protocol results in consistent lesion development. A representative photograph of a middle leaf of *Solanum tuberosum* cultivar Désirée inoculated with six droplets of *Alternaria solani* spore suspension was taken five days post inoculation

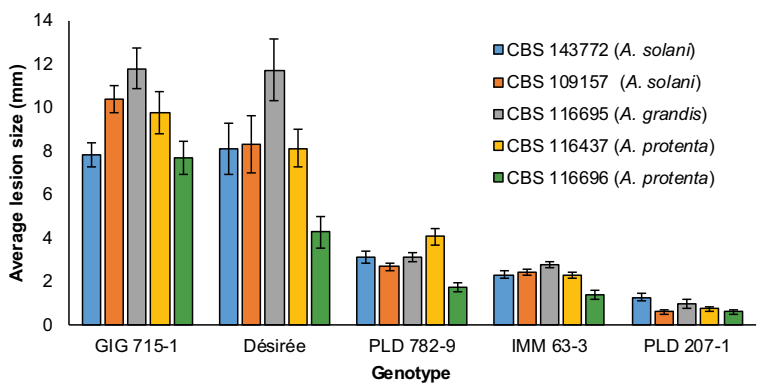

Fig. 2 Pathogenicity of different *Alternaria* isolates on *Solanum* genotypes. Average lesion sizes (at five days post inoculation) produced by five different *Alternaria* isolates/species on different *Solanum* genotypes (*S. microdontum* subsp. *gigantophyllum* 715–1, *S. tuberosum* cv. Désirée, *S. polyadenium* 782–9 and *S. immite* 63–3 and *S. polyadenium* 207–1, all leaf positions combined) are displayed. Error bars indicate standard error of the mean. All isolates are pathogenic on cultivated potato (*Solanum tuberosum* cultivar Désirée), but display differences in virulence. No difference in resistance pattern (ranking of *Solanum* genotypes from susceptible to resistant) is observed among the isolates tested

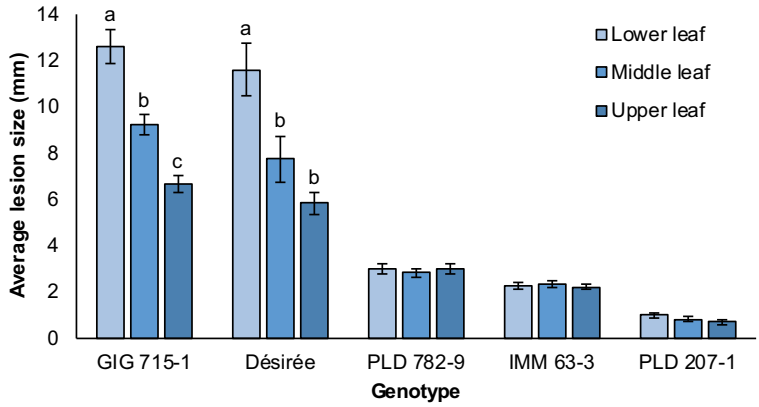

Fig. 3 Effect of leaf position on lesion size. The data for the different isolates and the two experiments are combined and average lesion sizes (at five days post inoculation) produced on lower, middle and upper leaves of different *Solanum* genotypes are displayed. Error bars indicate standard error of the mean. ANOVA showed a significant effect ($p < 0.05$) of leaf position on lesion size for *S. microdontum* subsp. *gigantophyllum* 715–1 and *S. tuberosum* cv. Désirée and the effect of leaf position on lesion size is analysed separately for these genotypes. Different letters are used to indicate significant differences between the average lesions size produced on leaves of different positions (Tukey's HSD test, $\alpha = 0.05$)

intermediate virulence. Despite the differences in virulence, the resistance pattern of the different *Solanum* genotypes was the same for all isolates (Fig. 2), which illustrates the reliability of the protocol.

It can be expected to find large variations in susceptibility or resistance to early blight in *Solanum* germplasm. Once promising resistant genotypes have been identified, a more detailed characterization of the resistance would be desired. For example, it would be useful to investigate the disease progress over time to get insight into the effect of maturity, investigate the effect of abiotic and other biotic stresses, test performance in the field, and test additional *Alternaria* isolates. These experiments are especially important when the goal is to introduce resistance in a potato cultivar, to make sure that the targeted resistance is of practical use.

The protocol that is described here will be useful for carrying out resistance screens and can provide a good starting point for subsequent studies. Such follow-up studies will help to clarify the mechanisms that underlie resistance or susceptibility to early blight and, ultimately, to develop resistant potato cultivars.

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Compliance with ethical standards

Conflict of interest The authors state they have no conflict of interest.

Human and animal rights Research did not involve Human Participants and/or Animals.

Informed consent Informed consent was obtained from all individual participants included in the study.

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