

# Sources of resistance to *Phytophthora* root rot within the genus *Beta*

Mozhdeh Kakueinezhad · Parissa Taheri · Bagher Mahmoudi · Saeed Tarighi

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**Abstract** *Phytophthora* root rot caused by *Phytophthora drechsleri* Tucker is one of the most devastating sugar beet diseases in tropical areas. To identify genetic resources resistant to this disease, an aggressive isolate of *P. drechsleri* was selected. Then, a screening method was optimized based on the standard scoring scales of 1–9 (1: no symptoms, 9: complete plant death). Finally, 19 sugar beet lines, three cultivars, and 14 accessions of the wild species *Beta vulgaris* subsp. *maritima*, *B. macrocarpa*, *B. procumbens*, and *B. webbiana* were evaluated for resistance to the most aggressive isolate of *P. drechsleri* by using the optimized method (inoculum included 20 g of rice seed together with superficial wound creation). The isolates of *P. drechsleri* had significant variation in aggressiveness, and Kv10 was the most aggressive isolate on the susceptible variety Rasoul. The lines O.T.201-15, SP85303-0 (resistant check), and S2-24.P.107 had the lowest disease index with scores of 3.09, 3.13, and 3.27 respectively; they were categorized into the resistant group. The

interaction between isolates and genotypes was not significant, which indicated the same response of each genotype to different isolates. Investigating the resistance of different generations of sugar beet revealed that progeny selection would be an effective method for increasing the resistance level of breeding materials to *P. drechsleri*. Among the wild species, the accession 9402 belonging to *B. macrocarpa* and the accession 7234 of *B. vulgaris* subsp. *maritima* had the lowest disease index (2.29 and 2.60, respectively) and were categorized into the resistant group.

**Keywords** Resistant · Sugar beet · Screening · *Phytophthora drechsleri*

## Introduction

Sugar beet (*Beta vulgaris* L.) is an important crop of temperate climates, which contributes up to 20% of sugar production worldwide (FAO 2009). *Phytophthora* root rot (PRR) of sugar beet, caused by *Phytophthora drechsleri* Tucker, is one of the destructive diseases of sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) in tropic climate areas (Tompkins et al. 1936). This disease has been reported from California, Colorado, Idaho, Montana, Oregon, and Utah states of the US (Tompkins et al. 1936; Jacobsen 2006), United Kingdom (Gates and Hull 1954), Greece (Karaoglani-dis et al. 2000), Ukraine (Sabluk et al. 2005), and Iran.

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M. Kakueinezhad · P. Taheri (✉) · S. Tarighi  
Department of Plant Protection, Faculty of Agriculture,  
Ferdowsi University of Mashhad,  
P.O. Box: 91775-1163, Mashhad, Iran  
e-mail: p-taheri@um.ac.ir

B. Mahmoudi  
Sugar Beet Seed Institute, Agricultural Research,  
Education and Extension Organization (AREEO),  
P.O. Box: 31585-4114, Karaj, Iran

In Iran, the disease has been reported from regions with different climates such as Behbahan, Isfahan, Qazvin, Hamedan, Karaj, Kermanshah, Khorassan, Khoozestan, Mamasani, and Yasooj areas (Sheikholeslami et al. 2006; Ershad 2009). This disease causes high damage in Iranian sugar beet farms during spring sowing in tropical areas, especially Fars province (Banihashemi 1998), and in autumn sowing at Khoozestan province (Mahmoudi et al. 2002; Zamani-Noor et al. 2004). The PRR causes reduction in root yield and sugar content, also increases postharvest respiration rate, sucrose losses, and decreases sugar accumulation during storage (Tompkins et al. 1936; Campbell et al. 2013). Heavy soil and frequent irrigation are favourable conditions for development of this disease (Tompkins et al. 1936; Fatemi 1971).

Four species of *Phytophthora*, including *P. drechsleri*, *P. cryptogea*, *P. megasperma*, and *P. nicotianae* have been reported as the causal agents of the PRR disease in sugar beet fields (Bennett and Leach 1971; Whitney and Duffus 1986; Ervin and Riberio 1996). However, *P. drechsleri* has high aggressiveness and occurs more frequently than the other species (Fatemi 1971; Habibi 1975; Mahmoudi et al. 2002; Ershad 2009).

Global warming changes agricultural production conditions, which is a potential threat that could increase the damage and spread of many thermophilic pathogens, especially different species of *Phytophthora* (Brasier and Scott 1994; Thompson et al. 2014; Panabières et al. 2016). Zapolska (2014) considers global warming as a reason for the spread of sugar beet PRR in Ukraine. This indicates the necessity for further studies to find effective approaches for disease management. The lack of effective fungicides for protecting the enlarging root zone during plant growth makes management of the disease very difficult (Asher 1993; Luterbacher et al. 2005). Therefore, the most effective and efficient method to control soil-borne diseases, such as PRR, is to breed and develop resistant varieties combined with cultural practices (Harveson et al. 2002; Büttner et al. 2004; Luterbacher et al. 2005).

Several investigations have been conducted on the evaluation and breeding of different field crops for resistance to PRR. They include soybean root rot caused by *P. sojae* (Zhang et al. 2014), pepper root rot

caused by *P. capsici* (Candole et al. 2010), pumpkin root rot caused by *P. drechsleri* (Mansoori and Banihashemi 1982), watermelon root rot caused by *P. capsici* (Kim et al. 2013), spruce pine root rot caused by *P. cinnamomi* (Frampton and Benson 2012), and safflower root rot caused by *P. drechsleri*, *P. cryptogea*, and *P. parasitica* (Da Via et al. 1981). Despite the importance of *P. drechsleri* in decreasing the yield and quality of sugar beet, limited research has been conducted on this pathogen in sugar beet, because in Europe (as the main region for sugar beet production), two Oomycetous pathogens including *Aphanomyces cochlioides* (not reported from Iran) and *Pythium ultimum* cause the highest damage on sugar beet (Luterbacher et al. 2005). Also, limited attention has been paid to resistance against this important phytopathogen. Fattahi et al. (2011) have studied resistance of four sugar beet breeding lines to root rot agents, indicating that the line SB-19-P.78 has the lowest infection to *P. drechsleri*. The line SP85303-0 has been reported as a *Phytophthora* sp. resistant line by the USDA-ARS (Panella et al. 2015a, b).

Very useful sources of resistance genes to different sugar beet diseases have been found in the wild species of *Beta* belonging to the sections *Beta*, *Corollinae*, and *Procumbentes* (Mesbah et al. 1997; Luterbacher et al. 2005). Previous studies have made it clear that there is high resistance to *A. cochlioides* and *P. ultimum* in the sections *Corollinae* (93% of the accessions), *Procumbentes* (10% of the accessions), and *Beta* (1–6% of the accessions) (Luterbacher et al. 2005). There have been good achievements in transferring resistance for Rhizomania (Beet Necrotic Yellow Vein Virus) and Beet Cyst Nematode (*Heterodera schachtii*) from the wild species of *Beta* to sugar beet. The *Hs1<sup>Pro-1</sup>* gene (the resistance gene for sugar beet cyst nematode) has been cloned from *B. procumbens* and transferred to *Arabidopsis* and tobacco (Cai et al. 1997). Resistance genes for Rhizomania, *Rz<sub>2</sub>* and *Rz<sub>3</sub>*, have been identified in *Beta vulgaris* subsp. *maritima* (Scholten et al. 1999). In future, development of resistance to all soil-borne diseases will likely be realized through identification and utilization of these new valuable sources, especially from the section *Beta* (Luterbacher et al. 2005).

The first step in resistance evaluation of a germplasm collection is the precise identification of the pathogen. In the past, identification and taxonomy

of *Phytophthora* were based on morphological traits (Waterhouse 1963; Stamps et al. 1990). Recently, Mostowfzade-Galamfarsa and Banihashemi (2015b) have designed the primer pair *ITS-DF2* and *ITS-DR2*, which amplifies a part of ITS1, ITS2 and the whole subunit of 5.8S and is highly specific in identification of *P. drechsleri* and *P. cryptogea*.

Diversity in aggressiveness of the isolates of *P. drechsleri* depends on the type of host that ranges from low to high (Olson and Benson 2013). Zimmer and Urie (1967) have studied the resistance of safflower cultivars to *P. drechsleri* and reported that the interaction of isolate  $\times$  cultivar was significant. However, the aggressiveness of the isolates of *P. drechsleri* on sugar beet has not been studied yet. Therefore, a part of current research was focused on aggressiveness of the pathogen and interaction of isolate  $\times$  cultivar. Also, the selection of a highly infective inoculum and a reliable evaluation method can play an important role in the accuracy of the results (Büttner et al. 2004). So far, there has been no research on different methods to evaluate sugar beet resistance to *P. drechsleri*. Evaluation of genetic materials in naturally infected field conditions could not be highly reliable to achieve resistant cultivars due to the non-uniform infection in the field. So, to evaluate the resistance of cultivars to *P. drechsleri*, there should be a simple, rapid, and efficient method in a controlled condition which could be used in a large scale. Researchers have used different inocula, such as zoospore suspension (Mansoori and Banihashemi 1982), vermiculite and hemp seed extract (Banihashemi and Fatehi 1989), rice seed (Zhang et al. 2014), culture medium (Fattahi et al. 2011), and corn seed (Nasr-Esfahani et al. 2012), to evaluate the resistance of different field crops to *Phytophthora*. Thus, it was necessary to find the most effective inoculum and inoculation method for precise evaluation of sugar beet resistance to *P. drechsleri*. The current research was carried out for the first time to: (1) introduce a protocol for the evaluation of sugar beet resistance to *P. drechsleri*, (2) screen sugar beet breeding lines for resistance to *P. drechsleri*, (3) screen wild *Beta* relatives for resistance to *P. drechsleri*, and (4) study the interaction between isolates of *P. drechsleri* and sugar beet lines. It is expected that the results obtained from this research will be used by plant breeders to develop cultivars resistant to this pathogen.

## Materials and methods

### Plant materials and sowing method

Seeds of 19 sugar beet lines, three commercial varieties, and 14 accessions of wild species of *Beta* were received from the Sugar Beet Seed Institute (SBSI), Karaj, Iran. The breeding lines were derived from the *Rhizoctonia* resistant populations SB-19 and SB-22 (FC201); the wild species belonged to *B. vulgaris* subsp. *maritima*, and *B. macrocarpa* from the section *Beta* Tranzchel; and the species *B. procumbens* (*Patellifolia procumbens*) and *B. webbiana* (*Patellifolia webbiana*) were derived from the section Procumbentes Ulbrich Tranzchel (Barocka 1985). The seeds used in each experiment were first washed for 3 h with running water. They were then disinfected by 0.5% sodium hypochlorite (NaOCl) for 2 min and washed three times with sterilized distilled water. Five disinfected seeds were sown in 1 kg pots containing sterilized clay-loam soil and free of any infection in greenhouse conditions with  $25 \pm 2$  °C temperature and 65% relative humidity (Mansoori and Banihashemi 1982). Thinning was done one week after germination, and the number of plants was reduced to one plant per pot. Two weeks after sowing, the sugar beet seedlings were fertilized with the Hoagland solution (Hoagland and Snyder 1933).

### Isolates of *Phytophthora*

The isolates of *P. drechsleri*, including two isolates from Fars province (Kv10 and Kv4) and two isolates from Khozestan province (Ph-17-22 and Ph-17-27), all of which had been isolated from sugar beet, were received from the collection of Shiraz University, Shiraz, Iran. In addition, one isolate, Ph-dr, was isolated from sugar beet fields of Kermanshah. Only the isolates Kv10 and Kv4 had GeneBank accession numbers (AY661873.1 and AY661874.1, respectively), whereas the other three isolates had been identified by using a morphological method. Therefore, considering the similarity of the species *P. drechsleri*, *P. cryptogea*, and *P. melonis*, along with the lack of code in the NCBI database for the other three isolates, the potato (*Solanum tuberosum*) pink rot assay at 20 °C (Mostowfzadeh-Ghalamfarsa et al. 2006) and inoculation of safflower (*Carthamus tinctorius*) seedlings (Banihashemi and Mirtalebi 2008)

were used to separate the two species of *P. drechsleri* and *P. cryptogea* (Mostowfizadeh-Ghalamfarsa and Banihashemi 2015b). To extract DNA, all isolates of *P. drechsleri* and one isolate of *P. cryptogea* were cultured on the pea broth culture medium at 20 °C. Then, DNA was extracted by the method of Dellaporta et al. (1983). In the PCR test, the specific primers for identification of *P. drechsleri*, known as ITS-DF2 (5'CTC TAT CAT GGC GAC CGC C 3') and ITS-DR2 (5'CAC CAG TCC ATC CCG CCG 3'), which are capable of amplifying a 567 bp fragment, were used (Mostowfizadeh-Ghalamfarsa and Banihashemi 2015b).

For each reaction, the final volume was 25 µl, which contained 2.5 µl of 10 × buffer, 100 mmol of BSA, 100 mmol of dNTPs, 1.5 mmol of MgCl<sub>2</sub>, 1 mmol of each primer, and 0.4 units of Tag DNA polymerase and 100 ng of template DNA. The PCR reaction was carried out in a thermocycler (Labcycler model, SensoQuest Company, Germany) with the following steps: 2 min initial denaturation at 95 °C, 30 cycles including denaturation for 20 s at 95 °C, primer annealing for 40 s at 65 °C, primer extension for 60 s at 72 °C, and one 10-min-step for the final extension at 72 °C to complete the length of amplified fragments. Electrophoresis of PCR products was performed in 1% agarose gel at voltage of 80 v. The gel was stained with SafeView™ (ABM Company, Canada) and photographed in the gel doc set. Finally, the banding pattern of the isolates was detected on the gel.

#### Optimization of a method for the screening of *Beta* germplasm resistance to *P. drechsleri* in greenhouse conditions

Two experiments were conducted to develop a reliable protocol for evaluating resistance of sugar beet lines to the PRR. In the first experiment, ten different procedures (five types of inocula times two application techniques) were compared on the susceptible variety Rasoul. Twelve-week-old sugar beet plants were inoculated with an aggressive isolate of *P. drechsleri*. Five types of inocula with two application methods, including wound creation at inoculation time and no-wound creation, were used. The inocula were rice seeds (Zhang et al. 2014), vermiculite with hemp seed extracts (Banihashemi and Fatehi 1989), and corn seed (Nasr-Esfahani et al. 2012) of 20 g each per plant, suspension of water and hemp seed (mixture of

sporangium, zoospore, and mycelia pieces) of 20 ml per plant (Fatemi 1971), and the CMA culture medium (Fattahi et al. 2011) at a volume of one petri dish of 9 cm size per plant. In the controls, the five above-mentioned sterile inocula were used in both wound and no-wound creation methods. For inoculation, the soil next to the root was pushed aside and one hole was created at the root tip by a heat-treated needle while the inoculum was placed in contact with the wounded tissue (Tompkins et al. 1936). After that, the inoculum was covered by soil. Drainage of the pots was sealed by using solid paraffin, and the pots were flooded for 24 h. Afterwards, they were irrigated daily. The greenhouse temperature was adjusted to 30 ± 2 °C. Two weeks after inoculation, each root was dug from the soil and the rate of root rot was determined by using the standard scoring scale of 1–9 (1: no symptoms, 9: complete plant death) as described by Büttner et al. (2004).

In the second experiment, 0, 10, 20, and 40 g of rice seed inoculum in both wound and no-wound creation conditions was applied to the susceptible variety Rasoul. Two weeks after inoculation, the rate of root rot was assessed by the standard scoring scale of 1–9. Then, the disease index (DI) (Büttner et al. 2004; Hanson and Panella 2006) and disease escape (percentage of healthy plants) (Panella et al. 2016) were calculated by using the following formula:

$$\text{Disease index (DI)} = \frac{\sum(\text{Disease score} \times \text{number of plants with that score})}{\text{Total number of plants tested}}$$

$$\text{Disease escape (\%)} = \frac{\text{Total number of healthy plants (score)}}{\text{Total number of examined plants}} \times 100.$$

#### Inoculum preparation, inoculation of 12 week-old sugar beet plants and scoring

To prepare inoculum, the method of inoculation with rice seeds was used (Zhang et al. 2014). Briefly, the rice seeds were poured into a 250 ml Erlenmeyer flask. In each flask, 25 g of white long seed rice and 20 ml of distilled water were poured and fully stirred. The flasks were then autoclaved for 40 min at 121 °C for three successive days. Then, ten agar discs (5 mm

diameter) taken from the actively growing edges of 5 day-old colony of *P. drechsleri* were added to each flask. The flasks were kept at 25 °C for four weeks and shook daily to prevent clumping and make sure that all seeds were uniformly colonized (Zhang et al. 2014). Before the use of inoculum, some of the rice seeds were cultured in the CMA culture medium and the colony produced was checked. The 12 week-old sugar beet plants were irrigated immediately after inoculation with 20 g of inoculum, together with wound creation, and kept saturated for 24 h. Then, the drainage hole of the pots was opened and irrigation was conducted normally. To make sure that *P. drechsleri* was present at the base of the plants, some pieces of the citrus leaf were placed into the drained water for 24 h. Then, the leaf pieces were washed with sterilized distilled water. After being dried, they were transferred to the semi-selective CMA-PARP culture medium containing 0.2 g Benomyl. Petri dishes were kept at 25 °C and checked daily to observe the pathogen growth. After formation of the colonies, sporangia were produced in the sterilized distilled water by using the seeds of hemp and then studied microscopically (Baniahashemi 2004). Two weeks after inoculation, the roots were dug from the soil and scored by the standard scale of 1–9 (Büttner et al. 2004). The DI was calculated as described before, while the percentage of plants with DI of 1 through 3 (Hanson and Panella 2006; Panella 1998) was calculated using the following formula.

$$\text{Plants with DI of 1 through 3 (\%)} = \frac{\text{Total number of plants with a score lower than 3}}{\text{Total number of examined plants}} \times 100$$

To complete the principles of Koch and confirm the presence of *P. drechsleri* in the rotted roots, a piece of the border of the infected tissue, which contained the healthy and infected tissues, was cultured on the CMA medium.

#### Investigating aggressiveness of *P. drechsleri* isolates

To find the most aggressive isolate, five isolates of *P. drechsleri*, along with a mixture of them, were inoculated on the susceptible variety Rasoul by using 20 g of rice seed inoculum together with the wound

creation method. Two weeks after inoculation, all the roots were dug from the soil and scored by the 1–9 scale (Büttner et al. 2004). Moreover, as previously mentioned, the DI and the percentage of plants with DI of 1–3 were calculated.

#### Screening of *Beta* germplasm resistance to *P. drechsleri*

Reaction of the lines and varieties, together with resistant (SP85303-0) and susceptible (Rasoul) checks to *P. drechsleri*, was studied at the 12 week stage. Inoculation was done by using 20 g of rice seed inoculum together with the wound creation method against the highly aggressive isolate identified in the current research. The negative control was inoculated with 20 g of sterilized rice seeds.

One-month-old plants of the wild *Beta* species, together with resistant (SP85303-0) and susceptible (Rasoul) checks, were investigated for their resistance to *P. drechsleri*. The inoculation condition was based on the method optimized in the current research with the difference that 10 g of rice seed inoculum together with wound creation (one superficial scratch was created at the tap root by a heat-treated needle) was used for inoculation. The negative control was inoculated with 10 g of sterilized rice seeds. Two weeks after inoculation, each root of the wild species and check plants was dug from the soil and scored in accordance with the 1–9 scale (Büttner et al. 2004) for investigating the progress of rot. The DI and percentage of plants with the DI of 1–3 were also calculated using the formulae mentioned before.

#### Determining interaction of the isolate and sugar beet genotypes

Considering the reaction of different breeding lines, five lines with resistant, relatively susceptible, and susceptible reactions were selected and their reaction to five isolates of *P. drechsleri*, together with a mixture of them, was studied at the 12 week stage. The inoculation condition was based on the method optimized in this research.

#### Experimental design and data analysis

The germplasm evaluation experiments for resistance to *P. drechsleri* were carried out as a completely

randomized design with eight replications and three repetitions. The data of the DI were analysed by the analysis of variance (ANOVA) and their means were separated by Duncan's multiple range test at  $P \leq 0.05$ , using the SAS software (SAS Institute, Inc., Cary, NC).

## Results

### Identification of *P. drechsleri*, *P. cryptogea*, and *P. melonis* isolates

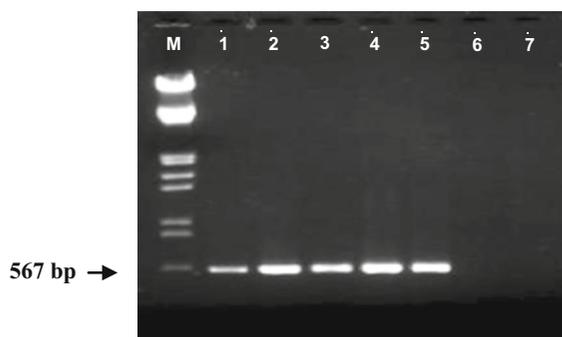
All isolates of *P. drechsleri* (numbers 1–5 in Fig. 1) produced the specific 567 bp band by using the primer pair ITS-DF2 and ITS-DR2 (Fig. 1). This band was not observed in the isolates of *P. cryptogea* (number 6 in Fig. 1).

In the potato pink rot test, pink rot was observed one week after inoculation on all potato tubers infected with the five isolates of *P. drechsleri* (Fig. 2). No symptom of rot was observed in control plants.

In Safflower seedlings' inoculation test, all isolates of *P. drechsleri* resulted in growth retardation and death of the safflower seedlings at five days after inoculation (Fig. 3).

### Pathogenicity test of *P. drechsleri* isolates on sugar beet

The isolates of *P. drechsleri* caused disease symptoms ranging from 1 to 9 in terms of the DI (Fig. 4).



**Fig. 1** The banding pattern of 567 bp band amplified by the primers ITS-DF2 and ITS-DR2. Numbers 1–5 isolates of *Phytophthora drechsleri* including Ph-17-22, Ph-17-27, Ph-dr, Kv4 and Kv10. Number 6 isolate of *P. cryptogea*. Number 7 negative control (Master mix without DNA). M size marker of DNA (Lambda DNA/EcoRI + HindIII Marker)



**Fig. 2** Symptoms of potato pink rot in potato tubers inoculated with the Kv10 isolate of *P. drechsleri* (right), as compared with the healthy check (left)

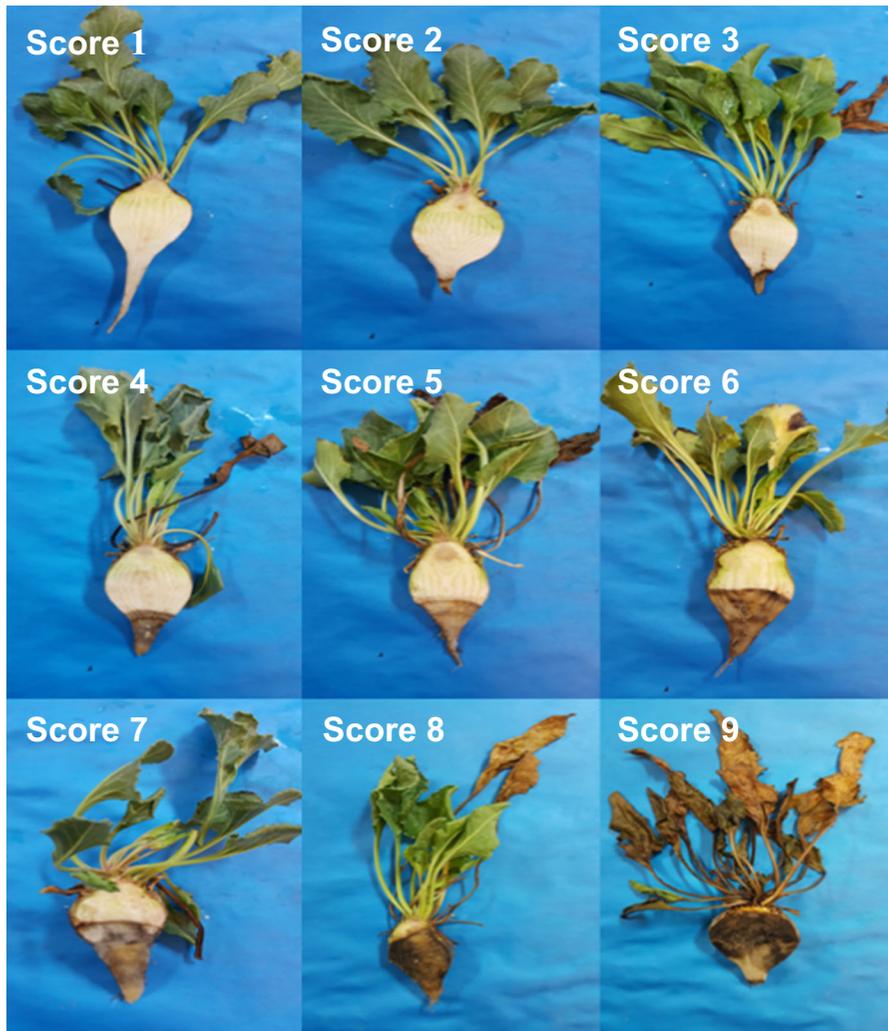


**Fig. 3** Reaction of safflower to *P. drechsleri*. Right healthy plant. Left plant infected with *P. drechsleri*

Symptoms of the disease in the above-ground plant parts included wilting and yellowing of lower leaves at initial stages and then wilting and death of the plant (Fig. 5). The symptoms on the roots included root brown rot starting from the root tip and developing towards the above-ground plant parts. Also, a black margin was observed between the healthy and rotted parts (Fig. 5). On this basis, the rate of root rot on each plant was scored by the 1–9 scale described by Büttner et al. (2004). There were significant differences at 5% probability level among the isolates for the rate of aggressiveness. The DI of the isolates of *P. drechsleri* ranged from 4.46 to 8, while the Kv10 isolate had the highest level of aggressiveness (8) and the lowest disease escape (0%) on the susceptible variety Rasoul (Fig. 6).

### Optimization of the screening method for evaluating *Beta* germplasm against *P. drechsleri*

The results of the analysis of variance in the first experiment indicated significant differences among the various methods tested ( $P$  value  $\leq 0.05$ ). The highest DI (8) and the lowest disease escape (0%) were

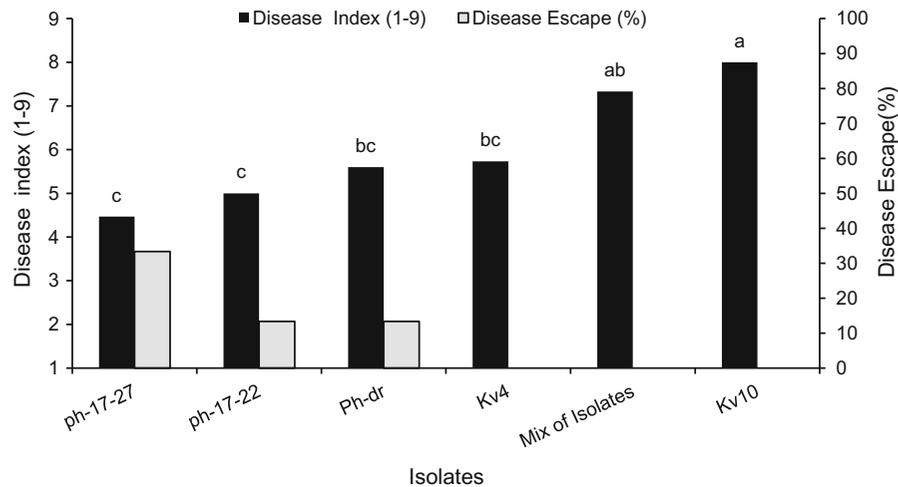


**Fig. 4** The scale of 1–9 in sugar beet lines infected with *Phytophthora drechsleri*



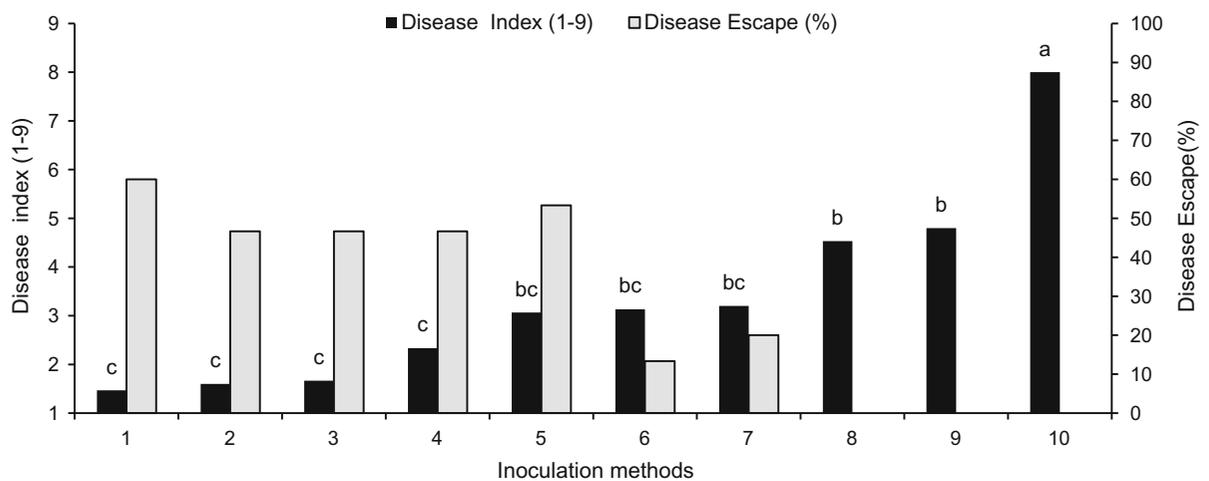
**Fig. 5** Symptoms of the *Phytophthora* root rot of sugar beet

observed in the method of inoculation with rice seeds on the wounded host tissues. Moreover, disease escape was not observed in inoculation methods with water suspension-hemp seeds and corn seeds (both were with wound creation), but they had a lower DI (4.80 and 4.53 respectively). Among the infected treatments, the method of infection with vermiculite without wound creation had the lowest DI (1.46) and the highest disease escape (60%) (Fig. 7). So, the results of this experiment indicated that the method of inoculation with rice seeds and wound creation resulted in a better development of sugar beet root rot disease than the other inoculation methods.



**Fig. 6** Comparison of pathogenicity rates of different isolates of *Phytophthora drechsleri* on the susceptible variety Rasoul. Bars with different letters for the disease index indicate significant differences at 0.05 probability level. Two weeks after inoculation, the rate of root rot was assessed by the standard scoring scale of 1–9 (1 no symptoms, 9 complete plant death). The disease index (DI) was calculated by the equation

DI = Sum of (number of observations in each grade)/(No. of plants assessed) (Büttner et al. 2004; Hanson and Panella 2006). The percentage of disease escape was calculated by the equation: Disease escape (%) = [Total number of healthy plants (score 1)/(No. of plants assessed)] × 100 (Panella et al. 2016)



**Fig. 7** The reaction of sugar beet to the infection caused by different methods of inoculation with the Kv10 isolate of *Phytophthora drechsleri*, 1 Vermiculite with hemp seed extracts without wound, 2 Hemp seed water cultures without wound, 3 CMA medium without wound, 4 Rice seeds without wound, 5 Corn seeds without wound, 6 CMA medium with wound, 7 Vermiculite with hemp seed extract with wound, 8 Corn seeds with wound, 9 Hemp seed water cultures with wound, 10 Rice seeds with wound. Bars with different letters for the disease

index indicate significant differences at 0.05 probability level. Two weeks after inoculation, the rate of root rot was assessed by the standard scoring scale of 1–9 (1 no symptoms, 9 complete plant death). The disease index (DI) was calculated by the equation DI = Sum of (number of observations in each grade)/(No. of plants assessed) (Büttner et al. 2004; Hanson and Panella 2006). The percentage of disease escape was calculated by the equation Disease escape (%) = [Total number of healthy plants (score 1)/(No. of plants assessed)] × 100 (Panella et al. 2016)

The second experiment was conducted to determine the best quantity of rice seed inoculum. The analysis of variance indicated that there was significant difference

at 5% level among the treatments in terms of infection. The DI of the control treatment without inoculum was found to be one in both wound and no-wound creation

treatments. Wound treatments with 40 and 20 g of inocula were grouped together and indicated the highest DI of 7.60 and 7.06 respectively, both without disease escape (Fig. 8). Owing to non-significant differences between these two treatments, the treatment of 20 g of inoculum with wound creation was used as an optimized inoculation method in the subsequent experiments for evaluation of resistance to PRR.

#### Evaluation of resistance to *P. drechsleri* in sugar beet breeding lines

Sugar beet breeding lines significantly differed at 5% probability level for resistance to *P. drechsleri*. The line O.T.201-15, resistant check (SP85303-0), and the line S2-24.P.107 had the lowest DI (3.09, 3.27, and 3.30 respectively) and the highest percentage of plants with DI of 1–3 (71.42, 72.72, and 66.66% respectively). These lines were placed in the resistant group. The lines S2-74 and S1-92-637 had the highest DI (8.21 and 8.16 respectively) and the lowest percentage of plants with the DI of 1 to 3 (4.34% and 5.26% respectively), which were placed in the susceptible group. The DI and percentage of plants with the DI of

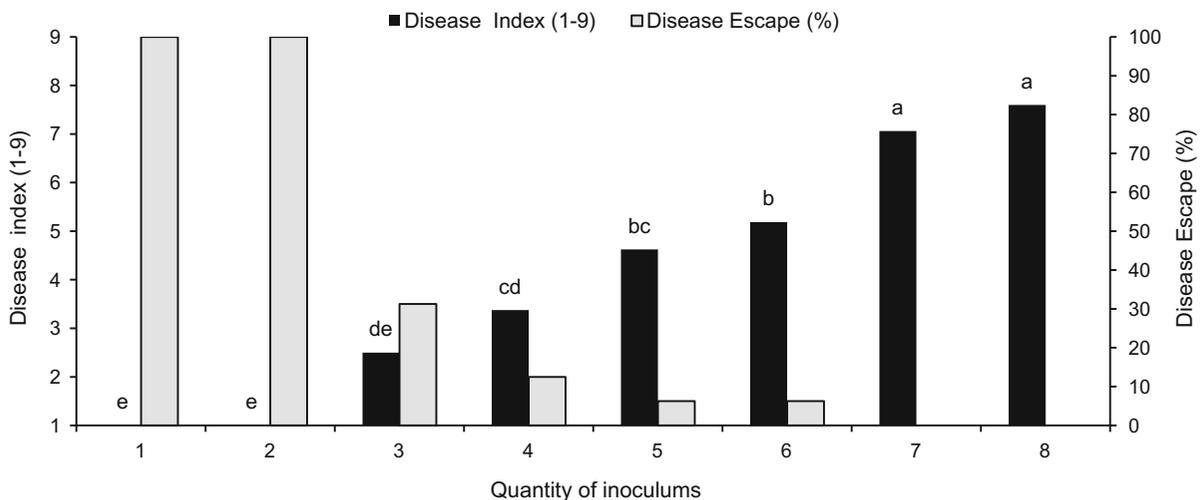
1–3 in the susceptible variety Rasoul were 7.54 and 9.09% respectively (Fig. 9).

#### Evaluation of resistance of wild *Beta* species to *P. drechsleri*

Owing to the annual habit and rapid flowering of wild relatives of those used in the current study, one month after sowing was the time used for inoculation. There were significant differences at 5% level among the wild species. Among the wild species studied, the accession 9402 belonging to *B. macrocarpa* and the accession 7234 from the species *B. vulgaris* subsp. *maritima*, both from the section *Beta*, had the lowest DI (2.60 and 2.29 respectively) and the highest percentage of plants with DI of 1 through 3 (82.35% and 80% respectively). These genotypes were placed in the resistant group. The susceptible and resistant checks had the DI of 7.26 and 3.06, respectively (Fig. 10).

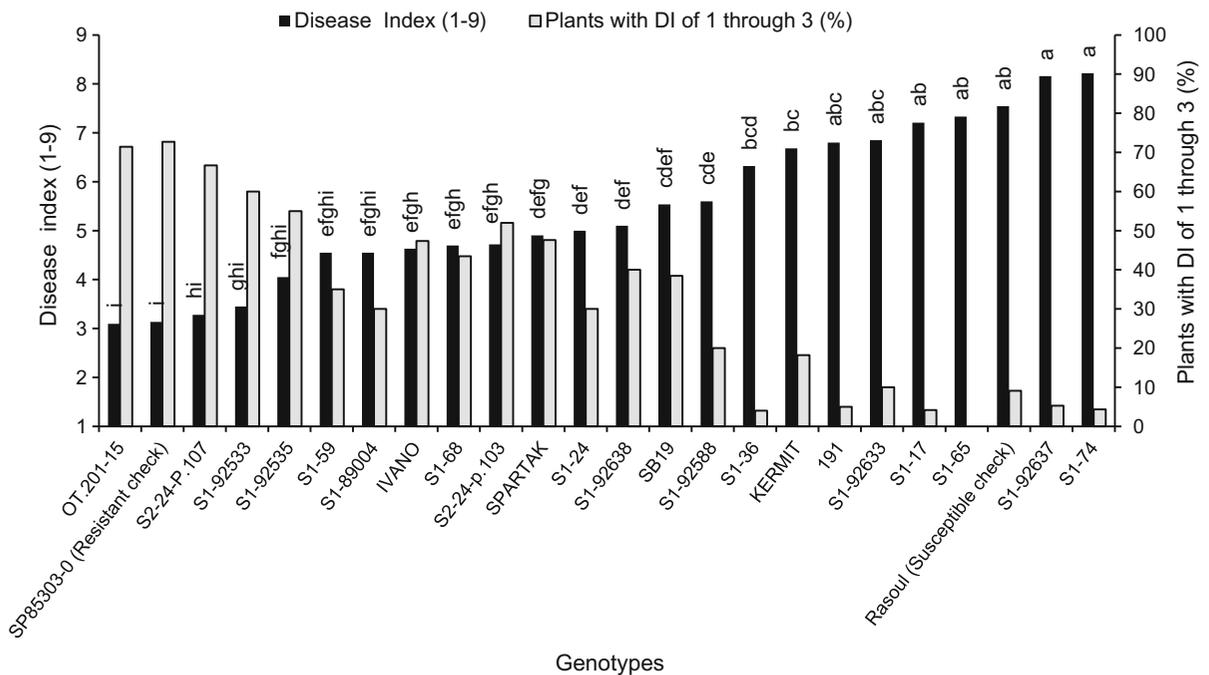
#### Study of isolate $\times$ genotype interaction

Results of the analysis of variance indicated that there were significant differences at 5% level among sugar



**Fig. 8** The reaction of sugar beet to the infection caused by different quantities of inoculum of Kv10 isolate of *Phytophthora drechsleri*, 1 Healthy check without wound, 2 Healthy check with wound, 3 10 g of rice seed without wound, 4 20 g of rice seed without wound, 5 10 g of rice seed with wound, 6 40 g of rice seed without wound, 7 20 g of rice seed with wound, 8 40 g of rice seed with wound. Bars with different letters for the disease index indicate significant differences at 0.05 probability

level. Two weeks after inoculation, the rate of root rot was assessed by the standard scoring scale of 1–9 (1 no symptoms, 9 complete plant death). The disease index (DI) was calculated by the equation  $DI = \text{Sum of (number of observations in each grade)} / (\text{No. of plants assessed})$  (Büttner et al. 2004; Hanson and Panella 2006). Percent of disease escape was calculated by the equation  $\text{Disease escape (\%)} = [\text{Total number of healthy plants (score 1)} / (\text{No. of plants assessed})] \times 100$  (Panella et al. 2016)



**Fig. 9** Reaction of sugar beet breeding lines to infection caused by the aggressive Kv10 isolate of *Phytophthora drechsleri*. Bars with different letters for disease index indicate significant differences at 0.05 probability level Two weeks after inoculation, the rate of root rot was assessed by the standard scoring scale of 1–9 (1 no symptoms, 9 complete plant death). The disease index (DI) was calculated by the equation  $DI = \text{Sum of}$

(number of observations in each grade)/(No. of plants assessed) (Büttner et al. 2004; Hanson and Panella 2006). The percentage of plants with DI of 1 through 3 was calculated by the equation  $\text{Plants with DI of 1 through 3 (\%)} = [\text{Total number of plants with a score lower than 3}]/(\text{No. of plants assessed}) \times 100$  (Hanson and Panella 2006)

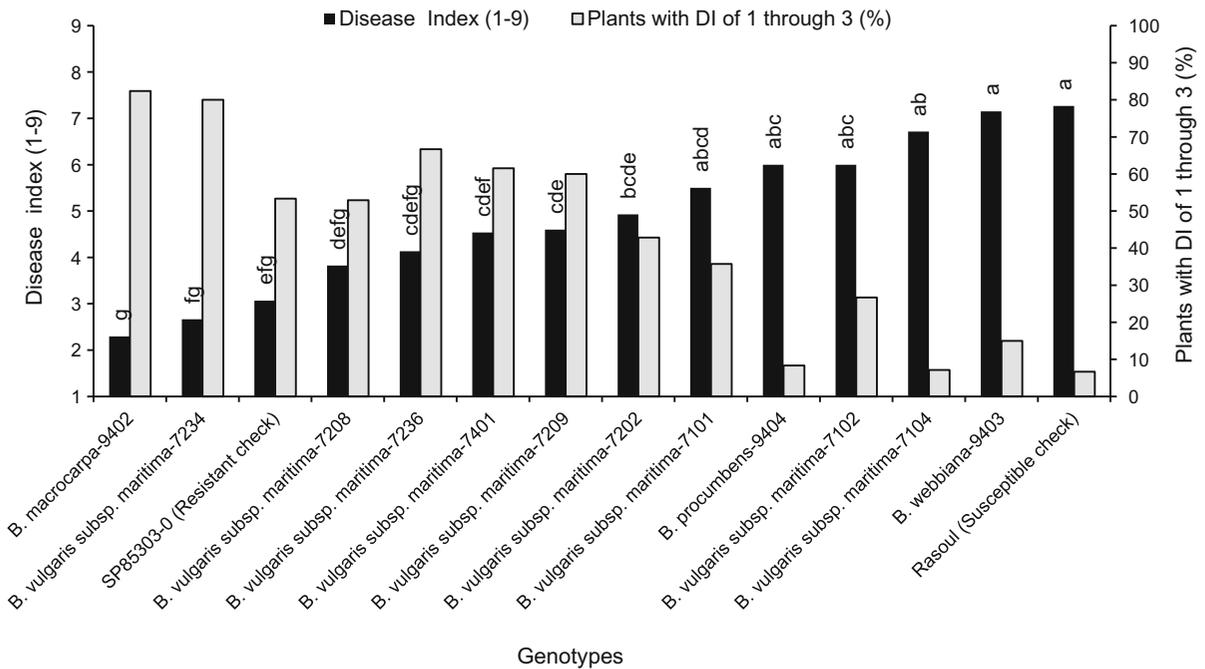
beet lines in resistance to the pathogen. Moreover, the isolates of *P. drechsleri* differed significantly at 5% level for pathogenicity. The line O.T.201-15 and the susceptible check Rasoul had the lowest (3.14) and highest (6.28) DI respectively (Fig. 11). The Kv10 isolate and the mixture of isolates resulted in the highest DI (5.98 and 5.44 respectively). Interaction of isolate  $\times$  genotype was found to be non-significant (Fig. 12).

## Discussion

In this research, two molecular and pathogenicity methods were used to distinguish the isolates of *P. drechsleri* from those of *P. cryptogea* and *P. melonis*. In molecular method, all the five isolates used belonged to *P. drechsleri*. The 567 bp band, amplified by the primer pair ITS-DF2 and ITS-DR2, was observed for all isolates of *P. drechsleri*, but it was not found in the isolate of *P. cryptogea*. This is in

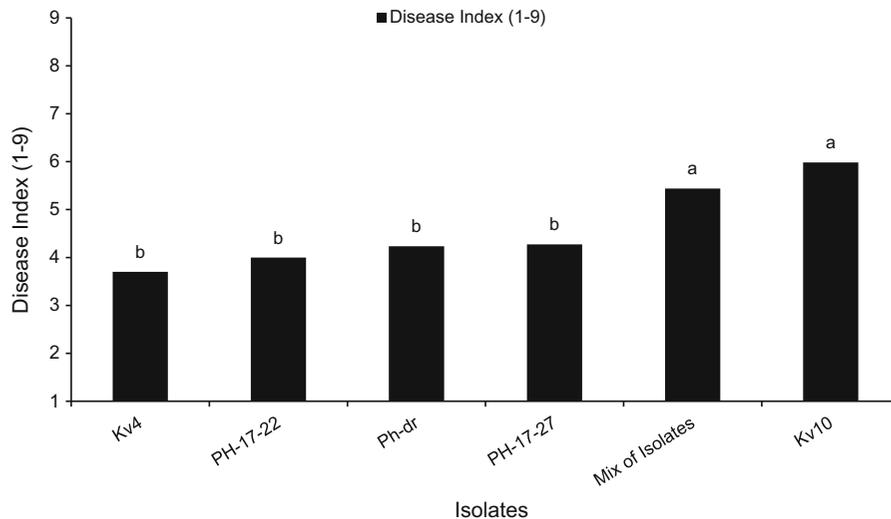
agreement with the findings of Mostowfizadeh-Ghalamfarsa and Banihashemi (2015b). Mostowfizadeh-Ghalamfarsa and Banihashemi (2015a) demonstrated that some isolates of *P. melonis*, obtained from cucurbits, pistachio, and sugar beet, were mistakenly identified as *P. drechsleri*, whereas their characteristics were not in accordance with those of *P. drechsleri*. Therefore, a highly specific marker for amplification of the ITS region was used to differentiate these two species.

In pathogenicity tests, all isolates of *P. drechsleri* caused potato pink rot, growth cessation, and damping-off on safflower seedlings, thus indicating that none of the isolates belonged to *P. melonis*. Other researchers have also reported that investigation of the ability of *P. drechsleri* to produce pink rot on potato tuber at 20 °C could be effective in differentiating *P. melonis* from *P. drechsleri* (Mostowfizadeh-Ghalamfarsa et al. 2006; Mostowfizadeh-Ghalamfarsa and Banihashemi 2015a). They have considered this method as a unique technique independent of cultivar,



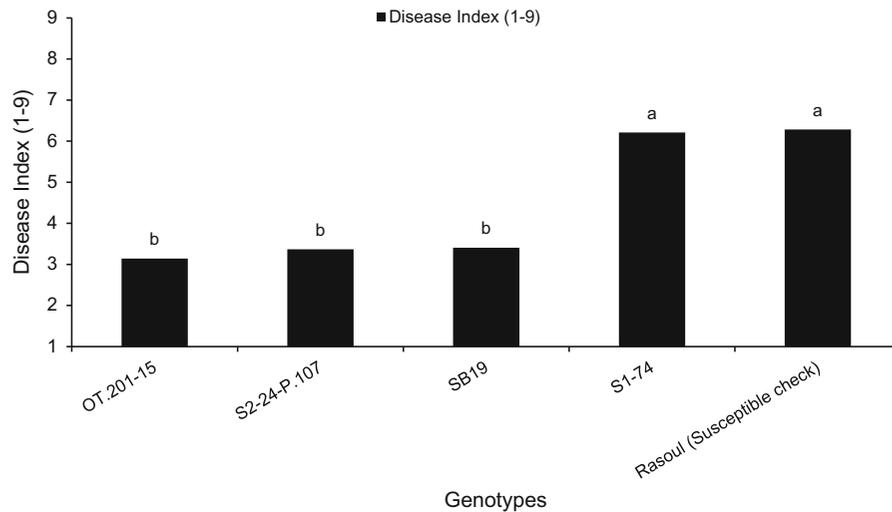
**Fig. 10** The reaction of wild relatives of *Beta* to the infection caused by the aggressive Kv10 isolate of *Phytophthora drechsleri*. Bars with different letters for the disease index indicate significant differences at 0.05 probability level. Two weeks after inoculation, the rate of root rot was assessed by the standard scoring scale of 1–9 (1 no symptoms, 9 complete plant death). The disease index (DI) was calculated by the equation

DI = Sum of (number of observations in each grade)/(No. of plants assessed) (Büttner et al. 2004; Hanson and Panella 2006). The percentage of plants with DI of 1–3 was calculated by the equation Plants with DI of 1 through 3 (%) = [Total number of plants with a score lower than 3]/(No. of plants assessed) × 100 (Hanson and Panella 2006)



**Fig. 11** The disease index of *Phytophthora drechsleri* isolates on five sugar beet lines. Bars with different letters indicate significant differences at 0.05 probability level. Two weeks after inoculation, the rate of root rot was assessed by the standard

scoring scale of 1–9 (1 no symptoms, 9 complete plant death). The disease index (DI) was calculated by the equation DI = Sum of (number of observations in each grade)/(No. of plants assessed) (Büttner et al. 2004; Hanson and Panella 2006)



**Fig. 12** The disease index of five sugar beet lines in response to *Phytophthora drechsleri* isolates. Bars with different letters indicate significant differences at 0.05 probability level. Two weeks after inoculation, the rate of root rot was assessed by the

easy-to-score, and a reliable method to differentiate *P. drechsleri* from *P. melonis*. Furthermore, Banihashemi and Mirtalebi (2008) have introduced the pathogenicity tests on safflower as a proper method to differentiate between the two species *P. drechsleri* and *P. melonis*. In this method, the inoculation of safflower seedlings with *P. drechsleri* results in growth retardation and damping-off on the seedlings, while the isolates of *P. melonis* do not produce the symptoms of damping-off on safflower (Esmaili-Shirazifard and Banihashemi 2008), which is in agreement with our results.

In our research, all isolates of *P. drechsleri* caused disease on the sugar beet and clearly showed the symptoms of PRR as described by Tompkins et al. 1936 and Jacobsen 2006. The isolate Kv10 was the most aggressive isolate on the susceptible variety. Other researchers have also reported diversity in the aggressiveness of the isolates of *P. drechsleri* (Olson and Benson 2013). The results of this experiment indicated the necessity of using an isolate with high aggressiveness in experiments related to the evaluation of sugar beet lines for resistance to *P. drechsleri*.

One of the important challenges for plant breeders in the evaluation of resistance to pathogens is to find a simple, reliable, and precise method for uniform infection (Mansoori and Banihashemi 1982). Owing to non-uniform infections and the inability to control

standard scoring scale of 1–9 (1 no symptoms, 9 complete plant death). The disease index (DI) was calculated by the equation  $DI = \text{Sum of (number of observations in each grade)} / (\text{No. of plants assessed})$  (Büttner et al. 2004; Hanson and Panella 2006)

environmental conditions in the field, one of the approaches was to conduct experiments in greenhouse conditions, which made it possible to differentiate plants for resistance to root rot. Consistent with our results, Luterbacher et al. (2005) have compared the results of greenhouse and field experiments to evaluate sugar beet resistance to *Rhizoctonia solani* and reported that greenhouse experiments had provided a proper environment for infection. Therefore, it was necessary to compare different methods of inoculation and different quantities of inoculum to find the best method for evaluating the resistance of genotypes to this disease.

Disease escape can often cause problems in resistance evaluation experiments in such a way that the susceptible plant seems to be resistant. Thus, to minimize the probability of disease escape in screening genotypes for resistance, it is necessary to provide conditions similar to natural condition for disease development (Bosland and Lindsey 1991). Therefore, we created an optimized method in the greenhouse by getting inspirations from the nature where this disease occurs in flooded and warm conditions together with wound creation by pests at the maturity stage of sugar beet (Tompkins et al. 1936). As the sugar beet root in the field is constantly exposed to damages caused by pests and physical injuries, which result in increased root rot, it is highly important to select cultivars, which

could maintain their resistance under these conditions. For this reason, the wound creation method was used to facilitate penetration of the pathogen for reliable selection. Moreover, the optimized quantity of inoculum plays an important role in decreasing disease escape (Vale et al. 2001). Our results indicated that wound treatments with 40 and 20 g of rice seed inoculum showed the highest DI and were classified in the same group. Therefore, 20 g of rice seed inoculum, which made it possible to differentiate genotypes two weeks after inoculation, was selected as the suitable quantity of inoculum. Wound creation provides proper condition for disease development and facilitates penetration of the pathogen into the root tissue. Thus, it ascertains that non-infection is due to resistance and isn't escape of disease. Under this condition, proper disease development and differentiation among the lines was observed. In no-wound creation treatment, a high probability for disease escape (47–60%) was observed. The no-wound creation treatment also caused the infection, but it required a longer time for development of disease symptoms with high score and differentiation of genotypes. In accordance with the results of this research, Tompkins et al. (1936) studied the pathogenicity of *P. drechsleri* on the sugar beet in the field, where both wound and no-wound treatments were used. They reported that despite infection occurrence in the no-wound treatment, only 41% of all plants were infected, but 82% of the plants had the infection in wounded tissue (Tompkins et al. 1936). Evaluation of strawberry resistance to *P. cactorum* with the zoospore suspension method indicated that inoculation of plants without wound creation in rhizome results in weak disease development, while wound creation saves time for screening of genotypes (Eikemo et al. 2000). Other researchers have also used wounded tissues to evaluate resistance to *Phytophthora* (Stirling and Irwin 1986; Quesada-Ocampo et al. 2009; Hajebrahimi and Banihashemi 2011).

Development of cultivars with desirable and durable resistance depends on access to resistance sources as well as sufficient knowledge about genetics of the pathogen and host plant. In this research, 19 breeding lines, three cultivars, and 14 accessions belonging to the species *B. procumbens*, *B. macrocarpa*, *B. webbiana*, and *B. vulgaris* subsp. *maritima* were evaluated. The DI and percentage of plants with the DI of 1–3 were calculated for each genotype.

Percentage of roots with a score lower than 3 is known as marketable or harvestable roots in sugar factories and also considered as a measure for resistance evaluation of a genotype (Panella et al. 2015a).

The O-type line O.T.201-15 had the highest resistance (DI of 3.09) at the mature plant stage. This line was derived from the mother line SB-22 (FC201), which contained the resistance genes for *Rhizoctonia*, *Aphanomyces*, and *Rhizomania* (Panella 2005). Also, the line S2-24-P.107 had resistance (DI of 3.27) at the mature plant stage. This line had been derived from the SB-19 population, which is resistant to *Rhizoctonia* root rot and was placed, by Mahmoudi et al. (2014), in the relatively resistant group with respect to *Pythium aphanidermatum* resistance. Consistent with our results, the *P. drechsleri* resistant cultivars of squash (*Cucurbita pepo*) were also resistant to *P. aphanidermatum* (Mansoori and Banihashemi 1982). Therefore, it is likely to find sources of resistance to *Phytophthora* in sugar beet populations resistant to *Rhizoctonia* and *Pythium*.

Lines of the second generation of selfing, S2-24-P.107 and S2-24-P.103, with an average DI of 3.27 and 4.72, respectively, had higher resistance from progeny selection than their parental line SB19-S1-24 (first generation of selfing) with an average DI of 5 and the earlier generation SB19 (initial population) with a DI of 5.53. This indicates that it is possible to enhance resistance to *P. drechsleri* by progeny selection in subsequent generations. Mirzaian et al. (2014) reported the progeny selection as an effective method to increase resistance to *P. drechsleri* and *P. ultimum* in safflower. Also, other researchers have shown that the level of resistance of the progenies could be increased by selection for resistance to *R. solani* in sugar beet (Hecker and Ruppel 1977; Ebrahimi-Koulaee and Mahmoudi 2010). In addition, not all progenies of a population or line have similar reactions. This can be attributed to the cross-pollination of sugar beet and the multigenic nature of the resistance to this disease, which is in accordance with the results of other researchers (Hecker and Ruppel 1977; Ebrahimi-Koulaee and Mahmoudi 2010).

In another part of this research, two accessions from the species *B. macrocarpa* and *B. vulgaris* subsp. *maritima* had high resistance to *P. drechsleri*. These belonged to the section *Beta*, and both crop wild relatives can easily be crossed with sugar beet. Consistent with our results, Luterbacher et al. (2005) have reported highly resistant accessions to the

Oomycetes *P. ultimum* and *A. cochlioides* in the species *B. vulgaris* subsp. *maritima* and *B. macrocarpa* of the section *Beta*. These two wild species are likely to contain genes for resistance to pathogenic Oomycetes of sugar beet.

The results of the isolate  $\times$  genotype interaction experiment indicated that there were different levels of resistance to *P. drechsleri* in the genotypes studied. Further, there were significant differences in aggressiveness of the isolates, but the isolate  $\times$  genotype interaction was not significant, which indicated that each genotype had the same reaction to all isolates. This implies that there is partial and multigenic resistance to *P. drechsleri* in sugar beet. This is consistent with the results of Windels et al. (1995) and Mahmoodi et al. (2004), who found non-significant isolate  $\times$  genotype interactions for *Rhizoctonia* root rot of sugar beet.

No sources of resistance to *P. drechsleri* exist in sugar beet. This research was conducted for the first time to identify resistant sources to this disease in breeding lines and wild relatives as well as to achieve a reliable method for screening *Beta* germplasm against this pathogen. Apparently, it is possible to find sugar beet lines with higher resistance by selecting progenies (developing S1 lines by selfing). Global warming has made it more important to develop sugar beet cultivars resistant to pathogens such as *P. drechsleri* and *P. aphanidermatum*. The results of this research can be used by sugar beet breeders to develop high-yield and high-quality cultivars containing genes for resistance to *P. drechsleri*.

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