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ORIGINAL ARTICLE

# *In vitro* evaluation of potato genotypes for resistance against bacterial soft rot (*Pectobacterium carotovorum*) – a new tool for studying disease resistance

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**Abstract**

*In vitro* screening techniques were used to evaluate 46 genotypes of Iranian potato collection for resistance to bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc). One month old *in vitro* rooted potato plantlets were inoculated by two inoculation techniques under *in vitro* conditions: 1) sterile toothpicks dipped into bacterial suspension and pressed into the crown of plantlets and 2) the freshly cut crown of plantlets were dipped into bacterial suspension of $10^8$ cfu · $ml^{-1}$ for 10 min. Typical soft rot disease symptoms, including the percentage of wilted leaves were recorded on inoculated plantlets 3, 6, 9, 12 and 15 days post-inoculation. The potato genotypes which were examined responded differently to Pcc and varying levels of resistance were observed. Potato genotype AG showed the highest level of resistance. Results obtained from *in vitro* screening were then verified by classical tuber slice assay. The verifications were conducted using five representative cultivars: Milva, Ramus, Picaso, Marfona and Agria which responded similarly to both *in vitro* and classical evaluation systems. Similar results obtained from these tests indicated that the *in vitro* screening technique developed in this study could provide a simple and rapid whole plant assay in selecting resistant potato genotypes against bacterial soft rot.

**Key words:** *in vitro*, potato soft rot, resistance, screening method

## Introduction

Potato is the third most important food crop after wheat and rice in Iran and is cultivated in many provinces of the country. Bacterial soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc; syn. *Erwinia carotovora* subsp. *carotovora*) is considered to be a major disease of potato crops across the temperate regions of the world (Pitman *et al.* 2010) including Iran (Baghaee-Ravari *et al.* 2011). Soft rot disease is often initiated through meristemic stolons, lenticels, or wounds. Production of pectolytic plant cell wall degrading enzymes by bacteria leads to macerated tissues which release bacterial inoculums spreading to other tubers. Rotting can occur in the field or in storage leading to significant losses of crops (Thangavel *et al.* 2014).

Pectolytic Erwinias, causing soft rot in crop plants, were classified in three species: *Erwinia cacticida*, *Erwinia chrysanthemi* and *Erwinia carotovora* (Young *et al.* 1996). These three species were later placed into the genus *Pectobacterium* (Hauben *et al.* 1998). However, based on recent studies, *E. chrysanthemi* is classified under genus *Dickeya* (Samson *et al.* 2005). *Erwinia carotovora* has a wider host range among these species (Avrova *et al.* 2002) and is considered to be

a destructive pathogen of potato crops in temperate regions, whereas *E. chrysanthemi* is a serious problem in warmer climates (Czajkowski *et al.* 2011). *Erwinia carotovora,* based on biochemical, pathological and molecular traits, has been divided into five subspecies namely: *E. carotovora* subsp. *carotovora, E. carotovora* subsp. *odorifera, E. carotovora* subsp. *atrosepticum, E. carotovora* subsp. *betavasculorum* and *E. carotovora* subsp. *wasabiae* (Goto and Matsumoto 1987; Gallois *et al.* 1992).

Chemical control and cultural practices including the use of certified seed potatoes have been relatively ineffective in reducing crop losses. Therefore, breeding for resistance remains the most effective method for controlling the disease caused by *Pectobacterium* on potato. Although potato accessions with complete resistance are not known, varying levels of resistance to soft rot have been reported (Czajkowski *et al.* 2011). In contrast, relatively high levels of resistance to soft rot have been observed (Austin *et al.* 1988) in wild species of potato and in tubers of somatic hybrids of *Solanum brevidens* and *S. tuberosum* and their sexual progeny. A reliable method of screening is crucial for the development of disease resistant commercial cultivars. Therefore, the use of disease-free *in vitro* plantlets for evaluation of potato germplasm could facilitate more precise screening and identification of reliable sources of resistance. In this study we have developed a new *in vitro* screening technique for evaluation of resistance in potato genotypes and we examined the validity of the technique in comparison to conventional tuber slice assay. We also report about some potato genotypes with relatively high levels of resistance to Pcc in *Solanum tuberosum* collection of Iran which can be used for further research and breeding new resistant cultivars.

## Materials and Methods

### Plant materials

One month old *in vitro* plantlets of 46 potato genotypes obtained from the National Plant Gene Bank of Iran were micro-propagated and established on a basic MS medium (Murashige and Skoog 1962). Explants were grown under *in vitro* conditions of 16 h light and 8 h dark periods, at 23°C.

### Bacterial strains

Bacterial strains of *Pectobacterium carotovorum* subsp. *carotovorum* used in this study were isolated from potato plants, showing typical soft rot symptoms, grown in potato fields of Khorasan Razavi Province, Iran. Isolates were identified and compared to a type strain of Pcc obtained from the Netherlands as positive control. All bacterial strains were grown on nutrient agar medium (NA) at 27°C for 24 h. The culture was then used to prepare suspension cells of $1 \times 10^8$ cfu · ml$^{-1}$ for *in vitro* screening assay.

### Pathogenicity test

Two strains of *P. carotovorum* subsp. *carotovorum* were used to carry out pathogenicity tests on potato tubers (*Solanum tuberosum* cv. *agria*). Tubers were washed in water and then surface sterilized with 96% ethanol for 2 min, air-dried and placed in jars containing 100 ml distilled water. The inoculations were performed by dipping sterile toothpicks into the bacterial suspensions which were prepared from fresh bacterial cultures. The culture medium was then adjusted to an absorbance with an optical density of 0.2 at 600 nm wavelength in order to reach a cell concentration of $1 \times 10^8$ cfu · ml$^{-1}$. The treated toothpicks were injected into each tuber to a depth of 1 cm. Several tips were used if the tubers were big enough and all were sealed to maintain high humidity at 27°C in the dark. After 24 h, tubers were inspected daily to record soft rot symptoms (macerated tissues).

### DNA extraction

Bacterial DNA was extracted from 24 h-old bacterial cultures grown on NA using Mahuko's (1994) method. A fresh bacterial colony was transferred to a sterilized 1.5 ml microtube containing 1,000 µl RB suspension buffer [0.15 M NaCl, 10 mM EDTA (pH 8)]. After centrifugation for 2 min at 9,000 rpm, then 300 µl of TES extraction buffer [0.2 M Tris-HCl (pH 8), 10 mM EDTA (pH 8), 0.5 M NaC1, 1% sodium dodecyl sulfate (SDS)] was added to the pellet and vortexed for 30 sec. A volume of 200 µl TES extraction buffer containing proteinase K (to a final concentration of 50 µg · ml$^{-1}$) was added to the suspension. After vortexing the mixture thoroughly, the tubes were placed in a water bath at 65°C for 30 min. One-half volume (250 µl) of 7.5 M ammonium acetate was added to the tubes. Then the samples were incubated at –5°C for 10 min and centrifuged for 15 min at 13,000 rpm. The supernatant was transferred to fresh tubes and an equal volume (500 µl) of ice-cold isopropanol was added to each tube. The tubes were incubated at –20°C for 1–2 h and centrifuged for 10 min at 13,000 rpm to pellet the DNA. Supernatant was decanted and the DNA pellet was washed with 800 µl of cold ethanol (70%) and air dried for 10–15 min. Extracted DNA was stored at –20°C until use.

Plant DNA was extracted from infected plantlets of cvs. Agria, Milva, Ramus and Picaso. The tissue was harvested from a location above the inoculation point on plantlets and ground in liquid nitrogen into a fine powder, and added to 300 µl of TES extraction buffer.

The extraction method described above was also used to extract DNA from potato plantlets.

## Polymerase chain reaction (PCR) detection assay

A pair of specific primers: EXPCCF/EXPCCR (Kang *et al.* 2003) for amplification of Pcc target sequence was used for molecular detection of Pcc. The sequences of the primers were as follows: EXPCCR – (5'-GCCG-TAATTGCCTACCTGCTTAAG-3') and EXPCCF – (5'-GAACTTCGCACCGCCGACCTTCTA-3'). PCR diagnostic assays were performed in 25 µl final reaction volume containing 1 µl of each primer 100 pmol · · $\mu l^{-1}$, 2.5 µl buffer 10X, 1 µl $MgCl_2$ 25 mM, 0.5 µl dNTPs 25 mM, 0.5 µl Taq DNA polymerase, and 2 µl of target DNA solution. PCR reactions were conducted using an Eppendorf thermocycler with one denaturation cycle of 4 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, 2 min at 72°C and one cycle of a final extension for 7 min at 72°C. PCR products were separated on a 1.2% agarose gel in Tris-acetate-EDTA (TAE) buffer, and visualized by staining with ethidium bromide (1 mg · $l^{-1}$).

## *In vitro* evaluation

One month old plantlets were inoculated with $1 \times 10^8$ cfu · $ml^{-1}$ Pcc in three replications. Two inoculation methods were used under *in vitro* conditions: 1) sterile toothpicks were dipped into bacterial suspension and injected into the crown of each plantlet (Fig. 1); 2) the crown of a 1 month plantlet was excised using a sterile scalpel and then dipped in bacterial suspensions for 10 min. Soft rot symptoms were recorded as the percentage of wilted leaves at time intervals of 3, 6, 9, 12 and 15 days post inoculation. Disease severity obtained from *in vitro* evaluation assays was scored from 1 to 6. This evaluation was based on the percentage of wilted leaves of the plantlet (Table 1).

## Tuber slice assay

To validate the findings of *in vitro* assays, further experiments were carried out using five representative genotypes which were selected on the basis of the results of *in vitro* screening as follows: Milva (resistant), Ramus and Marfona (moderately resistant), and Picaso and Agria (susceptible). Inoculation of tuber slices was performed as reported by Jellis with minor modification (Jellis 1992). Elite seed tubers were obtained, surface sterilized and cut into 5-transverse slices 0.7 cm thick. Slices were individually transferred to sterile 100 × 15 mm Petri dishes and inoculated in the center with Pcc suspension cells at 20 µl of $1 \times 10^8$ cfu · $ml^{-1}$. These Petri dishes were incubated at 27°C for 7 days and symptoms were recorded after the second day. The experiment was conducted using a completely randomized design with five replicates. After inoculation, the rotten tissue was removed and the diameter of the cavities was measured in two directions at right angles. The vertical and horizontal diameters (cm) of the cavities formed by the bacteria were measured.

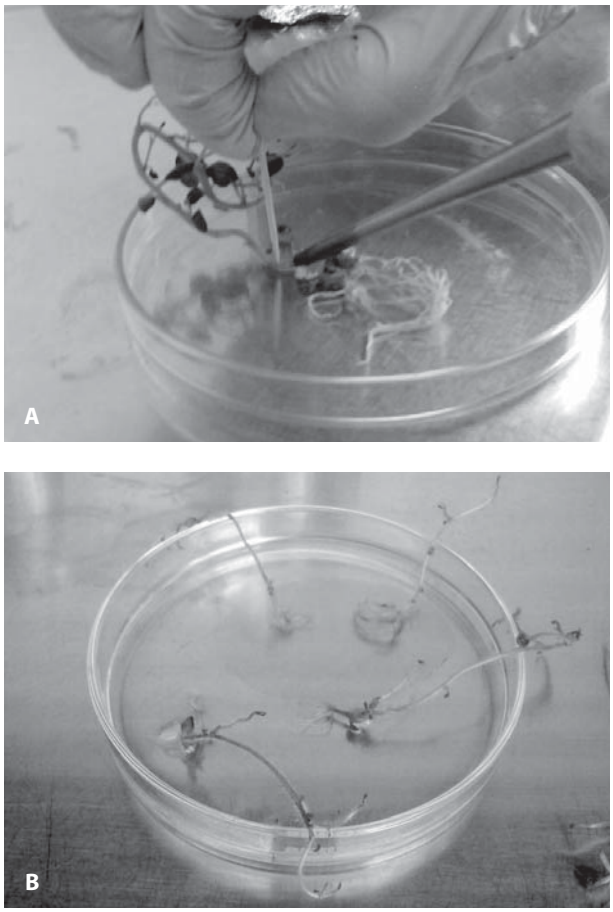


**Fig. 1.** Two inoculation methods used for potato plantlets against bacterial soft rot: A – using sterile toothpick for transferring inoculum to the plant tissue; B – dipping freshly cut crown in of the plantlet into bacterial suspension

**Table 1.** Scoring system for assessing disease severity developed by Pcc on potato plantlets

| Disease scores | Percentage of disease severity |
|---|---|
| 1 | No disease symptoms |
| 2 | < 25% leaves wilted |
| 3 | 50% ≥ leaves wilted > 25% |
| 4 | 75% ≥ leaves wilted > 50% |
| 5 | 75% < leaves wilted |
| 6 | 100% leaves wilted |

Pcc – *Pectobacterium carotovorum* subsp. *carotovorum*

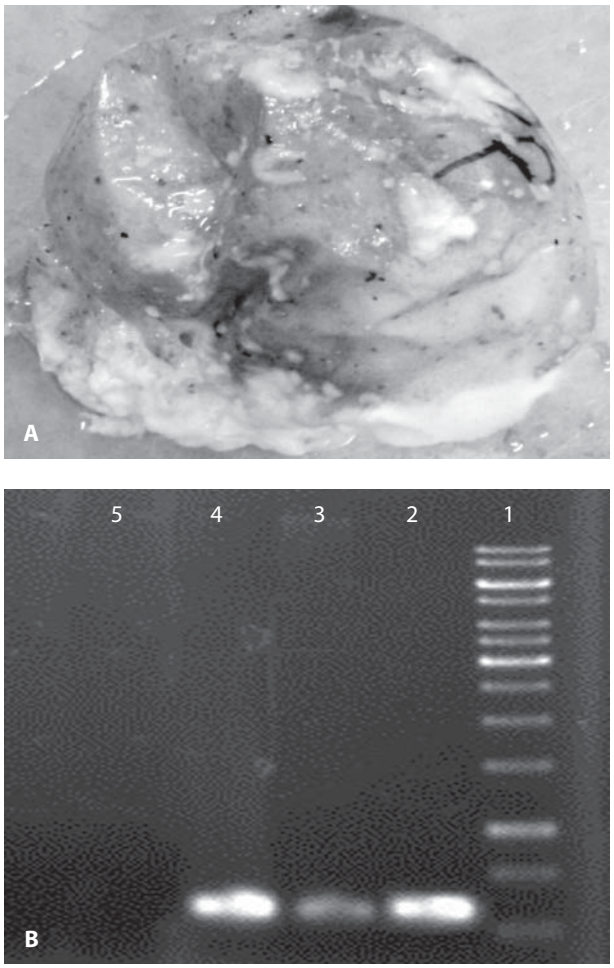


**Fig. 2.** Soft rot disease development and pathogenicity of *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) isolates used in this study: A – seed tuber of potato cv. Agria inoculated with isolate Pcc1; B – PCR detection of Pcc using specific primer pair, EXPCCF/EXPCCR. Lanes: 1) 1 kb DNA size marker; 2) isolate Pcc1; 3) isolate Pcc2; 4) Pcc type strain; 5) negative control (sterile distilled water)

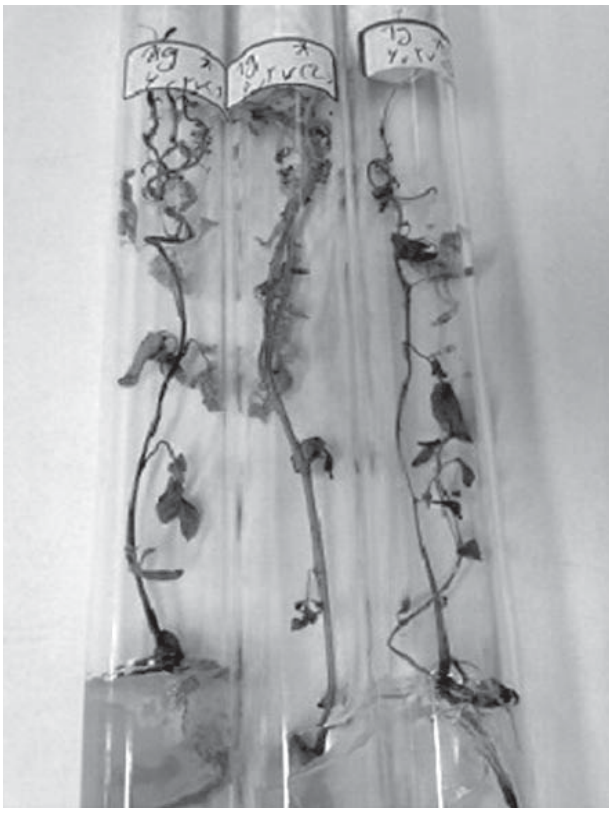

**Fig. 3.** Development of bacterial soft rot disease symptoms on the foliage of susceptible cultivar 1G, in three replicates

### Data analysis

Experiments were carried out using appropriate experimental designs. A factorial experiment with a completely randomized design was used to test the responses of genotypes to evaluation techniques. Statistical data analysis was performed with SAS® Statistical software. Analysis of variance was based on general linear model procedures, and means for different traits were compared with Fisher's Least Significant Difference (LSD) test. Correlations were estimated using Pearson correlation coefficients.

## Results

### Pathogenicity of isolates

All treated tubers of cv. Agria inoculated with two Pcc strains showed typical symptoms of the disease, including soft rot and tissue maceration. Soft rot symptoms appeared seven days post inoculation. Severe disease symptoms developed rapidly, especially when the bacterial suspension was injected into each tuber to a depth of 1 cm (Fig. 2A). Two representative isolates of Pcc collected from potato fields were successfully detected, producing the expected DNA fragment of 550 bp by the primer pair of EXPCCF/EXPCCR in a PCR assay. The bacterial isolate Pcc1 which produced a stronger DNA amplification signal was selected for artificial inoculation in the screening system (Fig. 2B).

### Response of potato genotypes to Pcc

Soft rot symptoms appeared at the sites of inoculation in almost all tested materials. Chlorosis and necrosis symptoms were developed on susceptible plantlets three days after inoculation followed by wilting (Figs 3 and 4). Thirteen genotypes showed varying levels of resistance (Fig. 4). Some of these genotypes such as AG, Milva and Pico were highly resistant and others such as Agria hybrid 2, Ramus, Els and Condoor were evaluated as being relatively/moderately resistant.

In addition to phenotypic evaluation, responses of representative potato genotypes were assessed by molecular diagnosis. A PCR product of 550 bp was amplified from the DNA of plantlet tissue harvested from a location above the inoculation point (Fig. 5).

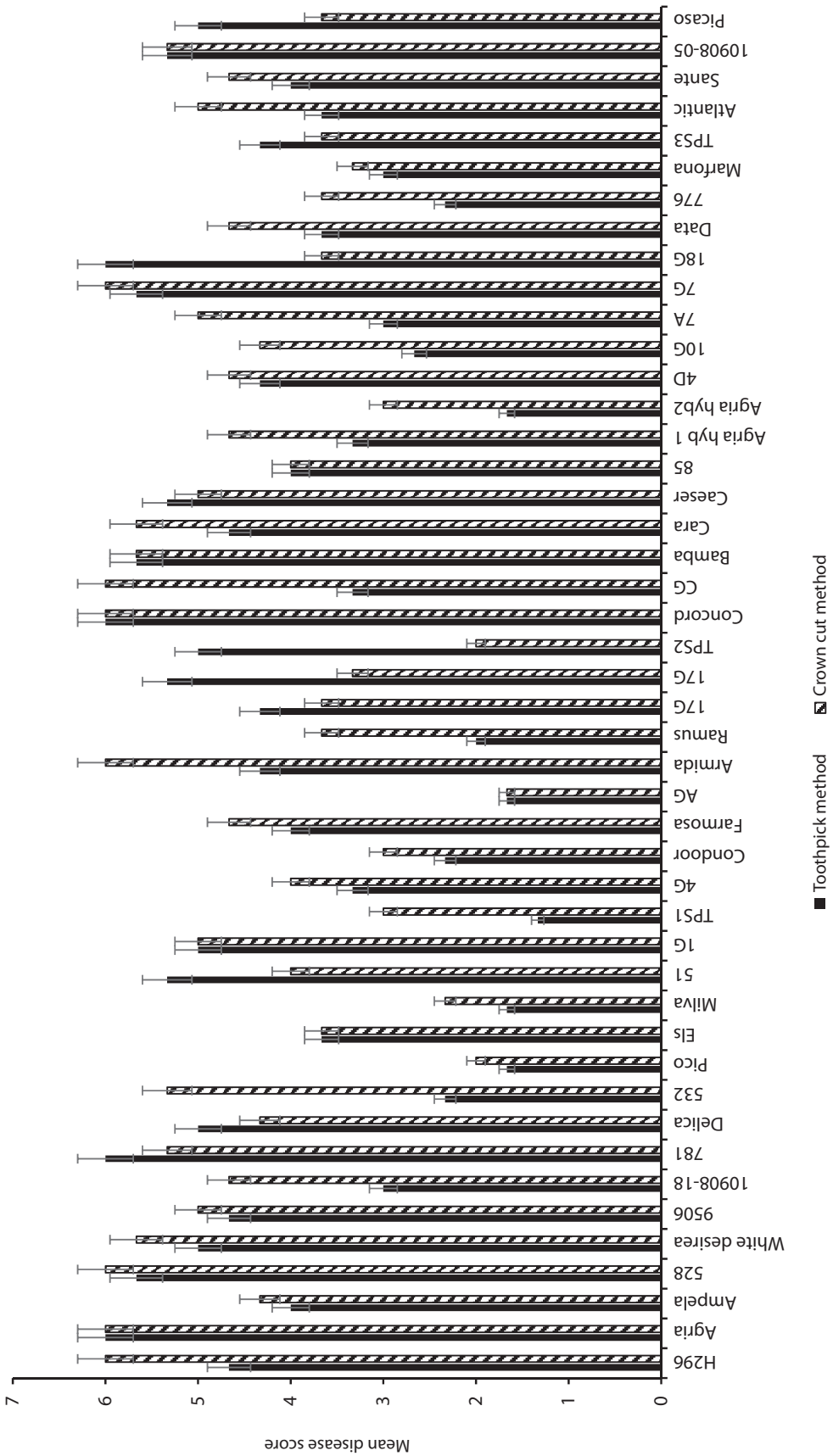


**Fig. 4.** Mean disease scores recorded on potato genotypes assessed based on the two *in vitro* inoculation methods: scores 0–2 (resistant), 2–4 (moderately resistant) and scores > 4 (susceptible)

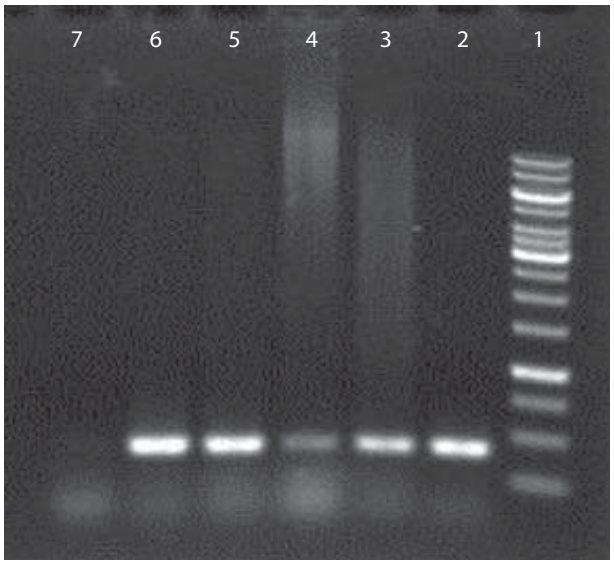


**Fig. 5.** Assessing *Pectobacterium carotovorum* subsp. *carotovorum* infection in *in vitro* plantlets by PCR. Lanes: 1) 1 kb ladder; 2) genomic DNA of isolate Pcc1 as positive control; 3) cv. Ramus; 4) cv. Milva; 5) cv. Picaso; 6) cv. Agria; 7) negative control (sterile distilled water)

### Validation of *in vitro* screening for potato soft rot

The results obtained from tuber slice assay (recorded as diameter of the cavity formed by tissue maceration) agreed with the results obtained from *in vitro* inoculation methods (Fig. 6). Potato cv. Milva which showed a high level of resistance against Pcc among all 46 genotypes under *in vitro* assessment was also determined to be the most resistance genotype in the classical tuber slice assay, since its tuber didn't macerate and no cavity was observed. Cultivar Ramus was assessed as being relatively resistant against Pcc by both *in vitro* methods and the tuber slice assay. It was also shown that cv Marfona, is moderately resistant and cv. Agria is highly susceptible to Pcc based on both screening systems. All five representative cultivars showed higher resistance in tuber slice assay, than *in vitro* screening techniques. It is notable that high levels of positive correlations were estimated among the three different assays and the highest correlation (0.752) was found between the two *in vitro* techniques (Table 2).

## Discussion

Different levels of resistance to *P. carotovorum* were detected among 46 genotypes of *S. tuberosum* collection of Iranian National Plant Gene-Bank, based on evaluation by two *in vitro* screening techniques. Interestingly, susceptible cultivars, Picaso and Agria, showed similar responses in both *in vitro* evaluation methods against Pcc. In contrast, cv. Ramus which was assessed as being moderately resistant responded significantly differently under the two *in vitro* inoculation techniques. Also in resistant cultivar Milva the severity of disease was higher when inoculated by the second *in vitro* method (Fig. 4). These results were consistent with studies by Allefs *et al.* (1995), who reported cv. Agria as a susceptible cultivar and Bagheri and Zafari (2005) who introduced Marfona and Condoor as moderately resistant.

Observing a varying spectrum of resistance across 46 genotypes examined here, some with no immunity response, suggests that resistance against Pcc in potato does not seem to be monogenic, qualitative or vertical. Earlier studies have also indicated that the genetic

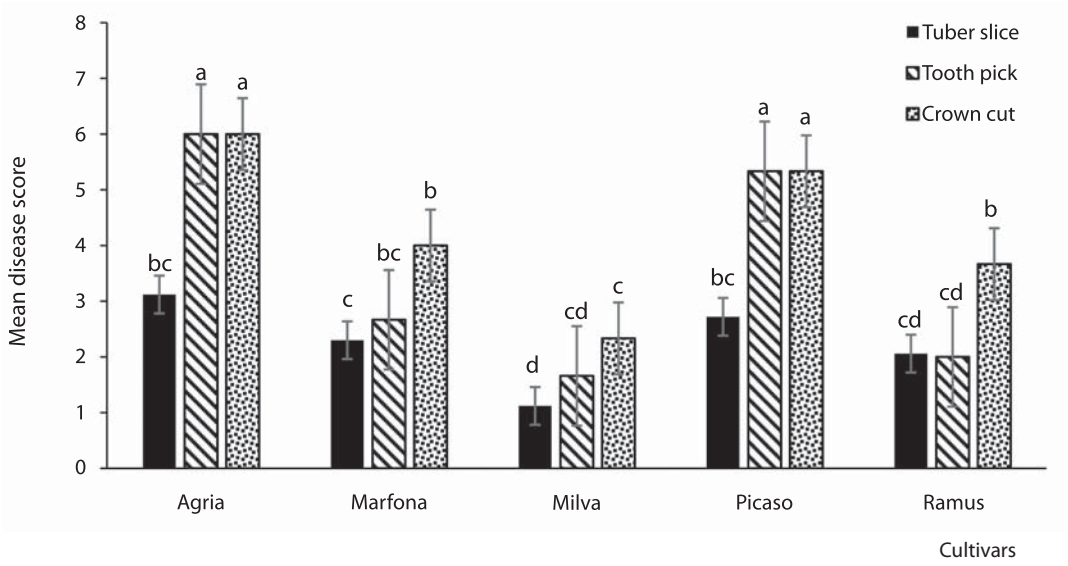


**Fig. 6.** Mean disease scores of Soft rot for selected potato genotypes based on three evaluation methods. Letters indicate significance of differences, where bars with common letters are not significantly different

**Table 2.** Pearson correlation coefficients between inoculation techniques for evaluation of potato genotypes against *Pectobacterium carotovorum* subsp. *carotovorum*

| Inoculation technique | Tuber slice | Tooth pick | Crown cut |
|---|---|---|---|
| Tuber slice | – | 0.723** | 0.516* |
| Tooth pick | – | – | 0.752** |

*correlation is significant at the 0.05 level
**correlation is significant at the 0.01 level

basis of resistance to soft rot is not well understood. Some studies described it as a complex phenomenon with no immunity response reported so far (Austin *et al.* 1988). Despite the striking phenotypic differences among resistant and susceptible genotypes in their responses to bacterial soft rot, the DNA of Pcc was amplified in genotypes with or without the phenotypic disease symptom. This indicated that Pcc was able to multiply at detectable levels (by PCR) in resistant and susceptible genotypes, alike. This agrees with results reported earlier for lack of immunity against Pcc in commercial potatoes.

Breeding potato cultivars with high levels of resistance against *P. carotovorum* subsp. *carotovorum,* requires an efficient screening technique. Such a technique should be sensitive, precise, simple, rapid and inexpensive in order to screen large numbers of genotypes requiring a minimum amount of plant material. The results of such a test should be in conformity with the results of classical methods of screening (Allefs *et al.* 1995; Vreugdenhil 2011).

There were significant differences between the two methods of in vitro inoculation. The toothpick method was more suitable and reliable, since it rendered more pronounced symptoms and helped to readily identify different levels of resistance. This inoculation technique resembled natural infection and produced more repeatable results which confirmed earlier research (Allefs *et al.* 1995). However, in our previous work on *in vitro* evaluation of potato cultivars for resistance to black leg disease (*Pectobacterium atrosepticum*), no significant differences were observed between the two inoculation methods (Azadmanesh *et al.* 2015). In addition it was also noticed that the degree of resistance to *P. carotovorum* subsp. *carotovorum* was similar in both *in vitro* and *in vivo* assays since the results of these tests were highly correlated. The highest correlation was detected between the two *in vitro* assays.

## Conclusion

Field screening methods are expensive in terms of both time and resources. Results produced by these methods may be variable and confounded due to uncontrolled environmental heterogeneity in temperature, humidity and the amount of inoculum. Apart from these, there are also other factors such as varying disease pressure under *in vivo* conditions, which may lead to variable and unreliable results. In contrast to the variability concerns of classical screening methods for resistance against Pcc, *in vitro* techniques such as the method developed and described here are considered precise, efficient and less costly for screening large numbers of germplasm in potato breeding programs. Our findings, more specifically, showed that the *in vitro* screening technique is also a reliable method for assessing resistance of potato plants against bacterial soft rot. The application of such a technique would speed up the screening procedure for disease resistance.

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