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In vitro evaluation of resistant of potato cultivars against black leg disease (*Pectobacterium atrosepticum*)

Azadmanesh Sima*, Javad Mozafari**, Hasanzadeh Nader* and Moslemkhani Cobra***

**Department of Plant Pathology, Faculty of Agriculture and Natural Resources,*
Science and Research Branch, Islamic Azad University, Tehran, Iran.
***National Plant Gene Bank Department, Seed and Plant Improvement Institute (SPII),*
Mahdasht Avenue, P.O. Box , Karaj, IRAN
****Seed and Plant Certification Research Institute,*
Agricultural Research Education & extension organization, Karaj, Iran.

(Corresponding author: Javad Mozafari)
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ABSTRACT: A screening system comprised of piercing and dipping was developed to evaluate the level of resistance in potato cultivars against potato black leg disease caused by *Pectobacterium atrosepticum*. For this, in vitro cultures of 45 genotypes were obtained from Iranian potato collection and established on MS medium. Healthy plantlets were wound with a sterile toothpick and inoculated with 108 cfu/ml bacterial suspension. Simultaneously, the crown cuttings were dipped in similar dense suspensions for 10 min. Diseased symptoms including leaves wilting and rotting of the stems were recorded after 3 days post-inoculation. Varying levels of resistance in both treatments were observed. In order of verified in vitro (laboratory) inoculation methods, a screening of resistance for representative cultivars 2704- Els-7A-Cara and 10908-18 in green house condition, adjusted and results were in accordance with laboratory methods. On resistant genotypes rather than the susceptible cultivars, it was also noticed the infection did not affect the plant normal growth. Comparing the resistant behavior of the same genotypes, it was concluded that these laboratory techniques are potent enough to screen potential resistant genotypes in order to use these trait-based cultivars against black leg disease under field conditions.

Keywords: Potato blackleg, resistance screening methods, disease index

INTRODUCTION

Potato (*Solanum tuberosum*) is a worldwide cultivated tuber-bearing plant which is the fourth main food crop in the world after maize (*Zea mays*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*) (Douches *et al.,* 1996). Potato does not require special growth conditions and it has been for a long time a major field crop in temperate regions, and increasingly in warmer climates (Haverkort, 1990).

Losses in production and storage of a potato crop are caused by several bacterial and fungal diseases. *Erwinia carotovora* subsp. *atroseptica* (van Hall) Dye, *Erwinia carotovora* subsp. *carotovora* (Jones) Dye, and *Erwinia chrysanthemi* (Burkh, *et al.*) are causal agents of two important potato bacterial diseases: stem blackleg early in the growing season and tuber soft rot in storage (Perombelon & Kelman, 1980).

Blackleg is a severe field disease leading to the development of an inky black and slimy soft rot of stems. Severely affected plants die, and tubers from diseased plants may show a black soft rot during storage. The pathogen is carried within diseased potato tubers or other plant debris, but it is usually dormant and does not cause disease symptoms unless environmental conditions are favorable (Reiter et al., 2002)

The classification of soft rot bacteria has been argumental and several studies have been conducted in order to clarify their taxonomy (Hauben *et al.*, 1998; Avrova *et al.,* 2002). Gardan *et al.* (2003) proposed that pectolytic bacteria of the genus *Erwinia* should be included in a separate genus, *Pectobacterium*, and the subspecies *atroseptica* be elevated to species level. However, both classifications are still valid in the scientific literature. Although bacteria in these three taxons can cause soft rot in plants, their host range and optimum temperatures are distinct. *E. carotovora* subsp. *atroseptica* infects mainly potato plants and tubers at an optimum temperature of 20ºC, whereas *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* have a wider host range and cause disease at higher temperatures, 20 to 35ºC (Costa *et al.,* 2006). Blackleg and soft rot are commonly occurring disease in field as well as during transit and storage. Due to its endemic nature, blackleg disease caused is prevalent in cool and temperate regions of Canada, the US, Western Europe India and Pakistan (Molina and Harrison, 1977 Caron *et al.,* 1979; Bain *et al*., 1990).

Erwinia carotovora subsp. *atroseptica* (Eca) and *Erwinia carotovora* subsp. *carotovora* (Ecc) are considered the main source of primary inoculum for blackleg and soft rot of potato. They are responsible for losses both quantitatively and qualitatively. Both subspecies are commonly associated with potato tuber soft rot, but Eca usually causes rot in the basal part of the stem (blackleg disease). *Erwinia carotovora* subsp. *atroseptica* occurs in both temperate and warm climates but mostly in storage. The rotting of mother tubers during the growing season has been reported as the major source of inoculum for contaminating progeny tubers, which later in storage, when conditions are favorable, could lead to losses due to soft rot of tubers (Perombelon, 1992).

The lack of chemical control against blackleg and soft rot diseases stimulates the interest in resistant cultivars. Genetic variation in resistance to *Erwinia* spp. was found among cultivars (Allefs *et al.,*1995). Sources of high resistance to *Erwinia* spp. were discovered in wild and primitive cultivated Solanum species (Lojkowska & Kelman, 1994; Rousselle-Bourgeois & Priou, 1995).

Several methods are used to screen potato genotypes for tuber soft rot and blackleg resistance caused by three *Erwinia* spp. namely *E. carotovora* ssp. *atroseptica*, *E. carotovora* ssp. carotovora and *E. chrysanthemi*. Resistance to tuber soft rot can be tested under laboratory conditions (Allefs *et al*., 1996) Blackleg resistance can be evaluated under greenhouse conditions on inoculated potted plants. Under field conditions yield reduction is evaluated after planting of artificially infested seed (Lapwood and Read.1984). The resistance of detached stems or leaves to *E. carotovora* spp. *atroseptica* or to *E. carotovora* spp. *carotovora* was evaluated by Lapwood & Read (1986a).

The goal of resistance breeding is the creation of cultivars resistant to blackleg disease. The positive correlation found between these traits suggests that this goal is possible reported that the ranking of tested cultivars was strongly related to the screening methods applied and environmental conditions. The understanding of the relationship between tuber and stem resistance to *Erwinia* spp. is rather poor (Lees *et al.,* 2000).

A very good and reliable method for evaluating resistance in plants is using in vitro explants. In vitro techniques have created new opportunities for the improvement of vegetative propagated plants (Ahlowalia, 1998), enabling the production of large mutant populations in comparatively a short time and small space. Since in vitro plantlets are free from viruses and fungi, this makes it a reliable tool for studying resistance responses.

Since the blackleg disease is one of the divesting bacterial diseases in most potato growing regions, in this study we focused on two in vitro screening techniques in order to find new sources of resistance in some species closely related to *Solanum tuberosum*.

MATERIAL AND METHODS

A. Potato Variants Generation and Maintenance

Somaclonal variants of 45 potatoes genotypes were obtained from national gene bank of Iran. Many of these genotypes were maintained as tissue culture plants micropropagated on potato multiplication (PM) medium comprised of MS salts and vitamins plus sucrose (30 g/litre), and 0.6% agar (Sigma-Aldrich, St Louis), adjusted to pH 5.8. Plants were routinely sub-cultured as two-node segments every 4 weeks and incubated at 22°C with a16h photoperiod under cool white fluorescent lamps (65 µmol m^{-2} s^{-1}).

B. Bacterial strains, media and cultural condition

The bacterial strains used in this study were isolated from diseased potato tubers and stem inky lesions. In brief, small amounts of tuber samples from the margin of healthy and diseased tissue were surface-sterilized and homogenized in 1-2 drops of sterile D.H_2O.

The test sample suspensions were plated on nutrient agar and incubated at 26°C for 24 h to allow isolation of P. atrosepticum. Single colonies were purified and selected strains were further characterized. All isolates were stored in sterile water at room temperature and in 20% (v/v) glycerol at -20°C.

The standard strain of *P. atrosepticum* was also obtained from Ferdowsi University of Mashhad - Khorasan Razavi province.

C. Bacterial DNA extraction

Total genomic DNA was extracted from 24-h cultures grown on NA using Mahuku method (Mahuku, 2004) and store -20°C until use.

D. Molecular identification

Two specific pairs of primers (Table 1) were used to detect *Pectobacterium* strains. Primers Y1 and Y2 were selected as PCR primers to amplify a 550 bp fragment of a pectate lyase encoding gene (pel gene) for detection of *Pectobacterium* species (Darrasse *et al.,* 1994). The other primer set i.e. ECA1f and ECA2r (De Boer and Ward, 1995) were used to detect specifically *P. atrosepticum* strains. PCR was performed in 25 µl of a reaction mixture containing 2.5 µl of 10×PCR buffer, 2 mM $MgCl_2$, 200 µM of deoxynucleoside triphosphates, 0.8 µM of each primer, 0.5 U of Taq polymerase (Roche Diagnostics) and 50 ng of template DNA.

PCR amplification was carried out using thermal cycler (Eppendorf, Germany) with the following thermal regime: initial denaturation at 94°C for 5 min, followed by 35 amplification cycles of 94°C for 30 s, 60°C (Y1/Y2) or 67°C (ECA1f/ ECA2r) for 45 s, and 72°C for 1 min, ending with incubation at 72°C for 10 min. In all cases, amplified DNA fragments were detected by electrophoresis in a 1.2% agarose gel stained with ethidium bromide (1µg/ml).

Table 1: Primers used in this study.

Primer Sequence	Sequence (5' to 3')
Y1	TTACCGGACGCCGAGCTGTGGCGT
Y2	CAGGAAGATGTCGTTATCGCGAG T
ECA1f	CGGCATCATAAAAACACG
ECA2r	GCACACTTCATCCAGCGA

E. Pathogenicity test

All three strains were tested for pathogenicity on 6 weeks-old potato plants cultivar Agria grown in 20 cm diameter pots in a 24°C regulated greenhouse. Inoculations were done by dipping sterile toothpicks into bacterial colonies grown for 24 h on NA at 26°C. Two stems per potato plant, 5 cm above the stem base, were immediately pierced with the contaminated toothpick after which the inoculated point was covered with biofilm. Inoculated plants were covered with plastic bags to maintain high humidity for 7 days in growth chamber at 24°C.

Control potato plants were pierced with sterile toothpicks and the wounds covered with biofilm. Potato plants were observed daily for visible blackleg symptoms.

F. In vitro Resistance Screening

For the screening purposes of resistance among potato cultivars, two methods of inoculation were performed.

Table 2: Assessment of black leg disease symptoms on potato plantlets under *in vitro* conditions.

Disease severity grading scale	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

In the first method, the sterile tooth pick were dipped in 108 cfu/ml of bacterial suspension (OD600 = 0.1) and then pressed in the crown of 1-month old in vitro plantlets. In the second method, the plantlets were cut from nod 2 and dipped in 108 cfu/ml of bacterial suspension and then placed on MS medium 6%. After 72h incubation, symptoms were recorded. To evaluate the individual response of in vitro plantlets to the bacterial inoculation, a 1-6 scale was used (Table 2).

G. Green house Evaluation Test

Six plantlets per 5 cultivars (2704- Els- 7A-Cara and 10908-18) were acclimatized in 20 cm diameter pots on a sand substrate for 14 days. After rooting, plantlets were transferred to bigger pots with dark brown carbonate soil and placed in a greenhouse. The plants were subject to artificial inoculation after 14 days maintenance.

To fulfill this, the plants were inoculated by piercing the stem with a sterile tooth pick contaminated with *P. atrosepticum* suspension, 5 cm above the soil level. Inoculation sites were immediately wrapped with parafilm to prevent desiccation. Plants were incubated in a glasshouse at 25°C at high humidity and blackleg lesions were evaluated daily for 21 days post inoculation (dpi).

A rating scale of 1 to 6 was used to indicate the degree of stem wilting. Initial symptoms appeared at the inoculation site on the stem base as shoe stinging and wilting of the stem (Fig. 5, Table 3) length of wilted stems were determined in 3 days intervals and statistical analysis performed(Table 4) .Data were analyzed using the SAS® program. Analysis of variance was determined using the general linear model procedures, and means were separated with LSD test.

Table 3: Assessment of disease resistance among potato cultivars against *P. atrosepticum* under greenhouse condition.

Symptomatic stems on a 1 to 6 scale	Description of symtoms
1	No disease symptoms
2	2-5 cm of the stem was striped
3	5-8 cm of the stem was striped
4	8-11 cm of the stem was striped
5	11cm of the stem was striped
6	Whole plant wilting

RESULTS

A. Molecular identification of Pectobacterium atrosepticum isolates

An expected 434 bp amplicon, corresponding to the conserved region of the *P. carotovorum* pectate lyase-encoding gene was obtained in all strains using Y1 and Y2 primers (Fig. 1).

These and the standard strain further amplified a 690 bp PCR product specific to *P. atrosepticum* using ECA1f and ECA2r primers (Fig. 2).

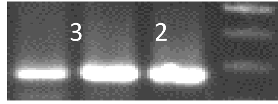

Fig. 1. Molecular detection of three *P. atrosepticum* strains by primers Y1 and Y2. lane1,1Kb size marker; Lane 2, standard strain of *Pa* ; Lane3, *Pa* isolate use in this study; Lane 4, weakly virulent *Pa* isolate.

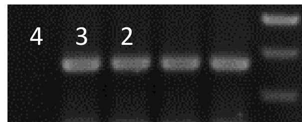

Fig. 2. PCR amplification of genomic DNA of *Pectobacterium atrosepticum* strains using specific primer set Eca1f/2r. Lanes: 1, Size marker (1kbp); 2, *Pa*1 (applied specifically in this section); 3, *Pa* strain 2; 4, *Pa* strain 3; 5, *Pa* (standard strain) and 6, negative control (water).

Pathogenicity test. In stem inoculation test, three bacterial isolates were used. Typical blackleg symptoms were appeared on inoculated stems within 3 dpi in the form of black lesions extending upwards and downwards from the point of inoculation (Fig. 3). Among these, one isolate with higher virulence was selected for in vitro assays.

B. In vitro evaluation of resistance of potato cultivars to Pa

Three days after inoculation, chlorotic and necrotic symptoms were developed in the in vitro plantlets. The population showed wide variation in susceptibility of potato cultivars to the bacterial inoculation.

Fig. 3. Typical blackleg symptom was appeared on potato cv. Agria stem after inoculation with *Pa* isolate.

A large proportion of individuals from the cultivars had high disease-symptom severity scores when assessed for black leg resistance when tested with both methods (Fig. 4). Of 45 genotypes for which results were obtained, 7 had disease-symptom scores between 0 and 4 on a scale of 1 to 6 in increasing susceptibility and another 38 genotypes had scores more than 4 in both methods of inoculation (toothpick method and crown cut method). Fig. 5 shows disease severity in all tested cultivars and differences between two methods. Also, area under progressive disease curve (AUDPC) was calculated for each cultivar in both methods (Table 4).

From about 100 ng of DNA extract from the Pa affected whole plantlet tissue, a PCR product of 690 bp was successfully amplified (Fig. 6).

Fig. 4. Severe disease incidence on susceptible cultivar 781 in three replicates. Wilting and necrosis of leaves are two most common visual symptoms.

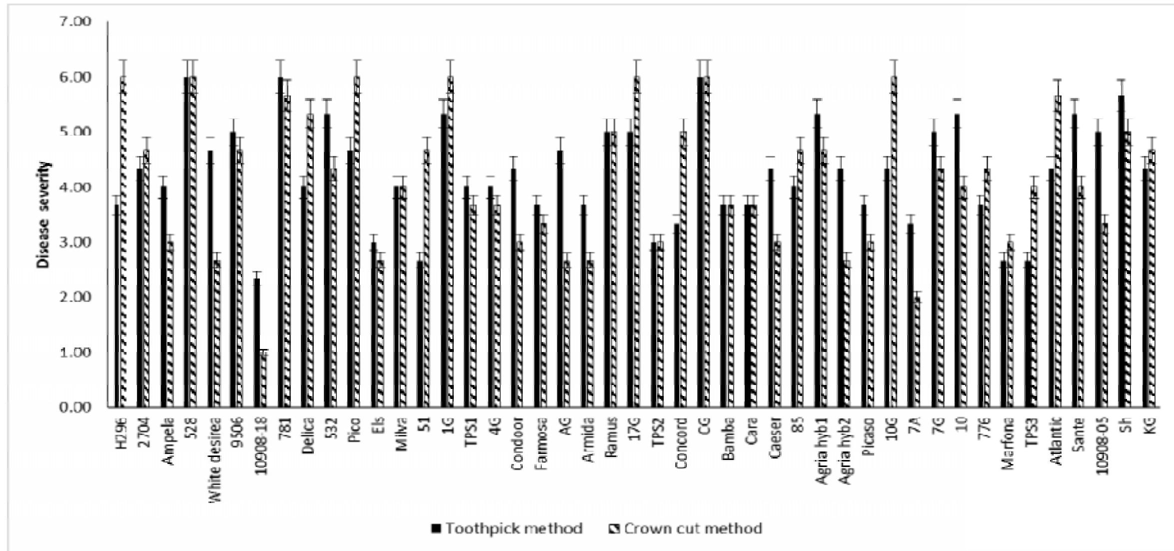

Fig. 5. Mean of disease progress in 46 tested cultivars obtained from two different *in vitro* assays.

Table 4: Analysis of variance for potato cultivar cultivar response to Pa inoculation under greenhouse condition.

Source	Df	Mean Square	F
Cultivars	4	10.488	5.948**
Error	75	1.763	

Table 5: Area under disease progressive curve, calculated for each cultivars in both method of inoculation.

Cultivars	Tooth pick	Crown cut
H296	67	92
Agria hybrid 1	89	89
Ampela	70	65
528	118	125
White desirea	77	60
9506	84	104
10908-18	43	26
781	101	96
Delica	78	100
532	74	67
Pico	70	94
Els	46	41
Ramus	59	58
Concord	53	75
1G	91	102
TPS1	37	41.96
4G	63	54
Condoor	83	54
Farmosa	76	64
AG	59	44
Armida	61.98	56
Milva	81	73
17G	103	104

Cultivars	Tooth pick	Crown cut
TPS2	42	55
Concord	72	99
CG	130	130
Bamba	52	48
Cara	75	71
Caeser	79	58
85	72	85
Agria hybrid2	107	89
Agria hybrid3	81	41
Picaso	67	49
10G	94	114
7A	55	40
7G	88	78
Data	93	81
776	48	58
Marfona	39	50
TPS3	53	63
Atlantic	67	91
Sante	75	72
10908-05	73	66
Sh	107	91
KG	86	93

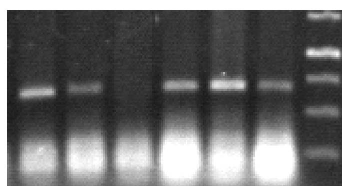

Fig. 6. Validation test for presence of Pa in 5 *in vitro* plantlets: Lane 1,1Kbp ladder; lane 2, positive control with Pa isolate used in the study; Lane 3, 2704; Lane 4, Els; lane 5, negative control with water; Lane 6, 10908-18; Lane7, Cara.

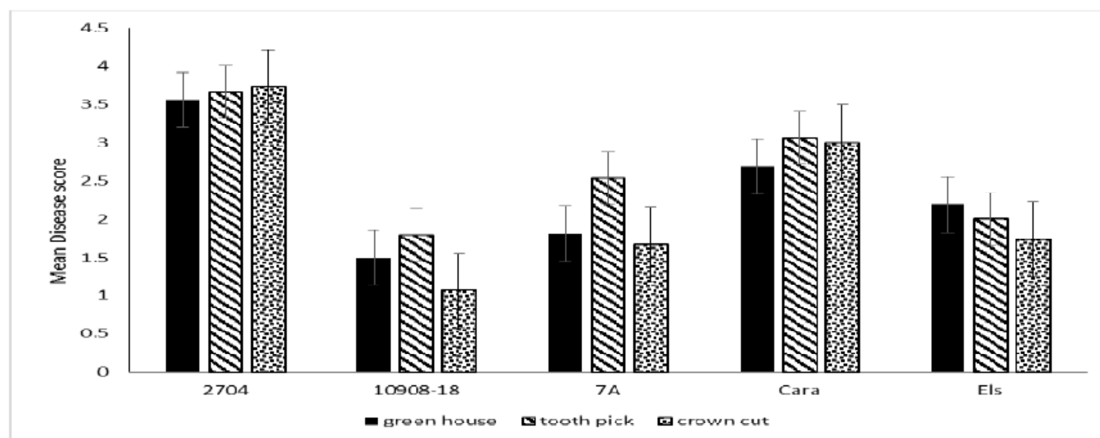

Fig. 7. Black leg mean severity scores in selected *S. tuberosum* genotypes tested with tuber slice assay.

C. Screening for stem tissue resistance and green house data

Potato stems grown in green house, inoculated with Pca strains developed blackleg symptoms and caused striping the stem in susceptible cultivar 2704. However, symptoms of blackleg were visible 3 days after inoculation in susceptible cultivar 2704 in contrast to stems of medium resistant cultivars Els and Cara. In resistant cultivar 7A and 10908-18, symptoms were not developed or were slight.

DISCUSSION

Potato blackleg causes significant crop losses in seed and ware potato production, both in the field and during storage. Because of no effective chemical control measurements and disease complexity, bacterial soft rot and potato blackleg pose a major problem to the potato industry. Genetic resistance is one of the best methods to control bacterial pectolytic of potato tubers (Wright *et al.,* 1991).

Screening methods should meet a number of requirements. They should be easy to perform and suitable for screening large numbers of genotypes in early vegetative generations, when there is only a limited amount of plant material available. Results must be reproducible and in agreement with the level of resistance that the screened clones will show under field or storage conditions (Allefs *et al.,* 1995).

In this study we tried to introduce two efficient and reliable methods for evaluating of potato cultivar resistance against potato blackleg caused by P. atrosepticum.

Since in vitro plantlets hypothetically are free from plant infection, this makes them good materials for studying breeding for resistance.

The methods described here simulates conditions under which blackleg is likely to occur in field condition. Our results clearly show that there is a very wide range of susceptibility to blackleg among potato cultivars.

We found no significant difference between two inoculation methods. In both methods similar levels of resistance were assayed.

Green-house evaluation test for 5 representative cultivars were in agreement with in vitro assays and approved the reliability of both methods.

Since the source of stem rotting is mother tuber (Allefs *et al*., 1996), it seems screening for resistance with tubers could be a good method for evaluation of resistance against *P. atrosepticum*.

The partial nature of resistance has often been regarded as a major hindrance to successful use of resistant cultivars to control soft rot, because of both insufficient performance and difficulties in breeding programs. However, the high level of resistance observed in some of the clones tested in this work, as well as the consistent increase of mean resistance levels over time we observed, open the door for a more general use of such resistant clones in the future.

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