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Original scientific article

COMPARING INOCULATION METHODS FOR *In vitro* EVALUATION OF RESISTANCE TO BLACKLEG DISEASE IN POTATO CULTIVARS

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To find the best inoculation method for evaluation of the resistance in potato genotypes against bacterial blackleg caused by *Pectobacterium atrosepticum* under *in vitro* conditions, five inoculation methods were compared. *In vitro* grown explants of five potato genotypes were inoculated with different inoculation methods, then placed on MS solid medium and incubated at 23˚C with 70% relative humidity under the light regime of 16 hours a day. After the appearance of symptoms, the efficiency of inoculation methods was then recorded based on the severity of disease symptoms in potato genotypes: Farmosa, Agria, Picaso, Marfona and a wild potato genotype '*Solanum phureja'*. Plantlets inoculated by piercing the crown with sterile toothpick inoculated in bacterial suspension of 10^8 cfu/ml showed the most severe symptoms. Based on all experiments, cultivar Marfona showed higher resistance among all cultivars and, cultivar Agria was the most susceptible. Finally, after witnessing the reactions of different varieties to inoculation methods and comparing them with previous evaluations of resistance in greenhouse conditions, the crown treatment employing sterile toothpick after infection in 10^8 cfu/ml

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bacterial suspension was selected and introduced as the best evaluation method of *in vitro* potato explants against blackleg.

Key words: bacteria, blackleg, disease resistance, *in vitro* evaluation, potato

INTRODUCTION

Blackleg, the decay of the shoots and the soft rot of potato as a result of soft rot agents, have been a dangerous disease of potato for many years. Bacterial agents of *Pectobacterium atrosepticum* (Pca), *Dickeya chrysanthemi* (Dch), *Pectobacterium carotovorum* subsp *carotovorum* (Pcc) cause blackleg in temperate and tropical regions, respectively. Being opportunistic, the bacterial agents use pectolytic enzymes, attacking the plant in appropriate circumstances (ALLEFS *et al.*, 1995) (PÉROMBELON, 1992; PASCO *et al.*, 2006). Since difference in resistance of agricultural cultivars and wild types, belonging to Solanum species, section Petata, such a kind of resistance evaluation can be effective in controlling the disease. Moreover, determining the relative resistance of various genotypes of potato against blackleg has numerous problems (ALLEFS *et al.*, 1995). Soft rot and blackleg, caused by *P. atrosepticum*, are expanded all over northwest Europe, leading to both soft rot, stem decay, and blackleg (PASCO *et al.*, 2006).

Symptoms of blackleg are appeared after the replication of large amounts of pathogens in the rotting mother tubers. The disease is not observed in microtubers which are not produced from mother tubers. Also, disease is not found in seeds free from Erwinia spp, even in highly polluted soil. Erwinia agents also favor condition suitable for infecting mother tubers. One of the main factors is soil water level (rainfall/irrigation) which if prolonged, induces the development of the anaerobic condition in mother tubers which is favorable for rotting initiation and bacterial replication (PÉROMBELON, 2002). A comparison of three taxa commonly found on potato showed that both *P. carotovorum* subsp. *carotovorum* and subsp. *brasiliensis* are more aggressive in causing tuber and stem soft rot than *P. atrosepticum* (MARQUEZ-VILLAVICENCIO *et al.*, 2011) But before, it was thought that *P. carotovorum* is more dangerous than other agents, especially in temperate regions (PASCO *et al.*, 2006) (PÉROMBELON and SALMOND, 1995) Stem decay starts from the stem's base where it is connected to the tuber, blackening some part or the entire stem. The symptoms are on the leaves or parts of the stem, due to airborne or waterborne factors (PEROMBELON and KELMAN, 1980). Apart from creating the symptoms at the beginning of the season, the disease might expand during the growing season, as well. In fully-grown plants, it starts by blackening the previously-healthy stems, followed by quick wilting of the plant or sometimes yellowed leaves (DE BOER and WARD, 1995). Most of the time, yield losses that occur in the store, after harvesting the yield can be more dangerous (HOSSAIN and LOGAN, 1983). As antibiotic is not allowed plant's protection, it has no function in controlling soft rot and bacterial blackleg.

As a consequence, disease management highly depends on preventive tools such as controlling the seed's health and agricultural hygiene, which are not always accountable for disease control when the disease inoculum is frequent. As a result, resistance breeding of potatoes is being pursued for many years (PASCO *et al.*, 2006). It has been observed in many types of *Solanum tuberosum* and has been employed in some studies. However, variation in *Solanum tuberosum*, complicated heritage and transfer of undesirable agricultural characteristics

from the parents to the progenies in time of crop improvement sometimes cause some obstacles (TEK *et al.*, 2004). Only a few genetic differences have been observed so far, indicating some resistance to polygenic and non-specific soft rot agents (PASCO *et al.*, 2006). Numerous methods for assessing resistance to *Pca* have been described (HOSSAIN and LOGAN, 1983; RABOT *et al.*, 1994; TAYLOR *et al.*, 2021; PASALARI, 2020). Choosing the appropriate method and protocol is essential to obtain reliable results. Artificial inoculation for the progress of blackleg symptoms needs an infected instrument that introduces the bacteria into the vascular system at the stem base or a leaf axil (ZIMNOCH-GUZOWSKA and ŁOJKOWSKA, 1993). Screening for disease resistance in the field is expensive, time-consuming and dependent on many variables such as environmental conditions, inoculum abundance, and disease development progress. Trees in field are also another main factor. Some pathogens and pests which attack trees are another problem in field screening (OSTRY, 1989). In order to evaluate the resistance, healthy plants are required. One way to create such plants is to produce *in vitro* explants from standard primary sources without any relevant disease. *In vitro* explants of a particular cultivar are genetically without essential changes and are similar to one another. *In vitro* propagation enables the production of a large amounts of population in comparatively short time and small space. Also, these explants are certified and free from relevant diseases. As a result, studying the disease on explants of a certain genotype brings trustable results based on a similar genotype basis.

The aim of this study was to determine the best method to assess *in vitro* potato resistance to *Pectobacterium* species based on finding a noteworthy relationship between *P.carotovorum* and *P.atrosepticum* both in resistance to blackleg and in resistance to tuber soft rot. It is vital that one of the resistance assessment issues is the contrast in same genotypes' resistance to different inoculation strategies and growth conditions. The moment issue, causing resistance assessment with challenges, to extend in vitro results to field conditions. To solve this issue, a vulnerable cultivar ought to be utilized as the control, and for this goal, we utilized cultivar Agria as a susceptible one.

MATERIALS AND METHODS

Plant material and in vitro cultivation

Shoots from in vitro grown plantlets of cultivars: Marfona, Agria, Picaso, Farmosa, and *S. phureja* were prepared from National Plant Genebank of Iran (NPGBI), Seed and Plant Improvement Institute (SPII). Each genotype was initially multiplied in MS medium (MURASHIGE and SKOOG, 1962) through single bud cultivation. In vitro reproduction was done in temperature, between 22 and 25 degrees centigrade, and appropriate lighting period that included 16 hours of light and 8 hours of dark. After a month, healthy explants with suitable growth were re-cultivated in 12 repetitions, to be used for the tests after four weeks.

Bacterial Isolates and Pathogenicity test

Three *P. atrosepticum* isolates were obtained from Plant Protection Department of the agriculture faculty of Ferdowsi University, Mashhad. The strains were cultured on Nutrient Agar (NA) medium, and to ascertain the characteristics of the isolates as Pca, PCR assay conducted with specific primers (Eca1F/Eca2R). One isolate selected for further studies. The pathogenicity of the selected isolate was tested via the susceptible cultivar Agria in the four-leaf stage,

employing sterile toothpick. At the height of 5 cm of the stem, inoculation performed, and symptoms were observed one week later. During the experiment, the plant was being kept at a humidity of 90%.

Inoculation Methods

First of all, four potato cultivars, including Farmosa, Agria, Picaso, Marafona, and one wild potato cultivar: *Solanum phureja*, in 3 replication of each with different reactions to the disease were selected (LEES *et al.*, 2000; BAGHERI and ZAFARI, 2005) Afterwards, five evaluation methods (Figure1), listed below, were carried out to determine the most appropriate *in vitro* inoculation method:

1. Crown cutting was performed then placed in a bacterial suspension of 10^8 cfu/ml (OD600= 0.1) followed by putting it in 6% agar MS medium.
2. The bacterial colony was directly placed at the end of the cut samples at the height of 4cm from the root followed by putting it in 6% agar MS medium, under 16 hours of light and 8 hours of dark
3. 10^8 cfu/ml bacterial suspension was sprayed on healthy explants, after that they were put in 6% agar MS medium under 16 hours of light and 8 hours of dark
4. The healthy explants were placed in 2% agar MS medium after a 10-minute treatment in the bacterial suspension of 10^8 cfu/ml and were kept in jars under 16 hours of light and 8 hours of dark.
5. Crown treatment was conducted employing sterile toothpick after infection in 10^8 cfu/ml bacterial suspension, placing the explants in MS medium, containing 6% Agar, under 16 hours of light and 8 hours of dark.

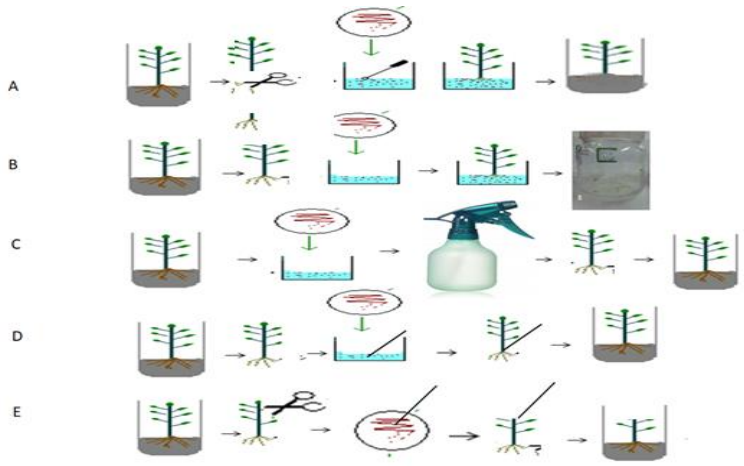

Fig. 1. Schematic of different inoculation methods: A: Plantlet inoculated with crown cut method, B: explants emerged in bacterial suspension then placed in 2% agar MS C: Plantlet inoculated by bacterial suspension spray, D: Plantlet inoculated by piercing the crown with sterile toothpick, and E: stem slices inoculated by direct bacterial colony

Evaluation and Scoring Methods

Each cultivar was being replicated three-time, and disease progress was recorded in 1-7 scale with 4 days interval as Table 1:

Table1. Scoring method based on disease development.

Disease severity	Score
Explants without symptoms	1
1/3 leaves wilted	3
2/3 leaves wilted	5
Whole plantlet ruined with the wilted leaves	7

The recording time about the symptoms was 20 days after the inoculation (with five stages of recording). Differences in the development of disease based on scoring were measured by analysis of variance. Regarding the score of disease symptoms, differences were measured by completely randomized design (CRD) in 3 replication (SPSS 21). After recording the symptoms, all treated explants removed by autoclave at 121˚C for 20 minutes.

RESULTS

PCR assay and pathogenicity test

PCR assay with specific primers (Eca1F/Eca2R), performed on all received bacterial isolates as *Pca* to ascertain all three were able to produce the considered 690 bp band specifically. The expected band was observed in all isolates, and all of them characterized as *Pca* by PCR assay. To prove pathogenicity of the isolates on the susceptible cultivar Agria, the extreme symptoms of the disease showed up a week after, and the isolate causing most extreme symptoms (entire crown's blackleg) was selected for further studies.

To select the best infection method, able to cause the most dispersion in a short time, five different methods of inoculation simultaneously were conducted. After data collection and statistical analysis via SPSS 21 (Table 2), the following results were obtained based on the disease progress, symptom scoring, and four-day reports of method's efficiency.

Based on statistical analysis, there were significant differences between inoculation methods. Firstly piercing the crown with sterile toothpaste, infected with a bacterial suspension of 10^{8}cfu/ml resulted in the highest disease rate. Then, crown cutting led to severe symptoms of the disease. Thirdly, 10^{8}cfu/ml bacterial suspension, which sprayed on the explants, showed vast amount of disease. After that Placing the healthy explant in the bacterial suspension of 10^{8} cfu/ml, followed by Placing the bacterial colony on 4-cm micro-explants directly, resulting in the minimum disease rate, and finally culturing the explants in 2% agar MS medium led to least disease symptoms (Figure 2).

The studies above showed that Marafona cultivar had the highest resistance, and Picaso, Farmosa, *S. phoreja* and Agria respectively placed in lower levels of resistance (Figure 3).

Table 2. Analysis of variances on the different methods of inoculation after the progress of disease symptoms on five cultivar explants with five recording times.

Source	Type III sum of squares	DF	Mean squares	F	Sig
Corrected Model	810.720[a]	149	5.441	9.716	.000
Intercept	2339.280	1	2339.280	4177.286	.000
Cultivar	126.946	4	31.741	56.681	.000
Date	178.427	5	35.685	63.724	.000
Method	185.276	4	46.319	82.712	.000
Cultivar*Date	25.462	20	1.273	2.273	.002
Cultivar*Method	170.80	16	10.655	19.027	.000
Date*Method	26.484	20	1.324	2.365	.001
Cultivar*Date*Method	97.627	80	1.220	2.179	.000
Error	168.000	300	.560		
Total	3318.000	450			
Corrected Total	978.720	449			

a. R Squared = .828 (Adjusted R Squared = .743)

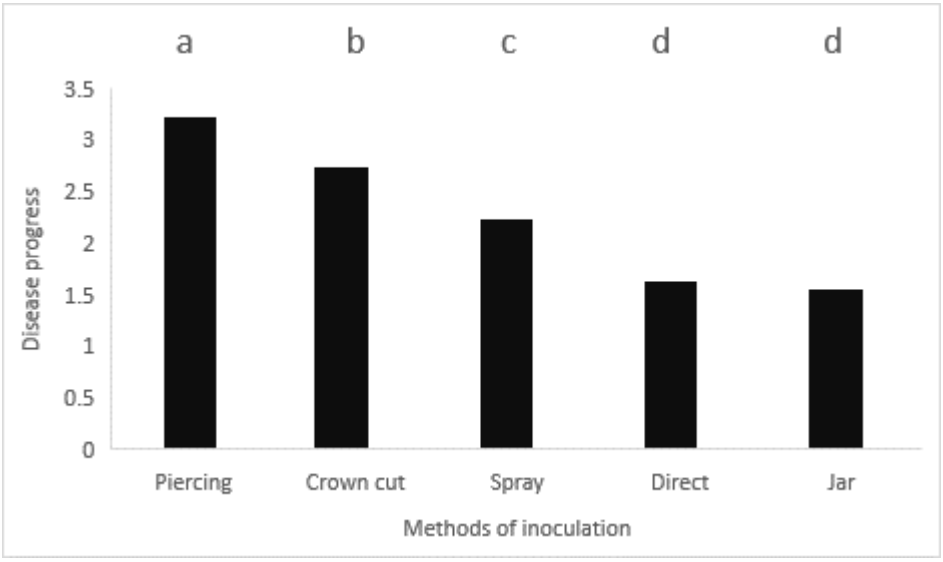

Fig. 2. Disease progress based on different inoculation methods. The same letter shows that means do not differ significantly according to Duncan's multiple range test at P=0.05

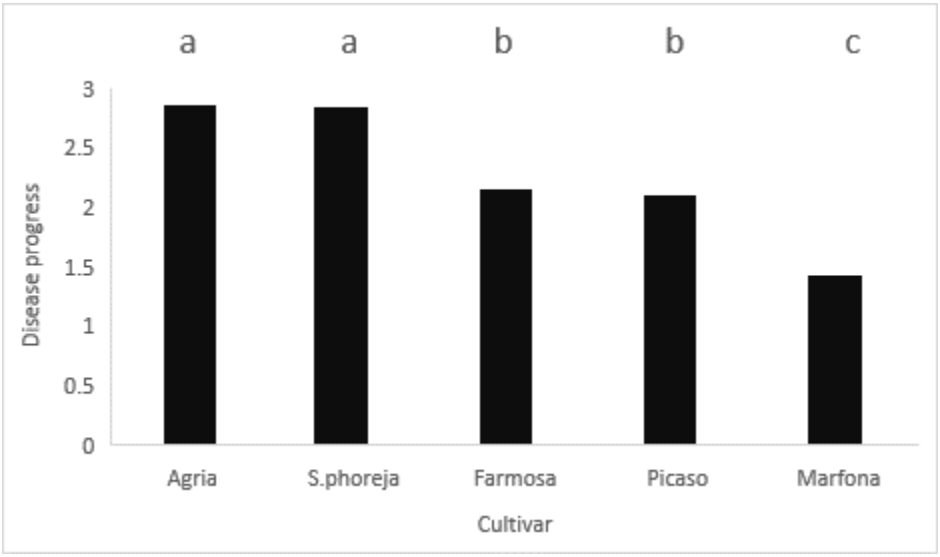

Fig. 3. Disease progress in cultivars examined with different methods of inoculation. There are no significant differences between varieties with common letters, based on Duncan's multiple range test at p=0.05.

DISCUSSION

Host resistance is the most important factor in controlling plant pathogens in epidemic circumstances (KRÖNER *et al.*, 2011). An appropriate method to reduce research costs when studying the resistance of cultivars to a disease is to evaluate theirs *in vitro* resistance. Since by *in vitro* culture, a great number of plants in the short term and small place are achieved, this method can be used to evaluate many plant's resistance in a short time and a small space. Also by using *in vitro* plantlets, large sample sizes are prepared, which is necessary for meaningful statistical analysis (CARPUTO *et al.*, 1997). The value, credit, and simplicity of this method to select *in vitro* cultivars depend on the sustainability and stability of such resistance in field conditions (MIRKARIMI *et al.*, 2013).

It is noteworthy that one of the resistance evaluation problems is the difference in same genotypes resistance to various inoculation methods and growth conditions. The second issue, causing resistance evaluation with difficulties, is to extend in vitro results to field conditions. For example, in a recent study (TAYLOR *et al.*, 2021) for ranking potato cultivar tolerance to Blackleg and relationship between laboratory studies and field examination, stem inoculation data were not correlated with blackleg incidence in field data.To solve this problem, a susceptible cultivar should be employed as the control (PASCO *et al.*, 2006).

The basis of pathogenicity in *Pectobacterium* genus is producing different enzymes, which decompose the essential compounds of the host plant like the cell wall (CZAJKOWSKI *et al.*, 2011). Hence, reviewing the resistance degree of different host plant cultivars is an appropriate way to study the interaction between this genus of bacteria and its hosts.

In the present study, after plantlets inoculated with different inoculation methods, the symptoms were observed in susceptible cultivars after two days. Once the reports were finished, it was seen that even Marfona cultivar, defined as the tolerant cultivar in previous studies, had overreacted against the pathogen, indicating that the considered inoculation method is the appropriate method to detect highly-resistant cultivars.

Since crown treatment employing sterile toothpick after infection in 10^8 cfu/ml, the bacterial suspension is closer to the infecting method in nature, having a vast expanse of resistive responses, resembling previous evaluation results more, it was selected as the effective, repeatable, and reliable method for *in vitro* studies. In cutting the crown by means of sterile scissor, dipping in 10^8 cfu/ml bacterial suspension, though there were no very excessive results, since the entire crown cutting might cause more stimulation of the plant and drying out of the vascular tissues and since the infection's nature in natural circumstances is not similar, it cannot be an appropriate inoculation method to evaluate the cultivar's resistance. In the method of 10^8 cfu/ml bacterial suspension spray on the explant's leaves, since the bacteria-less water, sprayed on the leaves, stimulates the defensive responses, causing necrotic symptoms on the leaves, the progress of the symptoms cannot be absolutely from the resistance against the pathogen. Also, how the aerial members are sprayed and the amount of the solution's spray in each experiment alters the results. Yet in direct inoculation method of bacterial colony to 4 cm micro-explants, it was seen that even in the susceptible cultivar Agria, in the inoculation place, the plants did not suffer from wilting and necrosis, and that the infected stem has made a bud from below the inoculation place and continued its natural growth without showing any sign of disease progress. In direct colony inoculation, there was a similar reaction in other cultivars of the test, except *S. phureja* explants. Thus the recent method was not considered to be an appropriate method for the studies, related to resistances. And finally in healthy plant's treatment, after placing them in bacterial suspension of 10^8 cfu/ml for 10 minutes and emerging them in closed glasses, containing 2% agar MS medium, we saw wilting effects on the plant and excessive yellowing but not wilting of the leaves, which might be due to lack of appropriate ventilation and shortage of oxygen . Thus, the above method cannot be an appropriate one to evaluate the resistance.

It was determined in these evaluations that among five tested cultivars, no genotype showed 100% resistance to the disease, and Agria cultivar had a very high susceptibility to the pathogen. On the other hand, Marfona cultivar was almost tolerable. Concerning the outstanding susceptibility of Agria cultivar, the results are in accordance with the results of Allefs (ALLEFS *et al.*, 1995). Regarding Marfona cultivar, the studies of BAGHERI and ZAFARI (2005) confirmed it. Although these researchers had demonstrated the resistance of the stem to *Pcc*, experiments of other researchers have shown that resistance to *Pcc* is correlated with resistance to *Pca* (CARPUTO *et al.*, 1997).

The only exception in our results is that long-day colonies of *S.phureja* and hybrids of this genotype with other *S.phureja* cultivars are illustrated as resistant to blackleg (LEES *et al.*, 2000) however, in all of our tests, this genotype showed huge susceptibility (Fig. 2). One possibility is that the colony of this genotype, tested in our study, has no resistance to blackleg or that due to the very susceptible nature of diploid *S. phureja* a smaller dose of primary inoculum should be injected to *in vitro* susceptible explants of this genotype, as in all five methods under study and even inoculation with water the explants of this genotype died after a short while,

proving the very susceptible nature of the explants of this potato genotype. It is assumed that successive *in vitro* reproduction of this cultivar leads to susceptibility against bacterial infection.

For choosing breeders line for resistance to blackleg, an easy and convenient method is required to eliminate the most susceptible cultivar. Between methods described above, the toothpick inoculation of plantlets with a diluted culture of *P. atrosepticum* could be recommended because following the development of the disease is less likely to be affected by environmental conditions. It must be mention that in our previous study (AZADMANESH *et al.*, 2015), two inoculation methods including cutting the crown following by incubation in 10^8 cfu/ml bacterial suspension and piercing the crown by infected toothpaste examined on 45 different potato genotypes. After conducting the tests, no significant differences found among disease progress between cultivars. Based on current research, these two types of inoculation led to close disease severity with meaningful differences. It is supposed that selected cultivars for a recent experiment caused this variation.

CONCLUSION

We noticed there were contrasts between five selected *in vitro* inoculation strategies based on statistical analyses. Referring to statistical studies, piercing the crown developed higher symptoms on the cultivars, and was leveled as the best method for inoculation. In order, crown cutting method, spraying the leaves, direct placement of bacteria on cut explants, and treatment of the healthy explants with the bacterial suspension followed by placement in 2% agar MS medium in jars ranked as efficient inoculation methods. In different tested cultivars, Agria and was the most susceptible one, and Marfona acted as the most resistant. Cultivars Picaso and Farmosa showed medium level of susceptibilities. At the end, it is necessary to mention that even though studying *in vitro* resistance is an appropriate method to quickly evaluate the resistance, eventually suitable to control the disease, due to some problems such as uncertain development of these results to field condition applying combined studding methods are recommended.

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POREĐENJE METODA INOKULACIJE ZA *In vitro* PROCENU OTPORNOSTI NA BOLESTI VLAŽNE TRULEŽI KROMPIRA

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Izvod

Da bi se pronašla najbolja metoda inokulacije za procenu rezistencije genotipova krompira na bakterijsku vlažnu trulež koju izaziva *Pectobacterium atrosepticum* u uslovima in vitro, upoređeno je pet metoda inokulacije. Eksplanti od pet genotipova krompira uzgojeni *in vitro* su inokulisani različitim metodama inokulacije, zatim stavljeni na MS čvrsti medijum i inkubirani na 23˚C sa 70% relativne vlažnosti pod svetlosnim režimom od 16 sati dnevno. Nakon pojave simptoma, evidentirana je efikasnost metoda inokulacije na osnovu težine simptoma bolesti kod genotipova krompira: Farmosa, Agria, Picaso, Marfona i genotip divljeg krompira „Solanum phureja". Biljke inokulisane probijanjem krune sterilnom čačkalicom inokulisanom u bakterijskoj suspenziji od 108 cfu/ml pokazale su najteže simptome. Na osnovu svih eksperimenata, sorta Marfona je pokazala veću otpornost među svim sortama, a sorta Agria je bila najosetljivija. Konačno, nakon što smo videli reakcije različitih sorti na metode inokulacije i upoređivali ih sa prethodnim procenama otpornosti u uslovima staklene bašte, tretman krune sterilnom čačkalicom nakon infekcije u 108 cfu/ml bakterijske suspenzije je izabran i uveden kao najbolji metod za procenu *in vitro* eksplantati krompira protiv vlažne truleži.

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