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PHENOLS AND PEROXIDASE ISOZYMES ACT AS BIOCHEMICAL MARKERS FOR RESISTANCE AGAINST LATE BLIGHT OF POTATO

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ABSTRACT

Effect of potato late blight pathogen *Phytopthora infestans* on phenol metabolism and peroxidase isozymes were studied. Two resistance cultivars and two susceptible cultivars were used for this study. In resistance cultivars, phenolic induction was high compared to susceptible cultivars. Six phenolic compounds were detected by using thin layer chromatography. Three were found all times, at and after inoculation, while two were found only in resistant cultivars, where as one was induced after 1 day of inoculation in all types of cultivars. The numbers of isoenzymes of peroxidase detected by polyacrylamide gel electrophoresis were changed after inoculation with spore suspension of *P. infestans*. The maximum number of isoenzymes occurred after infection in the two resistant potato cultivars. The potential use of the additional band only found in resistant cultivars as markers for Late blight resistance is discussed.

Keywords: Peroxidase isozymes, Phenolic compounds, *Phytopthora infestans*, *Solanum tuberosum*

INTRODUCTION

Late blight (LB) of potato caused by *Phytopthora infestans* is present in all the potato growing area of all India. This disease caused major loss in yield of potato worldwide. A few of *R*-genes and number of resistance varieties of potato have been identified for resistance source **[1]**, but success in breeding true resistance varieties

is yet to be achieved **[2]**. This is basically due to the inherent problem in resistance breeding, where an individual plant has to be inoculated for selection, which is tedious, expensive and labour-intensive. If molecular marker(s) for resistance could be found, the efficiency of the breeding programme would be increased.

Phenolics are well known antifungal, antibacterial and antiviral compounds occurring in plants **[3, 4]**. According to the Matern & Kneusal **[5]**, the first step of the defense mechanism in plants involves a rapid accumulation of phenols at the infection site, which restrict or slow the growth of the pathogen and play a important role in disease resistance **[2, 6, 7, 8, 9, 10, 11, 12]**. Thus, total phenol status of potato plant could be correlated with host resistance to a variety of disease, of potato.

Peroxidase oxidize phenols to form more toxic quinones, was also reported to play an integral part in disease resistance **[13, 14, 15, 16]**. The nature of peroxidase isoenzymes in different crops is a genetic character where different genomes control the synthesis of specific peroxidases **[17, 18, 19]**. Considering these observations, the present study was conducted to compare the biochemistry of resistant and susceptible varieties of potato to *P. infestans* in terms of changes in phenols, phenolic components and peroxidase activity and isoenzymes during early stages of infection, and to identify chemical or enzyme marker(s) of LB resistance.

MATERIALS AND METHODS

Plant and Pathogen

Four different cultivars of potato were obtained from Main Potato Research Station, Sardarkruishinagar Dantiwada Agricultural University, Deesa, North Gujarat, India, and were grown in departmental backyard. Among these, two were resistance varieties (Badshah and Bahar) and other two were susceptible varieties (Pukhraj and Laukar). *Phytopthora infestans*, causative agent of potato late blight, was isolated form infected potato tuber and maintained on V8 juice agar plates. Spores were harvested by the method of Queener and Capone **[20]** with some modifications. In short, fully grown V-8 agar plates were flooded with sterile distilled water having 0.1% sterile tween 80 and then the suspension was filtered through cheese cloth. Spores in the filtrate were counted by hemacytometer.

Treatment to Plant

Inoculum was prepared from 7- to 8-day-old cultures of *P. infestans* the concentration of the suspension was adjusted to approximately 10^4 spores per ml. The potato lines were inoculated to 40 days old four potato varieties (Badshah, Bahar Pukhraj

and Laukar) by injecting 0.5 ml of spore suspension following the method of Chona et al. **[21]**. Inoculated leaves were collected at 0, 1, 2, and 3 days after inoculation (d.a.i.) for the estimation of total phenols and their components (phenolics), peroxidase (POX) enzyme activity and isoenzymes and lignin estimation.

Extraction and Analysis of Phenolics

Estimation of total phenols and phenolics Plant phenols were extracted and purified by the method of Andersen & Pedersen **[22]**. The extract was suspended in acetone and made up to 10 ml with distilled water. Total phenols were estimated using the method of Malick & Singh **[23]** and expressed as mg phenols (in terms of catechol) per 1 g fresh tissue. Qualitative analysis of plant phenolics was done by high performance thin-layer chromatography (HPTLC) following the method of Sumere et al. **[24]**. Five standard phenolic components obtained from Sigma (St. Louis, MO, USA) were used. The solvent systems used and solutions for visualization of spots was n-hexane: toluene: diethyl ether: methanol: acetic acid (50: 25: 15: 5: 5) and visualized under UV as well as by using spraying reagent 20% Na_2CO_3. The R_f value and colour of the spots obtained from plant extracts were compared with the standards, and the phenolic compounds of the test samples were determined.

Peroxidase Assay

Peroxidase (POX) activity was determined using 1, 2-dianisidine as substrate in 0.1 M phosphate buffer (pH 6.0) **[25]**. Changes in absorbance of the test samples were recorded at 436 nm for up to 3 min at 30-s intervals. One unit (U) of enzyme activity was arbitrarily defined as the amount of enzyme that caused a change in absorbance of 0.1 per min (Δ OD per min) under the specified assay conditions. The specific activity of the enzyme was expressed as U per gram of fresh tissue. This enzyme activity was compared with postinfectional POX isozyme patterns using the same extract as was analyzed for POX activity.

Isozyme Assay

Tris- HCl (0.05 m, pH 7.4) was added at 5 ml per gram of leaves for extraction. The homogenate was centrifuged at 8000 g for 20 min at 48°C (Sigma 3K30 centrifuge). The clear supernatant was collected for polyacrylamide gel electrophoresis (PAGE) of POX isozyme. Native PAGE with 10% resolving gel was carried out at 30 mA current for 2-3 h until the dye front moved to the bottom of the gel. After turning off the power supply, the gel was carefully taken out and stained using 1, 2-dianisidin **[26]**. Immediately after resolution of bands, gel was recorded photographically.

RESULT AND DISCUSSION

The biochemical mechanism involved in plant disease resistance is a complex phenomenon. Since long time total phenols and phenolics has been considered as primary defense mechanism, whose levels are naturally high in the resistant varieties of many crops, including potato **[11, 27, 28]**. The present study indicate that phenols were present in all four varieties of potato, but were synthesized at higher than normal levels in the resistant varieties only during the early stages of infection by *P. infestans*, being enhanced for up to one day in the resistant varieties. Similar changes in phenol status upon infection were reported for *N. indica* in resistant lines of wheat **[8]** and for other plant-pathogen interactions **[29, 30, 31]**. A large range of lignin precursors including caffeic acid, ferulic acid, coumaric acid are freely available in plants, while like hydroquinone and scopoletin like compounds, which are simple phenols, are not widely distributed **[32, 33]**.

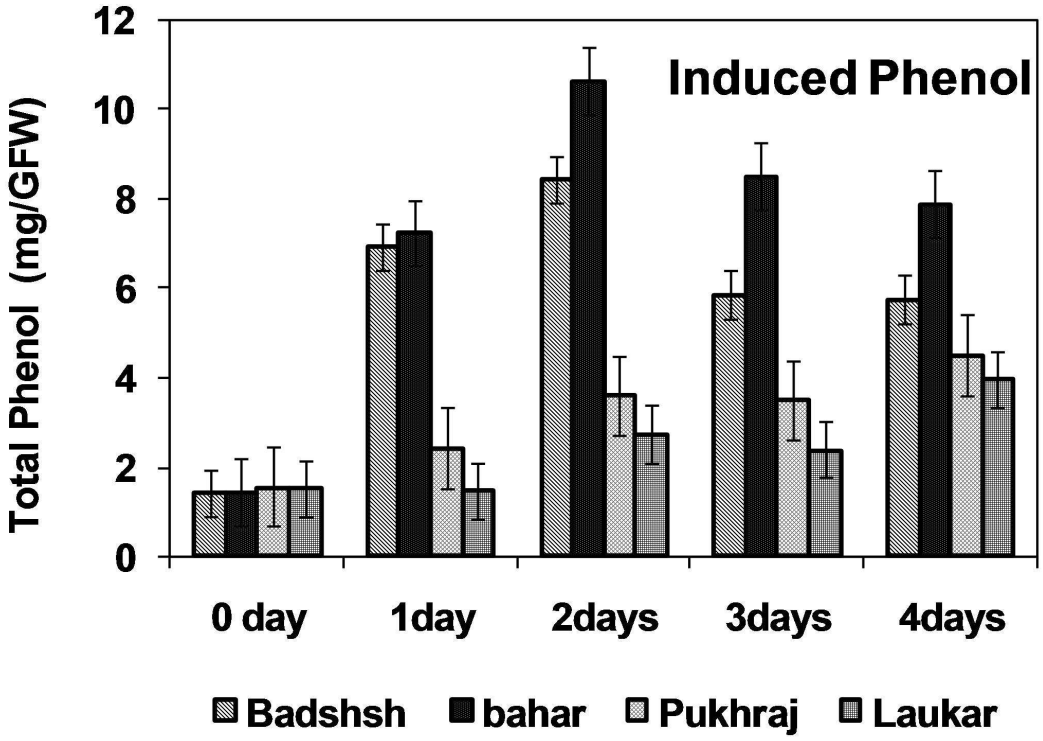

Figure 1: Induction of Total Phenol in Potato Varieties After Infection of *P. infestans*. Total Phenol was Higher at 1 d.a.i and Gradually Decreased. Total Phenol was Expressed as mg per gram of Fresh Tissue

Here we found the in resistance varieties,phenol level was high compared to susceptible varieties. Phenol level was suddenly increased in resistant varieties

after 24 hrs of infection. In susceptible varieties phenol level was also increased after 24 hrs but level was very less compared to resistant varieties. After induction on 24 hrs of infection, phenol level gradually decreased significantly in all four varieties of potato **(Figure 1)**. Phenylalanine, ferulic acid and salicylic acid were detected in all four varieties at various time intervals before and after inoculation **(Table 1)**. Coumaric acid was found to induce after inoculation in all four varieties. Hydroquinone was found only in resistant varieties before and after inoculation.

Table 1: Phenolic Profile of Resistant and Susceptible Varieties of *S. tuberosum*

			R_f							
Phenolic Standard			**Resistant Varieties**				**Susceptible Varieties**			
compound	**R_f**	**Colour**	**0***	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Phenylalanine	**0.56**	**Deep pink**	**0.56**	**0.56**	**0.56**	**0.56**	**0.56**	**0.56**	**0.56**	**0.56**
Ferulic acid	**0.71**	**Deep violet**	**0.72**	**0.72**	**0.72**	**0.72**	**0.72**	**0.72**	**0.72**	**0.72**
Hydroquinone	**0.73**	**Light grey**	**0.73**	**0.73**	**0.73**	**0.73**	**-**	**-**	**-**	**-**
Coumaric acid	**0.78**	**Orange**	**-**	**0.78**	**0.78**	**0.78**	**-**	**0.79**	**0.79**	**0.79**
Salicylic acid	**0.83**	**Violet**	**0.83**	**0.83**	**0.83**	**0.83**	**0.84**	**0.84**	**0.84**	**0.84**
Unknown (identified as scopoletin)		**Light grey**	**0.37**	**0.37**	**0.37**	**0.37**	**-**	**-**	**-**	**-**
Total no of spots			**5**	**6**	**6**	**6**	**3**	**4**	**4**	**4**

*days after infection

One new compound was also detected with R_f 0.36 and it was only observed in resistant varieties **(Figure 2)**. This new compound was scraped form TLC plate and purified by spin rotation using high-speed centrifuge. This compound was run on TLC plate with different solvent systems **(Table 2)** and it was observed that it was a single compound (not mixture of two or three compounds). Reviews related to phenolic like compound in plants were used for identification of this new compound **[34]**. It was observed that it was related to scopoletin and it was also compared with standard scopoletin. According to phenolic profile, Phenyl alanine, ferulic acid and salicylic acid were found all time in all four varieties of potato, while hydroquinone and scopoletin were only found in resistant varieties (Badshah and Bahar). It was reported that some antibiotic phenols occur in plants constitutively to function as preformed

inhibitors, while some occur in response to ingress of pathogens, exhibiting active pathogen defence in plants **[35, 36]**.

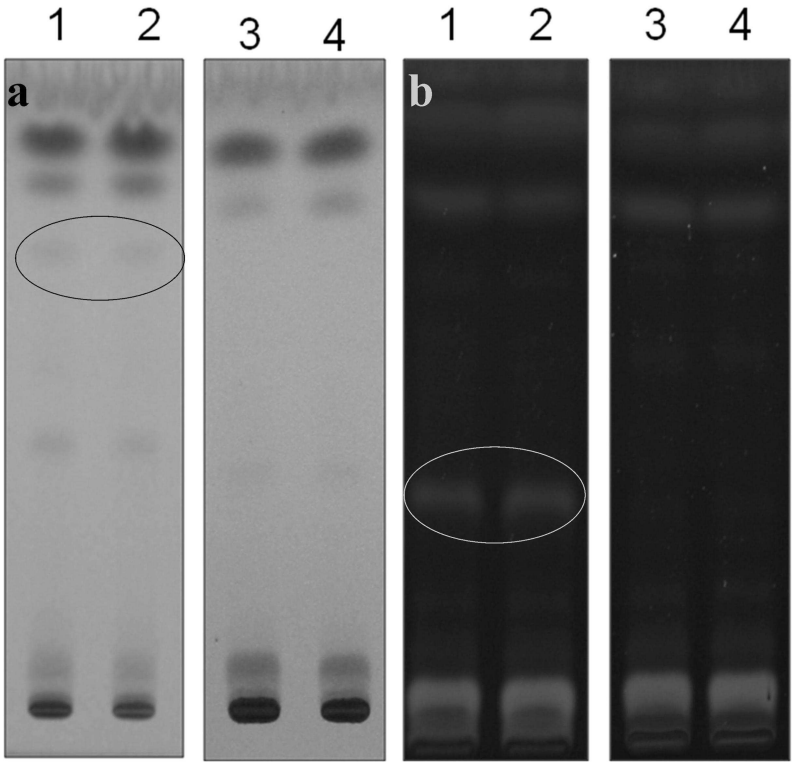

Figure 2: Phenolic Profile of Resistant and Susceptible Varieties of Potato. Number of Bands was Observed in Both Varieties. (a) TLC Plate Under Visible Light. One Additional Band only Observed in Resistant Varieties Indicated by Circle and (b) TLC Plate Under UV Light. One Unique Band Observed in Resistant Varieties Which were Absent in Susceptible One. 1: Badshah, 2: Bahar, 3: Pukhraj and 4: Lavkar

In this study, coumaric acid showed an inducible nature in potato following inoculation of spore suspension. Although the role of these estimated phenolics in disease resistance still needs confirmation, they may force the lignification process more in the resistant varieties than in susceptible plants. The deposition of lignin and other polymeric phenols has been implicated as a defence response in varieties of potato and tomato as well as in tobacco resistant to other diseases **[4, 7, 37]**. Deference between resistant and susceptible varieties in the phenol composition suggests their active role in resistance behaviour **[38, 39, 40]**. Scopoletin is a coumarin with phytoalexin activity **[41, 42, 43]** and precursors of lignin-like material thought to represent a defense reaction against pathogen invasion through its deposition into the cell wall **[44]**.

Table: 2 Solvents System Used for Identification of Scopoletin

Solvent System	Ratio of Solvents	Spray Reagents	R_f Value Obtained
Ethyl acetate: Isopropanol: Water	**65: 24: 11**	**Folin-Ciocalteu reagent**	**0.57**
Benzene: Methanol: Glacial acetic acid	**90: 16: 8**	**20% Na_2CO_3**	**0.72**
Chloroform: Ethyl acetate: Formic acid	**50: 40: 10**	**Diazotized sulphanilic acid**	**0.42**
Acetic acid: Chloroform	**1: 9**	**Vanillin HCl**	**0.56**
n-hexane: toluene: diethyl ether: methanol: acetic acid	**50: 25: 15: 5: 5**	**in fluorescent light**	**0.37 (R_f value matched with standard compound run in same solvent system)**

In this study, this phenol like compound was induced largly in resistant varieties than susceptible varieties of potato suggesting the important role in resistance against pathogenic organism. It is also a potent antioxidant substance and is used as a substrate to measure the oxidative burst in plant cell suspensions **[11, 45, 46]**. The phenol-oxidizing enzyme POX has been studied in many plant-pathogen interactions because of its significant role in the biosynthesis of plant cell wall components **[47, 48, 49, 50]** and, in turn, lignification and wall thickening, which offer defence responses in plants to pathogens, particularly to fungi **[49, 51, 52, 53, 54]**.

Here we observed that the activity of POX increased in all four varieties of potato after inoculation with *P. infestans*. Before inoculation, POX activity was greatest in the resistant potato. Following inoculation, maximum activity of POX in the resistant potato was at 2 d.a.i., while in the susceptible wheat it occurred at 2 d.a.i. but at low level (Fig. 3). Therefore, it is hypothesized that the sudden increase in POX activity in resistant potato varieties directed the reactions such as lignin synthesis and phenol accumulation to occur immediately in order to restrict the systemic growth of *P. infestans*. Similarly, Reuveni et al. **[55]** showed that the status of POX activity was enhanced in susceptible muskmelon plants after infection by *Pseudomonas cubensis*, but that the enhancement occurred too late to prevent disease development. Further, a more rapid increase in POX activity has been noted in low-infection type than in susceptible isogenic lines of wheat after inoculation with stem and leaf rust pathogens **[13, 56, 57, 58]**.

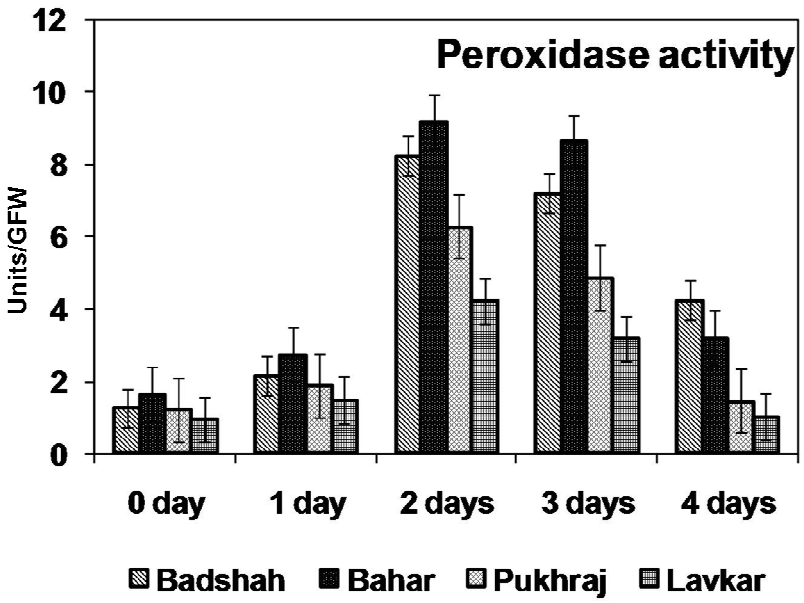

Figure 3: Peroxidase Activity in Different Varieties of Potato After Infection with *P. infestans*. Activity was Induces after 2 d.a.i. and then Decreased. Peroxidase Activity was Expressed as Units Per Gram of Fresh Tissue.

Although the two resistant genotypes Bahar and Badshah differed genetically, their POX isozyme pattern was similar and was different from that of the susceptible genotype Pukhraj and Laukar. A higher number of POX bands in resistant genotypes than in susceptible genotypes of wheat have also been found against powdery mildew **[2, 59, 60, 61]**. Bosch et al. **[62]** and Bruce and West **[63]** recorded the differences in POX banding patterns that resolved from suspension culture of caster been. Similar variation in POX isozyme bands in all four varieties of the potato was obtained in the present study. According to Yang et al. **[59]**, isozyme bands are the expression of individual genes. It seems that both resistant varieties shared some common loci, because two bands were absent in the susceptible varieties indicating the expression of KB-resistant gene(s).

Increase in total POX activity in host plants following pathogen invasion, as observed in the present investigation, has been reported by several other workers **[13, 56, 64]**.

However, the role of POX isoenzymes in the disease resistance response and the timing of their expression have not been thoroughly studied and its role was also unknown. Therefore, PAGE was carried out to correlate the time-course change in POX activity with the expression of POX isoenzymes during the pre and postinoculation period. Multiple isoenzymes of POX were present in inoculated leaves of the potato plants. Two isoenzymes were

common in noninoculated leaves while three common isoenzymes were detected in inoculated leaves of all four varieties. Gel was also analysed by AlphaEase FC software and it shows some insuced peaks in treated varieties. One additional peak was observed in resistant varieties of potato after treatment which is marked by * mark. **(Figure 4)**.

Isoperoxidase bands resolved in the all four varieties were exactly in the order of enhancement of POX activity at the same number of days after inoculation with *P. infestans* spore. It may be inferred that in Bahar and Bahar, isoperoxidases became active at maximum loci on 2 d.a.i, which resulted in the expression of a greater number of bands and reflected the peak of enzyme activity. Contrary to this, in susceptible Pukhraj and Laukar, the maximum number of isoperoxidase bands and highest POX activity were recorded at 4 d.a.i. Therefore, the number of isoperoxidase bands is proportional to the enzyme activity.

Yang *et al*. **[59]** observed higher POX activity in a resistant wheat cultivar than in a susceptible cultivar following inoculation with *Erysiphe graminis* f.sp. *tritici*. In addition, they observed an extra peroxidase isozyme band in the highly resistant cultivar, which could be the expression of a resistant gene. Appearance of new POX bands in resistant wheat cultivars after inoculation with fungal pathogens was also reported by many other workers **[65, 66, 67]**.

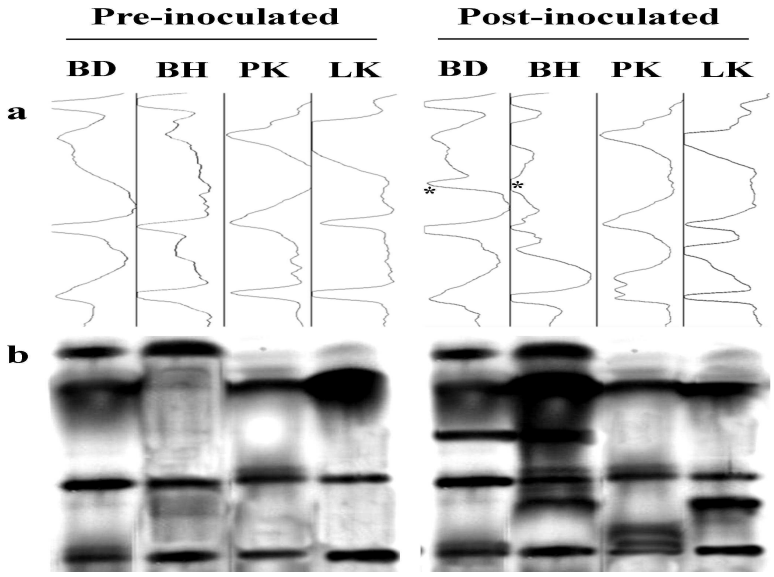

Figure 4: Peroxidase Isozyme Pattern. (a) Gel was Analyzed by Using AlphaEase FC Software for Band Analysis Showed a Unique Peak only Induced in Resistant Varieties which is Indicated by Asterisk (*). And (b) Peroxidase Isozyme Pattern on 10% Native Polyacrylamide Gel Stained by 1, 2-dianisidine Activity Staining. BD: Badshah, BH: Bahar, PK: Pukhraj and LK: Lavkar

It is noteworthy that phenols and activity of POX enzyme were naturally present in potato and seemed to be constitutive in nature. Although the initial levels of these biochemical compounds were found to be high in the resistant genotype, the levels, as such, do not appear to have any relation with host resistance. Rather, the mechanism, time and trend of their maximum induction as shown by the resistant genotypes suggest a significant role in governing plant resistance to disease. In this context, Rakshit et al. **[68]** also realized that the background level of chitinase activity in the absence of disease does not play a role in the ultimate resistance response of pea (*Pisum sativum*) to powdery mildew fungus.

CONCLUSION

The present study demonstrated that the biochemical basis of resistance in potato against *P. infestans* is the same in all genotypes. Such a defense mechanism in the resistant genotypes is activated immediately after inoculation or infection by *P. infestans*. The same mechanism may be operative in the susceptible varieties of potato, but it starts functioning quite slowly and reaches the effective level only after or just prior to establishment of LB disease. Elevated postinfectional phenomena such as total phenol status and POX activity, induced hydroquinone, scopoletin and increased number of isozymes bands may provide the possibility of identifying LB-resistant sources. However, before their utilization as reliable biochemical markers, further work on a range of resistant and susceptible potato varieties and genotypes, including testing of progeny from crosses of such potato, would be needed.

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