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ISSN: 2348-1900
Plant Science Today
www.plantsciencetoday.online

Research Article

Phytophthora infestans induced defense response in calli of wild and cultivated potato genotypes: Pathogen induced cell death in cultures - a marker for resistance

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Article history
Received: 02 July 2017
Accepted: 18 July 2017
Published: 22 July 2017

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Editor
K K Sabu

Publisher
Horizon e-Publishing Group

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Abstract

Late Blight caused by *Phytophthora infestans* (Mont.) de Bary is the most destructive foliar disease causing 30% yield losses in the potato (*Solanum tuberosum* L.) crop globally. Wild potato genotypes AC1 and AC4, and potato cultivar Kufri Girdhari are highly resistant, whereas wild genotype AC6, and cultivars Kufri Chandramuki and Kufri Jyoti are susceptible to Late Blight. In the current study, the calli of these six potato genotypes were used to understand the mechanism of cellular resistance to Late Blight. Exposure to *P. infestans* or its elicitors significantly induced peroxidase (POX) and superoxide dismutase (SOD) activities, and induced accumulation of phenolics and flavonoids, indicating the capability of the calli cells to mount a defense response. The study is the first to report the extracellular secretion of defense enzymes, SOD and POX when cells encounter the pathogen, implicating a similar whole-plant phenomenon of enhanced defense in the apoplast. Interestingly, the calli of resistant genotypes showed poor survival upon exposure to pathogen or when grown on elicitor medium, while the susceptible genotypes showed better survival. The percentage of calli cells accumulating intracellular H_2O_2 was high in resistant genotypes, and directly correlated with the observed higher cell death. The study shows that H_2O_2 accumulation in the cells of resistant genotypes is indeed self-destructive, a whole plant phenomenon termed hypersensitive response - cell death at site of infection. The potato callus system thus can be used to gain new insights into the plant-defense response to *P. infestans*.

Keywords: Potato; Late Blight; resistance; callus; peroxide; cell death

Aruna, K. N., V. S. Anil, and B. T. Krishnaprasad. 2017. *Phytophthora infestans* induced defense response in calli of wild and cultivated potato genotypes: Pathogen induced cell death in cultures - a marker for resistance. *Plant Science Today* 4(3): 105-120. http://dx.doi.org/10.14719/pst.2017.4.3.319

Introduction

The cultivated potato (*Solanum tuberosum* L.) is one of the world's principal food crops and is a tetraploid (4n). Late blight, a devastating foliar disease, caused by *Phytophthora infestans* is the major biotic constraint in potato cultivation leading to an annual loss in yield of 30% globally. The pathogen was named *Phytophthora infestans*, the Greek words for 'plant destroyer'.

Plants have several constitutive and inducible features that can protect them against infection by fungal and other pathogens. The induced defense mechanisms of plants include reactive oxygen species, phytoalexins, and other toxic, non-proteinaceous molecules, cell wall fortifications, defense-related enzymes and induced cell death (Heath, 2000). Reactive oxygen species (ROS) play a pivotal role in defense mechanisms of plants against

Plant Science Today (2017) 4(3): 105-120

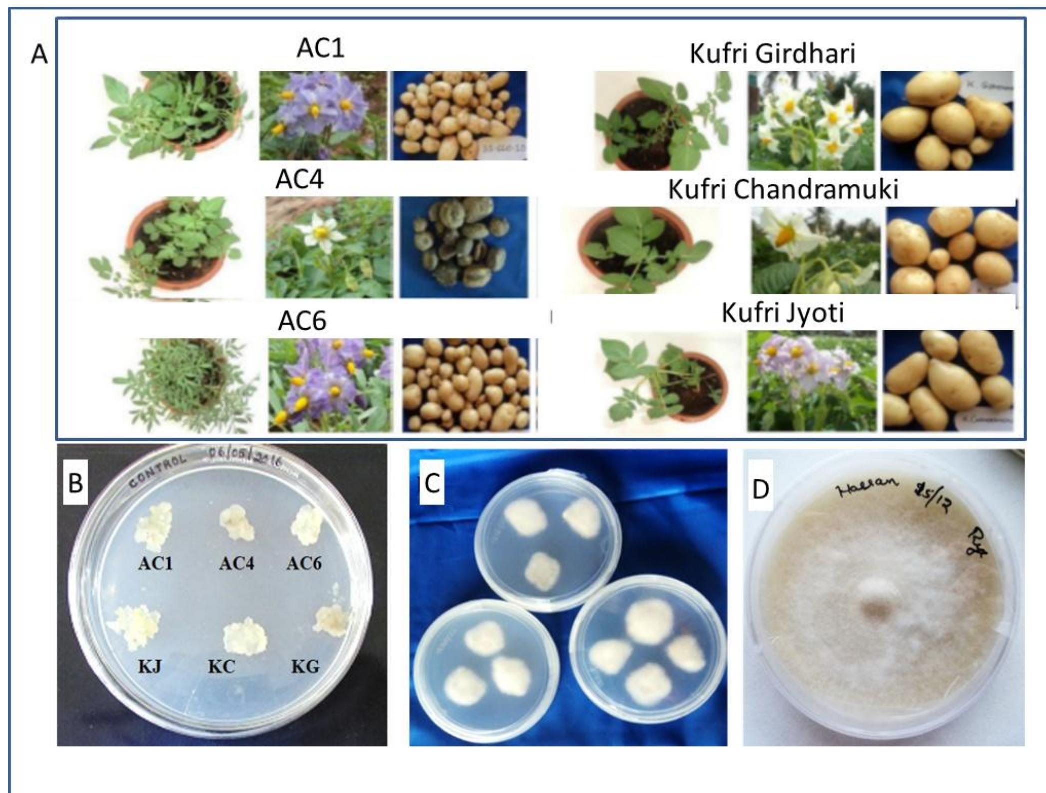

Fig. 1. Biological material used in this study: A. Wild and cultivated genotypes of potato; B. Leaf derived Calli of the six potato genotypes; C, *Phytophthora infestans* (ACH 1) isolate maintained on potato slices; C. *Phytophthora infestans* (ACH 1) isolate on Rye agar medium.

Table 1: Potato genotypes from which calli was derived for this study

Sl. No.	Genotype	Referred name	Species Name	Ploidy	Late Blight
1	Kufri Jyoti	KJ	*Solanum tuberosum*	*4n*	Susceptible
2	Kufri Girdhari	KG	*Solanum tuberosum*	*4n*	Resistant
3	Kufri Chandramuki	KC	*Solanum tuberosum*	*4n*	Susceptible
4	SS-660-10	AC1	*Solanum chacoense*	*2n*	Resistant
5	SS-1724-07	AC4	*Solanum sparsipillium*	*2n*	Resistant
6	SS-1725-54	AC6	*Solanum spegzinii*	*2n*	Susceptible

(H_2O_2) are examples of ROS that can cause the oxidative destruction of cells (Asada and Takahashi, 1987). Oxidative burst is a phenomenon that occurs at the site of infection due to the rapid accumulation of ROS (Lamb and Dixon, 1997) and can lead to a hypersensitive response (HR) that results in host cell death, which prevents further spread of biotrophic pathogens (Heath, 2000; Gechev *et al.*, 2006). ROS can also serve as second messengers for the activation of specific defense signaling pathways that can prevent disease (Dat *et al.*, 2000; Grant and Loake, 2000).

Plant resistance mechanisms are associated with up-regulation or down-regulation of oxidative enzymes such as superoxide dismutase, catalase, and peroxidase (Chaman *et al.*, 2001; Heng-Moss *et al.* 2004). These studies suggest that the increase in levels of peroxidases were a response to enhanced levels of endogenous peroxide. Oxidative enzymes are highly correlated with defense mechanism in resistant plants.

Earlier, nine genotypes of potato, six wild species (*Solanum chacoense* genotypes - AC1, AC2; *Solanum sparsipillium* genotypes AC3, AC4, AC5 and genotype of *Solanum spegzinii*, AC6) and three cultivars Kufri Jyoti (KJ), Kufri Chandramukhi (KC), and Kufri Girdhari (KG) obtained from CPRI, Shimla were screened for their resistance to Late Blight in lab and field experiments for over three years. Two of the wild species, AC1, AC4 and cultivar KG showed high degree of resistance against Late Blight disease in detached leaf assays and in three years of field evaluations (2012-2014). AC6, KC and KJ were susceptible to Late Blight. The resistant wild genotypes exhibited horizontal resistance (Anil *et al.*, 2013; Anil *et al.*, paper communicated).

This research was undertaken to evaluate cellular defense mechanisms in susceptible and resistant genotypes of potato using the callus system

as an experimental model. Calli generated from three potato genotypes resistant to Late Blight, AC1, AC4 and KG, and three genotypes susceptible to Late Blight, AC6, KJ, KC, were selected for this study. The study characterizes an in vitro callus model system for the study of cellular defense mechanism and the interactions of the potato - *P. infestans* host pathosystem.

Materials and Methods

Biological materials

Callus of potato genotypes: Callus cultures derived from leaves of three genotypes of wild species (AC1, AC4 and AC6) and three cultivars (KJ, KC and KG) of potato (Table 1) were earlier generated as per the tissue culture procedure developed by Anil and co-workers. In brief, callus was generated from surface sterilized leaves of potato genotypes on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 1 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2, 4-D) and 1 mg l^{-1} Benzyl amino purine (BAP). The callus induced at the cut edges of leaf discs was subcultured into MS medium containing 2 mg l^{-1} 2, 4-D for further callus proliferation and maintained in the same medium, till further experimentation. Fig. 1 shows the six genotypes of Potato and the respective calli.

***Phytophthora infestans* isolates**: *P. infestans* from Late Blight infested potato leaves was isolated earlier from fields at the College of Agriculture, Hassan (Anil *et al.*, 2013). Pure culture of *P. infestans* was maintained both on potato slices and on Rye medium and used for further experiments in this study. The Rye medium was prepared using Rye seeds according to the procedure described by Caten and Jinks (1968).

Preparation of *Phytophthora infestans* culture filtrate (CF): Rye broth, 100 ml, was inoculated with two mycelial plugs (0.25 cm^2 each) of freshly grown *P. infestans* grown on solid rye agar media. The flasks were incubated at 18°C with slow shaking (110 rpm) in a temperature-controlled orbital shaker for 30 days. The medium was separated from the mycelia by filtering through sterile filter paper (Watman 1) under vacuum. The medium was then centrifuged at 10000 rpm for 30 min and the supernatant filter sterilized.

Preparation of *Phytophthora infestans* for experiment: *P. infestans* was maintained on rye agar media for two weeks. Freshly produced sporangia were collected into distilled water, concentration of sporangial suspension adjusted to 2x 10^4 sporangia ml^{-1} and kept at 4°C for one hour to release zoospores and used for further experiment.

Experimental setup

Cellular defense response of calli cells of six genotypes grown on phytopathotoxic medium: Phytopathotoxic medium was prepared by adding culture filtrate (CF) of *P. infestans* to MS medium in 10, 20, 30% concentrations. The experiments was carried out with four treatments as follows:

Treatments	Description
C_0 -	Control (MS medium)
C_1 -	MS medium containing 10% CF
C_2 -	MS medium containing 20% CF
C_3 -	MS medium containing 30% CF

The calli were allowed to grow for 25 days on the phytopathotoxic medium and then evaluated for viability and defense response parameters.

Cellular defense response of calli exposed to *Phytophthora infestans* or its elicitors: The calli of six genotypes were exposed to *P. infestans* or its elicitors for a period of 24 hours and defense response, secreted protein analysis and cell death was monitored.

Treatments	Description
T_0 -	Control (callus alone)
T_1 -	Callus + *Phytophthora infestans* (PI)
T_2 -	Callus + Diffusible elicitors (PI inside a dialysis bag)
T_3 -	Callus + Culture filtrate (elicitors of PI)

Evaluation of cell viability: The viability of callus cells exposed to the different treatments was estimated by dye exclusion method using Trypan Blue vital stain. A packed cell volume (PCV) of 200 µl calli derived suspension cells were suspended in liquid MS media in a microfuge tube and placed in a rotary shaker for 10-20 min. After centrifugation at 4000 rpm for 5 min, the medium was discarded. 100 µl 0.4% Trypan Blue stain was added and placed again in rotary shaker for 10 min to allow the cells to take up the stain. The cells were washed and observed under the microscope (10 x). The nuclei of dead cells were stained Blue. The viability of cells was calculated by counting the dead cells in five microscopic fields. Per cent cell death was calculated as per the method described in Anil *et al*., 2007.

Enzyme extraction from callus: Calli from different treatments were frozen in liquid nitrogen to prevent proteolytic activity and homogenized using pestle and mortar. The homogenate was then suspended in extraction buffer (Phosphate buffer 0.1 M, pH 7.8, 1 mM PMSF and 0.1 % poly venyl pyrollidone] and held on ice for 15 min. The protein extracts were centrifuged at 14,000 rpm at 4°C for 30 min. The pellet was discarded and the supernatant containing the soluble proteins was used for further experiments. Protein level was estimated by the method of Lowry (1951) using BSA as standard.

Sample preparation and sample loading: Soluble protein extract from the samples of each treatment

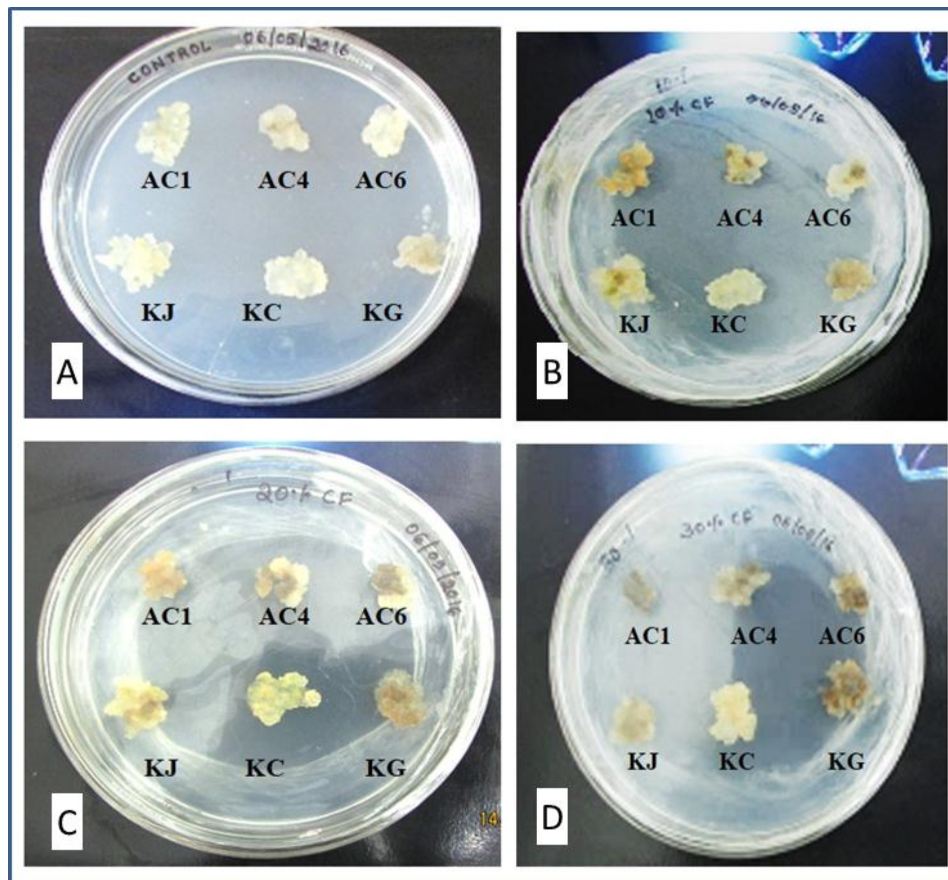

Fig. 2. Calli of six potato genotypes grown on phytopathotoxic medium. A. Control, C_0; B. 10 per cent CF, C_1; C. 20 per cent CF, C_2; D. 30 per cent CF, C_3

Table 2: Effect of phytopathotoxic medium on calli cell survival

Genotypes	Cell Mortality (%)				CD@1%
	C_0	C_1	C_2	C_3	
AC1	1.67	19.40	53.07	86.70	12.495
AC4	0.90	28.90	46.64	88.13	16.568
AC6	0.93	27.90	29.07	89.47	9.496
KJ	1.43	11.87	16.87	27.33	4.137
KC	0.97	3.50	10.13	16.27	3.828
KG	1.63	41.48	48.00	63.00	14.560
CD@1%	0.564	12.654	4.743	9.061	

were mixed with sample buffer, and boiled in water bath at 95°C for 1 min and allowed to cool at room temperature. In all experiments, 25 µg of protein extract was loaded per well of the PAGE gel.

Guaiacol peroxidase enzyme assay: Peroxidase activity was assayed as increase in optical density due to the oxidation of guaiacol to tetra-guaiacol as per the method by Castillo *et al.*, (1984). Absorbance due to the formation of tetra-guaiacol was measured at a time interval of 30 sec up to 1 min at 470 nm.

Native page analysis for peroxidase isoenzyme analysis: The protein samples (20 µg each) were separated by native gel electrophoresis at 6°C. For staining, the gel was incubated in a solution containing 0.1 M Potassium phosphate buffer (pH 6.1), 20 mM Guaiacol and 5.55 mM H_2O_2 for 10-20 min at room temperature until the bands appeared. The gel was then washed with 7.5% acetic acid and 1% glycerol to stop the reaction. The isoenzyme bands appeared in brick red color, which were stable for 24 h and the pattern was photographed.

Superoxide dismutase (SOD) enzyme assay: SOD activity was measured by the method described by Dhindsa *et al.*, (1981) with slight modifications. SOD activity in the protein extracts was monitored by its ability to inhibit photochemical reduction of nitroBlue tetrazolium. The Soluble protein fraction

25 µg, was assayed in a reaction mix of 1 mL, containing 100 mM Phosphate buffer (pH 7.8), 200 mM Methionine, 2.25 mM NitroBlue tetrazolium chloride (NBT), 3 mM EDTA, 60 µM Riboflavin, 1.5 mM Sodium carbonate. The test tubes containing assay mixture were incubated under light for 15 min and non-illuminated reactions without supernatant served as blank. Absorbance of samples along with the blank was recorded at 560 nm wavelength.

Enzyme unit: One unit of enzyme activity was defined as the quantity of protein required to reduce the absorbance reading of samples to 50 % in comparison with tubes lacking enzymes.

For the in-gel isoenzyme assay for SOD activity, the proteins were separated by Native PAGE as described earlier. The gel was incubated in a staining solution containing 100 per cent NBT (w/v), 0.2 M EDTA (w/v), 0.1M sodium phosphate buffer (pH 7.5), and 5 % riboflavin (w/v) for 30 min until the bands appeared. The isoenzyme bands appeared as white/colourless in a dark Blue background and the isoenzyme pattern was photographed.

Extraction of phenolics and Flavanoids: Phenolics and Flavanoids were extracted by a common protocol, essentially, 100 µg of calli was homogenized in 1 ml of 80% ethanol in a pestle and mortar. The homogenate was centrifuged at 10000 rpm for 20 min, the supernatant was collected and the residue re-extracted with five times the volume of 80% ethanol and re-centrifuged. After this the supernatant was collected and evaporated to dryness and the residue dissolved in 2 ml of distilled water.

Estimation of phenolics: Total phenolics levels was estimated using Folin-ciocalteu reagent (FCR) that results in the formation of a Blue colored complex which is measured at 650 nm spectrophotometrically (Ainsworth and Gillespie, 2007). In brief, phenolic extract (0.2 ml) was diluted with 3 ml distilled water and 0.5 ml of Folin-ciocalteau reagent was added. The contents were mixed thoroughly. Exactly after 3 min, 2 ml of 20% sodium carbonate was added. Then content were allowed to stand for 1 min in boiling water bath, cooled and absorbance was measured at 650 nm against the reagent blank. A standard graph was constructed with Gallic acid which was used as a standard phenolic. The total phenolic concentration present in the test samples were calculated using standard curve and the concentration is expressed as mg phenolics/g fresh weight.

Estimation of flavonoids: Flavanoid extract (0.2 ml) was diluted with distilled water to make the volume to 1 ml and then 1.8 ml 95% methanol, 0.1 ml of 1 M sodium acetate and 0.1 ml 10 % aluminium chloride were added. The absorbance was recorded at 415 nm using spectrophotometer against the blank. A standard graph was prepared using Rutin as the standard flavonoid. The flavonoid concentration present in the test samples were calculated using standard curve and the concentration is expressed as mg flavonoid/g fresh weight.

Separation of the Secreted protein fraction: The method to separate the secreted protein fraction was earlier developed by Anil and co-workers. The callus derived suspension cells of the six potato genotypes were placed in sterilized 150-ml flasks containing 20 ml of MS medium, pH 5.8 and subjected to treatments T_0, T_1, T_2, T_3 as described above. Appropriate calli derived suspension cells in MS medium were used as control (T_0). The flask was maintained on a rotary shaker at 160 rpm in the dark at 18˚C for three days. Following which the callus suspension cells in the beaker were centrifuged at 5000 rpm for 10 min to separate the cells and the medium. The supernatant (medium) obtained were sequentially centrifuged by using Contricon centrifuge tubes (10kD cut off) in an Eppendorf refrigerated centrifuge until the proteins got sufficiently concentrated. The secreted protein fractions from the different treatments were quantified by Lowry's method as before. The secreted proteins were evaluated for SOD and Peroxidase activity as per the methods elaborated above.

DAB staining for cellular H_2O_2 accumulation: The production of hydrogen peroxide (H_2O_2) in response to pathogen and/or elicitor treatment, was examined in potato calli of 6 genotypes with 3, 3'-diaminobenzidine (DAB) staining method described by Orozco-Cardenas and Ryan (1999) with slight modification. DAB precipitates and turns deep brown in the presence of H_2O_2.

The calli subjected to different treatments were placed in 10 ml of DAB staining solution (DAB – 1mg/ml DW, pH 3.6) for 2 hours to allow the callus to take up the DAB stain. The callus was washed with hot ethanol and the cells were then observed under microscope at 10X magnification. The dark brown stained cells indicated intracellular accumulation of H_2O_2. The total number of cells in a field and the cells stained by DAB were both counted in multiple microscopic fields and percentage cells accumulating H_2O_2 was recorded.

Statistical analysis

Microscopic cell counting for cell viability and Cells accumulating hydrogen peroxide were made with 5 microscopic fields in each slide for all the genotypes and for each of the treatments used, three replications were maintained. The statistical analysis of the data was carried out using Completely Randomised Design (CRD).

The biochemical assays were done in 3 replications for all the genotypes and for each of the treatments used. The statistical analysis of the data was carried out using Completely Randomised Design (CRD).

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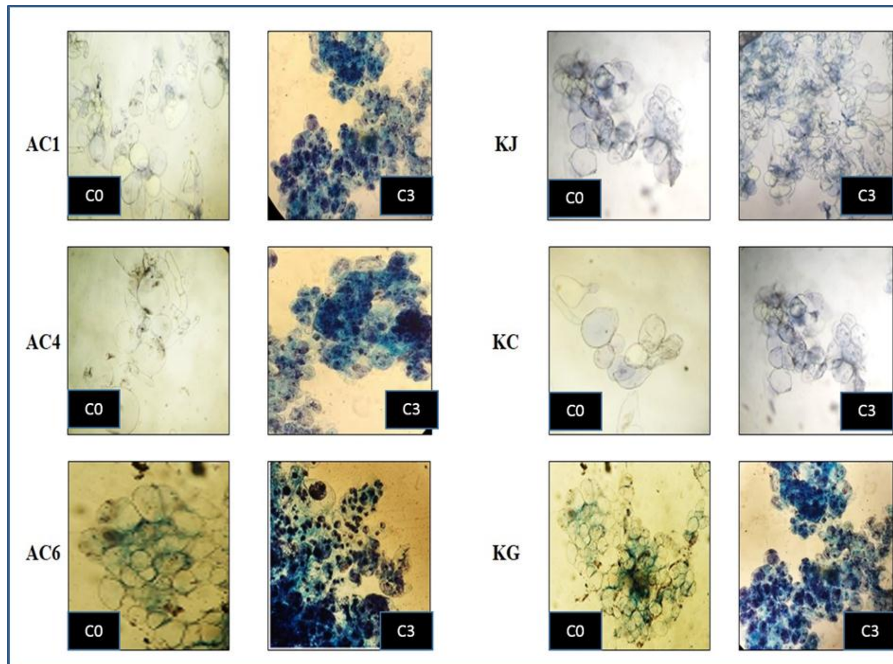

Fig. 3. Trypan Blue stained dead cells in calli grown on phytopathotoxic medium. C_0- Control; C_3- 30 per cent CF

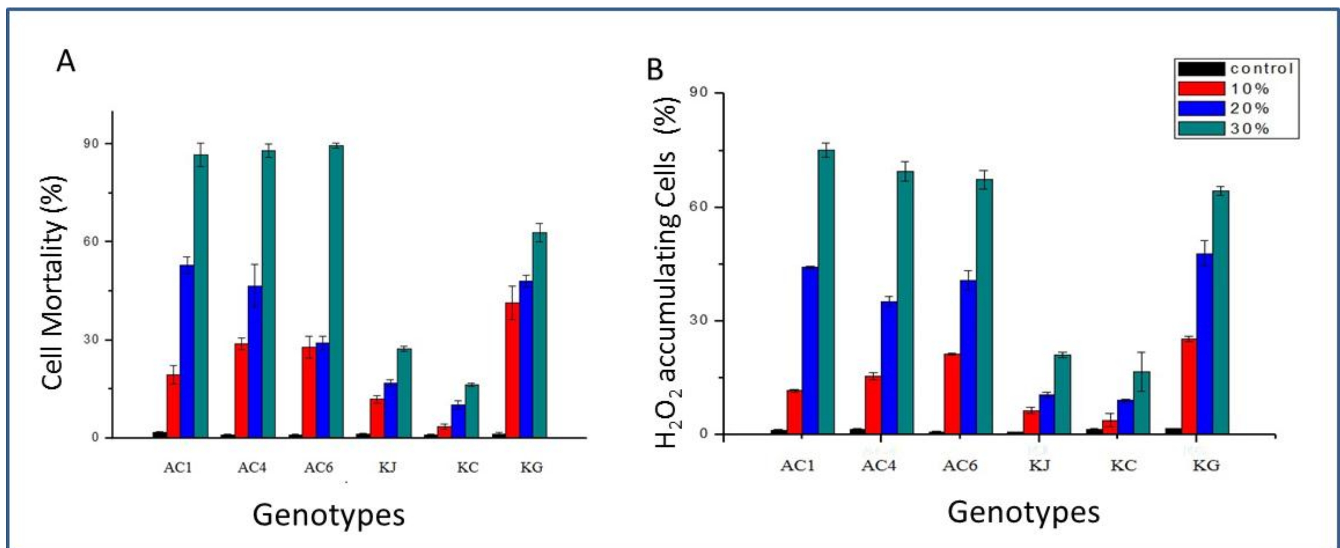

Fig. 4. Effect of growing Calli of potato genotypes on phytopathotoxic medium: A, Percent cell mortality in calli; B, Percent cells accumulating H_2O_2. Control, C_0; 10% CF, C_1 CF; 20 % CF, C_2; 30 % CF, C_3.

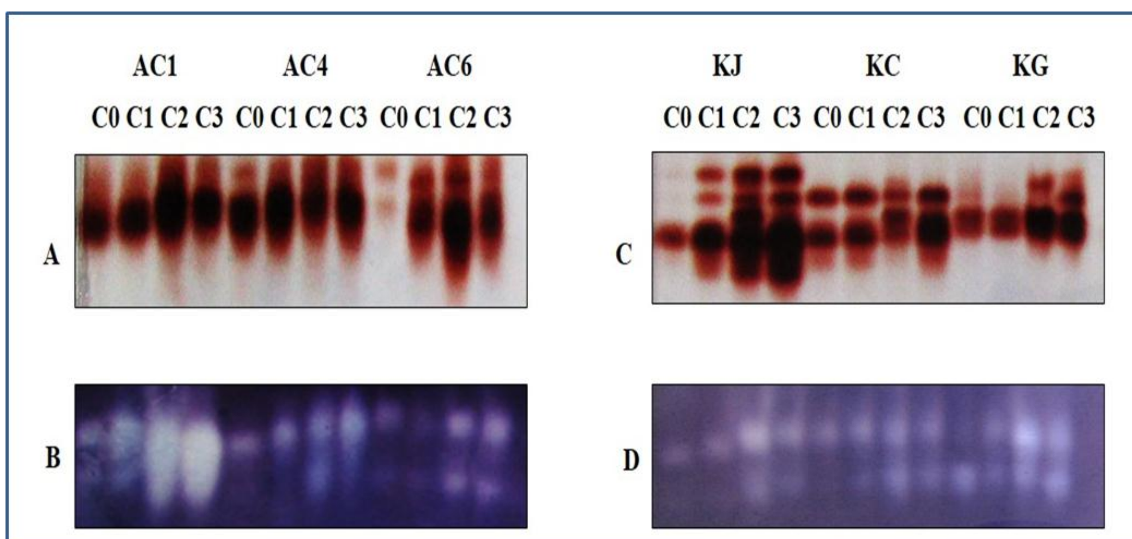

Fig. 5. Calli of six potato genotypes grown on phytopathotoxic medium. A. Control, C_0; B. 10 per cent CF, C_1; C. 20 per cent CF, C_2; D. 30 per cent CF, C_3

ISSN: 2348-1900

Results

Effect of phytopathotoxic medium on callus growth of six potato genotypes

Phytopathotoxic medium was prepared by adding filter sterilized culture filtrate (CF) of *Phytophthora infestans* to MS medium at a concentration of 0, 10, 20, 30% (C_0, C_1 ,C_2 , C_3).

Callus of the six genotypes were plated on each of the above treatments (C_0-C_3) in petriplates by placing 100 µL PCV (packed cell volume) each of callus equidistant from each other. The calli were allowed to grow for a period of 25 days (Fig. 2). The relative visual growth as compared to control plate, cellular defense enzyme activity and cell death were monitored on the 25th day post inoculation.

Cell viability of the callus

The Percentage mortality in calli cells grown on MS medium containing varying concentrations of Culture Filtrates (CF) of the pathogen was monitored by counting Trypan Blue stained nuclei of cells. The cell death was significantly higher with increase in the concentration of phytotoxic medium (10, 20 and 30% CF, C_1-C_3 treatments) in all the callus of potato genotypes over control (C_0) (Table 2) at 25 days post inoculation. The phytopathotoxic medium with 30 % culture filtrate induced highest mortality compared to other treatments. Thus the CF of *P. infestans* had a dose dependent effect on viability of calli cells of all genotypes (Fig. 3, Fig. 4).

When comparisons were made across genotypes, interestingly and surprisingly, the percent cell death was higher in resistant genotypes of potato calli plated on phytopathotoxic medium than susceptible ones. The variations in cell death were significant between the treatments and across the genotypes (Table 2, Fig. 4).

Defense response in calli cells when grown on Phytopathotoxic medium (C_0-C_3)

The peroxidase and SOD isoenzyme activity was analyzed in-gel by native PAGE. The gels were processed for SOD and Peroxidase assays as mentioned in materials and method. The banding pattern of peroxidase isozymes varied among genotypes, and peroxidase isoenzyme band intensity increased in calli of all the genotypes with increase in the concentration of phytopathotoxic medium (10, 20 and 30 per cent) (Fig. 5). The SOD activity band intensity increased in the calli of all the six genotypes grown on phytopathotoxic medium (Fig. 5).

Influence of Phytophthora infestans on callus cell viability

Callus cells were directly exposed to *Phytophthora infestans* and cell mortality was monitored by Trapan Blue staining followed regularly at 3 hr intervals for a period of 24 h. Potato callus of resistant genotypes AC1, AC4 and KG started dying at 12th h, AC6 at 15th h, KJ at 18th h and KC at 20th h following exposure to pathogen (Data not shown). The result also revealed that the callus of resistant genotypes were more sensitive to direct exposure to pathogen at the cellular level than the susceptible genotypes.

Effect of Phytophthora infestans or its elicitors on calli cells

The experiment was conducted with the following treatments:

T_0- Callus (Control)

T_1- Callus exposed to *Phytophthora infestans* (PI)

T_2- callus exposed to diffusilble elicitors (PI inside dialysis bag which is dipped into media containing callus)

T_3- callus exposed to culture filtrate (CF)

The callus taken per treatment was 200 µL PCV. The concentration of *P. infestans* was 20,000 sporangia/mL in treatments T_1 and T_2. The CF was obtained by centrifuging down a three week old *P. infestans* liquid culture. The clear supernatant was filter sterilized and used as CF. CF was used at 10% in T_3. Potato leaf derived calli of six genotypes thus exposed to different treatments T_0, T_1, T_2 and T_3 for 24 hrs were monitored for cell death by Trypan Blue vital stain as described in materials and methods. The dead cells that are stained Blue were counted under bright field microscope at 10X magnification.

The result indicate that in the callus of three established resistance genotypes (Anil *et al*., 2013) *viz,* AC1, AC4 and KG, the per cent cell death was higher in the treatments with exposure to *P. infestans* as compared to the cell death in established susceptible genotypes, AC6, KJ and KC. Resistant genotypes AC1, AC4 and KG when exposed to the pathogen showed higher level of mortality, *i.e.* 57.02, 46.80 and 48.21 % respectively as compared to the susceptible genotypes, KJ and KC, which when exposed to pathogen (T_1) showed only 19.27 and 12.67 per cent mortality after 24 hour exposure to the pathogen (Table 3). AC6, a susceptible genotype showed slightly higher percentage cell mortality, compared to KC and KJ. However it was significantly lower than the resistant genotypes AC1, AC4 and KG (Table 3).

The results indicate that the calli exposed to the pathogen directly (T_1) showed higher cell death compared to T_2 and T_3. However Elicitor treatments also induced significantly higher cell death as compared to control (T_0). The established resistance genotypes AC1, AC4 and KG exposed to pathogen showed higher cell mortality than the established susceptible genotypes.

Peroxidase activity

The results show that the peroxidase defense enzyme in the soluble proteins of all the genotypes exposed to pathogen showed increased activity compared to control. The elicitors of pathogen (T_2, T_3) also induced POX defense enzyme. However the direct exposure of pathogen (T1) showed

Table 3: Effect of *Phytophthora infestans* or its elicitors on calli cell survival

Genotypes	Cell Mortality (%)			
	T_0	T_1	T_2	T_3
AC1	2.47	57.02	13.4	17.14
AC4	1.97	46.80	17.94	24.4
AC6	2.30	38.50	14.08	20.3
KJ	1.08	19.27	5.02	7.14
KC	0.61	12.67	1.86	2.44
KG	1.63	48.21	30.44	40.56
CD@1%	1.164	6.375	4.946	3.574

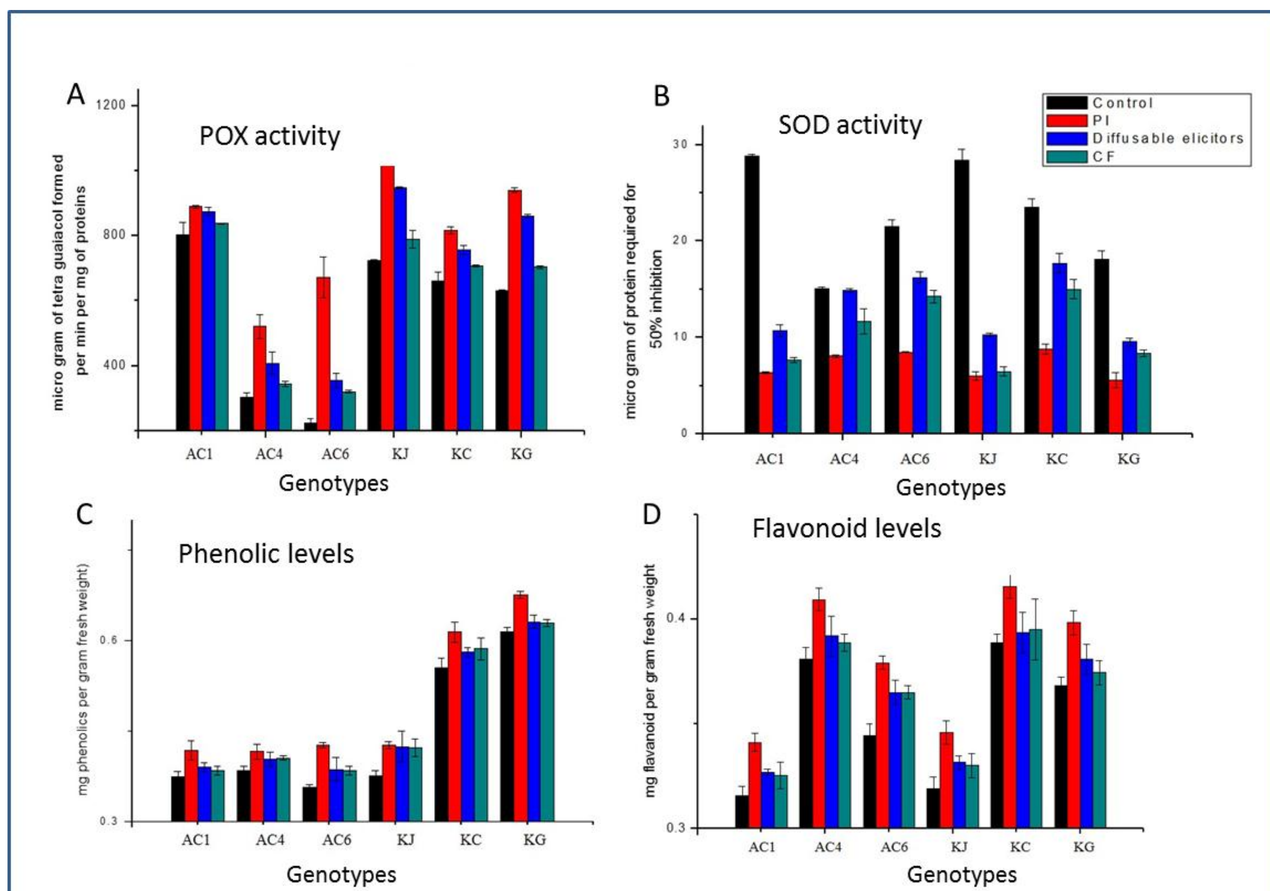

Fig. 6. Defense induction in calli of potato genotypes: A, Peroxidase activity; B, SOD activity; C, Levels of Phenolics; D, Levels of Flavanoids. Control, T_0; Direct exposure to *Phytopthora infestans*, T_1; Diffusible elicitor, T_2; CF, T_3.

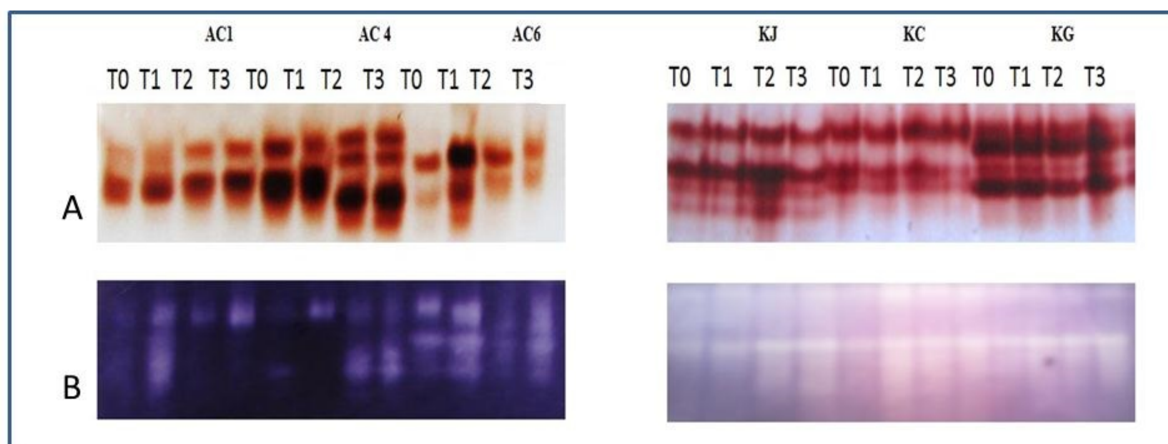

Fig. 7. In gel POX and SOD isoenzyme activity in soluble proteins of calli exposed to *P. infestans* or its elicitors: A. POX isozymes, B, SOD isozymes, Control, T_0; PI, T_1; Diffusible elicitor, T_2; CF, T_3

Table 4: Effect of to *Phytophthora infestans* and its elicitors on Peroxidase activity in calli

Genotypes	Peroxidase Activity (µg tetra guaiacol/ min/ mg of protein)				CD@1%
	T_0	T_1	T_2	T_3	
AC1	766.67	890.00	875.33	838.00	40.140
AC4	303.33	521.33	409.33	344.67	124.657
AC6	226.00	672.00	356.00	322.00	105.859
KJ	631.33	941.33	861.33	704.00	24.809
KC	662.00	816.67	757.33	708.00	79.670
KG	723.33	1023.33	947.33	790.00	104.323
CD@1%	64.438	94.605	99.775	49.686	

Table 5: Effect of *Phytophthora infestans* or its elicitors on Superoxide dismutase activity in calli

Genotypes	SOD activity (µg of protein required for 50% inhibition)*				CD@1%
	T_0	T_1	T_2	T_3	
AC1	28.80	6.34	10.72	7.67	1.640
AC4	15.11	8.08	14.90	11.69	3.175
AC6	21.52	8.48	16.24	14.27	2.578
KJ	28.42	6.05	10.30	6.47	3.138
KC	23.52	8.83	17.73	15.01	4.165
KG	18.11	5.57	9.58	8.39	3.084
CD@1%	3.282	1.867	2.402	3.313	

*Smaller values represent higher SOD activity

higher activity in all the six genotypes. The highest induction of POX was recorded in calli of resistance genotypes, KG and AC1 with exposure to *P. infestans* (Table 4, Fig 6A). Gel assays showed that *P. infestans* exposure enhanced POX isoenzyme activity in calli of all six genotypes. The direct exposure of calli to the pathogen (T_1) showed higher POX activity. However, diffusible pathogen elicitors (T_2) or elicitors in CF (T_3) also induced POX isoenzyme activity in all the genotypes. The resistant genotypes AC1, AC4 and KG showed higher intensity of POX isoenzyme bands than susceptible genotypes (Fig. 7).

Superoxide dismutase (SOD) activity

The soluble proteins extracted from the calli of all six genotypes with treatments T_0, T_1, T_2 and T_3 were assayed for SOD defense enzyme activity spectrophotometrically and the data are presented in Table 5. The results indicate significant variations in SOD activity among the treatments and also across the genotypes (Fig. 6B). SOD activity increased with *P. infestans*/elicitor exposure in all genotypes as compared to control (T_0). The highest activity was observed in KG (Table 5). The SOD isoenzyme activity was analyzed by native PAGE by using NBT as substrate. The SOD activity in calli of all genotypes increased with the pathogen/elicitor exposure over control (Fig. 7). The banding pattern of the isozymes varied among the genotype.

Phenolics and Flavanoid contents in calli exposed P. infestans/diffusible elicitors /CF

The exposure of calli of the six genotypes to *P. infestans* leads to the marked increase in phenolic content in all the genotypes over the control (T_0). The phenolic content was higher in the calli exposed directly to the pathogen (T_1) than those of other treatments (T_2 and T_3) (Fig.6C, Table 6).

Flavonoid content analysis

The flavonoid content in the callus of all the potato genotypes exposed to *P. infestans*/elicitors (T_0, T_1, T_2 and T_3) shows that there is an increase in flavanoid content in all calli of genotypes in treatments T_1, T_2 and T_3 compared to the control (T_0). The flavonoid content in all the calli exposed to *P. infestans* (T_1) was relatively higher than other two treatments (T_2 and T_3) (Fig. 6D, Table 7).

Analysis of secreted proteins for defense enzyme activity

Secreted protein of the calli derived suspension cells of six genotypes with treatments T_0, T_1, T_2 and

T_3 were collected in the surrounding medium, concentrated by repeated centrifugation using centricon centrifugation tubes. The protein thus concentrated was estimated by Lowry's method as described in materials and methods and the protein concentration was calculated by using BSA standard curve and analyzed first by SDS-PAGE (Fig. 8).

Peroxidase isoenzyme activity in secreted protein fraction

Gel assay shows that the POX isoenzyme activity significantly increased in the secreted protein fraction with direct exposure to *P. infestans* (T_1) in all the calli of potato genotypes (Fig. 8). POX also increased in secreted protein fraction of calli cells under exposure to diffusible pathogen elicitors (T_2) and in secreted proteins of calli subjected only to CF exposure (T_3). The pattern of POX isoenzymes varied among genotypes and in some cases across treatments (Fig. 8).

SOD isoenzyme activity in secreted protein fraction

Isoenzyme pattern of SOD in the secreted protein fraction from all six genotypes exposed to *P. infestans*/elicitors (T_1, T_2, T_3) shows that the SOD isoenzyme activity significantly increased in the secreted protein fraction with direct exposure to *P. infestans* (T_1) in all the calli of potato genotypes (Fig. 8). SOD also increased in secreted protein fraction of calli cells under exposure to diffusible pathogen elicitors (T_2) and in secreted proteins of calli subjected only to CF exposure (T_3). The pattern of SOD isozymes varied among genotypes and in some cases across treatments.

3, 3'-Diaminobenzidine (DAB) staining for H_2O_2 accumulation

To understand why resistant genotypes of potato showed higher cell mortality in calli, percent of cells accumulating H_2O_2 was evaluated when challenged with *P. infestans*/elicitors using DAB staining technique. Cell accumulating high H_2O_2 stained dark reddish brown in colour, whereas those cells with little or no H_2O_2 were colorless or very light brown. The calli of genotypes under study grown for 25 days on increasing concentration of phytopathotoxic medium (C_0-C_3) (Table 8, Fig. 4B) and directly challenged with the pathogen for 24 hrs (Table 9, Fig. 9), were stained with DAB as per the procedure in Materials and Methods. The result revealed that high accumulation of H_2O_2 was predominant in resistance genotypes, AC1, AC4 and KG with higher percent cells accumulating H_2O_2 (Table 8, Fig. 4B). Whereas fewer cells of the susceptible genotypes AC6, KJ and KC accumulated H_2O_2 (Table 8, Fig. 4B) that too at lower levels as indicated by the pale brown staining of cells when directly exposed to the pathogen or when grown on elicitor medium. Direct exposure to pathogen for 24 hrs also showed similar results of higher percent cells with H_2O_2 in resistant potato genotypes compared to susceptible genotypes (Table 9, Fig. 9).

Correlation between cell death and hydrogen peroxide accumulation

Direct linear correlation between cell death and cells accumulating high hydrogen peroxide in the calli cells of six potato genotypes was seen, independent of wild and cultivated genotypes. The genotypes indicated with red color are susceptible genotypes (AC6, KC, KJ) and genotypes in Blue color resistant genotypes (AC1, AC4, KG) (Fig. 9). The Resistant genotypes grouped together with higher percent cells accumulated H_2O_2 and higher percent Cell death.

Discussion

Late blight, caused by *Phytophthora infestans*, is one of the most devastating diseases of potato, causing yield loss of over 30% globally. In India, Late Blight has devastating effects on the potato crop, causing yield losses in the plains of India to the tune of over 50%. Popular cultivars such as Kufri Jyoti, were originally released as a Late Blight resistant variety, is now rendered highly susceptible. Late Blight also affects other economically important *Solanaceae* family crops like Tomato.

Due to domestication, the cultivated potatoes lost their genetic variation. Wild potato species represents diverse gene pool which have been used traditionally as a source of diverse trait for potato breeding (Hijmans *et al.*, 2007). These wild species can be used to breed potato against biotic stress like Late Blight and abiotic stresses such as salt, heat and cold stresses.

Understanding the molecular mechanisms of defense responses in resistant genotypes can identify novel mechanisms that could be exploited to enhance Late Blight resistance. Anil and Coworkers have earlier investigated nine potato genotypes- six wild type species (*Solanum chacoense* genotypes- AC1, AC2; *S. sparsipillium* genotypes AC3, AC4, AC5 and genotype of *S. spegzinii,* AC6) and three potato cultivars (KJ, KC and KG), for their response to Late Blight. Three years (2012-2014) of field testing and laboratory detached-leaf assays show that wild species, *S. chacoense* (AC 1) and *S. sparsipillium* (AC 4) and the cultivar Kufri Girdhari are highly resistant to Late blight and show heightened defense response. An understanding of resistance response at the cellular level, independent of influences and contributions from the tissue environment of the whole plant would shed light on cellular mechanisms. In vitro cultures can serve as a model system to understand such cellular mechanisms (Anil *et al.*, 2000).

Cells of calli represent dedifferentiated cells and the extent to which their physiology is similar to the whole plant varies between plant species. Calli of a salt-sensitive glycophyte and two salt-tolerant halophytes are reported to show similar salt sensitivity in culture. On the other hand,

ISSN: 2348-1900

Table 6: Effect of *Phytophthora infestans* or its elicitors on Phenolic content of calli

Genotypes	Phenolics (mg phenolics/g FW)				CD@1%
	T_0	T_1	T_2	T_3	
AC1	0.36	0.43	0.39	0.41	0.010
AC4	0.38	0.43	0.39	0.41	0.014
AC6	0.37	0.43	0.40	0.38	0.028
KJ	0.33	0.45	0.37	0.43	0.010
KC	0.55	0.64	0.58	0.59	0.056
KG	0.61	0.68	0.63	0.63	0.037
CD@1%	0.031	0.031	0.029	0.037	

Table 7: Effect of *Phytophthora infestans* or its elicitors on Flavonoid content of calli

Genotypes	Flavanoid (mg Flavanoids/g FW)				CD@1%
	T_0	T_1	T_2	T_3	
AC1	0.32	0.36	0.35	0.36	0.015
AC4	0.38	0.43	0.40	0.41	0.024
AC6	0.33	0.41	0.38	0.38	0.005
KJ	0.32	0.38	0.36	0.37	0.014
KC	0.39	0.43	0.40	0.41	0.018
KG	0.37	0.40	0.38	0.38	0.020
CD@1%	0.019	0.018	0.018	0.008	

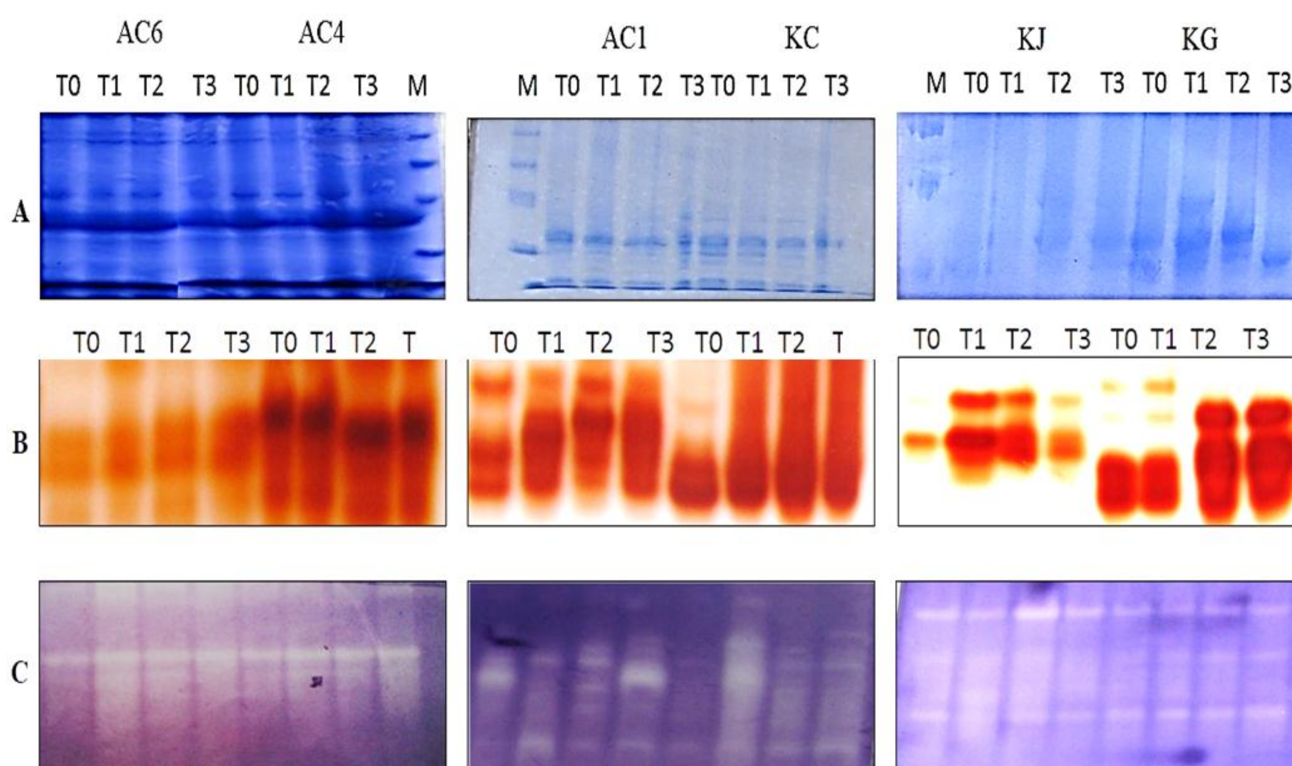

Fig. 8. Secreted protein fraction of Calli of the Potato genotypes: A, SDS- PAGE, B, POX isozyme; C, SOD isozyme banding pattern of AC1, AC4, AC6, KJ, KC and KG for secreted proteins. Control, T_0; PI, T_1; Diffusible elicitor, T_2; CF, T_3

the callus of salt-tolerant glycophyte, *Beta vulgaris* exhibited salinity tolerance comparable to the whole plant (Smith and McComb, 1981; Strogonov, 1973). Anil and coworkers have shown that suspension cultures of rice captured the salt-tolerance characteristics of the cultivars they are derived from (Anil *et al.,* 2007). Thus the cellular mechanisms observed *in vitro* cell cultures are indicative of the repertoire available to the genotype and can thus serve as a useful model system. Nonetheless, caution needs to be exercised in the extrapolation of the results obtained using isolated cells to the physiology of whole plants. The calli system facilitates the study of cellular mechanisms better, as conditions in the medium can be easily varied and the cells are more amenable to microscopic and cell biology techniques as compared to the whole plant/tissue systems.

In this study leaf derived calli of six potato genotypes, three derived from resistant genotypes (AC1, AC4, KG) and three derived from susceptible genotypes (AC6, KJ, KC), were used as a model experimental system to understand the cellular defense responses. The study has been able to show that the direct contact with pathogen (T_1), indirect contact with the pathogen (diffusible elicitor, T_2) and exposure to filter sterilized culture filtrate (T_3) all had the ability to induce significant defense response in all the six genotypes under study. The study also evaluates pathogen induced changes in secreted protein fraction of the calli. High levels of SOD and POX activity is detected in the secreted protein fraction, indicating that defense response goes beyond the cell itself and spills into the extracellular medium. The surprising observation of this study is the sensitivity of calli of established resistant genotypes to exposure to pathogen or its elicitor, while the susceptible genotypes fared much better under the same biotic stress treatments. Thus this created a paradox wherein low survivability levels at the calli level refuted the high resistance observed at the whole plant, the study attempts to decipher this paradox.

Plant defense mechanisms against pathogens are indeed complex. The plant cell on one hand has to produce ROS as a response to the biotic treat at the same time it has to mitigate pathogen-induced oxidative stress. Generally, a fine intracellular balance between ROS generation and scavenging exists in cells. Plants possess an array of antioxidants that can protect cells from oxidative damage by scavenging ROS (Noctor *et al.,* 1998). The enzymes involved in generation and scavenging ROS include SOD (generates H_2O_2) and POX (scavenges H_2O_2), which work together with other enzymes of the ascorbate-glutathione cycle maintain the appropriate balance of ROS (Hernandez *et al.,* 2000). SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2. POX is widely distributed in all higher plants and protects cells against the destructive influence of H_2O_2 by catalyzing its decomposition through the oxidation of phenolic.

POX and SOD play a very important role in plant defense response processes. The correlations between aggravation of diseases and enzymes activity are reported to be significant (Silva *et al.,* 2002; 2008). In the current study, significant increases in POX and SOD activities were detected both in spectrophotometric assays as well as in in gel assays that clearly showed enhanced activity of isozymes of calli (Fig. 5, 6, 7) of all genotypes when grown in phytopathotoxic medium (C_1, C_2, C_3), or when exposed to *P. infestans* (T_1) or its elicitors (T_2, T_3) (Fig. 5, 6, 7).

ROS are often detected in plant-pathogen interactions and are associated with symptom development. H_2O_2, a ROS, is generated by the activity of Superoxide dismutase (SOD) that converts a Superoxide anion into H_2O_2. H_2O_2 in the whole plant context is produced both within a cell and also in the apoplastic region as one of the primary defenses against pathogen attack. The toxic H_2O_2 induces cell death at the site of infection thus curtailing spread of disease, a phenomenon called as hypersensitive response. ROS in general can also act as a second messenger in the cell and play pivotal roles in cell signaling coupling various external stimuli to an appropriate response in plants (Alscher, *et al.,* 2002)

Earlier work (Anil *et al.,* 2013) clearly shows that resistant genotypes have high SOD activity, and accumulated high levels of H_2O_2 as compared to susceptible genotypes. This highlights the pivotal role of SOD and H_2O_2 in an effective defense mechanism in resistant potato genotypes (Anil *et al.,* 2013). In fact the current study also shows significant enhancement of SOD activity in calli of all genotypes when either grown on phytopathotoxic medium or when directly or indirectly exposed to pathogen or its elicitors (Table 5). SOD activity was induced and the activity was highest in the resistant genotype KG followed by AC1 and AC4. However, susceptible genotypes too showed enhanced SOD activity with the biotic stress imposed in this study. Among the treatments it was direct exposure to *Phytophthora infestans* (T_1) that induced highest degree of SOD activity, followed by exposure to filter sterilized CF (T_3) and indirect exposure to live pathogen (T_2) (Table 5, Fig. 5, 6, 7).

Interestingly, this study also shows that SOD is secreted out into the extracellular medium and higher levels of secreted SOD activity was detected when exposed to *P. infestans*/elicitors (Fig. 8). A plasma membrane bound enzyme (NADP dependent Reactive Oxidative burst homologe D) is implicated in this rise in ROS levels in the apoplastic fluid. This study along with an earlier study of secreted proteins with *in vitro* grown whole plants (Anil, unpublished) show that SOD is secreted out of the cell, suggesting its role in defense response in the apoplastic environment,

Table 8: Accumulation of Hydrogen peroxide in the calli grown on phytopathotoxic medium

Genotypes	Cells accumulating H_2O_2 (%)				CD@1%
	C_0	C_1	C_2	C_3	
AC1	1.64	11.63	44.17	75.17	8.961
AC4	1.61	15.50	35.13	69.50	7.250
AC6	0.83	21.30	40.70	56.53	7.873
KJ	0.80	6.43	10.57	21.10	3.229
KC	1.65	4.63	9.13	11.87	2.732
KG	1.63	25.20	47.87	64.30	8.430
CD@1%	0.305	2.592	9.691	7.488	

Table 9: Hydrogen peroxide accumulation in calli exposed to *Phytophthora infestans* for 24 hours

Genotypes	% H_2O_2 accumulated cells
AC1	44.17
AC4	40.70
AC6	35.13
KJ	10.57
KC	4.77
KG	47.87
CD @ 1%	9.800

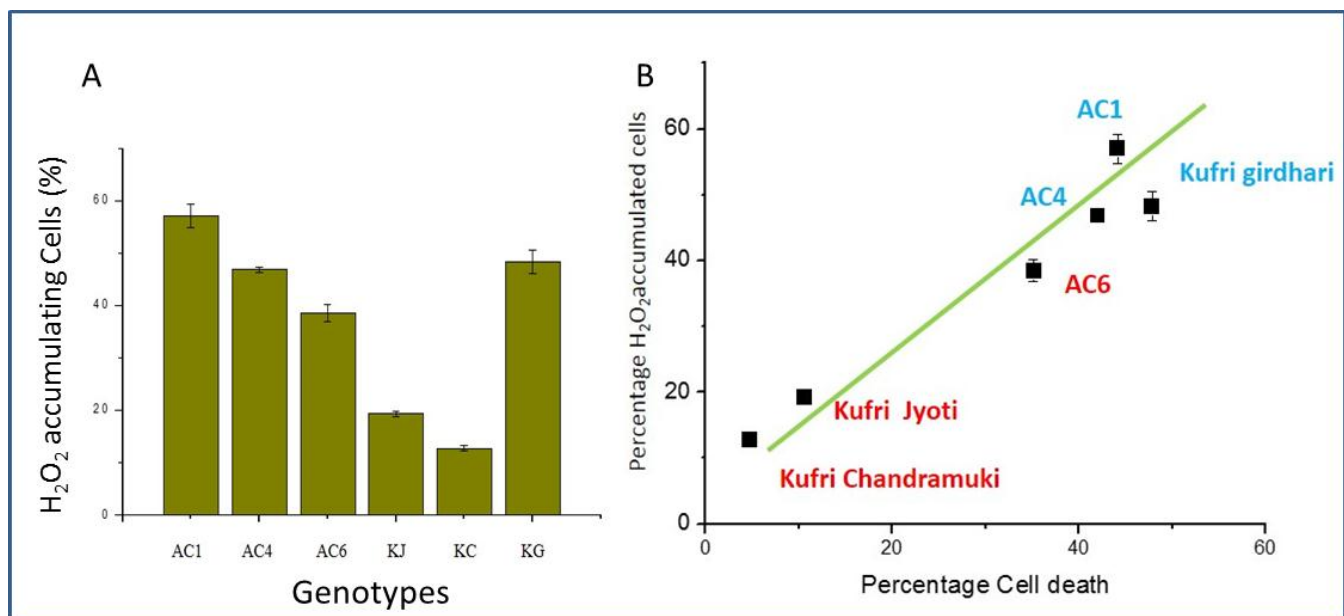

Fig. 9. Percent cells accumulating hydrogen peroxide in calli of six potato genotypes exposed to *Phytophthora infestans*

probably by creating a toxic environment to the incoming pathogen.

Peroxidases participate in a variety of plant defense mechanism and play an important role in the host pathogen interactions. Hydrogen peroxide is often supplied as an oxidative burst. Enzymes, peroxidase and catalases, are capable of eliminating the hydrogen peroxide formed during non-enzymatic or enzymatic dismutation (Chkhubianishvili *et al*., 2011). In addition, peroxidases (POX) play other important roles with an increased peroxidase activity being associated with environmental stresses on plants. POX is known in lignin synthesis and oxidation of the endogenous Indole acetic acid. Plant peroxidases act as catalysts for the polymerization of phenolic compound to form suberin and lignin in the cell wall, which act as barrier for the entry of pathogen (Kavitha and Umesha, 2008).

In the current study, calli from all six genotypes showed an induction of Peroxidase activity with direct exposure to *P. infestans* (T_1) or its diffusible elicitor (T_2). The exposure to filter sterilized CF (T_3) also showed POX induction but significantly lower than T_1 and T_2 (Table 4, Fig. 5, 6, 7). The general induction of POX corroborated with our earlier observations with the whole plant indicating that this is a common response mechanism to pathogen challenge in both susceptible and resistant genotypes of potato (Table 4). Interestingly, the calli cells show more than ten-fold higher basal peroxidase activity as compared to leaf tissues. The reason for this high basal peroxidase activity, is as of now, unclear. The results corroborate earlier studies that show inductions of POX isoforms at level of activity and gene expression correlate to disease resistance (Zhang *et al.*, 2009). Peroxidase activity was higher in resistant tomato cultivars than in susceptible cultivars after inoculation with the bacterial spot pathogen *Xanthomonas axonois pv*. vesicatoria (Kavitha and Umesha, 2008).

The current study evaluated the peroxidase activity in the secreted proteins of calli cells and found induction of peroxidase activity and changes in isozyme pattern in the secreted protein fraction when the cells where exposed directly or indirectly to *P. infestans* (T_1, T_2) or to the filter sterilized CF (T_3) (Fig. 8). This data correlated with our earlier findings that root extruded secretory proteins also accumulate higher POX when exposed to the pathogen (Anil, unpublished observation). The increase in secreted peroxidase from calli cells when challenged with pathogen has relevance at the whole plant scenario, peroxidases play role in lignification and suberization and thickening of cell wall, which in itself is a significant defense response. Indeed, we have earlier observed in the leaves of potato plants, callose formation and wall thickening around the infection site is a mechanism especially present in resistant genotypes (Anil *et al* 2013). Peroxidases in the extracellular environment may also play a H_2O_2 scavenging role, maintaining adequate levels of ROS in the extracellular medium in case of callus cells, and in the apoplastic fluid in case of the whole plant.

The secondary metabolites phenolics and flavonoids play important roles in defense response in plants. Comparative analysis of the genotypes and *P. colocasiae* infection showed that the phenol content was highest in the resistant genotypes than the susceptible genotypes. Phenolics act as substrates for the synthesis of compounds involved in disease resistance, like pterocarpanphytoalexins and hydroxycinnamic acid esters (Dixon and Lamb, 1990). Phenolics also facilitate synthesis of lignins and suberins at the site of infection, which act as a barrier to the penetration of the pathogen (Ebel and Grisebach, 1988).

Flavonoids are very important in plant resistance against pathogenic fungi. Flavonoids quench ROS generated both by the pathogens and the plant as a response to infection. Flavonoids play role in tightening of the plant structures such as walls and tissues by modulating auxin (IAA) activity, which can lead to the promotion of callose and tylose formation and closure of the vascular system to prevent pathogen spread (Beckman, 2000). They also inhibit the cell wall degrading enzymes of pathogens (Treutter, 2005) and have inhibitory effects on spore development and hyphae elongation and microbial adhesion.

Thus Flavanoid and phenolics contribute to resistance in multiple important ways in different plant species. In potato plants our earlier study shows enhancement of flavonoids and phenolics with *P. infestans* challenge. The current study corroborates the observation at the whole plant level as both flavonoid and phenolic levels rise significantly across the calli of genotypes tested. Highest levels of phenolics was detected in the resistant cultivar Kufri girdhri (Table 6, 7). The levels of phenolics and flavanoids varied across the treatments in each of the genotype as well, with highest levels observed with direct exposure to pathogen (Fig. 6). Phenolics play a role in lignification and in the production of phytoalexins. Flavanoids also contribute to wall thickening by callose formation. Our earlier observation with whole potato plants clearly shows wall thickening by callose formation around the infection site is a mechanism predominantly present in resistant genotypes (Anil *et al.*, 2013). Thus the induction of phenolics and flavonoids in calli when exposed to *P. infestans* reflects the repertoire of defense response mechanism that is available to the whole plant. The induction of SOD, POX and secondary defense metabolites in callus also suggest the callus system can be an effective model system to dissect the repertoire of cellular defense mechanisms available to the plants. In fact the culture filtrate elicitors also were toxic enough to induce cell death with time, but more importantly they were able to induce a robust defense response almost comparable to that observed with exposure to *P. infestans*.

Evaluation of cell viability of callus genotypes exposed to P. infestans

Thus it can be inferred, that the calli cells of potato genotypes retained much of the repertoire of defense responses of the whole plant. They robustly responded at a biochemical level by inducing SOD, POX, flavonoids and phenolics, also inducing secreted defense enzyme activity in the surrounding media when challenged with *Phytophthora infestans* (T_1) or its elicitors (T_2, T_3).

Calli cells of resistant genotypes survived poorly when grown on phytopathotoxic medium, or when exposed to pathogen or its elicitors (Table 2). This was an unexpected observation, which was reconfirmed by following cell death of calli at

regular intervals over a period of two days post-exposure to *P. infestans*. The data clearly suggest that susceptible genotypes especially among the cultivars survived exposure to *P. infestans* to a greater extent compared to resistant genotypes in the period monitored. The experiments pertaining to cell viability thus made clear that when exposed to pathogen, resistance at the whole plant level negatively correlated with cell survival at the cell culture level. To make sense of this difficult paradox, we hypothesized that the resistant genotypes could be producing higher levels of H_2O_2 as part of a robust hypersensitive response, levels that could be toxic to the cells and went on to test the hypothesis.

Hydrogen peroxide, a reactive oxygen species (ROS), plays an important role in plant defense responses (Christensen *et al.*,2001). To examine the role of plant H_2O_2 accumulation in cells, the percentage of cells accumulating high H_2O_2 in the calli of six genotypes was evaluated post 24 hrs of exposure to *P. infestans* using the DAB staining method. A higher percentage of cells stained strongly with DAB, indicating high H_2O_2 accumulation, in calli of resistant potato genotypes AC1, AC4 and KG (Fig. 9, Table 8, and 9). The result indicates that calli cells of resistant genotypes accumulate higher levels of H_2O_2, as part of a hypersensitive response; the cells die as H_2O_2 is toxic to the cells. Indeed, a correlation analysis (Fig. 9) by plotting percentage cell death as a function of percentage cells accumulating H_2O_2 clearly shows a linear correlation between these two parameters, which is found to be independent of ploidy (Wild 2n, Cultivars 4n). In both, wild and cultivated species, in the resistant genotype high percentage H_2O_2 accumulating calli correlated with high percent Cell death and *vice versa* for susceptible genotypes.

At the whole plant level hypersensitive response leads to cell death which restricts the spread of pathogen after infection. In the callus of resistance genotypes AC1, AC4, and KG, exposure to pathogen led to higher cell death. The hypersensitive response is a very effective defense mechanism in plants, the same robust response in an *in vitro* cell system lead to cell death of the dedifferentiated cell cultures. This study thus shows that what is an efficient mechanism of plant survival at the tissue/whole plant level, is reflected as a negative outcome (cell death) in the callus cells. Thus H_2O_2 induced cell death can be considered a marker or indicator of disease resistance of the genotype. Thus this study for the first time, uses callus cultures as a model system in demonstrating the toxic effect of robust H_2O_2 generation (as a result of interplay of SOD and ROS scavenging enzymes) on the cells of resistant potato genotypes. The potato callus model can be utilized to dissect and further understand cellular defense response including signaling pathways, second messenger, gene expression patterns and changes in membrane potential.

Acknowledgements

We thank CPRI, Shimla for providing the wild and cultivated genotypes of potato used in this study. This work was supported by the Innovative Young Biotechnologist Award (IYBA), Department of Biotechnology, Government of India to VSA.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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ISSN: 2348-1900