Susceptible and tolerant potato leaf-responses post challenge with *Pectobacterium carotovorum* subsp. *brasiliense* 1692

Short communication

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Abstract

Leaf responses of a susceptible potato cultivar (*S. tuberosum* cv. Valor) were compared to those of a tolerant potato cultivar (*S. tuberosum* cv. BP1) following challenge by *Pectobacterium carotovorum* subsp. *brasiliense* 1692 (*Pcb* 1692). Leaves of the susceptible cultivar showed excessive maceration and water soaking symptoms as well as increased proliferation of the bacteria. In leaves of the tolerant cultivar, bacteria appeared to be restricted to the point of inoculation,subsequently; there was very little multiplication of bacterial cells. Furthermore we demonstrated that disease is associated with the extensive spread of cell death in *S. tuberosum* cv. Valor while in *S. tuberosum* cv. BP1 cell death was observed to be associated with lack of disease development. Another response associated with challenge by *Pcb* 1692 in the susceptible and tolerant potato cultivar leaf tissue was the oxidative burst. Generally, the accumulation of hydrogen peroxide and superoxide in leaf-tissue appeared to correlate with tolerance levels.

Keywords:
Cell death Pectobacterium Potato Oxidative burst

Introduction

The contact between plants and pathogenic microorganisms triggers a chain of reactions in plants (Davidsson *et al*., 2014). Reactions triggered require a systematic communication of events within the plant, leading to either a compatible or incompatible interaction (Buonaurio 2008; Chisholm *et al*., 2006). Furthermore, the pathogen lifestyles, that is whether it is biotrophic, hemibiotrophic or necrotrophic plays an important role in how it interacts with the plant. These interactions can also be characterised according to phenotypes displayed by the host during the encounter with the pathogen. For example, host cell death during interactions with potential pathogens can represent either symptoms of disease or a form of defence strategy (Glazebrook 2005 and Shlezinger *et al*., 2011).

The hypersensitive response is a form of cell death host plants use to contain invading pathogens at the point of infection, thus preventing pathogen spread and associated disease symptom development. However, cell death can also facilitate disease symptom spread where dead host cells serve as a source of nutrient for the invading pathogen (Shlezinger *et al*., 2011). This is pertinent in necrotrophic pathogens as they depend on dead host cells for nutrient acquisition, thus dead host cells may aid indirectly in pathogen colonisation and spread (Glazebrook 2005; Shlezinger *et al*., 2011). However, the opposite can be observed in the case of both biotrophic and hemibiotrophic interactions. In these two types of interactions, cell death in host plants during initial contact with pathogens can lead to resistance.

Pectobacterium carotovorum subsp. *brasiliense* is a member of Soft Rot Enterobacteriaceae (SRE) group of pathogens. They cause tuber soft rot and blackleg wilt disease complex on potato plants and other plants worldwide (Onkendi *et al*., 2014; De Boer *et al*., 2012; Charkowski *et al*., 2012; Van der Merwe *et al*., 2010; Perombelon 2002). These necrotrophic pathogens employ plant cell wall degrading enzymes (PCWDEs), necrosis-inducing proteins (Nip) and toxins to macerate host tissues and feed on nutrients released (Mattinen *et al*., 2004; Charkowski *et al*., 2012). We previously demonstrated that *Solanum tuberosum* cv. BP1 is tolerant to blackleg but susceptible to soft rot disease while *S. tuberosum* cv. Valor is susceptible to both soft rot and blackleg disease (Kubheka *et al*., 2013). In this study, we investigated cell death in potato leaves of the tolerant potato cultivar (*S. tuberosum* cv. BP1) compared to those of a susceptible potato cultivar (*S. tuberosum* cv. Valor) challenged with *Pectobacterium carotovorum* subsp. *brasiliense* (*Pcb* 1692). Towards this end, phenotypic and

cellular responses to *Pcb* 1692 in potato leaf-tissue of these cultivars over time were documented.

Potato plants were grown in a greenhouse, with a photoperiod of 16 and 8 h (day and night, respectively) at temperatures between 22 and 26°C and 70% relative humidity. For virulence assays, leaves of the susceptible and tolerant potato cultivar were inoculated by infiltration with 10^8 CFU/ml (typically used for *Pectobacterium spp.* HR assays in tobacco leaves) overnight cultures of *Pcb* 1692 suspended in 10 mM $MgSO_4$ or 10 mM $MgSO_4$ as a negative control (Kim et al., 2009; 2011; Moleleki et al., 2012). Lesion development was recorded by measuring lesion diameter at 24, 48 and 72 hpi in centimetres. To estimate bacterial growth, entire leaves were removed, weighed, and then homogenized at 6, 12, 24 and 48 hpi. This was followed by dilution plating to estimate CFU of bacteria per gram of leaf-tissue. Each assay was repeated twice, with six leaves per replicate for each cultivar.

Leaves of the tolerant and susceptible potato cultivars infiltrated with *Pcb* 1692 exhibited visible cell death at the points of infiltration at 24 hpi (Fig. 1). Leaves from the tolerant cultivar did not develop soft rot disease symptoms, but displayed symptoms of localised cell death at the point of infiltration (Fig. 1A). In general, the lesions formed in the leaves of the tolerant cultivar were very dry (Fig. 1 Ai and ii). Moreover, these lesions, once formed during the initial stages of infection (at 12 hpi), did not expand but rather, remained localised at the point of inoculation (Fig. 1A-iii). Thus the symptoms observed in the tolerant cultivar resembled a type of programed cell death similar to hypersensitive response (HR). Initially, the leaves of the susceptible cultivar displayed the same lesion phenotype as the tolerant cultivar (6 hpi), but lesions started expanding substantially after 24 hpi (Fig. 1B i-iii). Furthermore, lesions formed on the susceptible cultivar were water soaked and resembled typical maceration or rotting symptoms that spread rapidly. This was accompanied by severe wilting and death of whole shoots at 48 hpi possibly due to bacterial cells clogging the vascular system (Fig. 1B- iii).

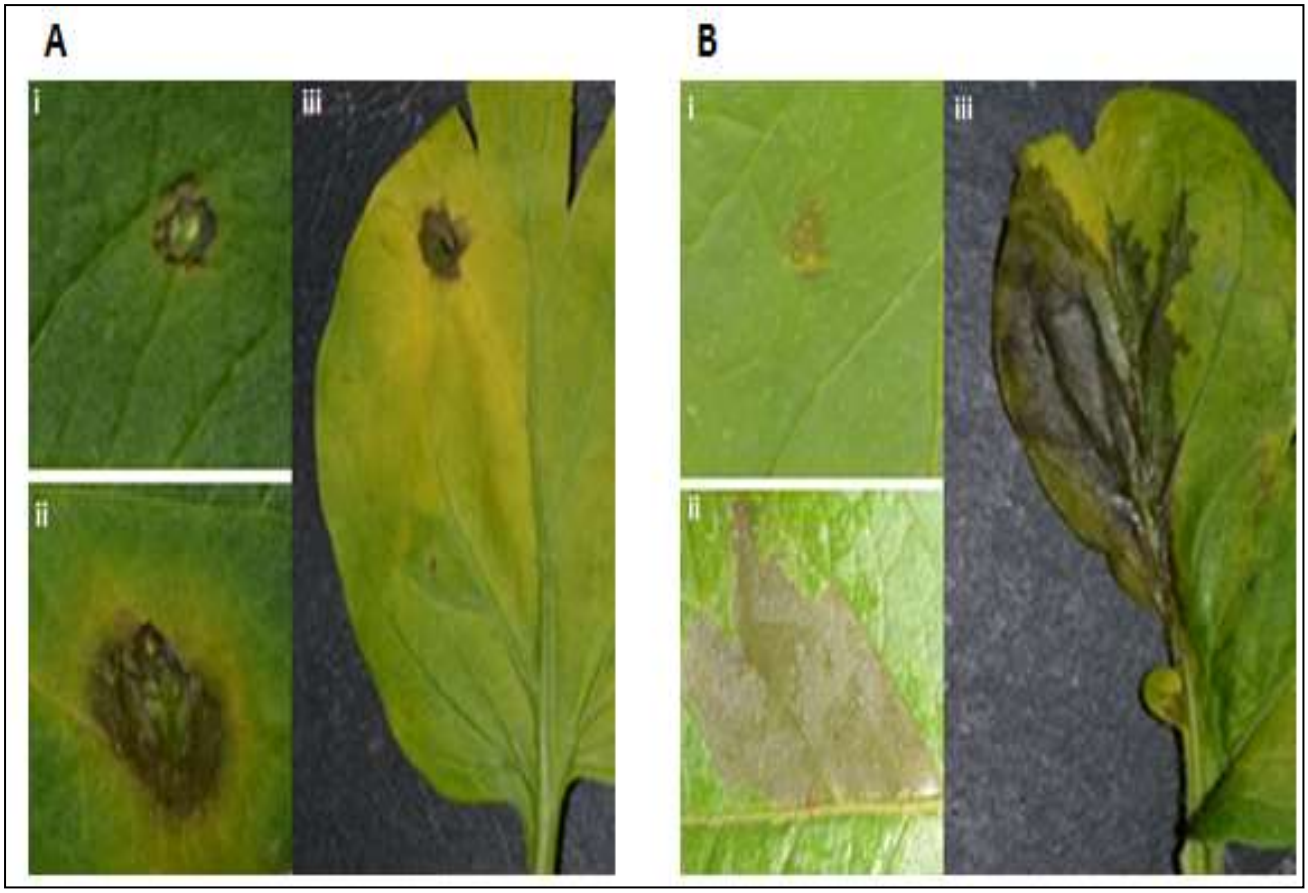

Figure 1: Leaves of the tolerent potato cultivar under go localised cell death compared to leaves of the suseptible potato cultivar that develop disease symptoms. A represents leaves of the tolerant cultivar and B- represents leaves of suseptible potato cultivar inoculated with (i) 10 mM $MgSO_4$, (ii) *Pcb*1692 at 24 hpi and (iii) *Pcb* 1692 at 48 hpi.

Lesion development in the tolerant cultivar remained constant from 48 to 72 hpi with no increase in diameter (Fig. 2 A), whereas, in the susceptible cultivar, lesions were observed to increase to 6 times the size of the tolerant cultivar and all leaves inoculated were macerated completely at 72 hpi. It was thus not surprising to observe high growth rates of *Pcb* 1692 cells in the susceptible cultivar compared to those in the tolerant cultivar (Fig. 2 B). The exponential increase in growth of *Pcb* 1692 in leaves of the susceptible cultivar from 6 hpi shows a direct correlation between lesion expansion and *Pcb* 1692 growth.

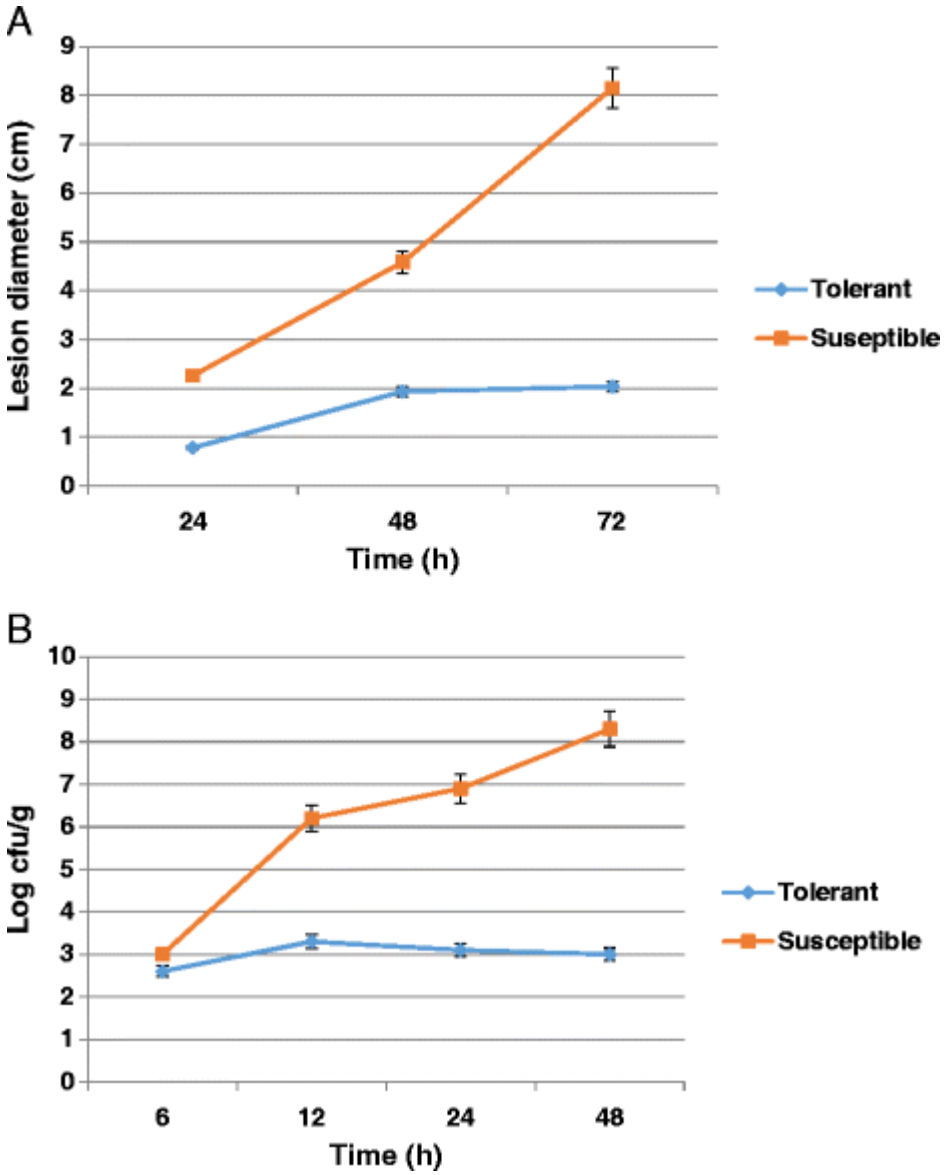

Figure 2: (A) Lesion development on potato leaves of susceptible compared to tolerant potato cultivar following inoculation of wild type *Pcb* 1692 over 72 hpi. (B) Bacteria proliferation in leaves of a susceptible and tolerant potato cultivar inoculated with *Pcb* 1692 Data points are means (cfu/g) from six randomly selected leaves per cultivar respectively over a 48 h period.

These observations lead us to hypothesise that in the susceptible cultivar, *Pcb* 1692 is able to actively promote its growth by exploring the surrounding dead plant tissue thus facilitating disease symptom spread. The dead host cells serve as a source of nutrients for the invading pathogen aiding, indirectly, in pathogen colonisation and spread (Glazebrook, 2005; Shlezinger *et al*., 2011). To test this hypothesis, we further investigated cell death in the two cultivars by monitoring loss of plant auto-fluorescence in plant tissue. In this regard, leaves were inoculated with *Pcb* 1692 cells, then at 24 hpi, leaf tissue from both cultivars was viewed under the

confocal laser scanning microscope using excitation filter 450 nm. In this assay, loss of plant auto-florescence was indicative of cell death as observed under a confocal laser scanning fluorescence microscope. Figure 3 indicates that in both cultivars, cells at the site of inoculation lost auto-florescence within the first 24 hpi. We also noticed that in the susceptible cultivar, the loss of auto-fluorescence was not confined to the infection point (indicated with white circles) but spread as the necrotic water soaked lesions expanded. On the contrary, the loss of auto-fluorescence in the tolerant cultivar was totally confined to the inoculation point.

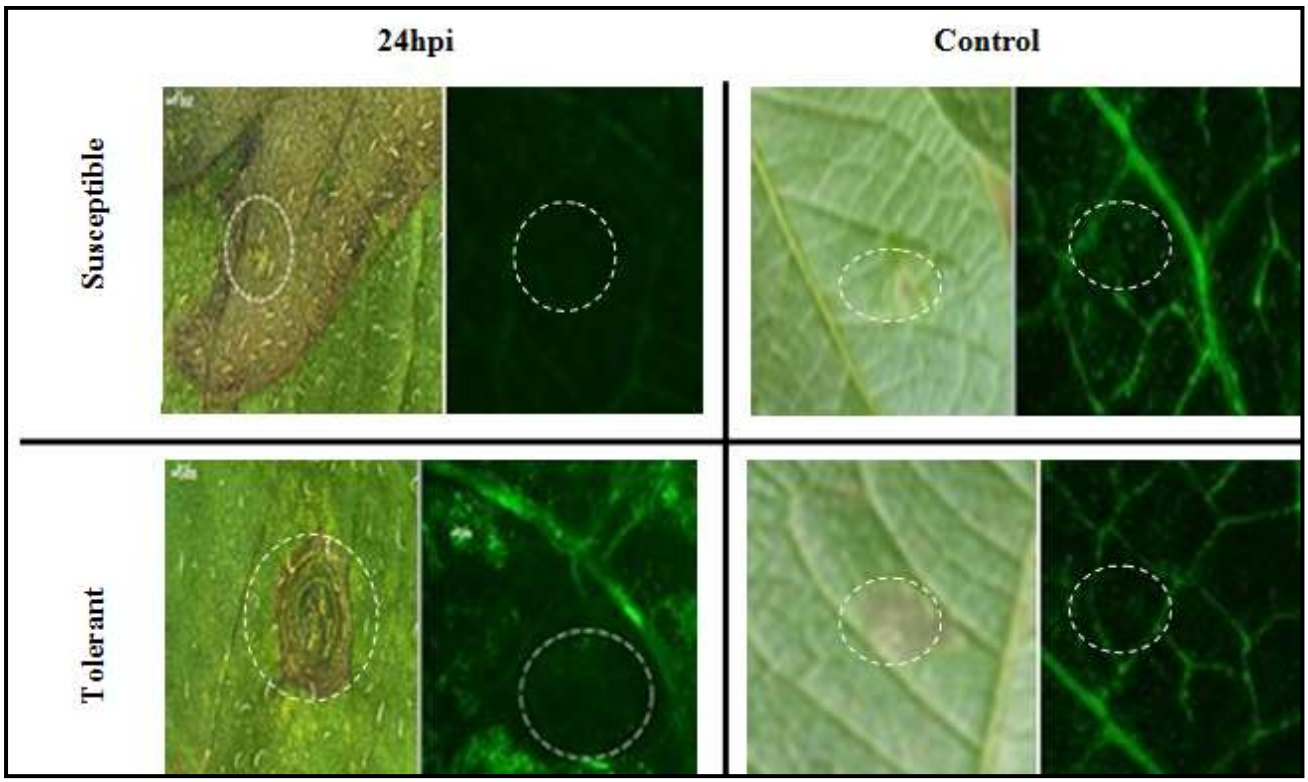

Figure 3: Cell death in leaves of a susceptible and tolerant potato cultivar challenged by *Pcb* 1692. Cell death is indicated by the loss of auto-fluorescence (areas within white circles indicate points of inoculation) when leaves are viewed under a confocal laser scanning microscope using an excitation filter at 450 nm.

Next, we investigated oxidative burst by visualising the accumulation of hydrogen peroxide and superoxide in leaf-tissue of the susceptible and tolerant potato cultivar. Both experiments were conducted as outlined by Wolgemuth 2002. Briefly, three fully expanded leaves were selected per time point (0, 6, 24 hpi) for each cultivar and infiltrated with *Pcb* 1692 as described above, the experiment was repeated at least twice. Superoxide production was observed by infiltrating leaves (0, 6, 24 hpi) with a solution of 10 mM sodium azide (NaN_3; Sigma) and 0.1 % (w/v) nitroblue tetrazolium solution. For hydrogen peroxide production, leaves (0, 6, 24 hpi)

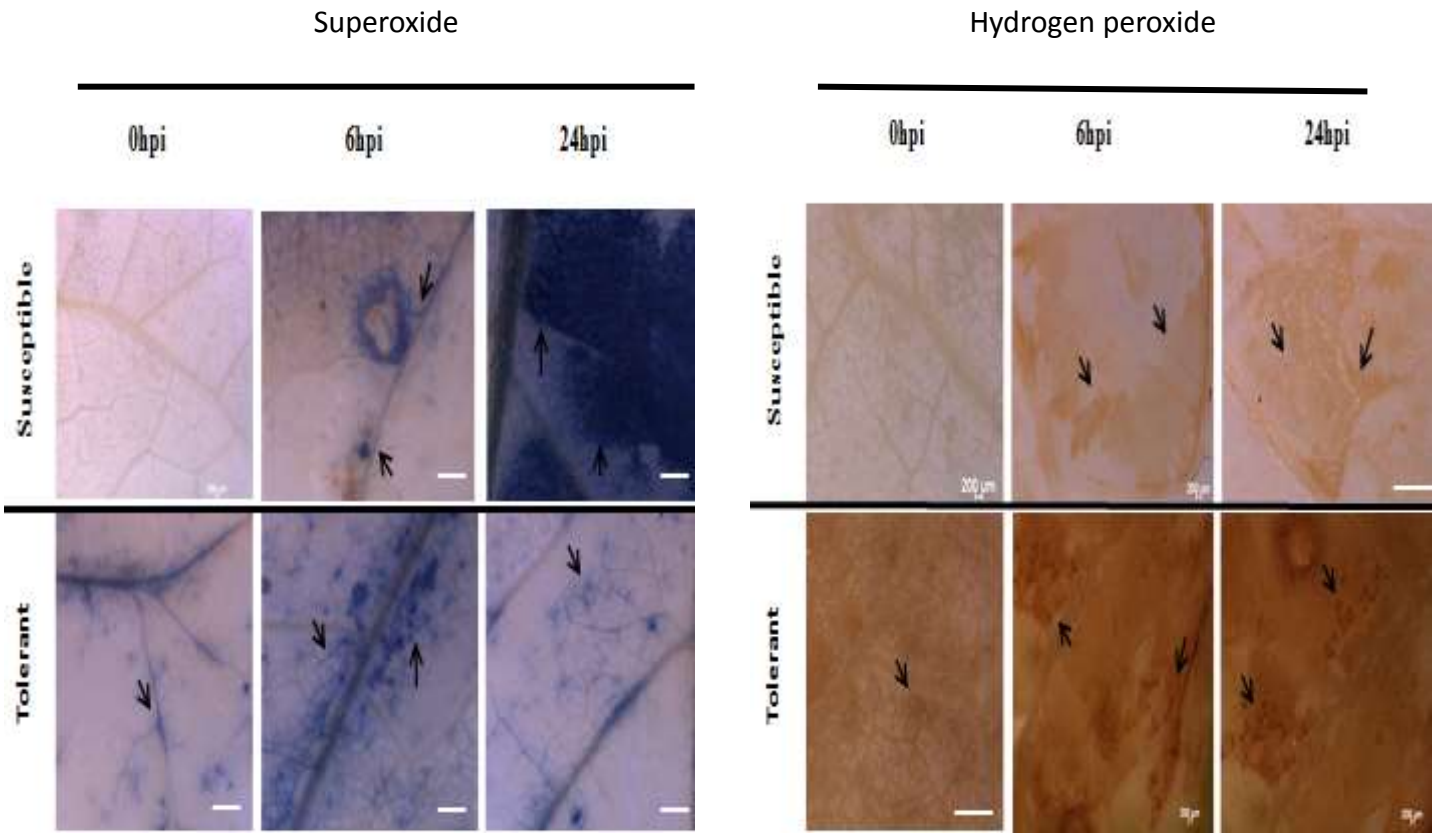

Figure 4: Superoxide and hydrogen peroxide (H_2O_2) accumulation in leaves of a susceptible and tolerant potato cultivar over a period of 24 hpi after inoculation with *Pcb* 1692. The blue colour indicates accumulation of superoxide (O_2-) in leaf tissue while the red-brown colour indicates the production of hydrogen peroxide (H_2O_2) and arrows indicate accumulation of compounds. Bars = 200 um.

were stained with a 0.1 % (w/v) diaminobenzidine solution (DAB; Sigma). The leaves from both treatments were then incubated for 8h at room temperature. This was followed by clearing staining solution from the leaves by boiling in 96 % (w/v) ethanol for 10 min and preserving them in 50 % ethanol. The leaves were viewed under the light microscope and superoxide was indicated as blue formasan formation and hydrogen peroxide was indicated as red–brown patches on leave tissue (Wolgemuth *et al.*, 2002). We observed that *Pcb*1692 induced distinctive accumulation of hydrogen peroxide and superoxide in both the susceptible and tolerant potato cultivar leaves (Fig. 4). Blue formasan precipitate or a deep brown colour indicated O^{2-} (superoxide) or H_2O_2 (hydrogen peroxide) production, respectively, as indicated by arrows in Fig. 4. There was an early increase in accumulation of O^{2-} in the tolerant cultivar at the point of inoculation (6 hpi) compared to the susceptible cultivar where the increase was

not as much. In the susceptible cultivar, we observed a gradual increase peaking at 24 hpi. Surprisingly the presence of O^{2-} at 24 hpi had dropped slightly in the tolerant cultivar while in the susceptible cultivar the presence of the blue formazan increased drastically (Fig. 4). The presence and increase in colour intensity for H_2O_2 in leaves remained constant for both cultivars from 6 and 24 hpi (Fig. 4). In the control leaves (0 hpi) we observed small amounts of H_2O_2 in the tolerant cultivar (shown by black arrows at 0 hpi) but not in the susceptible cultivar.

Plants are known to respond to pathogen invasion by activating defence responses associated with accumulation of a wide array of enzymes and inhibitors that prevent pathogens from invading plant host tissue. There are no studies that have looked at interactions of *Pcb* 1692 strain and potato plants in leaf tissue. However, there are studies that have investigated other *Pectobacterium* spp. interactions in leaf tissue of plants such as *Arabidopsis thaliana* and *Nicotiana benthamiana* (Ahn *et al*., 2007, Kim *et al*., 2011, Hogan *et al*., 2013). Our findings show that during the interaction of *Pcb* 1692 and leaves of the susceptible cultivar, *Pcb*-1692 is able to cause extensive tissue maceration and because of the resulting nutrients released, it is also able to multiply extensively. In interactions within leaves of the tolerant cultivar, *Pcb* 1692 remains confined to point of inoculation and not able to proliferate. Previously, host cell death in leaf tissue was reported to be induced by *P. carotovorum* through the use of DspE which is an effector protein delivered through the T3SS. Furthermore, mutations in the DspE/F operon and regulatory and structural genes of the T3SS resulted in the strains that were not able to cause cell death and or leaf maceration (Hogan *et al*., 2013). It is thus possible that recognition of DspE in the tolerant plant mounts increased defences leading to arrest of *Pcb* 1692 to the initial point of inoculation while lack of recognition of DspE in the susceptible cultivar results in easy spread of *Pcb* 1692 and the resulting tissue damage. This view could be supported by the observed increase in generation of ROS, particularly superoxide in the susceptible cultivar. Likely, this could increase cell wall crosslinking and iron influx promoting cell death (Ahn *et al*., 2007; Wolgemuth *et al*., 2007). It is also possible that *Pcb*1692 strain may be employing other known toxic compounds like the cell death inducing Nip to promote cell death in the susceptible cultivar (Davidsson *et al*., 2014; Mattinen *et al*., 2004). Timing seems to play an important role as ROS was mounted quite early in the interaction within the tolerant cultivar relative to the susceptible cultivar. The early response of the tolerant cultivar to Pcb 1692 challenge was also observed in our previous work on differential gene expression patterns stems of the two cultivars challenged with *Pcb* 1692 *(*Kwenda et al., 2016).

In conclusion, this study has demonstrated specific biochemical and phenotypic changes associated with the interaction between *Pcb* 1692 in leaves of a tolerant potato (*S. tuberosum* cv. BP1) cultivar relative to those observed in the susceptible potato cultivar and (*S. tuberosum* cv. Valor).

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