Comparative analysis of defence responses induced by the endophytic plant growth-promoting rhizobacterium *Burkholderia phytofirmans* strain PsJN and the non-host bacterium *Pseudomonas syringae* pv. *pisi* in grapevine cell suspensions

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are beneficial microorganisms that colonize the rhizosphere of many plant species and confer beneficial effects, such as an increase in plant growth. PGPR are also well known as inducers of systemic resistance to pathogens in plants. However, the molecular mechanisms involved locally after direct perception of these bacteria by plant cells still remain largely unknown. *Burkholderia phytofirmans* strain PsJN is an endophytic PGPR that colonizes grapevine and protects the plant against the grey mould disease caused by *Botrytis cinerea*. This report focuses on local defence events induced by *B. phytofirmans* PsJN after perception by the grapevine cells. It is demonstrated that, after addition to cell suspension cultures, the bacteria were tightly attaching to plant cells in a way similar to the grapevine non-host bacteria *Pseudomonas syringae* pv. *pisi*. *B. phytofirmans* PsJN perception led to a transient and monophasic extracellular alkalinization but no accumulation of reactive oxygen species or cell death were detected. By contrast, challenge with *P. syringae* pv. *pisi* induced a sustained and biphasic extracellular alkalinization, a two phases oxidative burst, and a HR-like response. Perception of the PGPR also led to the production of salicylic acid (SA) and the expression of a battery of defence genes that was, however, weaker in intensity compared with defence gene expression triggered by the non-host bacteria. Some defence genes up-regulated after *B. phytofirmans* PsJN challenge are specifically induced by exogenous treatment with SA or jasmonic acid, suggesting that both signalling pathways are activated by the PGPR in grapevine.

Key words: Defence genes, early signalling, grapevine, growth-promoting rhizobacteria, non-host bacteria.

Introduction

Plants strongly rely on an innate immune system to defend themselves against pathogenic microorganism invasion. This system is based on the capacity to perceive the intruder as ‘non-self’ and ends up in the activation of a wide range of defence responses. These defence responses are essential for the success of plant resistance and therefore contribute to plant immunity (Boller and Felix, 2009). Early responses in plant/microorganism interactions are characterized by...
signalling processes including ion fluxes, MAP kinase cascade activation, and the production of reactive oxygen species (ROS) (García-Brugger et al., 2006). Key signal molecules including salicylic acid (SA) and jasmonic acid (JA) are also produced within hours after pathogen challenge. These signal molecules participate in the regulation of downstream defence genes (Mur et al., 2006; Browse, 2009; Vlot et al., 2009). Ultimately, plant defence responses encompass the strengthening of cell walls and the production of antimicrobial compounds that altogether play a key role in pathogen restriction (Hammond-Kosack and Jones, 1996). In some cases, the interaction ends up in local plant cell death characteristic of the so-called hypersensitive reaction (HR) (Heath, 2000).

Plant defence responses can typically be triggered by the recognition of phytopathogenic microorganisms, but they can also be stimulated by non-pathogenic strains of plant growth-promoting rhizobacteria (PGPR) (Bloemberg and Lugtenberg, 2001; Van Wees et al., 2008; Lugtenberg and Kamilova, 2009). PGPR are able to colonize the rhizosphere of many plant species and to confer beneficial effects, such as increased plant growth and reduced susceptibility to diseases caused by plant pathogenic fungi, bacteria, viruses, and nematodes (Compant et al., 2008a). Some of these PGPR are endophytic since they can penetrate root tissues and sometimes diffuse through vessels to other plant organs (Rosenblueth and Martinez-Romero, 2006; Compant et al., 2008b; Hardoim et al., 2008). PGPR have been separated into two groups, (i) the extracellular PGPR (ePGPR) that colonize the rhizosphere, the rhizoplane or the spaces between cells of the root cortex, and (ii) the intracellular PGPR (iPGPR), which exist inside root cells, generally in specialized nodule structures (Gray and Smith, 2005). The biological control activity of PGPR is exerted either directly through antagonism of pathogen development or indirectly by eliciting a plant-mediated resistance response (Van Wees et al., 2008; Lugtenberg and Kamilova, 2009). PGPR are able to trigger induced systemic response (ISR), a defence state that takes place in the entire plant and prepares the host to respond to a broad range of pathogen attacks (van Loon et al., 1998; Bloemberg and Lugtenberg, 2001; Van Wees et al., 2008). In Arabidopsis thaliana, ISR triggered by root-colonizing strains of Pseudomonas fluorescens was shown to be ethylene- and JA-dependent but SA-independent (Knoester et al., 1999; Ton et al., 2002; Iavicoli et al., 2003). However, ISR triggered by some specific strains of PGPR may also involve SA signalling (Zhang et al., 2002). Although the systemic induction of resistance by PGPR is well documented, little data are available on local defence events taking place in plant cells directly in contact with these bacteria. Moreover, no direct comparison has been made between defence responses induced by PGPR and the typical defence reactions occurring during non-host or incompatible interactions triggered by bacteria.

Burkholderia phytofirmans strain PsJN, isolated from surface-sterilized onion roots (Frommel et al., 1991; Sessitsch et al., 2005), is a natural endophytic, non-
nodulating PGPR strain of potato and tomato (Frommel et al., 1991, 1993; Pillay and Nowak, 1997) and can be classified as a ePGPR (Gray and Smith, 2005). This bacterium is also able to colonize and diffuse inside grapevine tissues and to travel through xylem vessels in the different organs of the plant (Compant et al., 2005, 2008b). Moreover, B. phytofirmans PsJN colonization enhanced protection against Verticillium sp. in tomato (Sharma and Nowak, 1998) and Botrytis cinerea in grapevine (Ait Barka et al., 2000, 2002). In this paper, defence reactions induced in grapevine cells by B. phytofirmans PsJN and the non-host bacterium Pseudomonas syringae pv. pisi were compared. P. syringae pv. pisi is known to activate an innate immune response when infiltrated in Vitis vinifera plants (Robert et al., 2001, 2002). It is demonstrated here that, whereas both bacteria physically interact with plant cells, B. phytofirmans PsJN perception triggers a local immune response, which is significantly weaker in intensity than the one occurring during the non-host interaction.

Materials and methods

Plant cell culture

Cell suspensions of 41B (V. vinifera L. cv. Chasselas×V. berlandieri) were cultured in Murashige-Skoog medium (pH 5.8) containing vitamins (×1.5), sucrose (30 g l\(^{-1}\)), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.2 mg l\(^{-1}\)), 6-benzylaminopurine (BAP, 0.5 mg l\(^{-1}\)) and were propagated in the dark at 25 °C under shaking at 120 rpm. They were subcultured every 7 days to be maintained in exponential phase. For the experiments, 30 ml of cells subcultured for 6 days were used. Before any treatment, cells were allowed to adjust to the new condition overnight.

Microorganisms and plant assays

P. syringae pv. pisi and B. phytofirmans strain PsJN were grown in 100 ml King’s B liquid medium at 28 °C, on a rotary shaker (150 rpm). Escherichia coli that was used as a negative control in some experiments was grown in 100 ml Luria-Bertani liquid medium at 37 °C. Overnight cultures of bacteria were used for the experiments. Bacteria were collected by centrifugation (4500 g, 10 min) and washed with sterile MgCl\(_2\) (10 mM). After inoculation, the final bacterial concentration in cell suspension cultures was 10\(^{5}\) cfu ml\(^{-1}\).

Phytohormone treatments

Methyl jasmonate (MeJA) was purchased from Sigma-Aldrich-Chimie (Saint-Quentin-Fallavier, France). Sodium salicylate (SA) was purchased from Eurobio (Les Ulis, France). SA was dissolved in water and MeJA was dissolved in 10% ethanol prior dilution in water. Final concentrations of chemicals in cell suspension cultures were 1 mM for SA and 200 μM for MeJA. An equivalent volume of 10% ethanol was added to control cells to ensure that it did not interfere with the experiments. Final ethanol solutions did not exceed 0.1% (v/v).

Microscopy and cell death assay

Grapevine plant cells inoculated with B. phytofirmans PsJN. P. syringae pv. pisi or E. coli were observed under epifluorescence microscope during a 24 h time-course. Just before each microscopic observation, the challenged cell suspension cultures were incubated for 2 min in a solution of acridine orange (0.1%, pH 7) in order to visualize the bacteria (Monier and Lindow, 2004).
Results

B. phytofirmans PsJN and P. syringae pv. pisi physically interact with grapevine cells

Successful endophytic PGPR are known to approach plant roots via chemotaxis-induced motility and effectively colonize plant tissues via attachment (Rodriguez-Navarro et al., 2007; Hardoim et al., 2008). Plant cell attachment of phytopathogenic bacteria is also an essential step for colonization, especially allowing the translocation of effectors via a type III secretion system (T3SS) and pilus-like structure formation (Aldon et al., 2000; Buttner and Bonas, 2006). The behaviour of B. phytofirmans PsJN and P. syringae pv. pisi when added to grapevine cell suspensions was compared. E. coli was used as a negative control in these experiments. The physical interaction was observed by microscopy under epifluorescence over 24 h. At time 0, all the bacteria were in the vicinity of plant cells but not directly in contact (data not shown). Few B. phytofirmans PsJN or P. syringae pv. pisi bacteria were in contact with plant cells as soon as 5 min after challenge (data not shown). A large number of both bacteria were found to interact with plant cells at 1 h (Fig. 1A, C). Most of the plant cells were covered by B. phytofirmans PsJN or P. syringae pv. pisi 24 h after challenge (Fig. 1B, D). By contrast, E. coli did not stick to the plant cells even at 24 h post-inoculation (Fig. 1E, F).

Extracellular medium alkalinization of grapevine cells after B. phytofirmans PsJN and P. syringae pv. pisi perception

Among the early signalling events, extracellular alkalinization has been shown to be an essential component of ion fluxes involved in plant defence (Felix et al., 1993; van Loon et al., 2008). Moreover, alkalinization measurement has been used as an efficient method to monitor chemosensory perception in cultured plant cells (Felix et al., 1993). Grapevine cell suspensions incubated with B. phytofirmans PsJN produced a monophasic and transient burst of alkalinization that started within the first minute of the interaction and culminated at 2 h (Fig. 2A). By contrast, cell suspensions treated with P. syringae pv. pisi produced a two-phased alkalinization response with the first peak culminating within 1 h, followed by a second and sustained phase lasting for several hours (Fig. 2A).

P. syringae pv. pisi but not B. phytofirmans PsJN induces an oxidative burst and cell death in grapevine cells

Early signalling events in plant defence often include a rapid and intense production of ROS (Garcia-Brugger et al., 2007). Grapevine cell suspensions incubated with P. syringae pv. pisi produced a two-phased oxidative burst (Fig. 2B).
The first phase started after 15 min and culminated at 30 min. A second and sustained peak of H$_2$O$_2$ was detected after a few hours with a maximum at 3 h. This two-peak profile is reminiscent of the one observed for the extracellular alkalinization. Interestingly, no significant accumulation of H$_2$O$_2$ was detected in the cell suspension medium after challenge with _B. phytofirmans_ PsJN (Fig. 2B). The cell death process is often associated with the oxidative burst (Torres _et al._, 2006). As shown in Fig. 3, cell death in cell suspensions was detected as soon as 9 h and a plateau was observed at 15 h after _P. syringae pv. pisi_ inoculation. By contrast, no cell death was detected over the time-course after _B. phytofirmans_ PsJN challenge.

**Expression profiles of grapevine defence genes after signal molecule perception or bacterial challenge**

In order to compare grapevine gene expression profiles after PGPR and non-host bacteria challenge, several markers were selected covering a large set of defence classes. Three PR protein genes, an acidic chitinase (_chit4c_), a basic glucanase (_gluc_), and a protease inhibitor (_pin_) that are reliable defence markers in this plant system (Aziz _et al._, 2003; Varnier _et al._, 2009) were chosen for investigation. Transcript regulation of lipoxygenase (_lox_) was also monitored (Bézier _et al._, 2002; Aziz _et al._, 2003) since LOX are involved in the synthesis of oxylipins, ROS regulation, and play an important role in response to pathogen attack (Howe and Schilmiller, 2002). The expression profile of a new defence marker characterized through a differential display screen on _B. cinerea/grapevine_ interaction (Bézier _et al._, 2007), with unknown function and named _Vv17.3_ was also investigated. In order to correlate the defence gene expression to SA and JA signalling in our plant system, the expression of the defence markers was monitored first after treatment with exogenous SA and MeJA (Fig. 4). Interestingly, the _gluc_ gene is specifically induced after MeJA treatment. Conversely, _Vv17.3_ only responded to SA treatment. Both signal molecules stimulated _lox, chit4c_, and _pin_ expressions although the inductions were higher after the SA treatment. These results show that in our grapevine cell system, the _gluc_ and _Vv17.3_ genes are specific markers of the MeJA and SA signalling pathways, respectively.

The expression profile of all genes was then monitored at 9 h and 24 h after inoculation of the grapevine cells with _B. phytofirmans_ PsJN or _P. syringae pv. pisi_. All the defence markers were up-regulated after challenge with both bacteria (Fig. 5). _Gluc, lox_, and _pin_ expressions were significantly higher following _P. syringae pv. pisi_ challenge at both time points (Fig. 5A, B). Although _P. syringae pv. pisi_ also induced a stronger expression of _chit4c_ at 24 h, _B. phytofirmans_ was more efficient at stimulating this gene at 9 h. Up-regulation of _Vv17.3_ was similar at 9 h after...
inoculation with both bacteria but stronger in response to *P. syringae* pv. *pisi* at 24 h.

**PGPR and non-host bacteria perception by grapevine cells results in production of SA**

SA is a key molecule produced during non-host and incompatible interactions (Vlot *et al.*, 2009). The involvement of SA in plant/PGPR interactions is still under debate especially at the local level (Ton *et al.*, 2002; Zhang *et al.*, 2002; Pieterse *et al.*, 2009). Induction by *B. phytofirmans* PsJN of the specific SA-dependent gene marker *Vv17.3* suggested that SA should be produced in response to the bacterial challenge. In order to confirm this hypothesis, the amount of free SA was measured in grapevine cells challenged with *B. phytofirmans* PsJN. As shown in Fig. 6,
a significant level of free SA (265 ng g\(^{-1}\) FW) was detected 24 h after \(B.\) \textit{phytofirmans} PsJN inoculation, whereas the basal level in control cells remained very low (19 ng g\(^{-1}\) FW). As expected, an increase in the free SA level was also detected in grapevine cells treated with \(P.\) \textit{syringae} pv. \textit{pisi}.

**Discussion**

In a previous work, it was shown that \(B.\) \textit{phytofirmans} PsJN colonization of internal root tissues from grapevine \textit{in vitro} plantlets resulted in the accumulation of phenolic compounds and the strengthening of cell walls in the exodermis, two traits of typical host defence responses (Companet \textit{et al.,} 2005). Evidence is presented here that \(B.\) \textit{phytofirmans} PsJN perception by grapevine cells triggers a local immune response including ion fluxes, SA production and defence gene activation. Although a larger number of studies has been focused on the extensive description of ISR (Gray and Smith, 2005; Van Wees \textit{et al.,} 2008; Lugtenberg and Kamilova, 2009), only a few studies have deciphered local responses, especially early signalling events induced by ePGPR in plants. These studies are mostly focused on bacteria from the \textit{Pseudomonas} genus and \textit{Arabidopsis thaliana}. Global transcriptome analysis of \(A.\) \textit{thaliana} colonized by \textit{Pseudomonas thihevalensis} revealed that only nine genes were differentially expressed in root tissues that are in contact with the bacteria (Cartieaux \textit{et al.,} 2003). Among these genes, only one was associated with the stress response. Verhagen and co-workers also found that very few genes including transcription factors and genes related to ethylene regulation were differentially expressed in \textit{Arabidopsis} roots challenged with \(P.\) \textit{fluorescens} WCS417r (Verhagen \textit{et al.,} 2004). By contrast, a clear induction of defence responses including phenylalanine ammonia-lyase, peroxidase, and polyphenol oxidase activities was observed in cucumber roots after treatment with \(P.\) \textit{corrugata} and \(P.\) \textit{aureofaciens} (Chen \textit{et al.,} 2000). A recent work also demonstrated that phytoalexins including resveratrol and viniferin accumulated in grapevine cells challenged with three different strains of \(P.\) \textit{fluorescens} and by \(P.\) \textit{aeruginosa} (Verhagen \textit{et al.,} 2010). These results and ours reinforce the hypothesis that the patterns of local defence responses strongly differ from plant to plant and are closely dependent on the ePGPR species or strains (van Loon \textit{et al.,} 2008). Interestingly, only a few studies described the early defence signalling events induced by ePGPR. Our results show that \(B.\) \textit{phytofirmans} PsJN induced a transient extra-cellular alkalinization but no significant variation in \(H_2O_2\) levels was detected. By contrast, \(P.\) \textit{syringae} pv. \textit{pisi} induced two-phased alkalinization and ROS responses. Recently, Verhagen \textit{et al.} (2010) showed that grapevine cell suspensions challenged with \(P.\) \textit{fluorescens} CH0 and \(P.\) \textit{aeruginosa} 7NSK2 responded through a transient burst of \(H_2O_2\). The \(H_2O_2\) level was dependent on the bacterial species. To our knowledge, this work is the only one with the present study to describe local early signalling responses to an ePGPR living organism.

Differences in the range and the scale of early defence events induced by some PGPR could be related to the nature of the eliciting compounds. Van Loon \textit{et al.} (2008) tested lipopolysaccharides (LPS), flagellin, and pyoverdine siderophores from \(P.\) \textit{putida} and two \(P.\) \textit{fluorescens} in tobacco cell suspensions. They found that early defence responses vary greatly depending on the nature and the origin of these microbe-associated molecular patterns (MAMPs). Interestingly, flagellin from all strains triggered an alkalinization response, whereas only the flagellin from \(P.\) \textit{putida} induced an oxidative burst. Addition to tobacco cell suspensions of surfactin lipopeptide, but not fengycin or iturin, induced defence-related early events such as extracellular medium alkalinization and reactive oxygen species production (Jourdan \textit{et al.,} 2009). Only a few studies have been aimed at characterizing MAMPs from \textit{Burkholderia} species. To our knowledge lipopolysaccharides (LPS) are the main MAMPs isolated from \(B.\) \textit{cepecia} and \(B.\) \textit{plantarii}, which are active in tobacco and \textit{Arabidopsis} (Gerber and Dubery, 2004; Gerber \textit{et al.,} 2004, Zeidler \textit{et al.,} 2004). The majority of the eliciting activity from \(B.\) \textit{phytofirmans} could be recovered using a boiled extract of the bacteria (H Lacroix, unpublished data). Interestingly, this crude extract induced an oxidative burst, suggesting that live bacteria were probably regulating the levels of reactive oxygen species in the plant cell culture medium. Moreover, some experiments were done using proteinase K as described by Felix \textit{et al.} (1999) and it was found that the eliciting activity was mainly induced by a proteinous compound (H Lacroix, unpublished data). According to these results it is unlikely that LPS from \(B.\) \textit{phytofirmans} play the major role as elicitor in our system.

The two-peak alkalinization and ROS profiles with \(P.\) \textit{syringae} pv. \textit{pisi} are characteristic of HR-inducing bacteria and have been described as a typical signature (XR) for non-host and incompatible interactions (Atkinson

![Fig. 6. Free SA content analysis in grapevine cell suspensions challenged by \(B.\) \textit{phytofirmans} PsJN and \(P.\) \textit{syringae} pv. \textit{pisi}.

Free SA content was monitored in grapevine cells at 24 h after MgCl\(_2\) treatment (control) or after inoculation with \(B.\) \textit{phytofirmans} PsJN (Bp PsJN) and \(P.\) \textit{syringae} pv. \textit{pisi} (Ps pisi). SA was extracted and analysed by high-performance liquid chromatography. Data presented are means of duplicate experiments ±SD.](https://academic.oup.com/jxb/article-abstract/62/2/595/589368/Downloaded-from-hisst-academic-oup.com/journal-article-access/07 January 2019)
Interestingly, *P. syringae* pv. *pisi* responses in grapevine cells are comparable with those observed in tobacco cells suggesting similar mechanisms of perception and signalling (Atkinson et al., 1985). By contrast, the alkalization profile after challenge with *B. phytofirmans* PsJN is reminiscent of a compatible interaction profile (Glazener et al., 1996) albeit no oxidative burst could be detected. Moreover, no disease symptoms but only beneficial effects such as growth promotion and resistance to grey mould have been associated with grapevine colonization by this bacterium (Compant et al., 2005). Thus, although some aspects of *B. phytofirmans* PsJN/grapevine interaction are reminiscent of a compatible interaction usually characterized by disease symptoms, there could be a switch leading to a mutualist behaviour of both organisms. In agreement with this, expression levels of defence genes in grapevine cells after *B. phytofirmans* PsJN perception are significantly weaker than those occurring after *P. syringae* pv. *pisi*. Moreover, no cell death could be detected after perception of the ePGPR. Thus it can be hypothesized that although plant cells probably recognize *B. phytofirmans* PsJN as a potential intruder, the low level of induced defences may explain the ability of the ePGPR to colonize the roots and to migrate into the entire plant including inflorescences (Compant et al., 2005, 2008b). By contrast, *P. syringae* pv. *pisi* strongly induced the complete battery of defence genes that was tested and caused cell death. This bacterium is also known to cause necrotic lesions and a strong accumulation of PR proteins and phytoalexins when infiltrated in *Vitis vinifera* leaves (Robert et al., 2001, 2002). Altogether, our results demonstrate that *B. phytofirmans* PsJN perception by grapevine cells triggers a local immune response. However, the defence responses are significantly weaker than those occurring in a non-host interaction.

Timing and differences in early signalling events after microorganism perception by plants govern later responses (Garcia-Brugger et al., 2006). Early events are often followed by the production or mobilization of signal molecules such as SA and JA, which are essential for the regulation of defence gene expression (Hammond-Kosack and Parker, 2003). The involvement of these two signal molecules after the recognition of ePGPR by plant cells is still a matter of debate. Particularly, the involvement of SA in local defences induced by ePGPR is not fully documented and accepted (Van Wees et al., 2008; Pieterse et al., 2009). It is shown here that SA clearly accumulated in grapevine cells challenged with *B. phytofirmans* PsJN. In addition, we characterized a specific marker gene of the SA pathway in grapevine, named *Vvi7.3*, which is clearly and reproducibly induced by *B. phytofirmans* PsJN in our system, strongly suggesting that SA is involved in the activation of defence responses in plant cells perceiving the bacteria. SA accumulation in ePGPR/plant interactions has only been described in a few other studies. SA accumulation in tobacco plant in response to *P. fluorescens* 89B-61, *Bacillus pumilus*, and *Serratia marescens* has been proposed to play a role in ISR (Zhang et al., 2002). SA accumulates in bean leaves following *P. aeruginosa* 7NSK2 inoculation (De Meyer et al., 1999). *P. fluorescens* strain Pf4 and *P. aeruginosa* strain Pag were also shown to induce the synthesis of SA in chickpea seedlings (Singh et al., 2003). Recently, two different strains of *Streptomyces* and *Nocardoides albus* EN46 were found to stimulate the expression of SA-dependant genes in *A. thaliana* (Conn et al., 2008). Altogether these data and ours suggest that, depending on the plant/bacteria interaction system, SA may be produced and play a role in defence responses induced by ePGPR. A basic glucanase was also characterized as a specific marker of JA signalling. This gene is induced in response to *B. phytofirmans* PsJN, suggesting that, in addition to SA, JA signalling is also involved in the induction of defence responses by the bacterium in grapevine.

Attachment of PGPR to plant cells is the very early step required in the plant-microbe interaction (Rodriguez-Navarro et al., 2007). It was found that *B. phytofirmans* PsJN was sticking to plant cells within the first hours of the interaction and that plant cells could be covered by bacteria in 24 h. Bacterial attachment in ePGPR-plant interaction has been well described for *Azospirillum brasilense*, *P. fluorescens*, and *P. putida* and involves bacterial surface proteins, capsular polysaccharides, flagella, extracellular polysaccharides, and pili depending on the interactions (Rodriguez-Navarro et al., 2007). Attachment is also essential for the formation of biofilms. Some phytopathogenic bacteria also stick to host cells during plant tissue invasion. This contact is essential for the formation of T3SS and the translocation of effectors directly into plant cells (Buttner and Bonas, 2006). Interestingly, the profile and kinetics of adhesion of *B. phytofirmans* PsJN and *P. syringae* pv. *pisi* with plant cells are very similar. The mechanisms of attachment for both bacteria to grapevine cells are, however, not known and the incidence of this physical adhesion in the induction of the defence responses remains to be investigated.

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References


