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Cytochemical localization of acid phosphatase activity in tissues of *Pinus armandi* infected by *Leptographium qinlingensis*

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Abstract

Acid phosphatase activity was localized cytochemically in the sapwood of *Pinus armandi* naturally infected by *Leptographium qinlingensis*, a bark beetle vectored blue stain fungus. The results showed that acid phosphatase in the phloem and xylem of the host tree is induced by infection. Distribution of acid phosphatase activity was concentrated around the penetration point and the surrounding host cells. As the severity of infection increased, acid phosphatase activity gradually decreased, indicating a decline in the plant's defense ability. Depletion of the host's cytochemical defenses by *L. qinlingensis* may facilitate subsequent colonization of phloem and sapwood by the pathogen's bark beetle vector.

Keywords: *Dendroctonus armandi, Leptographium qinlingensis,* acid phosphatase, cytochemical localization

1. Introduction

Chinese white pine, *Pinus armandi* Fr., is indigenous to the Qinling, Bashan Mountains, and certain other mountainous areas in Southwestern China. This covers parts of Shaanxi, Gansu, Sichuan, Hubei, Hennan, Yunan and Guizhou provinces. It is mainly distributed at elevations between 1400 and 2300 m in the middle range of Qinling and Bashan Mountains, making up a mixed stand with *Betula* and *Quercus*. In this area, *P. armandi* does not only grow rapidly, but also produces a high-quality wood. But since 1953, *P. armandi* has been seriously damaged by the indigenous bark beetle Chinese white pine beetle, *Dendroctonus armandi* Tsai et Li, which primarily infests healthy *P. armandi* at the age over 30 years. The bark beetle can carry the blue-stain fungus, *Leptographium qinlingensis* Tang et Chen, in its mycangium and transmits the fungus during feeding, into the phloem and xylem tissues of the host, which results in sapwood blue stain and rapid tree death in the Qinling and Bashan Mountains.

The trilateral interaction between conifers, bark beetles and blue stain fungi plays a very important role in forest ecosystems. Therefore, this interaction has been widely studied in relation to the host tree's resistance, the bark beetle's brood development, and the pathogenicity of the blue stain fungi (Paine, 1997; Johnson, 1987; Berryman, 1972; Chen, 2000, 2003). Attack by bark beetles and blue stain fungi can induce a number of plant responses, including release and synthesis of pathogenesis-related proteins as well as nonprotein defensive chemicals. For example, secretion of resin can flush out bark beetles and inhibit fungal advancement into xylem and phloem tissues and cells (Brignolas, 1995; Rosner, 2004; Krokene, 1999; Lieutier, 2003; Raffa and Berryman, 1982; Franceschi, 1998, 2002; Hudgins, 2003). In addition, acid phosphatase has been implicated in induced defense responses of plants against pathogenic fungi.

The mechanism that regulates acid phosphatase distribution and activity is unclear, although the level of phosphate in the environment may regulate their abundance. Organic phosphates such as glucose-1-phosphate and trehalose-6-phosphate are found in high concentrations in tissues and cells infested by pathogens (Pannabecker et al., 1992; Wyatt, 1961). These compounds could provide an important source of energy and phosphate for the fungal pathogen. Efficient utilization of phosphorylated organic molecules, however, requires production of phosphatases, which hydrolyse phosphate from orthophosphate monoesters. Wounding of tobacco induced a gradual increase in acid phosphatase activity along with an accompanying loss of protein, this increase was restricted to tissue cores taken at, wound sites and absent from unwounded tissue on the same leaf (Kenton et al., 1999). The increase in activities of acid phosphatase in susceptible

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chilli varieties after infection reflected the response of the host to *Colletotrichum capsici* (Borua and Das, 2000).

The activity of acid phosphatase at the cellular or subcellular level related to defense by *P. armandi* and nutrient assimilation by *L. qinlingensis* and the bark beetle has not been studied in detail, nor have cell and subcellular structures been compared in host tree tissues at different levels of pathogen damage. This paper presents the first results of a cytochemical study dealing with the localization of acid phosphatase and phosphate assimilation in trees naturally infected by the blue stain fungus.

2. Materials and Methods

Plant materials and sampling

Plant tissue samples for this study came from 30-year-old trees growing in various forest stands of Huoditang Research and Teaching Experimental Station of Northwest Agriculture and Forestry University in the Qinling Mountains. It has pure stands of *P. armandi*, mixed stands of *P. armandi* and *P. tabulaeformis*, as well as mixed stands of *P. armandi*, *P. tabulaeformis* and *Betula* spp.

Samples were collected in June 2002, 2003, and 2004 from *P. armandi* trees that were either healthy or infected by *L. qinlingensis* at different stages of disease development. Attacked and/or infected sampling pines by Chinese white pine beetle and their associated blue stain fungus divided three stages, early stage attacked and/or infected by Chinese white pine beetles and their carried blue-stain fungus at the first year, withering stage infected by fungus at second year, and the later and/or dead stage infected at third year, and at withering and dead stages the phloem and sapwood tissues were obviously dyed. Sapwood samples of approximately 10 cm^2 in dyed area were cut from the trunk at breast height, and placed into liquid nitrogen for up to 20 hours until further processing.

Using a binocular microscope under ice bath conditions, the phloem and xylem samples were carefully dissected into three sections approximately 5×2×2 mm in volume using pointed forceps and a scalpel. At the same time, the phloem and sapwood samples were isolated using PDA and wort medium to confirmation infected by blue stain fungus.

Transmission electron microscopy

The sample materials were cut with a razor into slices, 0.3 mm in length, each specimens repeated thrice, and were floated in cold fixation buffer (Na-cacodylate-buffered 50 mM, pH 7.2, 2.5% glutaraldehyde and 4% formaldehyde) for 1.5–2 h at 4°C. The fixed slices were rinsed twice with Na-cacodylate buffer and twice with tris-maleate buffer in order to entirely eliminate the fixative. Appropriate sections, phloem and sapwood tissues intactness, were preincubated at 22°C for 2 h in a buffer solution (8 mM β-Na-phosphoglycerol, tris-maleate (40 mM, pH 5.2), 2.4 mM lead nitrate). This was followed by a 30 min incubation at 22°C in a water bath using a reaction mixture containing: 1 mM β-glycerophosphate, 2 mM $CeCl_3$ and 100 mM acetate buffer, pH 5.0.

Control treatments without sodium β-glycerophosphate and $CeCl_3$ were made. Incubated sections were rinsed with Na-cacodylate buffer (pH 7.2) twice to thrice during 1 h, they were fixed in a solution of Na-cacodylate buffer (pH 7.2) and 2% osmic acid for 2 h or overnight at 0–4°C. Subsequently, they were dehydrated in acetone and embedded in Epon. Blocks were sectioned on a Reichert ultramicrotome. Thin sections for transmission electron microscopy were collected on copper grids and stained with uranyl acetate followed by lead citrate and examined in a JEOL JEM 100 C transmission electron microscope after were cut by glass knives (Yang, 1991).

3. Results

Localization of acid phosphatase in the phloem and sapwood of P. armandi

The phloem of the trunk of *P. armandi* contains three major cell categories in a highly ordered arrangement: sieve cells, ray parenchyma, and axial phloem parenchyma. The sieve cells occur as concentric rows of rectangular cells, aligned with rows of xylem tracheids on the inner side of the cambial zone. The ray cells contained considerable numbers of lipid bodies and plastids with starch, but ray cells in the 30-year-old plus trees used for this study did not contain vacuolar polyphenolic bodies. Rays also give rise to radial resin ducts with large resin-containing cavities, or canals, towards the outer bark (older portions of the phloem). The resin ducts are lined with small, cytoplasmically dense, secretory parenchyma cells containing abundant lipid droplets.

The xylem consists of four types of cells along the longitudinal and transverse sections: tracheid cells, ray tracheid cells, resin ducts (including axial secretory resin cells and longitudinal secretory resin cells), and ray parenchyma cells (including axial ray parenchyma cells and longitudinal ray parenchyma cells). The tracheid cells and ray tracheid cells are mainly the support cells of stem tissue and the main route for transport of water, and there are large numbers of veins in the tracheids. The secretory resin cells, located in the resin duct, excrete resin as a defense response against harmful insects and pathogens. The ray parenchyma cells form orderly rows of rectangular cells in axial and longitudinal direction, and their physiological role is synthesis and storage of energy as well as allowing selective permeability to carbohydrate and lipids.

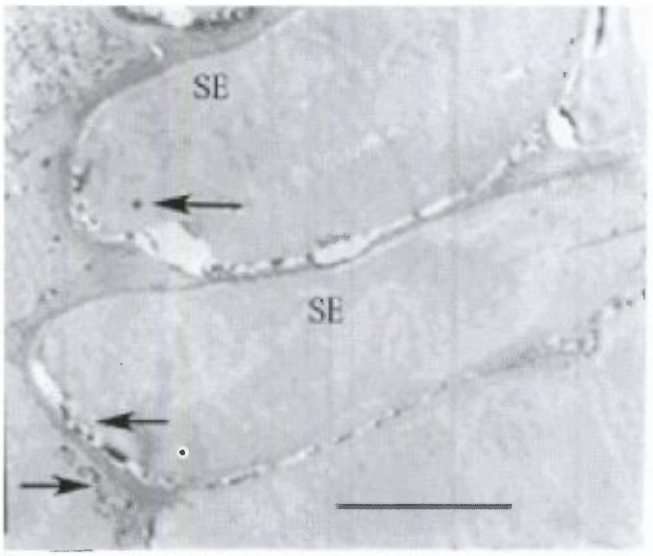
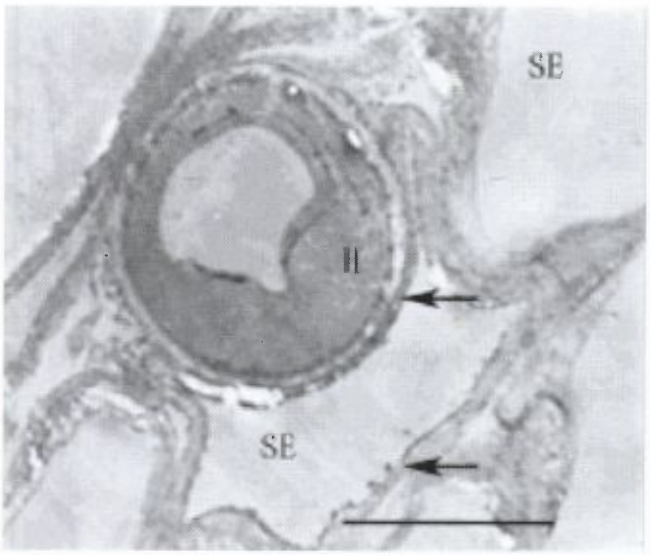


Fig. 1.



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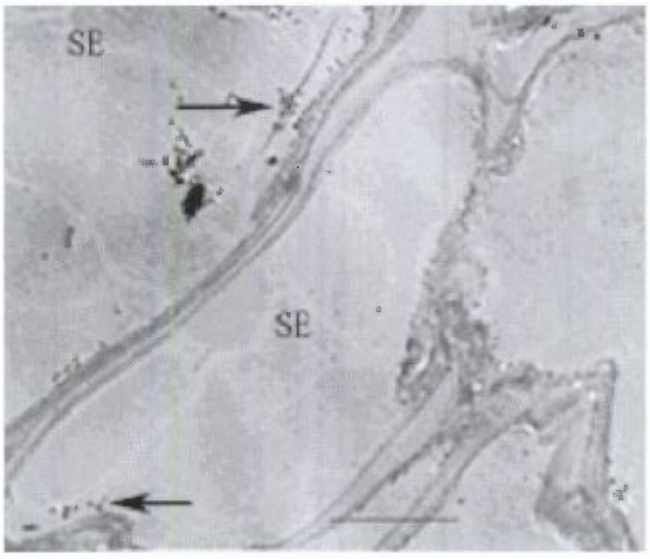

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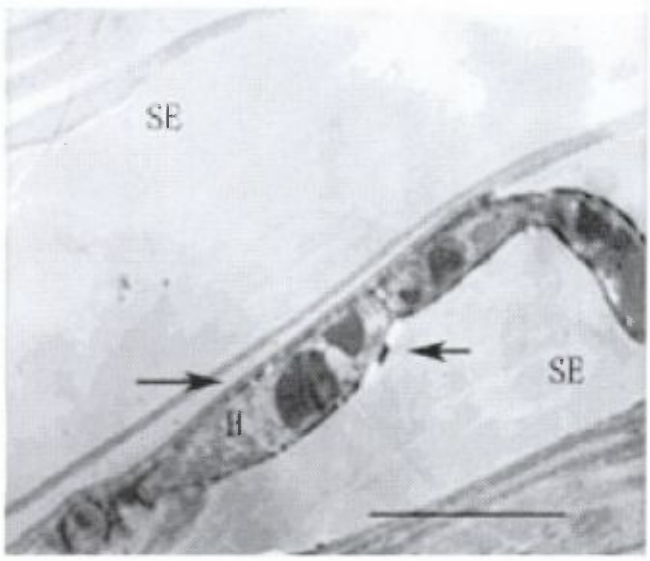

Fig. 4.

Figures 1–4. Localization of acid phosphatase in the phloem and sapwood of *Pinus armandi*. Samples are from 1.5 m up the trunk of 30-yr-old trees (Figs. 1–3 phloem tissue of withering tree; Fig. 4, xylem tissue). 1. Longitudinal section of phloem showing sieve cells (SE) in early stage of infection by the blue stain fungus *Leptographium qinlingensis*, dark deposits of acid phosphatase activity in sieve-cell wall's and intercellularly (arrow), scale bar 10 μm. 2. Cross section of phloem showing sieve cells (SE) penetrated by hyphae of *L. qinlingensis*, intense acid phosphatase activity in the hyphae (H) and sieve cell wall (arrow), scale bar 5 μm. 3. Longitudinal section of phloem showing sieve cells (SE) at advanced stage of infection, deposits of acid phosphatase activity in sieve cells wall and intercellularly (arrow). 4. Longitudinal section of host tree phloem infected by the blue stain fungus *Leptographium qinlingensis*. Sieve cells are clearly discernible, acid phosphatase activity intensity concentrated in hyphal wall (H) (arrow), scale bar 10 μm.

Localization of acid phosphatase in the phloem of P. armandi

Phosphatase activity was demonstrated in longitudinal and transverse sections of phloem and xylem by the staining of phosphate by externally supplied β-Na-phosphoglycerol phosphate.

As shown in Fig. 1, acid phosphatase activity in the phloem of *P. armandi* in very early stages of infection is located in the host cell wall. In severely infected phloem tissue, sieve cells are colonized by hyphae of *L. qinlingensis*, and acid phosphatase activity is located in walls of the surrounding tracheid cells (Fig. 2). Transverse sections of severely infected phloem tissues show

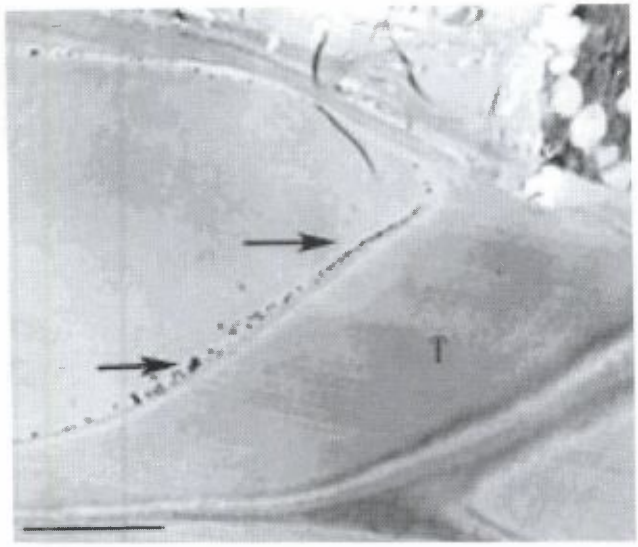

Fig. 5.

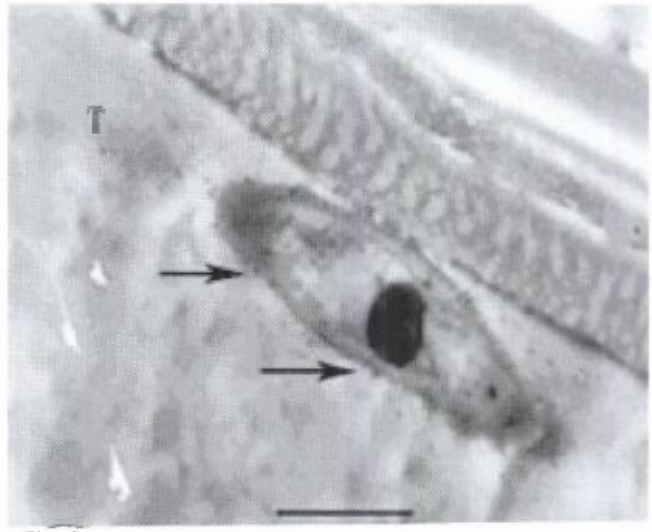

Fig. 6.

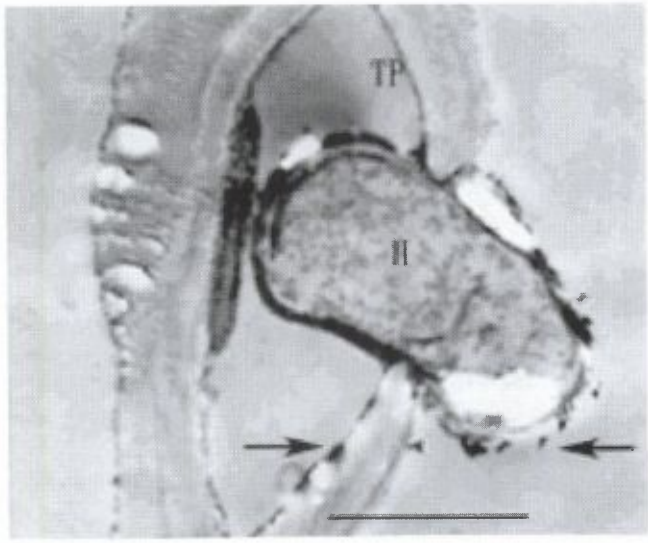

Fig. 7.

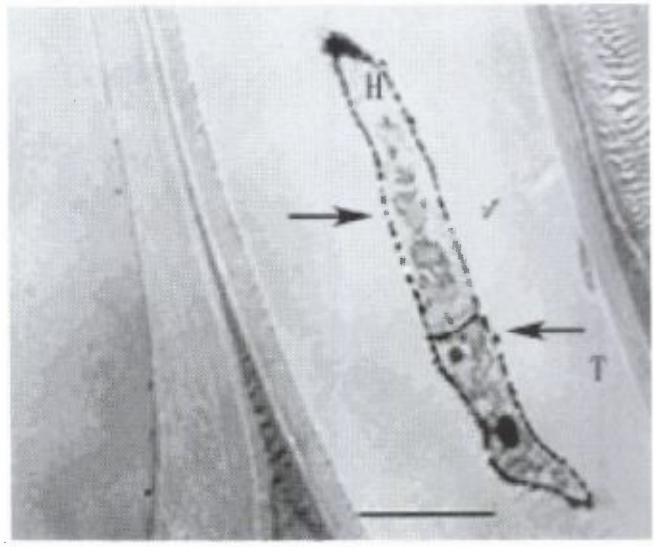

Fig. 8.

Figures 5–8. Localization of acid phosphatase in the xylem of *Pinus armandi*. Samples are from 1.5 m up the trunk of 30-yr-old trees 5. Longitudinal section of xylem in early stage of infection by *L. qinlingensis* showing acid phosphatase activity of tracheids (T) (arrow), scale bar 10 μm. 6. Longitudinal section of the xylem, tracheids penetrated by hyphae of *L. qinlingensis*, intense acid phosphatase activity in the hyphal wall (arrow), scale bar 5 μm. 7. Cross section of xylem of infected host tree at the withering stage showing acid phosphatase activity concentrated around the tracheids pits (TP) and around the fungal hyphae (H) (arrow), scale bar 10 μm. 8. Longitudinal section of xylem of infected host tree at the withering stage showing acid phosphatase activity of tracheids induced by invasion of *L. qinlingensis*, scale bar 10 μm.

ramification by fungal hyphae and an overall disorderly distribution (Fig. 3). Host cell walls and septa (or membranes) also have localized acid phosphate activity. In this damaged stage, there are also enzyme reactive deposits inside the plant cells (Fig. 3). In dead *P. armandi* phloem tissue in transverse and longitudinal sections, cells appear degraded and disorderly distributed. At this point, enzyme activity was weak in plant cells but very strong in fungal hyphae.

Localization of acid phosphatase in the xylem of Pinus armandi

During early stages of infection, longitudinal sections of the xylem of *P. armandi* showed acid phosphatase activity, in the interior wall of tracheids and in the intercellular space. Thus, enzyme activities are very strong in these vessels. Transverse sections of the xylem, contains both sections of hyphae, also have strong enzyme activity. At the time when

hyphae have invaded the entire plant cell, tracheid cell walls near the hyphae show more distinctive enzyme reactions (Figs. 4, 5). Acid phosphatase activity in the xylem of withering *P. armandi* is concentrated around the pore and its primary and secondary wall. Large numbers of pores are invaded by hypha, which leads to non-functional pores with altered structure and eventually cell death. The hypha in these invaded pores show strong enzyme reaction, as do the hyphal walls and the secondary walls of the pore (Figs. 6, 7). Moreover, there are numerous reactive deposits in the hyphal septum and around the hyphal walls in both transverse and longitudinal sections, and plant cell walls also show a weak enzyme reaction (Fig. 8).

4. Discussion

Acid phosphatase in the phloem of *P. armandi* is similar to that in the xylem. During early stages of infection, the plant grows vigorously, cell development and lysosome function are normal, and activity and content of acid phosphatase is very high. The tree's resin production is also very active at this stage. But in diseased trees, plant resistance is gradually weakened, the growth of tree slows, and the plant cells' acid phosphatase activity decreases. In withered trees containing numerous hyphae in infected tissues, enzyme activity is strong and mostly confined to fungal tissues. At this stage, the growth of hyphae is vigorous, they propagate extensively, and their quantity peaks, as does the hyphae-associated acid phosphatase activity. During tree death, plant cells are collapsed, the function of lysosomes is weak, contents and enzyme activities associated with the cell are reduced gradually, and acid phosphatase activity is low during the dying process.

Our observations show that in early stages of infection, acid phosphatase can be detected cytochemically mainly as an active host defense response in the phloem and xylem. The detection of strong enzyme reaction product during disease progression and during the withering stage suggests that resin cells and ray parenchyma cells are involved in the secretion of hydrolases required for the controlled hydrolysis of some cells and tissues, or for the utilization of this enzyme to regulate metabolic processes (Franceschi, 1998, 2001).

Acid phosphatase is stored in an inactive state and becomes active only during attack by pathogens and insects agents (Phillips, 1970; Baccetti, 1972; Bao et al., 1989; Ndiaye and Mattei, 1992). Therefore, we can suppose that the bark beetle-vectored blue stain fungus induces acid phosphatase activity to deplete the host's defense ability toward its vector which allows the latter to colonize the host tree stem. Acid phosphatase is apparently involved in the metabolism of phosphates, important for cell development and metabolism of the host. It is interesting to note that heterogeneity in intensity of reaction was observed in the different damaged stages. Our suggestion concerning this difference is that changes in acid phosphatase activity parallel both conifer death and the process of resistance depletion, both of which benefit bark beetle colonization and nutrient utilization, especially of ester and phosphates. With the invasion of *L. qinlingensis*, resin ducts are filled by fungal hyphae, which leads to a shortage of resin in *P. armandi*. Therefore, *D. armandi* on invaded plants can avoid the host's defense reactions, allowing *D. armandi* to feed and propagate in the phloem. Larvae of the bark beetles can block phloem while they feed, hence photosynthates of the host are held back and unavailable in the hosts's sink tissues. This further reduces the host's defense ability. Finally, the resistance system of *P. armandi* is impede to a level that leads to the death of the tree.

On the other hand, pathogens isolated from phloem and sapwood tissues also confirmed that in the dyed areas infected by *L. qinlingensis*, and was an agent caused blue stain of phloem and sapwood tissues. Therefore, we can suppose that the blue stain fungus changed metabolism process of host trees, it includes induces defense associated enzymes activities such as acid phosphatase in the infected areas to deplete the host's defense ability.

Acknowledgments

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