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The Role of the Extracellular Matrix (ECM) in Phytopathogenic Fungi: A Potential Target for Disease Control

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1. Introduction

Crop yield loss as a result of disease has an economic impact on many people. To protect against disease, plant pathologists have developed various fungicides and disease resistant cultivars. In parallel, pathogens have evolved to escape from disease protection measures through, for example, the emergence of fungicide resistant isolates and the breakdown of disease resistant cultivars. To resolve these issues, we should develop various disease protection strategies for different types of target sites. Therefore, we must improve our understanding of the infection mechanism of pathogens. Pathogens possess several classes of genes that are essential for causing disease, i.e., pathogenicity genes or virulence genes, the products of which are called pathogenicity factors. Phytopathogenic fungi have developed various pathogenic factors, e.g., adhesion molecules to the host cells, sensor machineries against host plants, invasion machineries into the host cells, adaptation ability on the host cells, and so on. These factors comprise potential targets for disease control. Fungal adhesion to host cells is an initial important step to establish infection, which is considered to be a universal mechanism across plant pathogenic fungi. In this chapter, we provide a review of the components required for fungal adhesion to the host cell, and propose fungal adhesion as a potential target for disease control.

2. Importance of fungal adhesion to the host cell

Pathogenic fungi have various strategies for propagation. Some fungi produce asexual spores, sexual spores, sclerotia, budding progenies, or just extending mycelia. These propagules comprise minimal compartmental units to transmit genetic information, and are highly replicable. These small propagules are able to travel great distances and colonize novel niches. Dispersing propagules may land on various types of environment. Each pathogenic fungus is capable of adapting to establish individuals on a specific environment. The mechanism of pathogenic fungal adhesion is considered to occur in a number of ways. For example, the hydrophobic interaction is a universal system of adhesion, i.e., most fungal surfaces are covered with hydrophobic components, such as hydrophobin. In addition, either protein-protein or protein-carbohydrate interaction also occurs.

The plant surface is coated with a cuticle (wax) to prevent desiccation, particularly when the environment is highly hydrophobic. When the spores of pathogens land on the plant surface, the spores elongate into germ tubes and have elaborate infection machineries, such as appressorium to infect plant cells. However, the spores must first settle on the plant surface to accomplish infection. If the spores are not able to adhere to the plant surface, they are easily dislodged by the wind or rain fall. Moreover, the spore germlings encounter the counterforce of infection pressure from the plant surface. Therefore, firm adhesion enables spore germlings to anchor tightly onto the host surface, and is important for the differentiation of specialized infection structures. Adhesion is required for infection in most plant pathogens, and this mechanism is also developing in phytopathogenic fungi. We showed that adhesion ability was variable depending on the living strategies of different fungal species (Fig. 1). For example, the saprotrophic fungus *Neurospora crassa* has weak adhesion ability on plastic surface. In contrast, phytopathogenic fungi, such as *Alternaria alternata* Japanese pear pathotype and *Magnaporthe oryzae*, adhered strongly to plastic surfaces. Since plastic surfaces are highly hydrophobic, they mimic the plant surface. Hence, phytopathogenic fungi might have evolved to settle on the surfaces of plants. However, the precise molecular mechanism of fungal adhesion on plant surfaces remains undetermined.
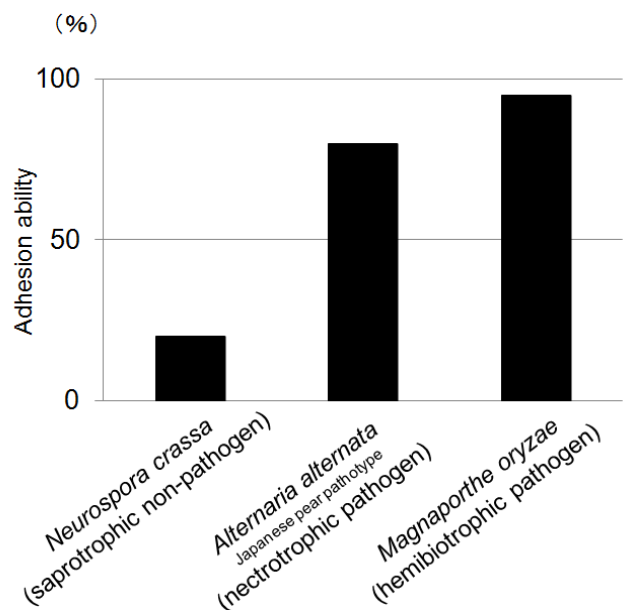

Fig. 1. Variation in the ability of fungal adhesion to plastic surfaces. The saprotrophic fungus *Neurospora crassa* has a weak adhesion ability. The phytopathogenic fungi, *Alternaria alternata* Japanese pear pathotype and *Magnaporthe oryzae*, have high adhesion ability. Adhesion ability was determined by total number of germlings was first counted under the microscope and then washed by dipping in distilled water 100 times vertically to remove the detached germlings.

In animal pathogenic fungi, adhesion to host cells is also important to manifest pathogenicity. However, because the animal cell has no cell wall and no wax layer, adhesion mechanisms to the host cell are supposed to be different to that of phytopathogenic fungi. Moreover, the environment within the animal body is different to that of the atmosphere, i.e., low oxygen concentrations, high carbon dioxide concentrations, as well as being filled with serum. Animal pathogenic fungi have evolved various adhesive proteins called

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adhesins (Sundstrom, 1999). In the next section, we describe various components of adhesion in animal and plant pathogenic fungi.

3. Components of adhesion strategies

3.1 Animal pathogenic fungal adhesion

Candida albicans causes severe oropharyngeal and esophageal mucositis in patients that have human immunodeficiency virus (HIV), and is the most intensively studied animal pathogenic fungi. *C. albicans* normally propagates budding yeast cells (blastospores). When the blastospores attach to the animal cells, the blastospores transform into a filamentous form to invade underlying tissues. The cell wall components between the blastospore and filamentous hypha are different. Therefore, the adhesion of *C. albicans* may be divided into two different steps, depending on morphological switching.

Blastospore adhesion is the first step of adhesion to the host epithelial and endothelial cells, especially to the extracellular matrix, and is important for the morphological switching of yeast cells from blastospore to filamentous forms (Gale *et al.*, 1998; Klotz *et al.*, 1993). This adhesion step is dependent on the presence of calcium ions (Klotz *et al.*, 1993). Ultrastructure analysis has revealed that the cell wall of blastospores is surrounded by a fibrillar reticulated layer (fimbriae) that contains mannose sugar, suggesting the importance of glycoprotein in this process (Bobichon *et al.,* 1994). The molecular approach elucidated the presence of several adhesive proteins, called adhesins. Since the blastospore adheres to the extracellular matrix of the animal cell, the involvement of the integrin-like component was suspected to be a component of adhesion. The homologous gene of vertebrate leukocyte integrins was cloned as *INT1* and characterized (Gale *et al.*, 1996; Gale *et al.*, 1998). When the *INT1* gene was introduced into *Saccharomyces cerevisiae*, the transformants producing INT1 exhibited enhanced aggregation (Gale *et al.*, 1996). Furthermore, the disruption of *INT1* suppressed blastospore adhesion to epithelial cells, hyphal growth, and virulence in mice (Gale *et al.*, 1998). In addition to INT1, agglutinin family proteins, ALA1 and ALS1, and the fibronectin binding protein, FLS5, were also involved in blastospore adhesion (Fu *et al.*, 1998; Rauceo *et al.*, 2006).

Once the blastospores settle on the host cells, morphological switching occurs to form filamentous growth (germ tubes, pseudohyphae, and hyphae). Proline and glutamine rich protein-encoding genes have been abundantly expressed in hyphae but not yeast forms, and were designated as *HWP1* (Staab *et al.*, 1996). The disruption of the *HWP1* gene reduced the stable attachment of blastospores to human buccal epithelial cells, and reduced their capacity to cause systemic candidiasis in mice (Staab *et al.*, 1999). The HWP1 protein also served as a substrate for mammalian transglutaminases, suggesting that the blastospore adherence mechanism may be involved in the cross-linking of HWP1 to unidentified proteins through transglutaminase activity (Staab *et al.*, 1999). Moreover, adhesin family proteins, ALS3 and HYR1, were also found to be involved in filamentous growth adhesion (Sundstrom, 1999).

Other adhesion components of animal pathogenic fungi have also been also studied. In *Aspergillus fumigatus*, a hydrophobin RODA was involved in adhesion (Thau *et al.*, 1994). In *Rhizopups oryzae*, the spores adhered to laminin and type IV collagen, but not to fibronectin, with adhesion decreasing during the elongation of germ tubes (Bouchara *et al.*, 1996).

3.2 Plant pathogenic fungal adhesion

Plant pathogenic fungi have several adhesion strategies. This review mainly focuses on the spore dispersal of plant pathogenic fungi. When the spores land on the plant surface, they germinate and develop appressoria to enter into the plant cell (Fig. 2). Appressoria adhere tightly to the plant surface. This process is divided into two steps, i.e., spore adhesion and germ tube adhesion. During germ tube elongation, the fungal surface is covered with *de novo* synthesized compounds collectively called the extracellular matrix (ECM), which might be involved in adhesion (Beckett *et al.*, 1990). The term ECM is confusing because the outer surface components of animal cells are also called the extracellular matrix (ECM). In this chapter, ECM corresponds to the secreted products from plant pathogenic fungi only. The adhesion strength and the adhesion components in each step are different for each plant pathogenic fungus. Here, we provide an overview of the typical adhesion mechanisms in plant pathogenic fungi.
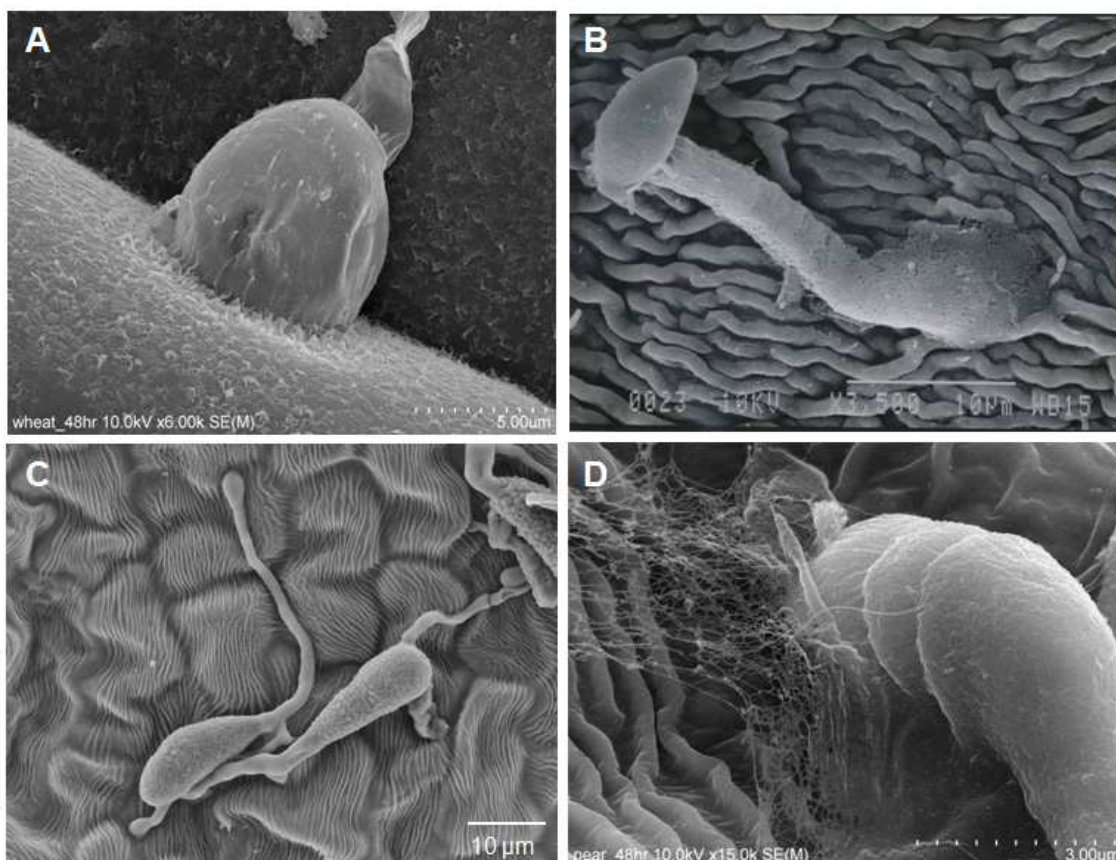

Fig. 2. Scanning electron microscope image of appressoria of phytopathogenic fungi on the host plant surface. (A) Appressorium of *Magnaporthe oryzae* on a wheat leaf. (B) Appressorium of *Venturia nashicola* on a Japanese pear leaf. Infection structure (C) and appressorium (D) of *Alternaria alternata* Japanese pear pathotype on a Japanese pear leaf.

3.2.1 *Magnaporthe oryzae*

The blast fungus *M. oryzae* is one of the most destructive fungal diseases in gramineous crop plants, especially rice, barley, and wheat. *M. oryzae* produces millions of conidia, asexual

spores, and disperses to distant areas. Successful infection by this fungus requires the following steps: (1) the spore to land on the host surface and elongate the germ tube from the apical spore cell and (2) the tip of the germ tube to differentiate into the appressorium, which is the infection machinery. Subsequently, the fungi penetrate into the host cuticle and generate a penetration peg from the bottom of the appressorium (Howard *et al.*, 1991). Although the conidia itself has low adhesion ability, the hydrated conidia attains strong adhesion ability within 30 minutes of incubation (Hamer *et al.*, 1988). Ultrastructural analysis has revealed that the spore tip mucilage (STM) is preserved at the apical spore and released after hydration (Braun and Howard, 1994). The attached spores on the surface start to germinate and secrete ECM. The secreted ECM supports the enhanced adhesion ability of the fungi on the plant surface. It is not known whether the STM and the ECM are the same component. During the infection process, some essential environmental signals have been identified as appressorium (infection)-inducing factors, such as the hardness (Xiao *et al.*, 1994) and the hydrophobicity of the attachment surface (Jelitto *et al.*, 1994; Lee & Dean, 1994), and the chemical components from the plant surface (Gilbert *et al.*, 1996). In addition, a number of up-regulating genes have also been characterized (Talbot, 2003). However, the available information was not sufficient to identify the principal components and regulation mechanisms of fungal adhesion in *M. oryzae*.

While the components of STM remain unclear, it was found that STM was reactive with lectin concanabalin A (ConA; α-D-glucose and α-(1,3)-/α-(1,6)-D-mannose binding), suggesting that STM consists of mannose containing glycoprotein (Hamer *et al.*, 1988). The ECM was also ConA-positive (Xiao *et al.*, 1994). The effect of three different specific sugar-binding lectins, namely ConA, PSA (α-D-glucose and α-(1,6)-D-mannose binding), and WGA (chitin binding), on appressorium formation and the adhesion ability of the *M. oryzae* germlings was evaluated (Fig. 3). High concentrations (50 μg ml^{-1}) of lectins, ConA, and WGA were found to inhibit appressorium formation and adhesion. These results indicate that these lectin treatments covered the cell wall surface, and inhibited the development of the cell wall architecture (data not shown). In contrast, low concentrations (10 μg ml^{-1}) of these lectins only affected adhesion, suggesting that low concentrations of lectins are preferentially bound to the adhesion components (Fig. 3). Moreover, although PSA was observed to bind to the cell wall surface, it did not affect the adhesion. ConA and PSA commonly bind to the α-D-glucose and the α-(1,6)-D-mannose moieties; however, only ConA binds to α-(1,3)-D-mannose, suggesting that α-(1,3)-D-mannose is a potential adhesion component (Fig. 3). Our unpublished study revealed that the incubation of *M. oryzae* spores in modified nutrient conditions produced less adherent spore germlings. Furthermore, the less adherent spore germlings were ConA-negative (K. Inoue, unpublished data), suggesting that mannose sugar may be important for adhesion.

Protein secretion via the Golgi pathway might be responsible for ECM production. In the treatment with cycloheximide at the early step of germination, the germ tube and the appressorium were not formed, and the germlings were easily removed (Fig. 4). In the treatments with monensin and tunicamycin, the adhesion rate was significantly lower than for the water control (Fig. 4). Hence, N-glycosylation of the mannose sugar moiety might be important for the maturation of the adhesive protein(s). This result also agreed with the conclusion of the lectin treatment experiments.
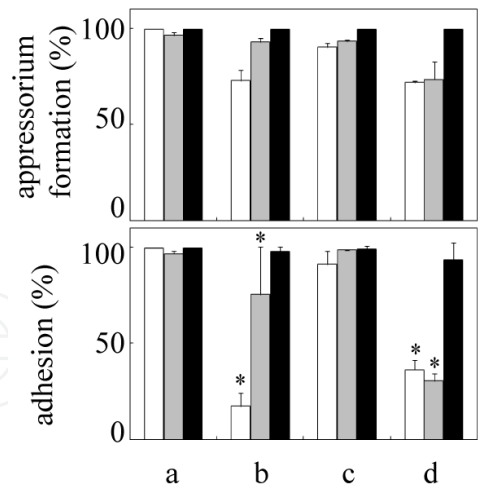
Fig. 3. Effects of lectins on appressorium formation and adhesion of *M. oryzae* germlings on the hydrophobic surface. The rate of appressorium formation (upper) and adhesion (lower) in *M. oryzae* germlings treated with the water control (a), 10 µg ml^{-1} ConA (b), 10 µg ml^{-1} PSA (c), and 10 µg ml^{-1} WGA (d) at 0 hour post-inoculation (hpi; white bars), 1 hpi (grey bars), and 6 hpi (black bars). Bars indicate standard deviation. * indicates a significant difference from the control ($p < 0.05$).
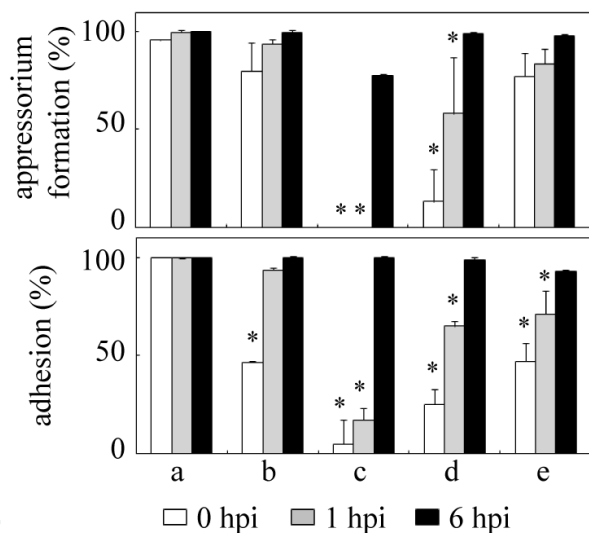

Fig. 4. The effects of inhibitors on appressorium formation and the adhesion of *M. oryzae* germlings on the hydrophobic surface. The rate of appressorium formation (upper) and adhesion (lower) of *M. oryzae* on the hydrophobic surface by inhibitor treatments with the water control (a), 50 µg ml^{-1} colchicine (b), 0.1 µg ml^{-1} cycloheximide (c), 1 µg ml^{-1} monensin (d), and 10 µg ml^{-1} tunicamycin (e) at 0 hpi (white bars), 1 hpi (grey bars), and 6 hpi (black bars). Bars indicate standard deviation. *, indicates a significant difference from the control ($p < 0.05$).

One of the candidate ECM components in *M. oryzae, Emp1*, was isolated from the cDNA library, and was homologous to the extracellular matrix protein *Fem1* in *Fusarium oxysporum* (Ahn *et al.*, 2004). The *emp1* disruption mutants showed a slight reduction in adhesion ability, but were largely involved in appressorium formation, suggesting that EMP1 was involved in surface recognition (Ahn *et al.*, 2004).

Ultrastrucutural analysis was used to elucidate the ECM components, and revealed that the ECM was an electron dense and fibrous structure (Fig. 5; K. Inoue *et al.*, 2007). This fibrous structure was supposed to be similar to the components of the cell adhesion factors (extracellular matrix in animal cells), such as collagen, fibronectin, and laminin. We then performed immunohistochemical analysis, using specific antibodies against mammal cell adhesion factors. Positive signals were detected from the treatments with antibodies against collagen, vitronectin, fibronectin, laminin, and integrin (K. Inoue *et al.*, 2007). Similar results have been reported by other research groups (Bae *et al.*, 2007; Dean *et al.*, 1994), and for other plant pathogenic fungi (Celerin *et al.*, 1996; Corrêa *et al.*, 1996; Gale *et al.*, 1998; Hyon *et al.*, 2009; Jian *et al.*, 2007; Kaminskyj and Heath, 1995; Manning *et al.*, 2004; Sarma *et al.*, 2005). Cell to cell adhesion mediated by integrin was involved in Arg-Gly-Asp (RGD) motifs (Giancotti & Ruoslahti, 1999). The germlings treated with RGD peptide reduced adhesion ability and appressorium formation (Bae *et al.*, 2007). The suppression effect by the RGD treatment was reversed by supplementing with cAMP, suggesting that RGD recognition may be involved in the signaling cascade for appressorium formation (Bae *et al.*, 2007). Based on this evidence, the functions and the components of fungal ECM resemble animal ECM. However, a similar nucleotide sequence to that of the animal ECM component was not detected from the fungal genome sequences. This suggests that only the restricted tertiary protein structure had a resemblance, with the amino acids sequences being highly diverged.

Melanin is also one of the cell surface components. The fungicide treatments, chlobenthiazone and tricyclazole, that inhibit melanin synthesis, have been shown to reduce germling adhesion (S. Inoue *et al.*, 1987).
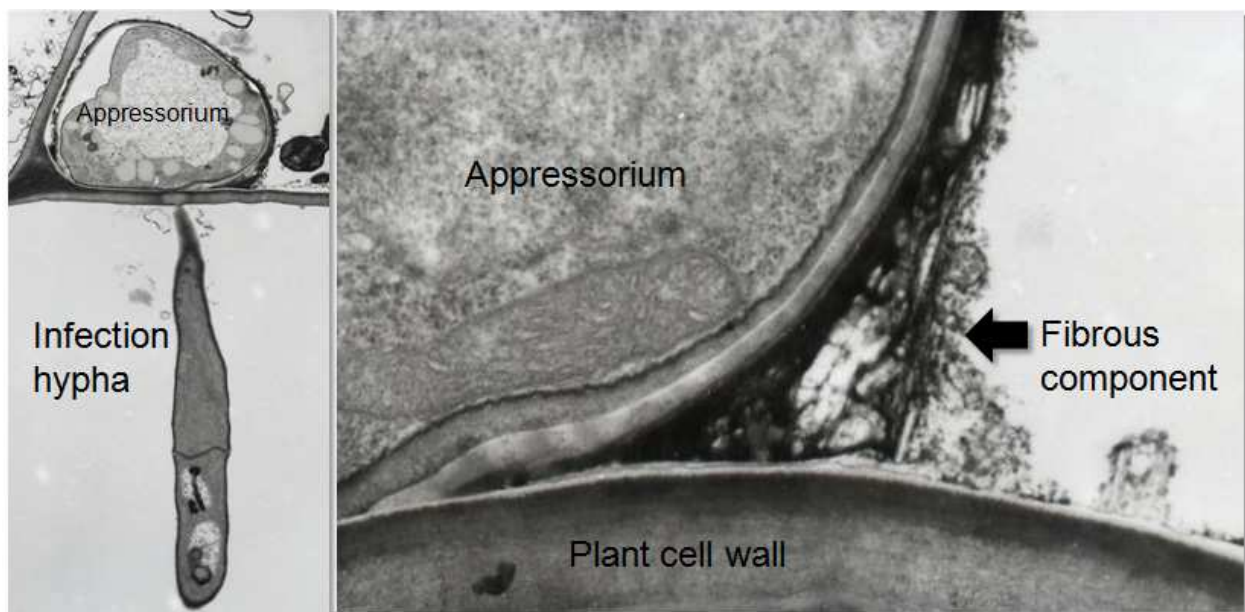

Fig. 5. Transmission electron microscopy images of *M. oryzae* appressoria on the leaf surface of wheat. Note: fibrous components were observed at the interface between the plant and fungal appressorium. Adapted from Inoue *et al.* (2007), with permission.

Hydrophobicity is one of the important factors for appressorium formation in *M. oryzae* (Jelitto *et al.*, 1994; Lee & Dean, 1994). To determine whether substrate hydrophobicity is essential for fungal adhesion and appressorium formation, fungal differentiation and

adhesion were compared on hydrophilic and hydrophobic surfaces. On the hydrophobic surface, most of germlings produced appressoria, with ca. 100% adherence over a six hour period (Fig. 6). On the hydrophilic surface, appressoria were not formed; however, over a six hour period ca. 100% of germlings adhered to the surface (Fig. 6). This result suggests that adhesion ability is dispensable for appressorium formation. The spore germlings may sense hydrophobicity, hardness, or chemical signals to form appressoria.
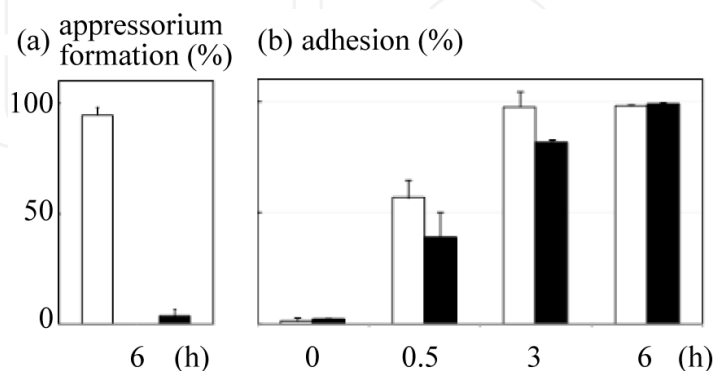

Fig. 6. The effect of surface characteristics on appressorium formation and the adhesion of *M. oryzae* germlings. (a) The rates of appressorium formation of *M. oryzae* germlings on hydrophobic (white bar) and hydrophilic (black bar) surfaces at 6 hpi. (b) The rates of adhesion to hydrophobic (white bars) and hydrophilic (black bars) surfaces at 0, 0.5, 3, and 6 hpi. Bars indicate standard deviation.

Fungal hydrophobins are cell-surface hydrophobic proteins with four disulphide bonds that are ubiquitous in filamentous fungi (Kershaw *et al.,* 2005; Wösten, 2001). Hydrophobins exist in multiple copies, and these proteins are divided into two groups based on their physical properties (Wessels, 1994). Hydrophobins seem to form part of the ECM. Class I hydrophobins form highly insoluble polymers, whereas class II hydrophobins form polymers that are soluble in some organic solvents (Sunde *et al.,* 2008). These hydrophobins are differentially expressed during the growth stage, and seem to have different functions (Nielsen *et al.,* 2001; Segers *et al.,* 1999; Wessels *et al.,* 1991; Whiteford *et al.,* 2004). In *M. oryzae*, two hydrophobins are well characterized: class I *Mpg1* (Talbot *et al.,* 1993) and class II *Mhp1* (Kim *et al.,* 2005).

In the *mpg1* null mutant, appressorium formation was reduced, suggesting the involvement of host-sensing (Beckerman and Ebbole, 1996; Talbot *et al.,* 1993; Talbot *et al.,* 1996). In a previous study, adhesion in the *mpg1* null mutant was not significantly affected in a conventional wash experiment, but was affected in a boiling-SDS wash experiment (Talbot *et al.,* 1996). In contrast, an RNA silencing experiment of *Mpg1* reduced adhesion (K. Ikeda, unpublished data). The *Mpg1* silencing mutants reduced ConA-positives (K. Ikeda, unpublished data), suggesting that Mpg1 is a ConA-positive glycoprotein, or that Mpg1 recruits ConA-positive glycoproteins to the cell surface. The complex formations involved in Mpg1 may strengthen adhesion and contribute to pathogenicity. The hydrophobin RolA in *Aspergillus oryzae* was associated with cutinase and allowed the substrate to degrade on the hydrophobic surface (Takahashi *et al.,* 2005). One of the cutinase genes *Cut2* in *M. oryzae* was involved in appressorium formation, but not involved in adhesion (Skamnioti & Gurr, 2007). Other cutinase genes may also be involved in adhesion.

The class II hydrophobin *Mhp1* disruption mutants affected pleiotropically, hydrophobicity, pathogenicity, and spore viability (Kim *et al.,* 2005). In our unpublished data (K. Ikeda), *mhp1* null mutants showed no alteration, except for reducing hydrophobicity. The class II hydrophobin cerato-ulmin in *Ophiostoma novo-ulmi* not only functioned in adhesion, but was also toxic to the host plant, as well as providing tolerance against desiccation (Temple *et al.,* 1997). Paradoxically, the *Cladosporium fulvum* hydrophobin HCf-6 suppressed adhesion ability (Lacroix *et al.,* 2008). Further analysis of the relationship between hydrophobin and adhesion is required.

3.2.2 *Colletotrichum* species

The genus *Colletotrichum* is one of the most important genera of plant pathogens. The genus *Colletotrichum* encompasses numerous species, and the key criterion for their identification is mainly based on plant host determination. The ungerminated conidium of *Colletotrichum* species possesses adhesion ability. Adhesion is more effective on hydrophobic than hydrophilic surfaces, and is required for *de novo* protein synthesis in *C. graminicola* and *C. musae* (Mercure *et al.,* 1994a; Sela-Buurlage *et al.,* 1991). However, the adhesion strength of *C. graminicola* conidia was not strong, with up to 30% of conidia adhering only, and was influenced by corn leaf age (Mercure *et al.,* 1994a). Attached conidia were ConA positive and were effectively detached by pronase E treatment (Mercure *et al.,* 1994b; Sela-Buurlge *et al.,* 1991). Ultrastructural analysis revealed that the ungerminated conidia of *C. lindemuthianum* and *C. truncatum* were coated with fibrillar ECM, suggesting that the conidia of *Colletrotrichum* species may be covered with adhesive ECM that is dissimilar to *M. oryzae* (Hamer *et al.,* 1988; O'Connell *et al.,* 1996; Van Dyke and Mims, 1991). To study the ECM molecular components in *C. lindemuthianum*, monoclonal antibodies (MAbs) were raised against conidia (Pain *et al.,* 1992). A MAb UB20 specifically recognized the conidium surface and UB20 treatment inhibited conidium adhesion (Hughes *et al.,* 1999). However, UB20 treatment did not affect appressorium formation and pathogenicity. The conidium adhesion deficiency was compensated after germination, suggesting that germ tube adhesion was involved in appressorium formation and pathogenicity (Rawlings *et al.,* 2007).

MAbs were also raised against conidium germlings of *C. lindemuthianum* (Pain *et al.,* 1994a,b). MAbs UB26 and UB31 were observed to bind to the ECM surrounding germ tubes and appressoria, but not to the conidia, suggesting that ECM compounds were different for conidia and germ tubes (Hutchison *et al.,* 2002). Moreover, the distribution of UB26 and UB31 positive signals was similar, but spatial distribution was different, i.e., UB31 antigens were located close to the cell wall, while UB26 antigens extended further from the cell wall (Hutchison *et al.,* 2002). These results suggest that multiple components exist in the ECM, and are involved in germling adhesion.

3.2.3 *Botrytis cinerea*

B. cinerea is an important fungal pathogen of a number of food and ornamental crops. Conidium adhesion to host surfaces is an important early event in the infection process. Adhesion ability is correlated with the water contact angle of the substrate, suggesting that hydrophobicity is important for conidium adhesion (Doss *et al.,* 1993). However, the percent adhesion of germinated conidia is larger than that of ungerminated conidia (Doss *et al.,*

1993). The adhesion ability of *B. cinerea* seems to be strengthened with the differentiation of germlings. Germling attachment was resistant to removal by boiling or by treatment with hydrolytic enzymes, periodic acid, or sulfuric acid, but was readily removed by a strong base, sodium hydrate (Doss *et al.*, 1995). The ECM components of germ tubes detected the presence of enzymatic activities such as polygalacturonase, laccase, and cutinase (Doss, 1999). Moreover, the ECM of germlings contained melanin (Doss *et al.*, 2003). These components were similar to *M. oryzae* and are assumed to be involved in adhesion. The class I hydrophobin *BcHpb1* was also partially involved in adhesion, although the disruption mutant of *BcHpb1* retained pathogenicity (Izumitsu *et al.*, 2010).

3.2.4 *Ustilago violacea* (*Microbotryum violacea*)

The anther smut fungus *Ustilago* (*Microbotryum*) *violacea* propagates yeast-like sporidial cells. The cell walls of smut contained a hair-like appendage (fungal fimbriae) that was similar to the Gram-negative bacteria cell wall appendage, fimbriae (Poon & Day, 1974). The fungal fimbriae might be essential for the later stages of conjugation (Poon & Day, 1975). The component of fungal fimbriae contained protein with motifs similar to collagen (Celerin *et al.*, 1996). Collagen is characteristic of the glycine-rich repeat motif, and functions in the cell adhesion of animal connective tissues. Collagen family proteins seem to have a conserved conformation between animal and fungi, although amino acid sequences are divergent. The relationship between the fungal fimbriae of smut and the fibrillar ECM of spore coat in other fungal species remains unclear.

3.2.5 Other fungi

Conidium adhesion was analyzed, and differences were found in various plant pathogenic fungi. In *Bipolaris sorokiniana* and *Venturia inaequalis*, conidium adhesion to the hydrophobic surface is weak, due to the hydrophobic interaction (Apoga *et al.*, 2001; Schumacher *et al.*, 2008). In *Nectria haematococca*, 90 kDa glycoprotein containing mannose sugar was associated with the development of the adhesiveness of macroconidia (Kwon & Epstein, 1997). In *V. inaequalis*, conidia released adhesive material that was termed spore tip glue (STG) (Schumacher *et al.*, 2008). The STG was ConA-negative, suggesting that it was different to STM found in *M. oryzae* (Hamer *et al.*, 1988; Schumacher *et al.*, 2008). These adhesion molecules of ungerminated conidia were pre-synthesized and preserved.

After the germination of conidia in most plant pathogenic fungi, the adhesion ability is increased by the production of ECM from germ tubes. In *Uromyces vicia-fabae* and *Blumeria graminis*, cutinase and esterase in the extracellular matrix (ECM) appeared to play a function in adhesion, either on the hydrophobic or hydrophilic surface, even during pre-germination (Deising *et al.*, 1992; Tucker & Talbot, 2001). Treatment with serine-esterase inhibitor diisopropyl fluorophosphate prevented adhesion (Deising *et al.*, 1992). This evidence suggests that adhesion may be partially involved in the combination of hydrophobin with cutinase as a molecular mechanism in *A. oryzae* (Takahashi *et al.*, 2005). However, the components of other ECM remain undetermined.

In the insect pathogenic fungus *Metarhizium anisopliae*, two proteins, MAD1 and MAD2, were involved in adhesion (Wang & Legar, 2007). MAD1 was involved in adhesion to the

insect surface, blastospore production, and virulence to caterpillars. In comparison, MAD2 was involved in adhesion to the plant surface, but showed no effect on fungal differentiation and entomopathogenicity (Wang & Legar, 2007).

4. Detachment of germlings from the host surface

Fungal adhesion to the host cells is expected to be one of the pathogenicity factors (K. Inoue *et al.*, 2007). The regulation of fungal adhesion is expected to lead to disease control. The adhesion mechanisms of pathogenic fungi are common, with specific features existing in each fungus. We attempted to detach pathogenic fungi from the host or artificial substrate by using enzymatic activity.

4.1 Effects of hydrolytic enzymes on appressorium formation and adhesion of *M. oryzae* germlings

Various types of hydrolytic enzymes were tested for detachment activity. In enzyme treatments of 1 hpi, germlings were detached without affecting appressorium formation, when using α-mannosidase, β-mannosidase, pronase E, collagenase N-2, collagenase S-1, and gelatinase B (K. Inoue *et al.*, 2011). In the enzyme treatments of 6 hpi, most germlings produced appressoria, with the inhibition of ECM production being difficult. In this situation, pronase E and all types of collagenase and gelatinase caused a significant detachment of germlings (K. Inoue *et al.*, 2011). Pronase E is known to be a mucoprotein-degrading enzyme, and had the ability of moderate removal effect in *B. sorokiniana* (Apoga *et al.*, 2001). In particular, collagenase type S-1 and gelatinase B seemed to be the most effective ECM target-specific enzymes, and had a minimal effect on appressorium formation, even at the early stages of application. Mannose moiety was also a target for ECM degradation. However, there are some discrepancies with a previous study; Xiao *et al.* (1994) reported that α-glucosidase and α-mannosidase are effective in removing germlings; however, we found that β-mannosidase is more effective (K. Inoue *et al.*, 2011). These time-lapse experiments clearly show that the timing of adding enzymes influenced the results, which might explain the discrepancies between the two studies. The mannose-degrading enzymes were effective at the early stage, but became ineffective with time. Most of the other substrate-specific enzymes, such as glycan-, protein-, and lipid-degrading enzymes, were difficult to degrade (K. Inoue *et al.*, 2011). These results suggest that the adhesive compounds of ECM consist of glycoproteins with mannose sugars, which gradually accumulate with time. In the pathogenicity test on the host plant, lesion formation was remarkably suppressed on the treatment with crude collagenase, collagenase S-1, and gelatinase B (K. Inoue *et al.*, 2011). A similar detachment effect by crude collagenase was also reported in the *Alternaria alternata* Japanese pear pathotype (Hyon *et al.*, 2009), suggesting universality in filamentous fungi.

4.2 SEM analysis of enzyme effects on the adhesion of *M. oryzae* germlings to the host leaf surface

To examine the effect of the enzymes on germling detachment to the leaf surface, each enzyme was applied to *M. oryzae* germlings at the appropriate time, and incubated for up to 25 hpi. The specimens were then observed by using SEM. It was difficult to ascertain

whether the absence of spores was the result of the enzyme treatments or the lack of spores at the onset of the experiment. *M. grisea* reportedly produces cutinases (Skamnioti & Gurr, 2007; Sweigard *et al.*, 1992). Therefore, this pathogen can degrade the wax of plant surfaces. The detached infection structure would be recognizable as vestiges of degraded wax on the wheat surface. In the treatment with cellulase or protease, the infection structures tightly adhered onto the surface (K. Inoue *et al.*, 2011). In contrast, the treatment with crude collagenase or gelatinase B (matrix metalloproteinases; MMPs) resulted in the detachment of germlings, and vestiges of the presence of germlings were observed (K. Inoue *et al.*, 2011). The detachment effect caused by the treatment with crude collagenase was validated at 18 hpi. In contrast, the detachment effect was cancelled out at 24 hpi, at which time fungal penetration into the host was established.

The SEM observation clearly shows the effects of the enzymes on the degradation of the interface between host plant and germlings. We demonstrated that the reduction in pathogenicity is attributable to the detachment of germlings based on treatment with effective enzymes. In this study, MMPs were confirmed to be useful for protecting wheat from *M. oryzae*.
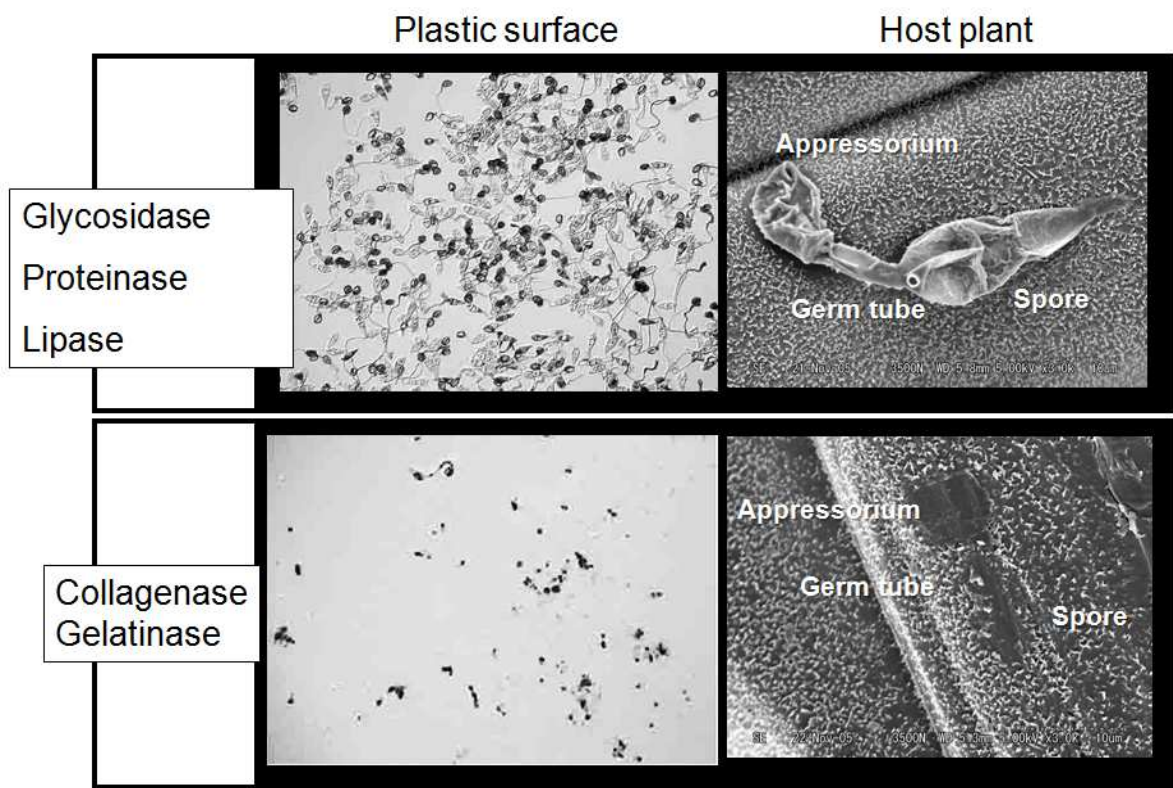

Fig. 7. Detachment effects of *M. oryzae* germlings following treatment with hydrolytic enzymes. Left photos: light microscopy; right photos: scanning electron microscopy. The treatments with collagenase were detached, and only the vestiges of germlings were observed (lower photos). Other hydrolytic enzymes, such as glycosidase were, not affected by germling detachment (upper photos).

4.3 Screening of gelatinolytic bacteria for biological control

To screen collagenolytic/gelatinolytic bacteria, we examined two different screening methods, i.e., (1) utilization of a leaf-associating bacteria library and (2) direct screening from the field, and incubation of samples with collagen (Fig. 8). Collagen is fibrous and difficult to catabolize for nutrient acquisition; therefore, we expected that direct screening with collagen incubation would allow the enrichment of high gelatinolytic genera (Shimoi et al., 2010).

The leaf-associated library was screened for gelatinolytic activity on a 96-microtiter plate (first screening) and Petri dish (second screening). In the case of direct screening samples from the field, five bacterial isolates, which showed especially high gelatinolytic activity, were further characterized as *Acidovorax* sp. (Ac1), *Chryseobacterium* sp. (Ch1 and Ch2), *Sphingomonas* sp. (Sp1), and *Pseudomonas* sp. (Ps1) (Shimoi et al., 2010). The effects of the proteinase inhibitors, EDTA, antipain, aprotinin, and PMSF on bacterial gelatinolytic activity were evaluated. The gelatinolytic activities of Ac1, Ch1, Ch2, and Sp1 were inhibited by EDTA in a dose-dependent manner (Shimoi et al., 2010). In contrast, gelatinolytic activity in Ps1 was not inhibited by EDTA (Shimoi et al., 2010). Bacterial gelatinolytic activities were not inhibited by antipain, aprotinin, or PMSF. These enzymatic characters suggested that all isolates, except Ps1, produced metalloproteinases. Therefore, we examined the effects of each metal cation on gelatinolytic activity in the presence of 5 mM EDTA. The gelatinolytic activities of Sp1 and Ac1 were restored by supplementation with 10 mM Ca^{2+} (Shimoi et al., 2010). In contrast, the gelatinolytic activity of Ch1 and Ch2 were restored by supplementation with either 10 mM Ca^{2+} or 1 mM Zn^{2+}, and to a lesser extent with 2 mM Mn^{2+} (Shimoi et al., 2010).
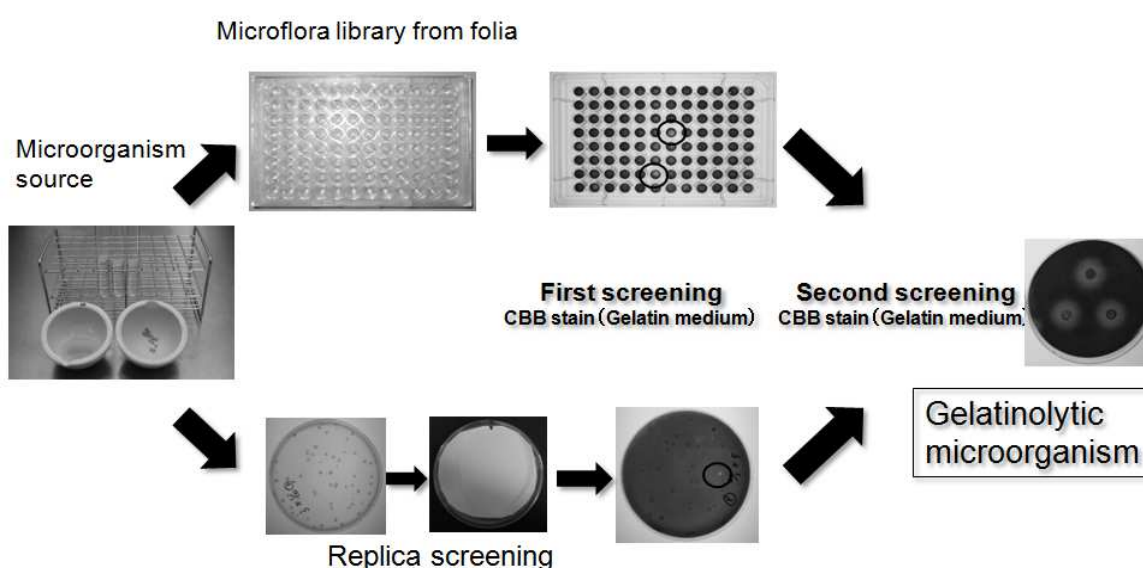

Fig. 8. Screening method to isolate gelatinolytic bacteria from nature. Adapted from Shimoi *et al*. (2010), with permission.

The effects of bacterial gelatinolytic activity on the germling adhesion of *M. oryzae* were evaluated. Treatment with each of the five bacterial isolates significantly decreased the percentage of germling adhesion (Fig. 9). In particular, the effects of Ac1 and Ch1 on germling detachment were comparable to the effects of commercial collagenase placed simultaneously on spore suspensions (Fig. 9). The detachment of germlings decreased when bacterial cultures were added 6 h after the inoculation of the spore suspension. Germling

adhesion was restored when EDTA was added to the mixtures of bacteria and spore suspension, except in the case of Ps1.
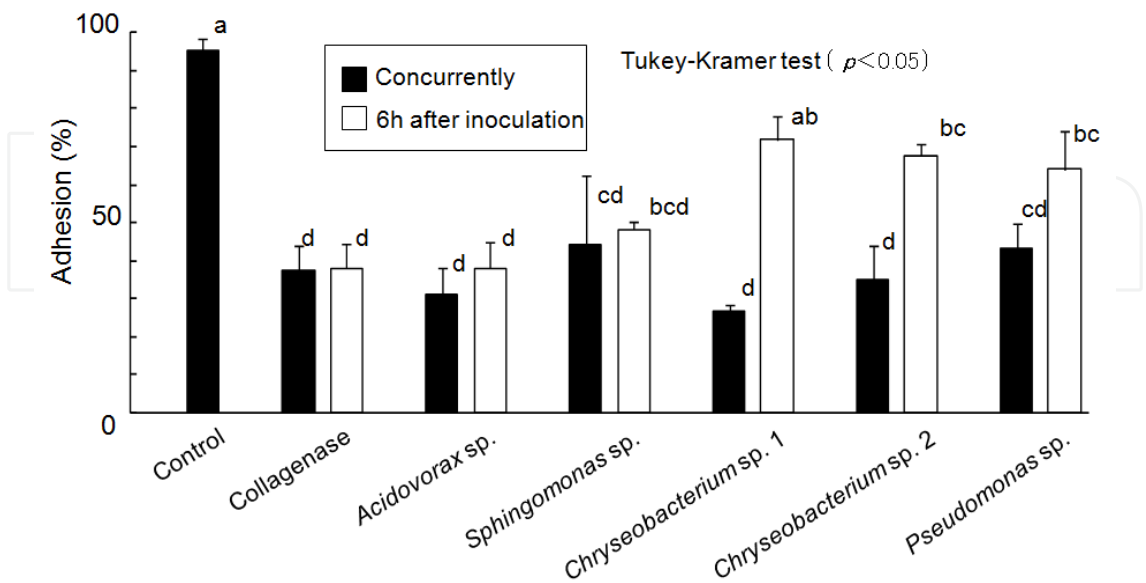

Fig. 9. Detachment effect of *M. oryzae* germlings treated with gelatinolytic bacteria or commercial collagenase. Gelatinolytic bacteria and collagenase were treated concurrently black bars) or 6 hours after inoculation (white bars) with *M. oryzae* spores. Adapted from Shimoi *et al*. (2010), with permission.

We evaluated the protective effects of the selected bacterial isolates on rice blast disease. Spore suspensions of *M. oryzae* and each bacterial culture were inoculated onto rice leaves (cv. Lijiangxintuanheigu; LTH). The treatment with all the bacterial isolates significantly decreased disease indices compared to the control (Fig. 10, Shimoi *et al*., 2010).
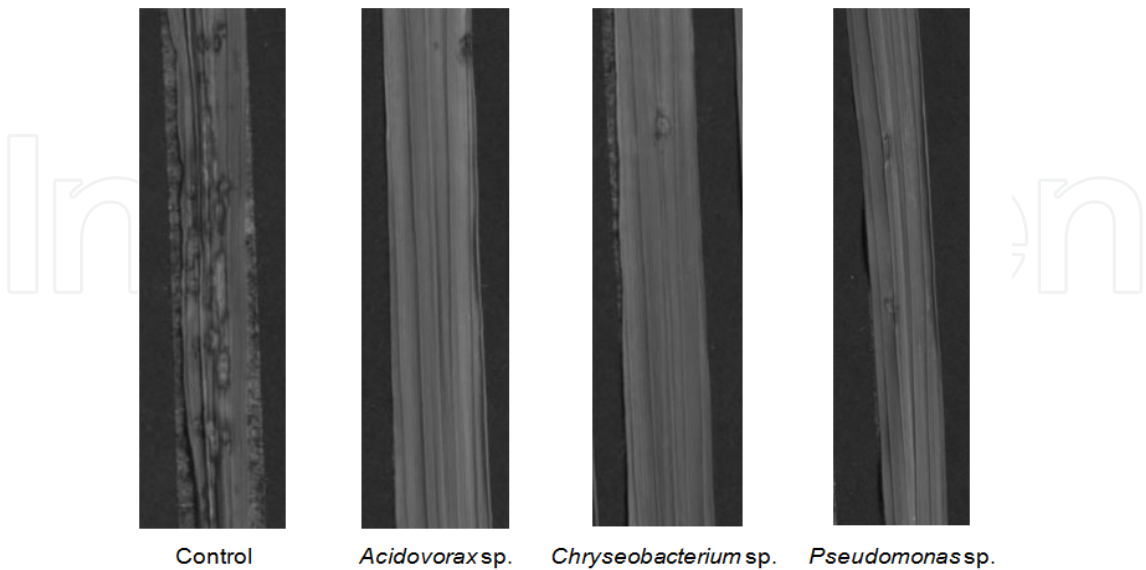

Fig. 10. Disease symptoms of rice blast inoculated with *M. oryzae* and gelatinolytic bacteria. Control: *M. oryzae* spore suspension alone. Adapted from Shimoi *et al*. (2010), with permission.

5. Conclusion

Germling adhesion is a general feature of plant pathogenic fungi, but its components are complicated. This adhesion ability is considered to be a promising a target for protection against disease. We found that glycoprotein degrading enzymes effectively detach germlings, while gelatinolytic bacteria showed a protective effect against disease. If these gelatinolytic bacteria can produce cell lytic enzymes, such as chitinase or glucanase, efficiency against disease protection would increase synergistically. Our future goals are to screen specific bacteria that produce multiple enzymes and provide a stable habitat in the foliar environment.

6. Acknowledgments

7. References

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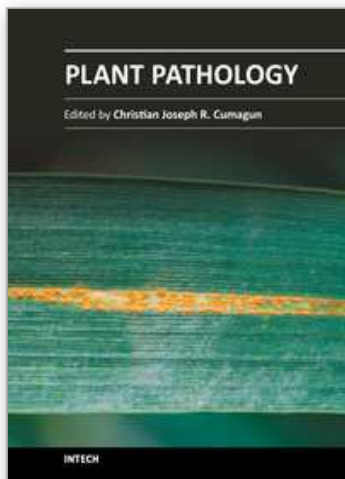
Plant Pathology

Edited by Dr. Christian Joseph Cumagun

Plant pathology is an applied science that deals with the nature, causes and control of plant diseases in agriculture and forestry. The vital role of plant pathology in attaining food security and food safety for the world cannot be overemphasized.

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