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Reaction of glucosinolate-myrosinase defence system in *Brassica* plants to pathogenicity factor of *Sclerotinia sclerotiorum*

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Abstract The glucosinolate-myrosinase defence system, specific to Brassicales plants, produces toxic volatile compounds during mechanical injury or pathogen attack. The reaction of this system to oxalic acid, known as a pathogenicity factor of *Sclerotinia sclerotiorum*, is not fully understood. The hydrolysis of glucosinolates was studied at varying conditions in the presence of oxalic acid in the substrate. In a bioassay, colonies of the pathogen were exposed to volatiles from hydrated mustard powder used as a myrosinase and glucosinolate source. The glucosinolate-myrosinase (GSL-M) system was activated in the presence of oxalic acid at a concentration and pH similar to that expected *in vivo*. Volatile production was inhibited only when the pH fell to 3 or below. It is unlikely that oxalic acid plays a significant role in disarming the GSL-M system during infection of *Brassica* hosts.

Keywords Oxalic Acid · *Sclerotinia sclerotiorum* · Glucosinolate-Myrosinase · *Brassica*

Sclerotinia sclerotiorum is a major pathogen of oilseed rape (*Brassica napus*) worldwide (Rahmanpour et al. 2009). During infection, the fungus must be able to overcome defence responses of the host. As a member of the Brassicaceae, oilseed rape contains a range of glucosinolates (GSLs) (Kirkegaard and Sarwar 1999). Upon wounding by infection or pest attack, GSLs are exposed to hydrolytic enzymes (myrosinases; M) (Mithen 2001). The myrosinase-mediated degradation of GSLs results in the production of isothiocyanates (ITCs), thiocyanates, nitriles and elemental sulphur. The specific hydrolysis product varies depending on the concentration of H^+ and other factors. ITCs are usually produced at neutral pH and have broad biocidal activity including fungicidal effects (Brown and Morra 1997). Researchers have reported toxic effects of ITCs on many plant pathogenic fungi including *S. sclerotiorum* (Manici et al. 1997; Smith and Kirkegaard 2002). In contrast, nitrile derivatives of GSLs occur at lower pH (Bones and Rossiter 1996) and in particular have very weak inhibitory effects on fungi (Brown and Morra 1997).

Manici et al. (1997) observed inhibition of pathogen growth by ITCs *in vitro*. Despite this, *S. sclerotiorum* is able to infect *Brassica* tissues. *S.*

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sclerotiorum has been shown to develop tolerance to ITCs after initial exposure to sub-lethal doses (Rahmanpour et al. 2009). However, it is possible that it may employ additional mechanisms to avoid the effects of the GSL-M system during infection. One such method could be an interaction between the system and oxalic acid secretion. It is possible that this may alter the final products of glucosinolate hydrolysis to less toxic substances, or reduce the toxicity of volatiles sufficiently to allow the pathogen to adapt to them.

Oxalic acid (OA) is a virulence factor of several phytopathogenic fungi, including *S. sclerotiorum.* A strong correlation between *S. sclerotiorum* pathogenesis, virulence, and OA secretion has been shown (Dutton and Evans 1996). Dong et al. (2008) transformed oilseed rape with a gene for oxalate oxidase from wheat. Transgenic plants had increased resistance to *S. sclerotiorum*, and also resisted the lowering of tissue pH when OA was applied to leaves. The results of Dong et al. (2008) confirm the importance of OA as a virulence factor. The role of OA secreted by *S. sclerotiorum* during pathogenesis has been reviewed by Bolton et al. (2006) and Hegedus and Rimmer (2005). According to Bolton et al. (2006), decreasing the ambient pH is considered to be the most important action of the acid after which some kinds of phytopathogenic activities by the fungus occur. In addition, Guimaraes and Stotz (2004) found that OA production by *S. sclerotiorum* deregulates stomatal closure. However, it is unclear whether OA prevents activation of the host resistance mechanisms (Prusky and Yakoby 2003). Given that OA production by the fungus is thought to interfere with the host's regulation of stomatal closure and decrease of ambient pH whereas myrosinase-driven breakdown of glucosinolate triggers the formation of volatile antifungal compounds, it is not clear in what way OA could have an impact on the glucosinolate-myrosinase system. To examine the possible role of OA, we studied the activity of the GSL-M system in presence of the acid and relevant pH levels.

In the present study, isolate RS5 of *S. sclerotiorum*, collected from canola fields near Orange, NSW, Australia (Rahmanpour et al. 2009) was used. The bioassay method was adapted from a protocol described by Sexton et al. (1999) with some modifications. Growth of the pathogen was evaluated in the presence of volatiles released from English mustard powder (Ward McKenzie, Melbourne, Australia). This is produced from a blend of brown (*Brassica juncea*) and white (*Sinapis alba*) mustards containing allyl GSL and myrosinase sources, respectively (Wickens 2001; Bellostas et al. 2007). Addition of water to this system allows glucosinolate hydrolysis to occur.

Agar plugs (4 or 7 mm diameter) containing hyphae of *S. sclerotiorum* were transferred from the margins of 3-day-old actively growing cultures to 85 mm diameter glass plates containing a thin layer of potato dextrose agar. Mustard powder was weighed into individual white plastic weighing boats (2 ml capacity). These were placed in the upturned lid of each plate, whilst the inverted bottom containing the fungal plugs was held aside. Water, buffer or OA solutions were pipetted onto the mustard powder depending on the experiment. The inverted bottoms of the plates were replaced, thereby positioning the fungal plugs over the containers of mustard powder. Plates were immediately sealed with Parafilm, and colony diameter, after subtracting the diameter of the inoculum plug, was recorded after 24 h incubation at 20°C in darkness. The growth inhibition by volatiles released from the mustard powder was calculated for each treatment using the following formula:

$$\mathrm{GI} = 100 \times \frac{\text{Control diameter} - \text{treatment diameter}}{\text{Control diameter}}$$

Mustard powder was used at 3 experimental quantities, 2, 5, and 10 mg. The mustard powder samples were mixed with distilled water, solutions of OA or buffer at a rate of 10 $\mu l mg^{-1}$. Initial experiments used a concentration of 10 mM OA with the pH adjusted to 4.0 as this has been reported to mimic conditions in infected tissue (Kolkman and Kelly 2000). The pH of OA solutions was adjusted to the stated value with NaOH. The effect of pH on the GSL-M system was investigated using 0.1 M sodium acetate buffer, pH 4.0, and 0.1 M sodium phosphate buffer, pH 7.0. The latter pH is considered optimum for release of ITCs (Bones and Rossiter 1996). Control plates for determination of growth inhibition had containers with the solutions (20 μl), but no mustard powder. The experiments followed a completely randomized design with three replications. Mustard powder mixed with sterile distilled water was used as a control and in addition, 0.1 M sodium acetate buffer at pH 4.0 was used to separate any effect of pH from that of OA. Results were analyzed

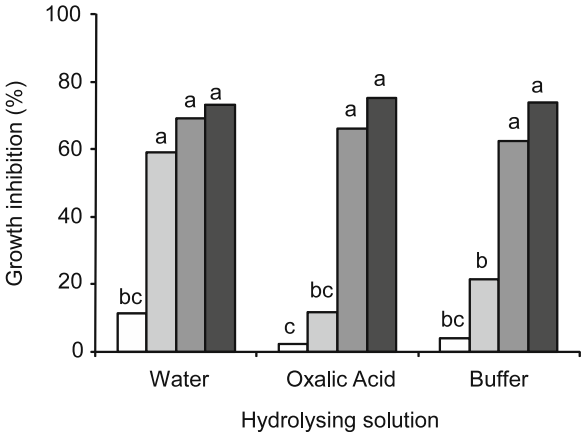

Fig. 1 Growth inhibition of *Sclerotinia sclerotiorum* by volatiles derived from 0 mg (□), 2 mg (▨), 5 mg (▩) or 10 mg (■) mustard powder hydrolyzed in oxalic acid (10 mM, pH 4.0), acetate buffer (0.1 M, pH 4.0) or sterile distilled water (SDW). Columns labelled with the same letter are not significantly different

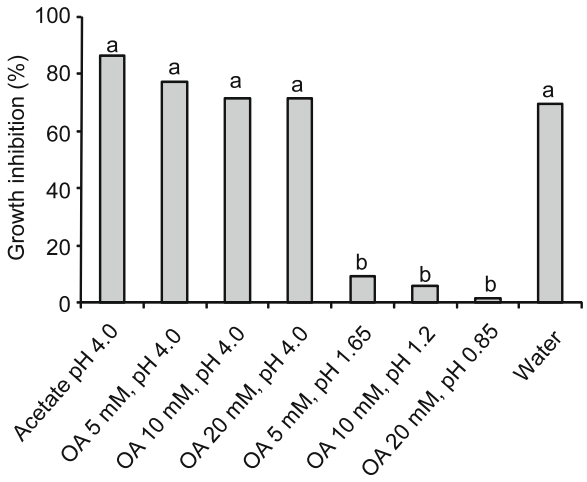

Fig. 3 Growth inhibition of *Sclerotinia sclerotiorum* by volatiles derived from mustard powder (5 mg) hydrolysis in three concentrations of oxalic acid buffered to pH 4.0 with 0.1 M acetate buffer, or left un-buffered. Columns labelled with the same letter are not significantly different. SDW, sterile distilled water

by ANOVA, using the SPSS statistical program and significant ($P<0.05$) differences separated using the Student-Newman-Keuls test.

Toxic volatiles were produced, reflected by significant inhibition of the radial growth of *S. sclerotiorum* (Fig. 1). Only the effect of mustard powder quantity was significant; there was no difference between OA, distilled water and acetate buffer treatments and no interaction with the quantity of mustard powder.

As distilled water itself is often acidic (pH 6.6–6.8), a further experiment was carried out in which volatile production at pH 4.0 was compared to that with OA or 0.1 M phosphate buffer at pH 7.0. There was no significant difference between inhibitory volatiles produced at pH 4.0 and 7.0 with either buffer or 10 mM OA (Fig. 2). In a third experiment, growth inhibition by volatile production from mustard powder was monitored in the presence of OA at three different concentrations with no pH adjustment, and

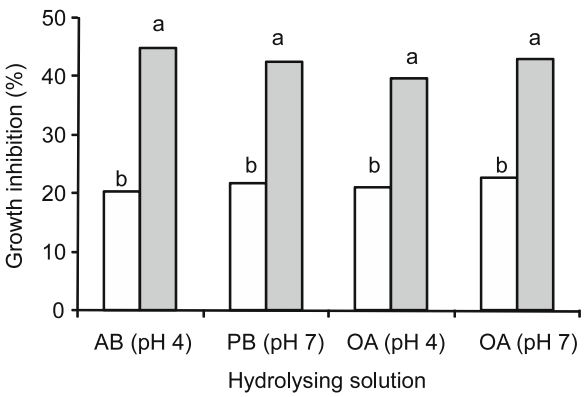

Fig. 2 Growth inhibition of *Sclerotinia sclerotiorum* by volatiles derived from 2 mg (□) or 5 mg (■) of mustard powder hydrolyzed in acetate buffer (AB) (0.1 M, pH 4.0), phosphate buffer (PB) (0.1 M, pH 7.0) or 10 mM oxalic acid adjusted to pH 4.0 or pH 7.0 with NaOH. Columns labelled with the same letter are not significantly different. SDW, sterile distilled water

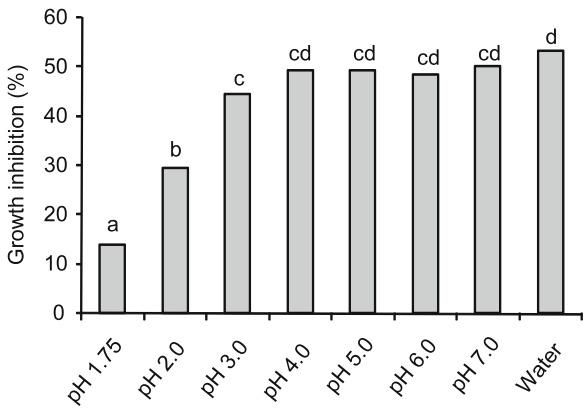

Fig. 4 Growth inhibition of *Sclerotinia sclerotiorum* by volatiles derived from mustard powder (5 mg) hydrolysis in 10 mM oxalic acid adjusted to different pH values with NaOH. Columns labelled with the same letter are not significantly different. SDW, sterile distilled water

with pH adjusted to 4.0 with sodium acetate buffer (Fig. 3). The pH of OA at 5 mM, 10 mM and 20 mM was measured to be 1.65, 1.2 and 0.85, respectively. In this experiment, all three concentrations of OA almost totally prevented production of inhibitory volatiles from the mustard powder. The effect of presence or absence of buffer in the OA treatments was highly significant, but there was no significant difference between concentrations.

To determine the pH at which volatile production was affected, the experiment was repeated with mustard powder added to 10 mM OA with pH values adjusted to a range from 1.75 to 7.0. A highly significant effect of pH on growth inhibition was found, indicating production of volatiles from mustard powder (Fig. 4). The inhibition of *S. sclerotiorum* growth in the presence of mustard powder at acidic pH was significantly less than at higher pH. No differences were seen in growth inhibition in the presence of buffers between pH 4.0, and 7.0. However at pH 3.0, inhibition of fungal growth was significantly reduced, with further reductions at pH 2.0 and pH 1.75.

The experiments indicated that release of toxic volatiles happens in the presence of OA at a physiological concentration (10 mM) and pH (4.0) expected to mimic conditions in infected tissue (Kolkman and Kelly 2000). This indicates that OA would have a neutral role on hydrolysis of GSLs leading to production of toxic volatile ITCs. Furthermore, this also suggests that GSLs are hydrolyzed to produce significant quantities of toxic volatiles in the presence of the acid. Release of inhibitory volatiles during infection of oilseed rape leaves was also observed by Rahmanpour et al. (2009). Inhibitory quantities of volatiles were produced under conditions both favourable for the pathogen activity in host tissues (pH 4.0) and optimum for myrosinase (neutral pH). The lack of effect was not due to saturation of the fungal response as differences were observed with different quantities of mustard powder.

Inhibition of the GSL-M system was observed only when OA with a pH of 3 or below was used. The inhibitory effect of substrate conditions increased as pH decreased below 3. When compared to previous experiments, the results suggest that the pH of the OA solutions was a more important factor than OA itself. Borek et al. (1994) reported that decomposition of sinigrin, the main GSL component of mustard seeds, yields nitrile of allyl-GSL as the major reaction product in acid solutions below approximately pH 4.0. Decomposition of sinigrin in less acid solutions with pH values above 4.0 yielded allyl ITC as the major reaction product. A significantly reduced fungal growth inhibition by mustard powder-derived volatiles was restricted to pH<3. A largely reduced total amount of toxic volatiles released under these non-physiological conditions must be considered as a likely explanation for the effects observed. The results from Borek et al. (1994) are in agreement with a significant effect of pH 3.0 and below on production of inhibitory volatiles. This suggests that myrosinase isoenzymes in mustard powder are able to produce effective amounts of ITCs through GSL hydrolysis under a wide range of pH conditions.

Whereas acidification by OA reduced the production of toxic volatiles in the bioassay, the pH required for significant reduction was considerably lower than has been reported in plant tissues infected with *S. sclerotiorum* (Bolton et al. 2006). This suggests that it is not likely to be an important mechanism for avoiding toxicity of GSL breakdown products and the pathogen growth-inhibiting volatiles are produced at a wide range of pH levels regardless of OA itself.

In conclusion, the GSL-M defence system of *Brassica* plants reacts to produce antifungal volatile compounds in substrates affected either directly or indirectly (effect on pH) by OA. In other word, this pathogenicity factor, produced by *S. sclerotiorum* during infection, does not overcome the system to protect the pathogen. Therefore, the pathogen is likely to employ another pathway to disarm the host defence mechanisms. Furthermore, it has been documented that GSLs are required as components of the plant defense response against microbial pathogens (Clay et al. 2009). The conjugation of the ITCs with glutathione through the proposed activity of glutathione-S-transferase or peptidase enzymes *in vivo* (Bednarek et al. 2009), appears more likely than direct or indirect effect of oxalic acid on the GSL-M system.

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