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Correlations between disease severity, glucosinolate profiles and total phenolics and *Xanthomonas campestris* pv. *campestris* inoculation of different Brassicaceae

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ABSTRACT

Many Brassicaceae species are economically important crops and *Xanthomonas campestris* pv. *campestris* (*Xcc*), the causal agent of black rot, is considered one of the most important necrotrophic plant bacterial diseases occurring worldwide on these and many other crops. Therefore identifying resistance mechanisms and genes is crucial. Researchers continue to investigate the role of phytochemicals (plant secondary metabolites) in protecting plants against diseases and pathogens. Glucosinolates (GLS), and more specifically their hydrolysis products, are known to have various biological effects including antimicrobial activity. From the positive results of initial *in vitro* studies with *Xcc* and other pathogenic bacteria new experiments were designed to evaluate the possible in planta role of GLS, and also phenolics, in the interaction with *Xcc*. The in planta studies, with various Brassicaceae seedlings, have shown a correlation between GLS profiles, and therefore the subsequent hydrolysis products, and the inhibition of *Xcc* growth. There were no significant correlations between *Xcc* infection and total phenolics. Positive correlations were found between specific and total GLS contents and the severity of disease. Further *in vitro* and in planta studies need to be performed to evaluate the role of GLS and other defense mechanisms in *Xcc* and other important bacterial infections of Brassicaceae crops.

1. Introduction

Exposure of plants to bacterial pathogens involves complex interactions between the bacteria and the plants; leading to compatible (susceptible), resistant (hypersensitive reactions) or non-host reactions (Leach and White, 1996; He, 1998; Dow et al., 2000; Glazebrook, 2005; Beattie and Lindow, 1999; Göhre and Robatzek, 2008). The mode of bacterial infection, necrotrophic or biotrophic, also affects the type of host reactions in relation to defense (Glazebrook, 2005). The Gram-negative aerobic bacteria *Xanthomonas campestris* pv. *campestris* (*Xcc*) is the causal agent of black rot, and is considered one of the most important necrotrophic bacterial diseases occurring worldwide on all Brassica, other members of the Brassicaceae, and a few wild Capparales species (Leyns et al., 1984; Williams, 1980; Alvarez, 2001). *Xcc* predominantly infects plants through the hydathodes (water-pores) on the leaf margins and from there infects the vascular tissues (Alvarez, 2001). Some races can also infect through the stomata and colonize the apoplastic spaces before penetrating the vascular tissues, but optimal conditions for this type of infection are rare because it requires modifications to the cuticular waxes around the stomata (reduced hydrophobicity) (Alvarez, 2001). Other sites of infection include the roots and often through wounds, in both cases the ultimate site of infection is the vascular tissues (Alvarez, 2001). Symptoms include V-shaped necrotic lesions on leaves, rots, darkening of vascular tissue with extensive wilting and necrosis, and also chlorosis (Leyns et al., 1984; Williams, 1980; Alvarez, 2001; Kamoun et al., 1992). *Xcc* was originally divided into five races designated 0, 1, 2, 3 and 4 (Kamoun et al., 1992). Later studies further refined the designation

Abbreviations: *Xcc*, *Xanthomonas campestris* pv. *campestris*; GLS, glucosinolate[s]; 3-MSP, 3-methylsulfinylpropyl (glucoiberin); 4-MSB, 4-methylsulfinylbutyl (glucoraphanin); SIN, 2-propenyl (Sinigrin); 4-MERC, 4-mercaptobuthyl (glucosativin); BENZYL, glucotropaeolin; 4-MTB, 4-methylthiobutyl (glucoerucin); 3-IM, 3-indolylmethyl (glucobrassicin); 4-MIM, 4-methoxy-3-indolylmethyl (4-methoxyglucobrassicin); NIM, *N*-methoxy-3-indolylmethyl (neoglucobrassicin); SFN, sulforaphane; BITC, benzylisothicyanate; PEITC, 2-phenylethylisothiocyanate; GAE, gallic acid equivalents.

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of *Xcc* races into 1–6; the designation "0" became "6" to avoid the implication that race "0" was avirulent (Vicente et al., 2001). It was also found that *Xcc* races 1 (62%) and 4 (32%) were predominant and that the other races were rare and often host-specific (Vicente et al., 2001).

Brassica oleracea is highly susceptible to *Xcc* whereas 71 *Brassica juncea* is highly resistant (Taylor et al., 2002).

In recent decades researchers have been evaluating the roles of phytochemicals (secondary plant metabolites) in protecting plants against pathogens (bacteria, fungi, and nematodes) and pests (various phytophagous insects) (e.g., Cowan, 1999; Bennett and Wallsgrove, 1994). Plants, including the Brassicaceae crops, produce phytochemicals as a part of their normal program of growth and development (inbuilt chemical barriers; structural barriers such as lignin, and pre-formed phytoanticipins such as glucosinolates) or *de novo* synthesis in response to pathogen attack or stress (phytoalexins) (Bennett and Wallsgrove, 1994). Numerous studies have suggested that plant–pathogen interactions are partially mediated via phytochemicals production, despite inconsistencies revealed by some works on the ability of particular compounds to provide resistance to a specific pathogen (Bennett and Wallsgrove, 1994).

Glucosinolates (GLS) are a group of secondary organic anionic plant metabolites containing β-D-thioglucose and sulfonated oxime moieties and have been implicated in plant defense in Brassicaceae (syn. Cruciferae) crop plants, comprising 96 a variety of aliphatic, aromatic and indole-based side chains (Table 1) (Rosa et al., 2007; Halkier and Gershenzon, 2006). They are synthesized from amino acids and the biosynthetic pathways have been elucidated and recently major progress has been made on the identification of the genes responsible for the biosynthesis of the core GLS structures (Halkier and Gershenzon, 2006). GLS co-occur with the enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), which is responsible for GLS hydrolysis, during infection and physical damage (Rosa et al., 1997). The enzymatic catabolism can yield several hydrolysis products including isothiocyanates, nitriles, epithionitriles, and thiocyanates depending to the respective GLS present in the tissue, and conditions under which hydrolysis occurs (Rosa et al., 1997). The increasing interest in GLS is based on the biological properties exhibited by the various hydrolysis products e.g., antimicrobial and anticarcinogenic activities (Rosa et al., 2007; Juge et al., 2007). GLS hydrolysis products, such as the isothiocyanates, have been recognized for their potential as "biofumigation" agents due their ability to suppress several soil-borne pathogens and phytophagous nematodes (Gimsing and Kirkegaard, 2006; Warton et al., 2001). Another group of plant secondary metabolites associated with plant defense mechanisms are the (poly)phenolics, a very diverse class of phytochemicals with roles in cell wall function and active defense mechanisms against bacterial and fungal pathogens (Nicholson and Hammerschmidt, 1992; Hammerschmidt, 1999). Despite promising initial results with phytochemicals, and different studies conducted with plant pathogenic organisms, fewer studies have been conducted with plant pathogenic bacteria, and much less with *Xcc* (Tierens et al., 2001). A recent study has shown that several classes of GLS hydrolysis products were active *in vitro* against *Xcc* and several other important plant pathogenic bacteria (Aires et al., 2009).

The aim of the current study was to evaluate 121 the potential role of GLS, and their respective enzymatic hydrolysis products, against *Xcc* infection. Thus in planta experiments were designed to evaluate the variations in profiles and contents of GLS and total phenolics in young plants of common crop Brassicaceae before and after inoculation with *Xcc* in order to evaluate how the initial profiles, and post-inoculation profiles, were correlated with the development of the disease.

2. Materials and methods

2.1. Plant material and chemicals

For the in planta experiments different crop Brassicaceae were selected – cress (*Lepidium sativum*), salad rocket (*Eruca sativa*), broccoli (*Brassica olerecea* L. var. *italica* cv. Marathon), white cabbage (*B. olerecea* L. var. *capitata* cv. Coração de boi), tronchuda cabbage (*B. oleracea* L. var. tronchuda cv. *Tronchuda* Portuguesa) and; seeds were obtained from local commercial sources. The seeds were sown in 3 l pots with previously sterilized peat and sand mixture in the proportion of 3:1 (v/v). The pots were placed in a growth chamber (Fitoclima 2500 EDTU, Aralab, Portugal) with a 14 h day photoperiod, a photosynthetically active radiation (PAR) of 340 μmol m-2 s-1137, supplied from 5 lamps (Osram HQI-BT 400w, Osram Sylvania, Inc., Danvers, MA, USA), a temperature regime of 25/18 °C day/night, and a constant relative humidity (70%). These conditions were chosen based on the best development of plants and bacteria. When the plants had 3–5 true leaves the plants were inoculated. All chemicals were of analytical grade and were obtained from Sigma/Aldrich (Poole, UK). All water used was ultra-pure (distilled and de-ionized).

2.2. X. campestris pv. campestris inoculation method

The *Xcc* isolate used in the current work was 145 supplied by the Instituto Superior de Agronomia (ISA), Universidade Técnica de Lisboa, Portugal; it had been isolated from Portuguese Brassica but the Race is still being determined (it is probably either Race 1 or Race 4) (Vicente et al., 2001). Isolated colonies were picked from the cultures grown overnight, inoculated into 4.0 ml of 0.9% NaCl solution. The suspensions were prepared by adjusting the turbidity to match 0.5 McFarland standards. When the plants had produced 3–5 true leaves, corresponding to stage 2 of 2 of the Andaloro scale (Andaloro et al., 1983), the oldest fully expanded leaf was inoculated with a bacterial suspension (25 μl) that was injected into the intracellular spaces of the petiole using a syringe (Shaw and Kado, 1988; Sousa et al., 2003). The development of the disease was monitored using a scale adapted from one previously used to express disease severity (Shaw and Kado, 1988). The scale comprises of 5 distinct values: 1 – plant without disease symptoms; 2 – plant with 15% of leaves with symptoms (one or two small lesions at maximum of 1.5 cm diameter), 3 – plant with 15–35% of the leaves with symptoms (three to five lesions between 1.5 and 4.0 cm in diameter), 4 – plant with 35–70% of the leaves with symptoms (five or more lesions well developed, ≥4.0 cm in diameter), 5 – plant with 70–100% of leaves with symptoms (coalescence of the lesions, necrosis of the leaf margins and scorch symptoms on the leaves) and 6 – plant death, with or without soft rot. In the initial studies of disease progress monitoring was done during 20 days for the Brassica species (broccoli, white cabbage and tronchuda) and 10 days for the cress and wild rocket. In the studies to evaluate changes in phytochemicals all species were harvested 10 days post-inoculation. Negative controls were always used (un-inoculated seedlings). The experiments were conducted in triplicate. At the end of the experiments the whole aerial tissues (leaves and stems) were harvested, frozen and freeze-dried for the phytochemical evaluations.

2.3. Glucosinolate determinations

Samples were freeze-dried and reduced to a fine powder. Glucosinolate (GLS) extractions and analyses were done using a previously validated and reported method (Bennett et al., 2007). Essentially 3 ml boiling 90% (v/v) methanol containing 200 μl of 1 mg ml^{-1} benzyl GLS, as an internal standard, was added to 0.2 mg DW of each sample. Boiling was continued for 2 min, to inacti-