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Formation of biphenyl and dibenzofuran phytoalexins in the transition zones of fire blight-infected stems of *Malus domestica* cv. 'Holsteiner Cox' and *Pyrus communis* cv. 'Conference'

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A B S T R A C T

In the rosaceous subtribe Pyrinae (formerly subfamily Maloideae), pathogen attack leads to formation of biphenyls and dibenzofurans. Accumulation of these phytoalexins was studied in greenhouse-grown grafted shoots of *Malus domestica* cv. 'Holsteiner Cox' and *Pyrus communis* cv. 'Conference' after inoculation with the fire blight bacterium, *Erwinia amylovora*. No phytoalexins were found in leaves. However, both classes of defence compounds were detected in the transition zone of stems. The flanking stem segments above and below this zone, which were necrotic and healthy, respectively, were devoid of detectable phytoalexins. The transition zone of apple stems contained the biphenyls 3-hydroxy-5-methoxyaucuparin, aucuparin, noraucuparin and 2′-hydroxyaucuparin and the dibenzofurans eriobofuran and noreriobofuran. In pear, aucuparin, 2′-hydroxyaucuparin, noreriobofuran and in addition 3,4,5-trimethoxybiphenyl were detected. The total phytoalexin content in the transition zone of pear was 25 times lower than that in apple. Leaves and stems of mock-inoculated apple and pear shoots lacked phytoalexins. A number of biphenyls and dibenzofurans were tested for their *in vitro* antibacterial activity against some *Erwinia amylovora* strains. The most efficient compound was 3,5-dihydroxybiphenyl (MIC = 115 μg/ml), the immediate product of biphenyl synthase which initiates phytoalexin biosynthesis.

1. Introduction

Erwinia amylovora is the causal agent of fire blight, known as one of the most destructive diseases of apple (*Malus domestica*) and pear (*Pyrus communis*) cultivars worldwide (Bonn and van der Zwet, 2000). The pathogen can kill trees or entire orchards within a single growing season, leading to devastating economic losses. The total world production of apples and pears in 2009 was around 71 and 22 million tons, respectively (FAO, 2009). The two pome fruit trees belong to the rosaceous subtribe Pyrinae (formerly subfamily Maloideae; Potter et al., 2007), species of which form biphenyls and dibenzofurans as phytoalexins (Kokubun and Harborne, 1995).

Initially, the biphenyls aucuparin (**2**; Fig. 1) and 2′-methoxyaucuparin were isolated from the heartwood of *Sorbus aucuparia* (Pyrinae; Erdtman et al., 1961, 1963). The first dibenzofurans to be detected were α-, β-, and γ-pyrufurans in fungus-infected sapwood of *P. communis* (Kemp et al., 1983; Kemp and Burden, 1984). In 1995, Kokubun and Harborne published a survey of biphenyl and dibenzofuran induction in Pyrinae. A total of six biphenyls and 15 dibenzofurans were isolated from various species after fungal inoculation or natural infection. The accumulation site of the phytoalexins was the sapwood, as observed previously with other classes of phytoalexins formed in trees after wounding, injury or fungal attack (Kemp and Burden, 1986). No Pyrinae species was found to simultaneously accumulate both classes of phytoalexins (Kokubun and Harborne, 1995). *Malus* species were biphenyl producers and *Pyrus* species were dibenzofuran producers. Later, co-occurrence of the two classes of phytoalexins was observed in cell cultures of the *M. domestica* cultivar 'Liberty' treated with an elicitor (Hrazdina et al., 1997; Borejsza-Wysocki et al., 1999). In addition, different patterns of phytoalexins comprising either biphenyls or dibenzofurans as major components were induced by different types of elicitors in cell cultures of *S. aucuparia* (Hüttner et al., 2010). Simultaneous formation of biphenyls and dibenzofurans in intact apple and pear plants after inoculation with the bacterial pathogen, *E. amylovora*, is reported here.

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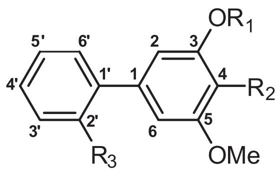
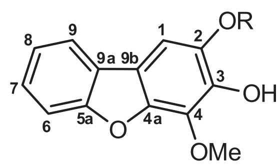


	biphenyl	R_1	R_2	R_3
1	3-hydroxy-5-methoxybiphenyl	H	H	H
2	aucuparin	Me	OH	H
3	noraucuparin	H	OH	H
4	2'-hydroxyaucuparin	Me	OH	OH
5	3,4,5-trimethoxybiphenyl	Me	OMe	H



	dibenzofuran	R
6	eriobofuran	Me
7	noreriobofuran	H

Fig. 1. Chemical structures of biphenyls and dibenzofurans detected in *E. amylovora*-inoculated shoots of the apple cultivar 'Holsteiner Cox' and the pear cultivar 'Conference'.

As *de novo*-formed phytoalexins, biphenyls and dibenzofurans are restricted to the Pyrinae (Kokubun and Harborne, 1995). However, a number of plant families contain the compounds as constitutively formed products (Gottstein and Gross, 1992). Co-occurrence of constitutive biphenyls and dibenzofurans was reported for fruits of *Pyracantha fortuneana* and roots of *Pourthiaea lucida* and *Rhaphiolepis indica* (Dai et al., 2006, 2008; Abd El-Razeka et al., 2007; Lin et al., 2010). The presence of either biphenyls or dibenzofurans as preformed phytoanticipins was found in several plant families (Song et al., 2006; Kim et al., 2009; Shiu and Gibbons, 2009; and literature cited therein).

The carbon skeleton of biphenyls and dibenzofurans is formed by biphenyl synthase (BIS; Liu et al., 2007). Recently, the BIS gene family in the genome sequence of the apple cultivar 'Golden Delicious' was analyzed and the genes were grouped into four subfamilies (Velasco et al., 2010; Chizzali et al., 2012). In the apple cultivar 'Holsteiner Cox', the *BIS3* gene was found to be expressed in stems in response to inoculation with *E. amylovora*, with maximum transcript levels in the transition zone between necrotic and healthy stem segments. Here, we report accumulation of the products in the transition zone.

2. Results and discussion

2.1. Morphological changes in fire blight-infected shoots

Kemp et al. (1983) isolated two dibenzofurans (α- and β-pyrufurans) from a 'dark-pigmented reaction zone' between healthy and diseased sapwood segments of the pear cultivar 'Hendre Huffcap' and also found γ-pyrufuran in the cultivar 'Thorn' (Kemp and Burden, 1984). The authors then detected the biphenyl aucuparin (**2**) in a similar region of the apple (*Malus pumila*) cultivar 'Cox's Orange Pippin' (Kemp et al., 1985). All cultivars were inoculated with the fungus *Chondrostereum purpureum*, the causative agent of the silver leaf disease, and wood samples were analyzed for phytoalexins 8 weeks after inoculation. The present study was carried out with the *P. communis* cultivar 'Conference' and the *M. domestica* cultivar 'Holsteiner Cox', the latter being a seedling of *M. pumila* cv. 'Cox's Orange Pippin' studied by Kemp et al. (1985).

Shoot tips were inoculated with *E. amylovora*, the fire blight-causing bacterium. In both fruit tree cultivars studied, necrosis of the infected shoot tips developed 4 d post-inoculation and a dark-pigmented interphase appeared between the infected and the healthy stem segments. Shain (1979) designated this interphase between the necrotic reaction zone and healthy sapwood as 'transition zone'. In the apple cultivar 'Holsteiner Cox' which was studied here, the transition zone advanced from the infected shoot tip downwards the stem until the 6th week after inoculation and then stopped migrating (Fig. 2). At this time point, the transition zone was about 4 cm long and therefore three stem segments of the same size, namely the transition zone itself, a 4-cm-segment above (necrotic) and a 4-cm-segment below (healthy) were collected for phytoalexin analysis.

While the transition zone in 'Holsteiner Cox' stopped after 6 weeks, shoots of the pear cultivar 'Conference' were entirely necrotic after this time span. As with apple, the 4-cm-transition zone and the 4-cm-segments above and below were analyzed at various times after inoculation until the advancing necrosis embraced the whole shoot.

In both cultivars, necrosis of leaves paralleled necrosis of the stem. At various times, three leaves of 'Holsteiner Cox' and 'Conference' were collected for phytoalexin analysis, namely the leaf with a necrotic vein located closest to the stem's transition zone, the necrotic leaf above, and the healthy leaf below.

2.2. Phytoalexin accumulation in fire blight-infected apple shoots

GC-MS analysis of methanolic extracts from the transition zone and the flanking segments revealed the presence of biphenyls and dibenzofurans exclusively in the transition zone (Fig. 3a). The flanking segments above and below the transition zone lacked detectable quantities of defence compounds. In mock-inoculated shoots, the stem segment corresponding to the transition zone was also devoid of phytoalexins (Fig. 3b). The same was true for leaves of both *E. amylovora*-inoculated and mock-inoculated shoots. These observations agree with previous findings that leaves of 130 species of the Rosaceae including numerous Pyrinae species lacked biphenyl and dibenzofuran phytoalexins after treatment with biotic and abiotic stimuli (Kokubun and Harborne, 1994, 1995; Hrazdina, 2003). However, the defence compounds were found in the sapwood of fungus-infected Pyrinae species including members of *Malus* and *Pyrus* (Kokubun and Harborne, 1995; Grayer and Kokubun, 2001). Recently, leaves of fire blight-infected 'Holsteiner Cox' shoots were found to contain transcripts for BIS2 but lacked immunodetectable amounts of BIS protein, suggesting that the BIS2 transcripts are not translated into enzyme protein which could form phytoalexins (Chizzali et al., 2012).

Identification of biphenyl and dibenzofuran phytoalexins was based on a GC-MS library comprising the previously structure-elucidated phytoalexins from elicitor-treated *S. aucuparia* cell cultures (Hüttner et al., 2010). The compounds accumulating in the transition zone of fire blight-infected apple shoots were 3-hydroxy-5-methoxybiphenyl (**1**), aucuparin (**2**), noraucuparin (**3**), 2′-hydroxyaucuparin (**4**), eriobofuran (**6**), and noreriobofuran (**7**) (Fig. 1). Accumulation started 28 d post-inoculation when low