EFFECT OF PROHEXADIONE-CALCIUM ON NECTAR COMPOSITION OF POMACEOUS FLOWERS AND ON BACTERIAL GROWTH

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ABSTRACT

Prohexadione-calcium is an acylcyclohexanedione that reduces incidence of fire blight, caused by *Erwinia amylovora*. To determine whether prohexadione-calcium can be used in conjunction with biological control agents, its effect on the secretion and composition of nectar from apple and pear flowers was evaluated. Plants treated with prohexadione-calcium produced more nectar, but the sugar concentration of the nectar was lower. The ability of *E. amylovora*, and of two biological control agents of fire blight, *Pantoea agglomerans* and *Pseudomonas fluorescens*, to grow in a sugar solution corresponding to the composition of nectar from treated or untreated plants was determined in the laboratory. All bacteria including *E. amylovora* grew better in the sugar solution corresponding to the nectar from treated plants. The concentration of sugar in the non-treated flowers may be too high for bacterial growth. These results also suggest that prohexadione-calcium can be used alongside biological control agents for reduction of fire blight incidence.

Keywords: fire blight, biological control, *Pantoea agglomerans*, *Pseudomonas fluorescens*, acylcyclohexanediones.

INTRODUCTION

Growth-regulating acylcyclohexanediones, such as prohexadione-calcium (calcium 3-oxido-4-propionyl-5-oxo-cyclohexene carboxylate; ProCa) and trinexapac-ethyl [4-(1'-cyclopropyl{1'-hydroxy} methylene)-3,5-dioxocyclohexane-1-carboxylate; TrixE] represent an alternative to antibiotics or heavy metal for the control of fire blight (Spinelli et al. 2005). ProCa and TrixE are structurally similar to 2-oxoglutaric acid and consequently they block 2-oxoglutarate-dependent dioxygenases, which are involved in the formation of growth-active gibberellins and in the metabolism of flavonoids (Rademacher 2000).

Although ProCa does not have any antimicrobial activity, its protective effect against fire blight infection on shoots has been previously reported (Costa et al. 2001; Bazzi et al. 2003). However, its effect against flower infection, which is a key step in the fire blight epidemiology (Thomson 2000), has not been fully investigated. Preliminary data suggest that ProCa and TrixE reduce fire blight incidence on apple and pear flowers (F. Spinelli, unpubl. data). Therefore, these compounds could also be used in conjunction with biological control agents, such as *Pantoea agglomerans* and *Pseudomonas fluorescens*.

Since *Erwinia amylovora*, the fire blight pathogen, gets into the plant through the nectararhodes (Wilson et al. 1990), the nectar composition may play an important role in the infection process. Under a certain threshold concentration, the sugars present in

the nectar may be a valuable source of energy for the bacteria (Wilson et al. 1989), thus facilitating the infection (Thomson 1986; Wilson et al. 1990; Campbell et al. 1991). On the other hand, a sugar concentration that is too high may inhibit bacterial growth (Billing 1976; Smith 1990; Smith 1996; Steiner 1990; Thomson et al. 1982; Pusey 1999). Therefore, variation in the sugar concentration of the nectar may influence the epiphytic population of the pathogen and the biological control agents. Variations in the ability of these different bacteria to grow in the nectar could translate into different levels of infection or control. This is why this project aimed at determining the effect of ProCa on nectar secretion and sugar composition, and subsequent effects on bacterial growth *in vitro*.

MATERIALS AND METHODS

Plant material and nectar collection

Apple plants (cv. Golden delicious) and pear plants (cv. Abbé fetèl) at the pink button stage, were sprayed until run-off with 250 ppm of ProCa. Plants treated with water were used as a control. For each treatment, 3 blocks of 8 trees each were sprayed. After 15 days, the nectar was collected from the nectaries using a glass pipette especially designed for nectar sampling (Fig. 1).

FIGURE 1: Pipette used for collecting nectar. The design helps to minimise the dilution of nectar with humidity present in human breath.

The nectar was collected on two consecutive days with similar weather conditions; during sampling the temperature varied between 18°C and 22°C, and the humidity varied between 60 and 70%. To minimise the possible differences due to differential secretion of nectar during the day, nectar was collected from control and treated plants at the same time. Daily production of nectar, total sugar concentration, ratio between sugars and dry matter were determined with a refractometer and by gas chromatography (Bagdanov et al. 1997).

Nectar composition analysis

A 100 µl aliquot of nectar of was added to 4 ml of distilled water and mixed with 40 µl of a 10% mannitol solution (internal standard from Fluka). One hundred µl of the solution was transferred to a conical-bottomed test tube and dried out in a current of nitrogen at 50°C. Once dried, 200 µl of oxime reagent (pyridine solution containing 12 mg/ml hydroxylamine hydrochloride) were added and the tube sealed with a screw-on cap. After mixing, the tube was heated at 70–75°C for 30 min. When the sample cooled to room temperature, 100 µl of hexamethyldisilazane and 10 µl of trifluoroacetic acid were added, the tubes were then well-mixed and left to stand for 30 min. The sample

was then centrifuged at 5000 rpm for 2-3 sec. For analysis by gas chromatography, 0.6–1 µl of the supernatant were used. The gas chromatograph was fitted with an SE 52 capillary column and a flame ionisation detector. The fused silica gel capillary column was 25 m, 0.32 mm diameter, 0.1–0.15 µm film thickness. A carrier gas flow of 4 ml/min of hydrogen was used.

Effect of nectar sugar composition on bacterial growth

The growth of *E. amylovora* EaDCA 289/01 (Spinelli 2002), two strains of *P. agglomerans*, P10c (Vanneste et al. 2002) and EhDCA 296/01 (Spinelli 2002), and *P. fluorescens* PfA506 (Lindow 1982) was tested in the laboratory. The amounts of sucrose, glucose and fructose necessary to obtain the same percentages as those found in the nectar of apple or pear flowers treated or not with ProCa were dissolved in phosphate buffer 50 mM pH 7.3. These solutions, called artificial nectar, were sterilised by filtration through a 0.22 µm membrane. Each bacterial strain was grown in 30 ml glass vials containing 5 ml artificial nectar; 10 µl of an overnight culture in Luria broth was used to inoculate the artificial nectar. The bacterial cultures were incubated for 5 days at 27°C with moderate shaking. The bacterial populations were assessed at 0, 4, 24, 48 and 72 h after inoculation by tenfold sequential dilutions and plating on appropriate medium agar plates. The experiment was repeated 4 times.

RESULTS

Effect of ProCa on the quantity and composition of apple and pear nectar

Apple and pear nectars were mainly composed of sucrose, fructose and glucose (Table 1). However, the relative ratios of these sugars differed between the two plant species. Pear nectar was much less concentrated than apple nectar (Table 1). Nectar from apple and pear flowers treated with the acylcyclohexanediones contained significantly less sugar than the nectar from the control plants. In addition, ProCa treatment enhanced the production of nectar per flower (Table 1).

TABLE 1: Glucose, fructose, sucrose and total sugar as a percentage of the total weight of nectar collected from apple or pear flowers treated or not treated with Pro-Ca and the number of flowers needed to collect 100 µl of nectar.

Source of nectar	Fructose	Glucose	Sucrose	Total sugar	No. flowers
Pear – not treated	7.73a ¹	8.64a	1.01a	17.56a	273a
Pear treated with Pro-Ca	5.88a	6.63b	0.84a	13.35b	197b
Apple – not treated	9.26a	9.58a	30.90a	50.34a	442a
Apple treated with Pro-Ca	6.38b	6.68b	27.06b	40.12b	331b

¹Percentages or numbers for either apple or pear nectar followed by the same letter were not significantly different. Means separation by Duncan's test ($P < 0.05$).

Effect of sugar concentration on bacterial growth

The concentration of *E. amylovora* and the three biological control agents grown in the four artificial nectars are presented in Figure 2. *Erwinia amylovora* EaDCA 289/01 was less sensitive than the other strains to the sugar concentration present in the artificial nectars, while *P. fluorescens* PfA506 was the most affected by the variation in sugar concentration. When grown in artificial nectar corresponding to nectar from untreated apple flowers, the concentration of the three biological control agents decreased rapidly, and they were not detectable 24 h after inoculation. In artificial nectar corresponding to nectar from apple flowers treated with Pro-Ca, *P. agglomerans* (EhDCA 269/01 and P10c) populations grew up to ca 10⁵ cfu/ml after 72 h. In the same artificial nectar the population of *P. fluorescens* PfA506 decreased rapidly and was not detectable 24 h after inoculation.

FIGURE 2: Population concentration (cfu/ml) of *E. amylovora* EaDCA289/01, *P. agglomerans* EhDCA269/01 and P10c, and *P. fluorescens* A506 grown in phosphate buffer containing sugar corresponding to (a) nectar from non treated apple flowers, (b) ProCa-treated apple flowers, (c) non treated pear flowers and (d) ProCa-treated pear flowers. Vertical bars represent the standard error of the means.

The sugar concentration in the pear nectar was less than that in the apple nectar, and therefore, its effect on bacterial growth was not as pronounced as that of apple nectar. Nevertheless, the artificial nectar corresponding to nectar from ProCa-treated pear flowers supported a higher bacterial population than the nectar corresponding to nectar from untreated pear flowers. In ProCa-treated pear nectar, *P. agglomerans* EhDCA 269/01 and P10C multiplied rapidly, whereas *P. agglomerans* EhDCA 269/01 seemed to only just survive in untreated pear artificial nectar. As with apples, *P. fluorescens* PfA506 was not detected in either of the two artificial pear nectars after 72 h. *Erwinia amylovora* EaDCA 289/01 could multiply on artificial pear nectar representing nectar from treated or untreated plants.

DISCUSSION

The lower sugar concentration in the nectar and greater amounts of nectar collected from flowers treated with ProCa might be due to a dilution effect as a consequence of a higher availability of water, possibly due to a greater root extension. In fact, after treatment with acylcyclohexanediones, plant assimilates are generally shifted into the roots, which are then stimulated to grow (Fletcher & Arnold 1986; Guak et al. 2001). Acylcyclohexanediones can also decrease the nectar sugar concentration by inhibiting the gibberellins and ethylene biosynthesis. After application of gibberellin (1500 ppm) to *Cucumis sativus* flowers, Kamler & Tronickova (1980, 1982) observed an increase in the sugar content of the nectar. In addition, ethylene promotes mobilization of substrates and an efflux of material from petals to the gynaecium, nectar and stem

(Nichols & Ho 1975). Nichols & Ho (1975) observed that the levels of ethylene that caused irreversible wilting of petals also promoted an accelerated transfer of sucrose to the nectar. Therefore, it is likely that the application of acylcyclohexanediones reduced both ethylene and gibberellins level in plant tissues, and consequently decreased the sugar content of the nectar.

The nectar from ProCa treated flowers had a lower osmotic potential than nectar from control flowers due to a lower concentration of sugar. Therefore, nectar from treated flowers was more suitable for bacterial growth. *Erwinia amylovora* was less affected by sugar concentration than the other bacterial strains. It has been suggested that *E. amylovora* could use the sucrose present in the nectar for the synthesis of levan, which is a polymer of fructose (β -2,6-fructan). Levan synthesis would result in a reduction of the osmotic potential such that it became suitable for the growth of *E. amylovora* (Gross et al. 1992). In the artificial nectar corresponding to untreated apple or pear flowers nectar, the population of two of the three strains of biological control agents rapidly decreased below level of detection; *P. agglomerans* P10c could multiply on artificial nectar from untreated pear flowers. In contrast, in artificial nectar corresponding to nectar from ProCa-treated apple or pear flowers, the strains of *P. agglomerans*, and in particular P10c, were able to multiply to relatively high levels. This suggests that potential biological control agents may be able to colonise the nectarial cup of plants treated with ProCa, and potentially with other acylcyclohexanediones, and therefore increase the level of control of fire blight.

Further studies are needed to determine whether the effect observed *in vitro* on bacterial growth also occurs *in vivo* and to investigate the physiological mechanisms underlying the reduction of sugar concentration.

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