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Effects of pyrazole compounds from melon on the melon aphid *Aphis gossypii*

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Abstract

Two secondary compounds of the genus *Cucumis*, pyrazole and β(pyrazol-1-yl)L-alanine (βPA), were found and measured in the phloem sap of melon. The effects of these compounds on attractivity, growth inhibition and mortality were tested on aphids from cucurbit and noncucurbit hosts. βPA had no noticeable effect on the aphids tested. In contrast, pyrazole had highly deleterious effects on *Acyrthosiphon pisum*, which does not feed on melon. In addition, the strain of *Aphis gossypii* which is adapted to melon appeared to be much more tolerant to pyrazole than the cotton strain of the same species. Both strains of *A. gossypii* were stimulated to feed by moderate concentrations of pyrazole, whereas *A. pisum* was not. The resistance to *A. gossypii* due to the *Vat* gene in melon cannot be explained by the distribution of pyrazoles in the two susceptible/resistant lines tested: βPA was present in these two lines at comparable levels and pyrazole at levels too low to mediate any behavioural effect.

Keywords: Homoptera; Aphididae; *Acyrthosiphon pisum*; *Cucumis melo*; Cucurbitaceae; Phloem sap; Nonprotein amino acid; β pyrazolylalanine; Toxicity; Insect–plant interaction

1. Introduction

Aphis gossypii Glover is one of the most widespread species of aphids, and displays a large range of host-plants, covering very different families (Blackman & Eastop, 1984). This impressive polyphagy makes it a major pest of numerous crops, including many species in the families Cucurbitaceae (Daiber, 1992) and Solanaceae (Petitt, Loader, & Schon, 1994; Owusu, Brempongyeboah, Horiike, & Hirano, 1994), while being also the main aphid pest of cotton (Leclant & Deguine, 1994). However, this extended host-range probably covers infra-specific taxonomic variations that are still poorly evaluated. There are many strains or 'host races' of *A. gossypii*, including strains living on cotton or on cucurbits (Blackman & Eastop, 1984; Leclant & Deguine, 1994; Guldemond, Tigges, & Devrijer, 1994). This single species also displays extensive insecticide resistance (Furk & Hines, 1993), but the determinants of the adaptability of this aphid are still poorly understood.

Host–plant resistance is an attractive tool for integrated pest management, and was explored extensively for the control of *A. gossypi*, especially in cucurbits (Pitrat & Lecoq, 1982; Kennedy, Mac Lean, & Kinsey, 1978; Collins, Perkins Veazie, Maness, & Cartwright, 1994; Shinoda, 1993). Some mechanisms governing such resistances were investigated (Kennedy et al., 1978; Chen, Martin, Rahbé, & Fereres, 1997a), but the chemical determinants of resistance in cucurbits are still unknown. A recent analysis of a set of isogenic lines differing by the *Vat* resistance gene (Pitrat & Lecoq, 1982)[1] showed that this resistance was constitutive (Chen, Rahbé, Delobel, Sauvion, & Febvay, 1997b) and mediated by still unidentified chemicals (Chen et al., 1997b; Chen, Delobel, Rahbé, & Sauvion, 1996).

Among the sources of plant resistance against insects, nonprotein amino acids have an important role either because of their unpalatability or because of their toxicity (Bell, 1978). The genus *Cucumis* is characterised by one family of such compounds, based on pyrazole, which were identified in seeds of many members of the Cucurbitacae (Dunnill & Fowden, 1965). They are also abundant in the seedlings of these plants, at concentrations depending on the cultivar (Brown & Diffin, 1990). Free pyrazole was incorporated into β-pyrazolylalanine, pos-

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[1] *Vat*, for virus aphid transmission, a gene governing a specific resistance to the vector *A. gossypi* and to nonpersistent virus transmission.

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sibly as a detoxification mechanism, the general high toxicity of pyrazole being well documented (Magnusson, Nyberg, Bodin, & Hansson, 1972).

In view of this and our interest in finding the chemical basis of the *Vat* resistance in melon, we investigated the effects of pyrazole derivatives as possible candidates for the mediation of resistance towards *A. gossypii*. *Acyrthosiphon pisum* Harr., an aphid with a specialised host range and not adapted to melon, was used as a reference species for our behaviour and toxicology experiments.

2. Results

2.1. Phloem content

In total unhydrolysed extracts, both γ-glu-βPA and βPA eluted in a very crowded portion of the chromatogram (Ser and Asn areas) and were not amenable to direct quantification. Actually, βPA and γ-glutamyl-βPA were unambiguously detected only in the pellets of the 80%-ethanol precipitation of phloem samples (due to partial insolubility). These pellets were free of most protein amino acids, and contained many peptides and other unidentified ninhydrin positive peaks. βPA eluted with asparagine (retention time (rt) ≈ 18 min), but was characterised by a wavelength ratio $W_{R570/440\,nm} = 7.35$ (Asn: 0.73; standard amino acid: ≈10) and a colour factor of 0.754 (at 570 nm, towards the internal standard used, which was glucosaminic acid); γ-glu-βPA eluted at rt ≈ 15.1 min (approx. rt of serine) and displayed a $W_{R570/440nm}$ of ≈9; its relative colour was not accessible due to absence of a synthetic standard. Identification was obtained by peak collection after ion-exchange chromatography, then acid hydrolysis and cochromatography with βPA standard. Trials of FMOC derivatisation, followed by HPLC, also gave a poor separation of βPA and γ-glu-βPA from other amino acids (Fig. 1), compared to the clear chromatograms of standards. Hydrolysis appeared to be the only way to recover βPA in a non crowded zone of a chromatogram. Quantification of total βPA was therefore obtained through ninhydrin reaction after hydrolysis of sample extract, giving the sum of free βPA plus glutamic acid-bound βPA (Table 1).

Quantification of pyrazole was more difficult: good results were easily obtained through FMOC-derivatisation with standard solutions in water, but although the solubility of pyrazole in organic solvent should allow its extraction from phloem by partition, large amounts of solvent were required. The subsequent need to reduce solvent volume led to losses of pyrazole. This was overcome by direct derivatisation of phloem samples. However, this also led to the derivatisation of a lot of other FMOC-reactive compounds (mainly amino acids), resulting in a much more complex chromatogram Fig. 1. The FMOC-pyrazole derivative eluted in a clean portion of the chromatogram, and was identified by its retention time and a characteristic shoulder in its 200–400 nm diode-array spectrum (vs. spectra of most FMOC-amino acid adducts).

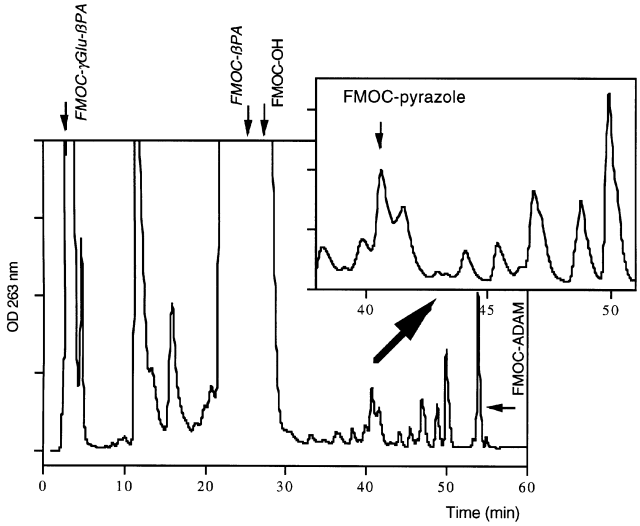

Fig. 1. HPLC chromatograms of phloem sap samples of melon for assay of pyrazole after FMOC precolumn derivatisation: retention times of standard FMOC-derivatives are indicated (arrows), with a window enlargement of the pyrazole region (identified and quantified by cochromatography, UV spectrum and spiking of standard).

Table 1
Pyrazole and β-pyrazol-1-yl alanine content in phloem sap of melon

Compound	Genotype	
	R (μg ml^{-1} ±S.E.)	S (μg ml^{-1} ±S.E.)
Pyrazole	2.75±0.13	9.7±2.3
Total βPA	37.2±1.4	35.2±1.3

The pyrazole concentrations in phloem sap of resistant and susceptible melon cultivars are shown in Table 1. βPA levels were always higher than pyrazole levels. For βPA, the concentrations were not different in the resistant and in the susceptible melon lines, while the difference for pyrazole was significant between lines. Molar levels of βPA in both lines were ≈0.25 mM, which is in the range of a minor amino acid of the phloem sap, such as glycine or alanine (Chen et al., 1996); pyrazole is ≈45 μM in R line, and ≈150 μM in S line.

2.2. Toxicity

Even at the highest concentration employed, βPA had no significant effect on growth or mortality of any aphid population tested (Table 2 and Fig. 2a). Growth variability was quite high for both *A. gossypii* strains, and the

Table 2
Mortality of aphids after seven days on artificial diets containing different levels of pyrazole and β-pyrazolylalanine ($n=60$)

Aphid	Compound	Dose control	Concentration				Trend test[a]	
			50 μg ml^{-1}	200 μg ml^{-1}	500 μg ml^{-1}	1000 μg ml^{-1}	ε	p
Aphis gossypii (melon strain)	βPA	3%	–	2%	–	2%	−0.603	NS
	Pyrazole	18%	34%	26%	39%	42%	3.119	<0.01
Aphis gossypii (cotton strain)	βPA	12%	–	15%	–	22%	1.329	NS
	Pyrazole	7%	13%	15%	14%	23%	2.632	<0.01
Acyrthosiphon pisum	βPA	10%	–	2%	–	3%	−1.720	NS
	Pyrazole	10%	21%	10%	41%	98%	13.178	<0.001

[a] Statistics and probability of a non parametric comparison of treatments (Ho, no effect of dose on mortality; trend test of Gros & Chessel, 1982).

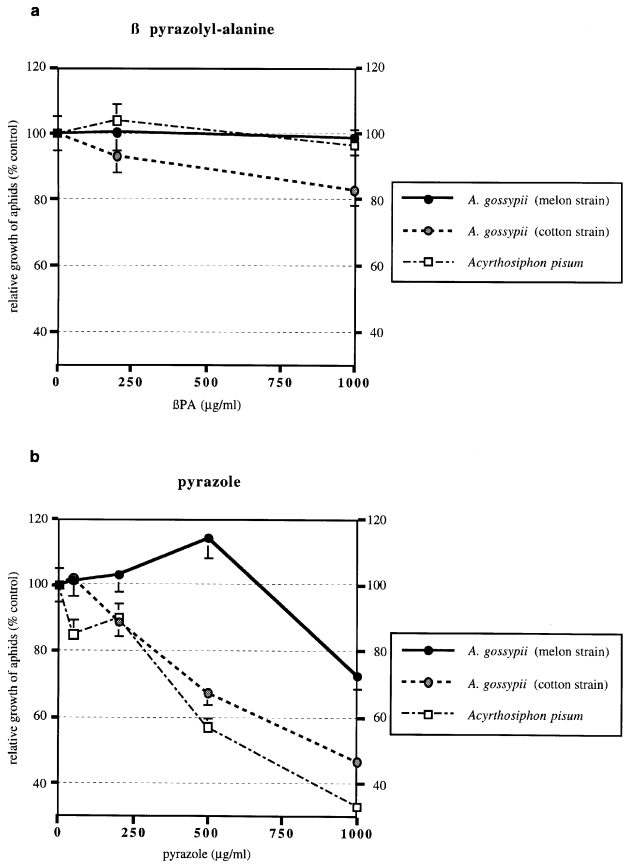

Fig. 2. Influence of β pyrazolyl-alanine (a) and pyrazole (b) on growth of the three aphid clones tested. Values are given as % of mean weights of controls on day 7 (±S.E.).

18% decrease for the cotton strain at 1000 μg ml^{-1} was not significant.

At the lowest concentration tested (50 μg ml^{-1}), pyrazole had no effect on the growth of *A. gossypii*. The growth of *A. pisum* was already significantly reduced by 16% at this concentration (Fig. 2b). At higher concentrations, growth was dramatically reduced for the 'cotton strain' of *A. gossypii* and even more for *A. pisum*. No significant effect was shown for the 'melon strain', except at the highest dose (1000 μg ml^{-1}). The calculated growth inhibition indices (IC_{50}, μg ml^{-1}: >1500 for *A. gossypii*$_{melon}$, 920 for *A. gossypii*$_{cotton}$ and 670 for *A. Pisum*) also show that to obtain a 20% reduction in aphid weight, the concentration of pyrazole required is 3× higher for the 'melon strain' than for the others. Pyrazole induced a significant concentration-dependent mortality in the three strains Table 2, the strongest effect being on *A. pisum* (98% mortality at 1000 μg ml^{-1}).

2.3. Phagostimulation

βPA appeared largely ineffective on food choice whatever the dose or the aphid strain, except for a phagodeterrent effect on *A. pisum* at the dose of 40 μg ml^{-1} (Fig. 3a). This weak effect was checked twice and was repeatedly significant (total number of replicates for this point is 80). A slight stimulant effect on the cotton strain of *A. gossypii* was also observed at dose 200 μg ml^{-1}, but was not checked further (as this dose exceeded by far normal levels of βPA).

Pyrazole exhibited a significant attraction for both strains of *A. gossypii* between 10 and 100 μg ml^{-1}. No effect was detected at higher concentrations (200, 1000 and 5000 μg ml^{-1}). *Acyrthosiphon pisum* showed no preference, except at the highest concentration which was strongly antifeedant (Fig. 3b), but an influence of toxicity may be suspected in this case, since the choice tests were scored after 12–14 h of contact.

3. Discussion

Our chromatographic data revealed that the three previously reported pyrazole compounds (Dunnill & Fowden, 1965) were present in the phloem sap of melon, and were therefore available to aphids. We did not assay β-pyrazolylalanine and its γ-glutamyl derivative separately, but total βPA content of phloem sap was shown to reach that of a normal protein amino acid. It may be noted that standard ion-exchange amino acid analyses

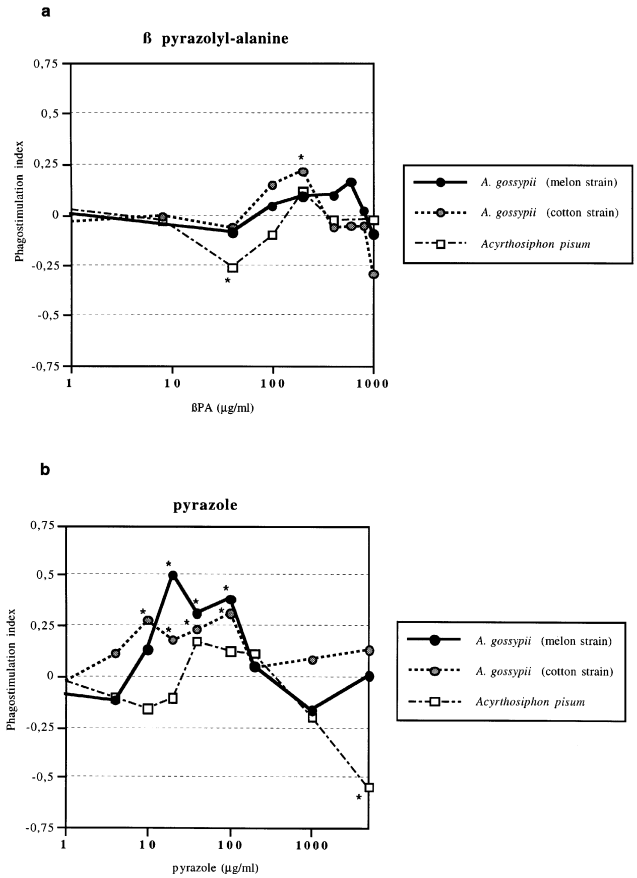

Fig. 3. Phagostimulatory response of the three aphid clones to a range of doses (log scale) of β pyrazolyl-alanine (a) and pyrazole (b). The phagostimulation index is: $N_{\text{aphids on treated diets}}$-$N_{\text{apids on control diets}}$/total $N_{\text{aphids fixed}}$. N is the number of feeding aphids, and stars indicate significant response over control (sign tests).

of intact cucurbit phloem sap would probably lead to misidentification of asparagine (in fact mostly free βPA). In our analyses, comparison of hydrolysed and intact samples indicated that Asn was the minor component of the Asn-βPA peak. Similarly, the major nitrogen carrier in a *Cucurbita maxima* phloem sap was reported to be aspartic acid (Richardson, Baker, & Ho, 1982), but our data clearly showed that the peak co-eluting with Asp in our sample was a major peptide, and that the main N carrier, at least in melon, was in fact glutamic acid (second largest in the cited paper), in addition to many unidentified peptides and nonprotein ninhydrin positive compounds (Chen et al., 1997b).

Our behavioural assays demonstrated that neither pyrazole nor βPA were able to promote any phagodeterrency to *A. gossypii*, as it might have been expected from a putative chemical factor underlying the *Vat* resistance in melon (Pitrat & Lecoq, 1982; Chen et al., 1996). The only clear deterrent effect was displayed by βPA towards *A. pisum* at physiological levels, and only at this dose (40 µg ml^{-1}, 0.26 mM). This may be related to the restricted host-range of this aphid, which lives mainly on legume hosts, and is much more sensitive to nonhost secondary compounds than polyphagous insects, as this has already been shown with other aphids (Dreyer & Jones, 1981; van Emden, 1978). It is also interesting that pyrazole exhibited significant attraction to *A. gossypii*, in a ten-fold physiological concentration range (10–100 µg ml^{-1}). Since the 'cotton strain' did respond similarly to the melon one, this trait is not due to learning or previous experience, and it would be interesting to check whether this trait is characteristic of the species as a whole, or of some related subgroups within the species. As a consequence, *A. gossypii* may use pyrazole as one of the cues for host characterisation when probing on *Cucumis*, although there is no behavioural differentiation between aphid 'host races' at this level.

A further step in host–plant adaptation is the metabolic fitness to the host's secondary chemistry (Dowd, Smith, & Sparks, 1983). It is quite clear at this level that the 'melon strain' of *A. gossypii* is better adapted to the presence of pyrazole in the diet, and this clone coped with doses up to 500 µg ml^{-1} without deleterious effects. The physiological concentrations of pyrazole in melon phloem sap (4–10 µg ml^{-1}) did not appear to harm the 'cotton strain' either, but higher doses produced significant growth alteration. We carried out our measurements in one physiological condition however, and pyrazole levels may vary with plant development or history, including daily variations and stress situations, as was shown to occur with other phloem constituents (Cull & van Emden, 1977). In contrast to *A. gossypii*, *A. pisum* was highly sensitive to pyrazole toxicity, even at low doses. Serious mortality and growth reduction occurred, and the toxicity threshold was much lower than the phagodeterrent threshold (no choice against pyrazole for *A. pisum* under 5000 µg ml^{-1}).

Finally, we found no chemical differences in pyrazole compounds between the two R/S melon lines that could directly mirror the resistance phenotype of these plants towards *A. gossypii* (a strong antixenosis). This was our working hypothesis, on the basis of unidentified peak correlations with resistance genotype (Chen et al., 1997b). Although differences were found in pyrazole levels between the two lines (but not βPA), this work rules out the involvement of this class of compounds in *Vat* gene expression.

4. Experimental

4.1. Insects

The 'melon strain' of *A. gossypi* (clone Ag-LM02) originated from INRA Montfavet and has been reared on melon cv. Vedrantais in our laboratory since 1991. The 'cotton strain' of *A. gossypi* was introduced from a field population collected in Burkina Faso in 1994 (clone Ag-

LB03) and was reared on cotton cv. Coker seedlings. Our *Acyrthosiphon pisum* clone originated from a field sample collected on lucerne in 1986 (INRA Lusignan, clone Ap-LL01) and was reared on broad bean seedlings. Aphids were all reared in ventilated Plexiglas cages (21°C, 70% r.h., L16:D8). Mature aphids were allowed to deposit their larvae on plants for 24 h, and the resulting apterae were grown to maturity at low density on healthy seedlings; they were then transferred to new plants for 24 h, and the resulting offspring (aged 0 to 24 h) were used for all experiments.

4.2. Plant material

Melon seeds (*Cucumis melo*) cv Vedrantais (S, susceptible variety) or cv. Margot (R, resistant variety, *Vat*) were germinated in compost at 31°C. Margot was obtained from 9 generations of backcrosses of *Vat* resistant plants into the Vedrantais genotype, and thus the two lines were considered to be highly isogenic (Pitrat, pers. comm.). The seedlings were then kept under the same conditions as the insects, under horticulture fluorescent light (3000 lux). Plants were used for sap collection when reaching leaf stage 5/7. Cut edges of petioles from fully expanded leaves resulted in natural exudation at the distal/leaf side, which is considered to be pure phloem sap in cucurbits (Richardson et al., 1982). This sap was collected within 1 min in a microtube and immediately processed for analysis.

4.3. Chemicals

Pyrazole was commercial (Aldrich), and β-(pyrazol-1-yl)-L-alanine (βPA) was synthesised from N-benzyloxycarbonyl-serine (N-CbZ Ser) through a modification of a published procedure (Arnold, May, & Vederas, 1988; Pansare, Huyer, Arnold, & Vederas, 1991). From 2.5 g of N-CbZ Ser, the β-lactone was obtained, and purified by flash chromatography on silica with ethyl acetate/pentane (60/40) as eluant (Pansare et al., 1991). The lactone (1.1 g) was transformed to N-CbZ-βPA (0.63 g), and the CbZ moiety hydrogenolysed in 85 ml methanol with 50 mg of catalyst (Pd/C, 10%). β-pyrazolylalanine was crystallised in water/methanol (221 mg), the purity of which was evaluated by automated ion-exchange chromatography (major ninhydrin peak; less than 2% of serine; one trace unknown peak).

4.4. Chromatography

4.4.1. βPA assays

An exactly weighed amount of phloem sap (≈75 μl), pooled from 20 R or 10 S melon plants, was collected and immediately diluted in 5 volumes of ethanol 95%, kept overnight at 4°C to precipitate phloem proteins, and centrifuged. The pellet was extracted 3× with water (100 + 50 + 50 μl), ultrafiltered on Millipore Ultrafree-MC membranes (10,000 MW cut-off) and the aqueous low-molecular weight fraction was added to the supernatant of the former alcoholic precipitation. The combined supernatants were dried under vacuum and were subjected to acid hydrolysis (Pico-Tag work Station Waters). The resulting amino-acids were analysed by ion-exchange chromatography on Beckman 6300 Li following the standard 120 min Beckman procedure for biological fluids. Elution time and wavelength ratio (ninhydrin detection) were used to identify βPA by comparison with synthetic standard. Due to the co-elution of βPA with asparagine, direct quantification of βPA in alcoholic extracts was not possible, and hydrolysis (preserving βPA but not asparagine) was necessary to assay total βPA in the phloem.

4.4.2. Pyrazole assays

After several trials under different conditions of extraction of pyrazole from samples, and due to persistent yield inconsistencies, derivatisation of raw samples appeared to be a far more reliable strategy (because of pyrazole volatility). The 9-fluorenyl-methoxy-carbonyl derivatisation method of Gustavsson and Betner (1990) was modified to take into account the influence of the various substances present in the sap, as well as the slower reaction speed of pyrazole compared with α-amino acids. Ten microliters of sample (phloem sap) and 5 μl of a pyrazole solution (0, 20 or 40 μg ml^{-1}) were mixed with 15 μl of borate buffer (0.5 M boric acid solution adjusted to pH 7.7 with sodium hydroxide). Thereafter, 30 μl of FMOC reagent (9-fluorenyl-methyl chloroformate; 45 mM) in acetone were added and mixed immediately. After completion of the reaction (21°C, 10 min), 30 μl of 1-amino adamantane (ADAM) (40 mM) in water–acetone (1:3, v/v) were added and mixed, then left standing for 2.5 min and centrifuged for 3 min (7000 g). The supernatant was analysed using a Kontron MT2 HPLC system with a diode array detector. The 300 × 3.9 mm column was packed with Lichrosorb C18 10 μm. Elution was performed with a gradient of solvent A (7 ml HOAc, 1 ml NEt_3, water qsp 1000 ml after adjustment to pH 4.2 with NaOH) and solvent B (pure methanol). Gradient started with 45% B for 10 min, then went up to 60% B in 20 min, to 100% B in 25 min and back to 45% B in 20 min. Flow rate was 1 ml min^{-1}, detection wavelength was 263 nm and spectra (200–400 nm) were continuously recorded for identification purposes. Due to competition with N-compounds more reactive to FMOC (mostly amino acids), pyrazole in the sample was quantified by an internal calibration curve ('*y*'-intercept of the 0 point in the 0, 20, 40 μg ml^{-1} co-injection series).

4.5. Toxicity assays

UV-sterilised Parafilm sachets enclosing 0.75 ml of diet were made under sterile conditions and placed on PVC

rings ($h=1.5$ cm, $d=4$ cm). A standard aphid diet (Febvay, Delobel, & Rahbé, 1988, diet A5) with a sucrose content adjusted to 20% was sterilised by filtration after addition of pyrazole or βPA. Toxicity was tested on a one-week cycle. Three groups of 20 neonate larvae were deposited on artificial diets for each treatment (day 0). The percentage of settling was noted after one hour as an indication of the short-term phagostimulatory status of the diet (Rahbé, Febvay, Delobel, & Bournoville, 1988). Sachets were replaced on day 3 or 4, and mortality was scored in each ring on days 1, 3 and 7. Aphids were individually weighed on day 7 (except for the few winged adults or pre-alate larvae), when most aphids on the control diet were adults.

Mortality data were statistically tested following the non parametric trend test of Gros and Chessel (1982).

Individual weights on day 7 were submitted to one-way ANOVA to determine if there was any effect of concentration of the two chemicals, and then analysed by fitting the data to a Michaelis–Menten model to determine the concentrations that inhibited growth by 50% or 20% (IC_{50} or IC_{20}).

4.6. Choice tests

As fully described previously (Chen et al., 1996), 6 neonate aphids were deposited in one small tube cage (5 mm large, 3–5 mm diam.), both ends of which were covered with stretched Parafilm sachets containing the substances to be tested in solution in artificial diets. The previously cited A5 diet was used for *A. pisum*, and a 'Melo 1' diet was used for *A. gossypii*, which was a 15% solution of sucrose containing all protein amino acids at the concentrations found in melon phloem sap (Chen et al., 1996) adjusted to pH 7.0 with KH_2PO_4. Aphids were left in contact with diets overnight in a dark box, and choice was scored and tested through a sign test (test > control = '+'; test < control = '−'; test = control not used). At least 20 replicates were performed for each species and clone of aphid, and on every dose tested.

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