Clones of pea aphid, *Acyrthosiphon pisum* (Hemiptera: Aphididae) distinguished using genetic markers, differ in their damaging effect on a resistant alfalfa cultivar

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Abstract

CUF 101, a resistant cultivar of alfalfa, was exposed to 15 clones of *Acyrthosiphon pisum* Harris collected from alfalfa fields in three regions of France (east, south, central west) to determine whether the level of resistance varied across the different clones. The survival of alfalfa seedlings infested at the cotyledon stage was assessed using a standardized method. Although no difference in seedling mortality was detected between clones grouped by region, there was a significant variation among the 15 pea aphid clones. In particular, two clones of southern origin were more aggressive. In addition, the different pea aphid clones were characterized using allozyme and RAPD-PCR markers. Among the 15 clones, seven allozyme genotypes (plus one when adding colour polymorphism) and 12 RAPD-PCR genotypes were distinguished. The two southern clones differing by their aggressiveness on the resistant alfalfa belonged to the same allozyme and RAPD genotype which was distinct from the other pea aphid clones. Our results reinforce the need to take into account aphid genetic diversity in breeding programmes for resistance in cultivated plants.

Introduction

Aphids are the most frequent insect group for which formation of host-adapted races (biotypes) and rapid adaptation to resistant crops have been observed (Caillaud *et al*., 1995; Dixon, 1998). For instance, the specialization of the pea aphid *Acyrthosiphon pisum* Harris (Hemiptera: Aphididae) on herbaceous Fabaceae has been reported in Europe by Müller (1980) for populations living on *Vicia* species, pea, alfalfa, red clover, and in North America by Via (1989, 1991) for populations found on alfalfa and red clover. In Australia, Sunnucks *et al*. (1997) found two host-adapted forms of *Therioaphis trifolii* Monell (Hemiptera: Aphididae) restricted to either clover or alfalfa. For *Aphis gossypii* Glover (Hemiptera: Aphididae), Vanlerberghe-Masutti & Chavigny (1998) showed evidence for genetic divergence between aphids according to host-plant, with samples collected on cucurbit differing from those on non-cucurbit plants. Similarly, De Barro *et al*. (1995a,b) found genetic differences between individuals of *Sitobion avenae* Fabricius (Hemiptera: Aphididae) from wheat and cocksfoot. In *Schizaphis graminum* Rondani (Hemiptera: Aphididae) which feeds on cereals, the fast emergence of biotypes following deployment of new genes for plant resistance is well documented and its economical impact has received considerable attention (e.g. Puterka & Peters, 1989; Puterka & Burton, 1990).

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When considering resistance of plant varieties to aphids, the distinction of new biotypes is based on their ability to feed and damage plants resistant to some other biotypes (Claridge & Den Hollander, 1983). These variations in aphid/host-plant relationships are important as they may alter breeding programmes for crop resistance to aphids. Blackman (1981) considered aphid populations on a given crop to be composed of a variable number of clones with numbers of each clone present at any time depending on immigration, and on the fitness of each clone in relation to the host. He concluded that tests for resistance of plants to aphids should include a sample of naturally occurring aphid genotypes as broad as possible.

In the case of the pea aphid, the variability in host-plant interactions may occur at the local level, either between different plant species (Bournoville, 1977; Via, 1991) or between clones on a single host (Sandström, 1994). Bournoville (1981) showed a similar magnitude of variation in reproductive rate among clones originating from the same field of alfalfa and among clones collected from geographically distant alfalfa fields when tested on a susceptible cultivar of alfalfa.

The first aim of this study was to examine the range of variation in ability to overcome host plant resistance among pea aphid clones collected in the main alfalfa growing areas in France. In North America, many resistant cultivars to the pea aphid were developed during the 1970s (Sorensen *et al.*, 1988). According to the French Plant Variety Review Board, none of the alfalfa cultivars are currently resistant to the pea aphid in France. However, one cultivar of USA origin, CUF 101, does demonstrate resistance to *A. pisum* in our tests in France (Bournoville *et al.*, 1999; Girousse *et al.*, 1999). We have therefore used CUF 101 to compare resistance across *A. pisum* clones.

The second aim of this study was to identify the genotypes of the different *A. pisum* clones using both allozymes and random amplified polymorphic DNA (RAPD) markers (see Loxdale & Lushai (1998) for a recent review on molecular markers applied to entomology). While aphids do not exhibit much in the way of allozyme polymorphism (Suomalainen *et al.*, 1980; Simon *et al.*, 1982; Dalmasso & Bournoville, 1983), the variation present may still indicate significant variation in regard to host utilization (Hales *et al.*, 1997). The RAPD technique usually enables the detection of greater levels of genetic diversity than allozymes. The technique has been successfully applied in several aphid species to examine, for example, population structure (De Barro *et al.*, 1995a; Martinez-Torres *et al.*, 1997), discrimination between host-adapted races (De Barro *et al.*, 1995b; Sunnucks *et al.*, 1997; Vanlerberghe-Masutti & Chavigny, 1998) and in the search for markers linked to life cycle variation (Simon *et al.*, 1996).

Materials and methods

Plant material

In the USA, the alfalfa cultivar CUF 101 carries multiple resistance to pests and pathogens (Lehman *et al.*, 1983), and is highly resistant to three aphid species (*Acyrthosiphon kondoi* Shindji, *A. pisum* and *Therioaphis trifolii* f. *maculata* Buckton). In standard tests to characterize alfalfa accessions, this cultivar is used as a check for assessing resistance to pea aphids (Berberet *et al.*, 1991). In France, CUF 101 is not cultivated commercially, although it is occasionally sown in plant breeding nurseries.

Table 1. Origin of the clones of *Acyrthosiphon pisum* used in this study.

Clones	Regions	Department name	Sampling dates	Colour
33	CW	Vienne	23 II 95	G
29	CW	Vienne	02 III 95	R
30	CW	Vienne	09 III 95	G
32	CW	Vienne	09 III 95	G
31	CW	Vienne	09 III 95	G
A4	S	Tarn et Garonne	28 III 95	G
B3	S	Tarn	28 III 95	G
C2	S	Aude	29 III 95	G
D1	S	Herault	30 III 95	G
E1	S	Tarn	30 III 95	R
U1	E	Marne	11 IV 95	G
V2	E	Marne	11 IV 95	G
X3	E	Aube	11 IV 95	G
Y1	E	Côte d'Or	12 IV 95	G
Y4	E	Côte d'Or	12 IV 95	G
Clone 'R'	CW	Vienne	29 X 92	G

French regions: CW, central west; S, south; E, east. Colour of the lines: G, green, R, red.

Aphid material

Fifteen clones of *A. pisum*, collected by sweeping plants from alfalfa fields in three regions of France, were established between 23 February and 12 April 1995. Each clone was derived from a single female (table 1). Five clones were collected from the central west of France at Poitou (fields at the INRA Research Centre of Lusignan, 30 km from Poitiers), five from eastern France at Champagne and Burgundy (fields 25 to 70 km from Troyes), and five from southern France (four fields 25 to 50 km from Toulouse; one close to Montpellier). These regions were chosen as being representative areas of alfalfa production. Poitou and southern France are traditional alfalfa growing areas, while Champagne is an area of intensive production of dried alfalfa pellets. The three collection areas were 500 km from each other. In addition to these clones, we also included a reference clone 'R' which was collected from an alfalfa field (29 October 1992) at the INRA Research Centre, Lusignan and reared on alfalfa.

As *A. pisum* exhibits a range of colour variation, in alfalfa fields in France (Bournoville, 1973), two of the clones were red (one from the central west, the other from the south), the others, including the reference clone, were green. The cultures were maintained in a constant temperature chamber (20°C, 16L/8D, 200 μmol m^{-2} s^{-1}), on potted susceptible alfalfa cv. Milfeuil under screen cages (33 × 20 × 55 cm).

In order to determine whether the sampled females were fundatrices (first parthenogenetic generation after egg) or other forms resulting from either short- or long-term parthenogenetic reproduction, their antennal segments were measured. The idea was to assess rapidly the life-cycle (e.g. holocyclic or anholocyclic) of some of the clones. Hille Ris Lambers (1947) reported that the relative length of the *processus terminalis* to the basal part of the sixth antennal segment was shorter for the fundatrix of *A. pisum* than for its

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apterous virginoparae. The two morphs were additionally discriminated by the ratio between the third and sixth antennal segments which is higher for fundatrices.

Effect of aphid clones on resistant alfalfa

The effect of aphid clones on CUF 101 was evaluated under controlled conditions using a method previously described by Bournoville *et al*. (1999). This involved challenging seedlings at the cotyledon stage with a fixed biomass of aphids and then assessing seedling survival. Seedling survival was tested through three experiments, an experiment consisting of the test of the five aphid clones from the same region. To enable comparison between clones from different regions, the reference clone 'R' was included in each of the three experiments. Six replicates of 50 seedlings were used to test each clone and one control of 50 non-infested seedlings was set to assess background seedling death. The initial aphid infestation (150 mg per 50 seedlings) was renewed six days later with an additional further 150 mg of aphids to maintain high numbers of aphids. After 13 days, the infestation was ended by the application of an insecticide. The weight of 20 wingless adults from each clone was measured prior to each infestation using a microbalance (±10 µg precision). After 27 days, wilted and dead seedlings were counted in each replicate. Data analysis was carried out in order to compare the effects of the 15 aphid clones on seedlings. The number of wilted and dead seedlings per replicate was corrected to take into account variation between the three experiments. For that, mean number of wilted plus dead seedlings for clone 'R' was calculated over the three experiments (m_R) and for each of the three experiments (m_{Rj}, with $j = 1, 2, 3$). Then deviations between means of clone 'R' in each experiment and general mean of clone 'R' were calculated ($e_j = m_{Rj} - m_R$). For each clone i from experiment j, counts of dead plus wilted seedlings for replicate k, x_{ijk} , was corrected such as: $y_{ijk} = x_{ijk} - e_j$. Two way (region and aphid clone) analysis of variance was carried out on corrected values of wilted and dead seedlings, and clones were compared by Scheffé's mean comparison test (Scheffé, 1953). The relationship between aphid individual weight at infestation times and the mean corrected number of wilted and dead seedlings for each clone, was studied through linear regression analysis in order to check whether the individual weight of the aphids had an effect on alfalfa seedlings.

Molecular characterization of the pea aphid clones used in biological tests

From time to time, adults from each clone of *A. pisum* were frozen (−70°C) for allozyme and RAPD–PCR analyses. Allozyme analysis was performed on single individuals from each of the 15 clones as well as from the reference clone. Electrophoresis was performed on cellulose acetate gels using the method described by Hebert & Beaton (1989). A total of 36 loci from 25 different enzymatic systems were screened for polymorphism (table 2).

Total DNA was extracted from single individuals of *A. pisum* lines by using the Isoquick kit (Microprobe Corporation ™) according to manufacturer's recommendations and resuspended in 40 µl of TE buffer (10 mM Tris pH 7.5, 0.1 mM EDTA). In a preliminary analysis, DNA from each line was subjected to PCR amplification using 20 different random oligonucleotide primers (Operon Technologies, Alameda, California). Of these, four primers (OPA3, OPA18, OPA20 and OPL11) were chosen because of their high reproducibility and rate of polymorphism. For each of the four selected RAPD primers, the PCR reaction was repeated three times, using a different individual

Table 2. Names, E.C. numbers, and number of loci of enzymes analysed for variation in clones of *Acyrthosiphon pisum*.

Enzyme name	EC number	Number of loci
Aconitase	4.2.1.3	1
Adenylate kinase	2.7.4.3	1
Alkaline phosphatase	3.1.3.1	1
Aminoaspartate transferase	2.6.1.1	2
Arginine phosphokinase	2.7.3.3	2
Esterase	3.1.1.1	2
Fumarase	4.2.1.2	1
Glucose phosphoisomerase	5.3.1.19	1
Glyceraldehyde-6-phosphodehydrogenase	1.2.1.12	1
Glycerol-3-phosphate-dehydrogenase	1.1.1.8	1
Glucose-6-phospho-dehydrogenase	1.1.1.49	1
Isocitrate dehydrogenase (*IDH*)	1.1.1.42	2
Leucine aminopeptidase	3.4.11.1	2
Malate dehydrogenase	1.1.1.37	2
Malic enzyme	1.1.1.40	1
Mannose phosphate isomerase	5.3.1.18	1
Dipeptidase Phe-Pro	3.4.13.11	1
Dipeptidase Phe-Leu	3.4.13.11	1
Dipeptidase Gly-Leu (*PEP-GL*)	3.4.13.11	1
Tripeptide aminopeptidase Leu-Leu-Leu	3.4.11.4	3
Tripeptide aminopeptidase Leu-Gly-Gly (*PEP-LGG*)	3.4.11.4	3
Phosphoglucomutase	5.4.2.2	1
6-Phosphogluconate dehydrogenase	1.1.1.43	1
Sorbitol dehydrogenase (*SDH*)	1.1.1.44	1
Superoxide dismutase	1.15.1.1	2

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Table 3. Mean and standard error of aphid individual weight at the two infestation periods (D and D+6), and of the corrected number of dead and wilted seedlings per replicate (D+27) in seedling tests with the 15 aphid clones.

Regions	Clones	Individual aphid weight in mg (S.E.)		Corrected number (y) of dead and wilted seedlings (S.E.)	Scheffé's group for y
		D	D+6		
C-West	29	2.16 (0.09)	1.51 (0.12)	21.61 (1.75)	abc
	30	1.87 (0.09)	1.88 (0.10)	19.94 (4.09)	abc
	31	2.18 (0.11)	2.16 (0.18)	18.94 (1.78)	abc
	32	1.63 (0.11)	1.49 (0.07)	17.27 (1.11)	abc
	33	2.05 (0.09)	1.92 (0.09)	13.77 (0.88)	bc
East	Y1	1.70 (0.07)	1.97 (0.07)	19.77 (2.60)	abc
	U1	1.81 (0.10)	1.88 (0.09)	19.44 (1.65)	abc
	Y4	1.32 (0.08)	2.32 (0.17)	18.10 (1.78)	abc
	V2	1.80 (0.08)	2.52 (0.14)	16.11 (1.19)	abc
	X3	1.30 (0.08)	1.90 (0.15)	11.77 (1.81)	c
South	C2	1.30 (0.07)	1.65 (0.07)	29.44 (0.67)	a
	B3	1.27 (0.08)	1.71 (0.09)	27.44 (2.47)	ab
	E1	1.86 (0.09)	1.45 (0.07)	13.94 (1.39)	bc
	D1	1.29 (0.08)	1.76 (0.08)	10.61 (2.38)	c
	A4	2.54 (0.14)	2.23 (0.17)	9.94 (1.57)	c

Results of mean comparison of the corrected number of dead and wilted seedlings, by Scheffé's method (5%).

belonging to the same clone. RAPD amplifications were performed in a volume of 25 µl containing 1.5 mM of $MgCl_2$, 2.5 µl of 10× Appligene-Oncor reaction buffer, 1 µl of a 10 mM solution of dNTP's, 1 µl of 10 µM of primer, 1 unit of *Taq* polymerase (Appligene-Oncor), 1 µl of a 1:10 dilution of resuspended DNA and ultrapure water. Thermal cycles consisted of an initial denaturation step of 94°C for 5 min, followed by 40 cycles of 1 min at 92°C, 1 min at 40°C and 2 min at 72°C. A final extension period of 10 min at 72°C completed the reaction. PCR products using 20 µl of the RAPD reaction were resolved by electrophoresis in 1.5% agarose gels in a 0.5× TBE buffer at 100 V.

Results

Morphs of the initial adults

In five out of the 15 clones of *A. pisum*, the founder females had antennae shorter than the body, and so were presumably fundatrices: one was from the central west (33), and four (U1, V2, Y1, Y4) from Champagne or Burgundy. The mean ratio of the *processus terminalis* to the basal part of the sixth antennal segment was 2.36 and the ratio of the third to the sixth antennal segments, 0.98. The corresponding values for the other individuals with antenna longer than the body were 3.09 and 0.80, respectively.

Biological evaluation of the level of alfalfa resistance

The mean weights of the adults of *A. pisum* at the two infestation times are shown in table 3. Although the different clones of aphids were reared under similar controlled conditions during the five to six week period separating sampling from testing, mean weights ranged from 1.27 mg to 2.54 mg at the first infestation (D) and from 1.45 mg to 2.52 mg at the second infestation (D+6). The three tests were conducted over a two month period. During that time, the mean weight of the reference clone 'R' varied from 1.00 mg to 2.12 mg. From our experience, that kind of variation is not rare, depending on the level of aphid multiplication, although mean weight less than 1.0 mg had to be considered as less favourable. In such a case, the breeding of the clones had to be prolonged to make sure that the weight was representative of the characteristic of the clone.

On day 27, background seedling mortality was null in the non-infested replicates, and correction was applied using only clone 'R' as described before. The mean corrected number of dead and wilted seedlings (among 50 seedlings per replicate) ranged from 9.9 to 29.4 (table 3), which represents from 19.8% to 58.4% of seedling mortality and wilting. The analysis of variance showed that there was no significant difference in seedling mortality and wilting between the three regions where clones originated from, while the 15 aphid clones differed significantly (table 4). Among them, three groups were identified based on Scheffe's (1953) tests (table 3). All the clones from the central west of France had similar effects on seedlings, and it was the same for the aphid clones from the east. Clonal differences were essentially due to clones from the South: the minimal effects were observed for clones A4 and D1, while the maximal effects were observed for two other clones of the same region (B3 and C2), which induced about three times more seedling wilting and mortality. According to linear regression analyses, no significant relationship was found between individual weight of the aphids and seedling mortality/wilting at D (r^2 = 0.096; F = 1.38; d.f. = 1,13; P = 0.26) or at D+6 (r^2 = 0.069; F = 0.97; d.f. = 1,13; P = 0.34).

Molecular characterization of pea aphid clones

Allozyme polymorphism was detected at only four of the 36 loci (11.1%) with two alleles at each variable locus (*SDH, IDH, PEP-LGG, PEP-GL*). These four loci allowed us to distinguish between seven allozyme genotypes among the 15 aphid clones plus the reference clone. Taking into account colour polymorphism led to the characterization of one more genotype, i.e. E1r (table 5).

Based on RAPD analysis, 34 reproducible bands were scored within our sample of pea aphid lines. Thirty eight per cent of these RAPD fragments were polymorphic leading to the characterization of 12 genotypes among the 15 clones and 13 genotypes when including clone 'R'. Three genotypes

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Table 4. Results of the two-way ANOVA (region and clone) on the effect of aphid clones on alfalfa seedlings.

Source	d.f.	Sum of squares	Mean square	F value	Pr>F
Model	14	2637.02	188.35	7.98	<0.01
Error	75	1770.50	23.61		
Total	89	4407.52			
Source	d.f.	Type I SS	Mean square	F value	Pr>F
Region	2	31.35	15.67	0.66	0.51
Clone	12	2605.67	217.14	9.20	<0.01

Dependent variable is the corrected number of wilted + dead seedlings per replicate.

were found twice each among all samples. Two copies of the same genotype were found within the same region: they were collected in central west and south (32–33 and B3–C2). One copy of the same genotype was also detected in two different regions (D1–V2). When colour, allozyme and RAPD variations were combined, 12 genotypes were still detected among the 15 lines.

Discussion

A high level of genetic variability may be found within aphid populations, even when they reproduce asexually (Simon *et al*., 1999 but see Sunnucks *et al*., 1996). Mackay & Lamb (1988) observed that genetic variation occurred in two species of aphids, *A. pisum* and *A. kondoi*, developing on alfalfa in Australia where asexually reproductive lines have been introduced recently. By measuring responses to three alfalfa cultivars, these authors determined that 12 lines of *A. kondoi* were probably made up of three to seven clones, and three or four among 12 lines of *A. pisum*. Among our 15 clones, genetic variability was found between regions as well as within the same geographical region. Molecular characterization of our pea aphid clones made possible the detection of seven genotypes using allozyme markers and 12 with RAPDs. Combining colour, allozyme and RAPD polymorphisms did not increase the number of genotypes, so that two pairs of pea aphid clones still shared the same multi-locus genotype (clones 32–33 and B3–C2). Although clones 33 and 32 were collected from the same region (central-west), it is unlikely that they shared the same genotype. Indeed, clone 33 was initiated from a fundatrix, as demonstrated by the ratio of the *processus terminalis* to the basal part of the sixth antennal segment, while clone 32 derived either from another fundatrix or from a long-term asexually reproducing population, and therefore had a different genotype. Less ambiguous was the case of clones B3 and C2, collected in the south of France (fields 40 km apart) and similar at both allozyme and RAPD markers. First, the morphological measurements made on antennae indicated that these clones were not initiated from fundatrices. Moreover, they were collected from the south of France where anholocycly is expected to occur at a high rate so they are more likely copies of the same genotype. Use of hypervariable markers such as microsatellites would certainly help to confirm this hypothesis (Sunnucks *et al*., 1996; Simon *et al*., 1999).

Genetic markers linked to insect aggressiveness are of great interest for plant resistance management. Guirao *et al*. (1997) determined two types of the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) by phytotoxic disorders in plants, esterase patterns and molecular techniques, but there was no evident link between genotypes and their action on plants. For our own part, we have shown that two *A. pisum* clones (B3 and C2) with specific RAPD and allozyme profiles also displayed difference in aggressiveness on the resistant alfalfa. Indeed, these clones induced the maximal effect on seedling survival when considering the 15 clones. This difference was not caused by the aphid individual weight of these clones as no relationship was found between aphid individual weight

Table 5. Allozyme genotypes of the 15 pea aphid clones and clone 'R' used for biological tests.

Clones	Colour	*SDH*	*IDH*	*LGG*	*GL*	Genotype
29	Red	SS	SF	SS	FF	I
30	Green	SS	SS	SF	FF	II
31	Green	SS	SS	SF	FF	II
32	Green	SS	SS	SF	FF	II
33	Green	SS	SS	SF	FF	II
A4	Green	SF	SS	SF	FF	III
B3	Green	SS	SS	SF	SS	IV
C2	Green	SS	SS	SF	SS	IV
D1	Green	SS	SS	SF	FF	II
E1	Red	SS	SS	SF	SF	V
U1	Green	SS	SS	SS	SF	VI
V2	Green	SS	SS	SF	SF	VII
X3	Green	SS	SS	SF	FF	II
Y1	Green	SS	SS	SS	SF	VI
Y4	Green	FF	SS	SS	FF	VIII
Clone 'R'	Green	SS	SS	SF	FF	II

SS, homozygote for the slow allele; FF, homozygote for the fast allele; SF, heterozygote genotype. Genotype column: composite genotype at four allozyme loci + colour.

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and seedling mortality and wilting. Differences between France and USA in the ranking of alfalfa cultivars tested for pea aphid resistance were reported by Bournoville (1980) who assessed pea aphid net reproductive rates under controlled conditions. The present study reinforces the necessity to check the resistance of plants against aphids differing in origins and genotypes. But caution is required before making conclusions for plant breeding applications, because our results concerned aphid clones and not populations. However, it could be valuable to check whether such aggressive clones occur in populations or strains used in alfalfa breeding programmes and also to specify their patterns of spatial and temporal variation in natural populations of *A. pisum*.

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