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Hydroxamic acids in *Aegilops* species and effects on *Rhopalosiphum padi* behaviour and fecundity

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Abstract

As an environmentally compatible alternative to the use of conventional insecticides to control cereal aphids, the possibility of exploiting natural resistance to insect pests in wheat species was investigated. Previous work, comparing the antibiotic and antixenotic effects of hexaploid wheat (*Triticum aestivum*, AABBDD), tetraploid wheat (*Triticum durum*, AABB) and some A genome diploid species on the bird cherry-oat aphid, *Rhopalosiphum padi* L., found little difference between accessions in the higher ploidy plants, but the diploid species contained attributes that could be important in the breeding for resistance against aphids in the future. This study concentrated on wild accessions of diploid *Aegilops* species to which the closest ancestor of the B genome donor of hexaploid wheat belongs. The aphid *R. padi* showed reduced attraction and an increase in the intrinsic rate of population growth on the B genome species tested by compared to the hexaploid control. Investigation of a group of secondary metabolites, the hydroxamic acids or benzoxazinones showed that leaf tissue of one of these (*Aegilops speltoides*) contains high levels of DIMBOA-glucoside and of the main aglucone, 2,4-dihidroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) while no hydroxamic acids were found in the leaf tissue of *Ae. longissima*, *Ae. bicornis* and *Ae. sharonensis* and only trace levels in *Ae. searsii*. In those species, an unknown compound was present, which may have an effect on aphid behaviour. The effect of aphid feeding on levels of hydroxamic acids in *Ae. speltoides* and *Ae. sharonensis* was also examined. While a localised defence reaction to aphid feeding had been identified in the hexaploid and tetraploid species, a more systemic effect was observed in the diploid *Ae. speltoides*.

Key words: *Aegilops* species, *Aegilops speltoides*, aphid behaviour, *Rhopalosiphum padi*, hydroxamic acids, plant resistance.

Introduction

Modern wheat belongs to two species, the hexaploid *Triticum aestivum* (AABBDD, 2n = 42 chromosomes) and the tetraploid *T. durum* (AABB, 2n=28 chromosomes). These species are grown around the world in all temperate zones and are economically the most important of the small grained cereals. Under the influence of ancient and modern human cultivation, wheat has developed through a series of speciation events driven by amphiploidy (hybridisation) between diploid *Triticum* species and *Aegilops* species (Nevo *et al*., 2002).

The major grass subfamilies radiated 50-80 million years ago and the Triticeae tribe - where the ancestral species of hexaploid wheat belongs - diverged within the Poaceae about 35 million years ago (Huang *et al.*, 2002; Carver, 2009). Within the Triticeae, the B genome diverged before the separation of the A and D genomes (Carver, 2009). A number of potential speciation events have been proposed, but it is most likely that tetraploid wheat (AABB) first arose from hybridisation of *T. urartu* (AA) or *T. monococcum* ssp. *boeoticum* (AA) with *Ae. speltoides* (BB). Subsequent hybridisation of a cultivated tetraploid with *Ae. tauschii* (DD) led to the bread and spelt hexaploid wheats (AABBDD).

Insect pests are ubiquitous and diverse throughout the cultivation zones for wheat, but the most economically significant aphid pest of European cereal crops is the bird cherry-oat aphid (*Rhopalosiphum padi* L.). Factors such as crowding, temperature, photoperiod and poor host plant quality can cause an increased production of alatae (winged morphs) in aphid populations. Widespread dispersal of the alatae may lead to greater spread of transmitted diseases, particularly barley yellow dwarf virus (BYDV). Even low levels of antibiosis can be important in limiting infestation levels of *R. padi* (Hesler *et al.*, 1999). Several potential sources of host plant resistance have been identified against bird cherry-oat aphid in Europe including leaf phenolic content and phenylalanine ammonia-lyase activity (Smith *et al.*, 2004). In addition to these, the importance as an aphid resistance factor of a group of secondary metabolites, the hydroxamic acids (HAs), was recognised in the 1990s. Experiments showed aphids prefer to settle on plants that contain a lower concentration of HAs (Givovich and Niemeyer, 1991; Nicol *et al*., 1992) and that in artificial diet an increased level of HA concentration decreased the survival rate of cereal aphids (Barria *et al*., 1992).

HAs were first discovered in the 1960s in rye, in relation to fungal diseases, and later found in maize where they were associated with resistance to the European corn borer, *Ostrinia nubilalis* (Hubner) (Klun and Robinson, 1969; Niemeyer, 2009). HAs can be found in the cultivated monocotyledons maize, wheat (Nicol *et al*., 1992) and rye. The HA 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) can be found in the wild Hordeum species (Barria *et al*., 1992) although no HAs present in the modern cultivated barley varieties.

The maximum recorded HA level in cultivated wheat is between 1.4 to 10.9 mmol/kg fresh weight (FW) (Copaja *et al*., 1991) and nearly 40 mmol/kg FW was found in wild rye (Nicol *et al*., 1992; Nicol *et al*., 1993).

2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the main HA aglucone to be found in wheat extracts, has been shown to cause antibiosis, feeding deterrence, decreased performance and reduced reproduction in aphids (Figueroa *et al*., 2004). In addition, it has mutagenic effects and affects the level of genetic polymorphism in aphid populations (Figueroa *et al*., 2004).

As the concentration of HAs increases, aphids need a longer time to search for suitable phloem vessels (Givovich and Niemeyer, 1994; 1995) because they feed from a single sieve tube and the HA concentration can be different between sieve tubes (Givovich *et al*., 1994). They also spend a longer period ingesting xylem fluid (Ramìrez and Niemeyer, 1999), which does not contain HAs (Givovich and Niemeyer, 1995). The HA level also affects virus transmission because aphids take a longer time to attach to the phloem and are therefore less able to transmit BYDV (Givovich and Niemeyer, 1991; Nicol *et al*., 1992).

In a preliminary antixenotic study we investigated the host plant selection / settling behaviour of *R. padi*, on a very diverse set of hexaploid (*T. aestivum*) and tetraploid (*T. durum*) wheats, a few cultivars showed a moderate reduction in aphid preference while several were more preferred than the hexaploid control variety, Solstice (Elek *et al*., 2009; Elek *et al*., 2013). Most of the diploid species tested, such as the A genome *T. monococcum* and *T. boeoticum*, were less preferred by aphids than the hexaploid control (Elek *et al*., 2012), but the greatest resistance was found in the B genome diploid species *Ae. speltoides* and *Ae. sharonensis*. This study investigated the antibiotic and antixenotic effect of different B genome diploid species against *R. padi* together with HPLC analysis to evaluate HA levels in the plant tissues.

Materials and methods

Plant material

Accessions of *Ae. sharonensis* (2170003, 2170004), *Ae. bicornis* (2190001, 2190003), *Ae. longissima* (2150001, 2150002), *Ae. speltoides* (2140008) and *Ae. searsii* (2210001, 2210003) were supplied by the John Innes Centre, and the hexaploid variety Solstice by Rothamsted Research.

Aphids

R. padi was collected from volunteer wheat plants from the field in Thriplow, Herts, UK in September 2007 and again (refreshing the colony) in 2008 and 2009. The colonies used in this study were established from one aphid using the mildew resistant spring wheat variety Tybalt as the culture plant. Aphids were kept in a glasshouse in a temperature range of 12-25 °C and 16:8 L:D, in 25 × 25 × 70 cm insect cages. Colonies were checked regularly to avoid contamination with other aphid species and diseases.

For the settling test, mixed age alate aphids were collected from the top of the rearing cage with a small electric potter. For the fecundity test and the aphid feeding experiments, wingless aphids (apterae) were gently shaken off or removed from the culture plants with a paint brush.

Settling test of aphids on wheat plants

This was a choice test in a small ventilated cylindrical chamber (12 cm diameter by 20 cm high) between one 7 day old seedling each (growth stage 11, Tottman and Makepeace, 1979) of the test (*Ae. speltoides* 2140008 or *Ae. sharonensis* 2170003) and of the control hexaploid plant (Solstice). Plants were growing in Levington F1 seedling compost in 2.5 × 2.5 cm cells. To set up the experiment the cells were moved onto a wet sand tray to keep the humidity high during the testing period. Twenty randomly allocated *R. padi* alatae were placed in each cage and there were 14 to 17 replicates per plant species. Alatae, which had settled on each of the two plants, were counted and recorded after 2, 5 and 24 hours. The test was conducted in the glasshouse at 20 °C, 16:8 L:D. For each observation the difference in the number of alatae settling on Solstice compared to the test variety was calculated and the full dataset was analysed using the linear mixed model routine with residual maximum likelihood estimation (REML) in GENSTAT (13th edition). Occasions and cages were used as random terms, with test variety, time (2, 5 or 24 hours) and their interaction used as fixed effects. No transformation was required.

Aphid fecundity tests on wheat plants

This was a no choice test used to determine the intrinsic rate of population increase (r_m) (Wyatt and White, 1977) of *R. padi* on the test varieties (*Ae. speltoides* 2140008, *Ae. sharonensis* 2170003, and *Ae. longissima* 2150002) compared to the control plant (Solstice).

In a separate experiment, the r_m was determined on *Ae. searsii* (2210003) compared to Solstice.

Seven alatae were put in a cage with one plant of each test variety to produce similarly aged neonate nymphs for the experiment. After 24 hours the alatae were removed and the nymphs were allowed to develop on those plants for 3 days until they had moulted to 2nd instar. At this age the nymphs were large enough to transfer easily onto 7 day old test seedlings (GS11) which were grown in Levington F1 seedling compost. One 3 day old nymph was placed on the middle part of the first leaf of each of 8 to 10 replicate seedlings and enclosed in a 2 cm diameter clip cage (MacGillivray and Anderson, 1957). The developing aphids were monitored at the same time each day until they produced their first nymph (time d). From the first day of production, the new nymphs were removed and their numbers recorded daily over a period at least equivalent to time d (equivalent to the days from birth to the first nymph produced). The experiment was carried out in a glasshouse at ≈20 °C and 16:8 L:D. From the data the intrinsic rate of population increase was calculated using the formula [1] of Wyatt and White (1977).

[1] Intrinsic rate of population increase:

$$r_m = c\,(\log_e Md) / d$$

Where c is a constant = 0.74, d = pre-productive period in days (from birth to the first nymph produced), Md = number of nymphs produced in the reproductive period equal to d.

The intrinsic rates of population increase from the two experiments were analysed using the linear mixed model routine (REML). Since no significant differences were found between the experiments, the results of *Ae. searsii* are presented together with the rest of the test varieties.

The intrinsic rate of population increase, average number of nymphs produced per day and the time taken from birth to produce the first nymph were analysed using ANOVA. Variation among varieties was partitioned into an overall comparison of diploid species against Solstice plus differences between the diploid species.

Hydroxamic acid analysis of different plant tissues

Levels of HAs were measured in replicated 6 day (GS 11), 9 day (GS 11-12) and 12 day old (GS12-13) seedlings of *Ae. sharonensis* (2170003, 2170004), *Ae. bicornis* (2190001, 2190003), *Ae. longissima* (2150001, 2150002), *Ae. speltoides* (2140008) and *Ae. searsii* (2210001, 2210003) grown in compost under controlled conditions (20 °C and 16:8 L:D), with two replicates per time point per variety, each replicate consisting of 3 plants. Leaf, coleoptile and root tissue samples, frozen in liquid nitrogen, were ground in a pestle and mortar, ensuring the sample remained frozen during preparation. The ground tissue was transferred to an Eppendorf tube, which was frozen to avoid the sample melting and sticking to the side of the tube. 25 mg of frozen sample was weighed into an Eppendorf tube containing 0.5 ml of a 98% methanol + 2% acetic acid mixture. It was sonicated for 10 minutes and centrifuged for 10 minutes on 21.5 g at 4 °C. Supernatant was transferred into a glass vial for analysis by HPLC. A reversed phase HPLC procedure was adapted from previously published methods (Baumeler *et al*., 2000).

A thermal hypersil C-18, (5µm, 4.6 × 250 mm) column was used, with mobile phase (A) HPLC grade water and (B) methanol/isopropanol (95/5) + 0.025% acetic acid. The gradient profile of solvent A and B was: 0-2 min, 10% B; 2-11min, 10-50% B; 16-17min, 50 to 10% B. Injection volume was 20µl, the flow rate was 1 ml/min and the run time 17 minutes. From the HPLC it was possible to determine the levels of HAs found in the plant tissues in mmol/kg fresh weight (FW) by using a range of standard DIMBOA and DIMBOA glucoside concentrations. The synthetic standards were provided by Prof. Dieter Sicker, University of Leipzig.

The HPLC method used could not separate DIMBOA and HDMBOA-glucoside (2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside), and therefore these compounds are presented as combined values throughout, although levels of the latter are known to be low in wheat (Oikawa *et al*., 2002).

HA levels were compared across species, varieties within species and time points using two-way ANOVA in GENSTAT. A square-root transformation was required to stabilize the variance.

Aphid feeding experiment on plants and sampling for HA analysis

To determine the effects of *R. padi* feeding on HA levels in leaf tissues of *Ae. speltoides* (2140008) and *Ae. sharonensis* (2170003), batches of twenty *R. padi* alatae were put into replicated cages with one seedling (GS 11) for 24 hours to produce pre-conditioned nymphs for the experiment after which the alatae were removed. Nymphs were allowed to develop on those plants for 3 days until they moulted to 2nd instar and then they were transferred to 7 day old test plants (GS 11-12). Batches of twenty-five 3 day old nymphs were put in a clip cage on the middle part of the first leaf of each of fifty 7 day old (GS 11-12) test plants, which were kept in a controlled environment (20 °C and 16:8 L:D). Fifty equivalent plants with clip cages, but no aphids were also kept under the same conditions as controls. The first sampling was done on approximately half of the replicate plants after 24 hours of aphid feeding, harvested in 3 replicates of 8 plants each. The aphids were removed from the leaf using a small paint brush, and the above ground part of the plant was harvested and separated into three parts; 1) the leaf area immediately under the clip cage where the aphids had been feeding, 2) the coleoptile and 3) the remainder of the leaf material. The tip of the first leaf was not included. These were wrapped in foil, labelled and frozen immediately in liquid nitrogen. Control plants were sampled in the same way as the test plants. After 48 hours of aphid feeding the sampling was repeated on the remaining plants. Plant samples were prepared for HPLC analysis as described in the previous section.

Data were analysed using three-way ANOVA in GENSTAT. A square-root transformation was required to stabilise the variance.

Results

Settling test of aphids on wheat plants

There was evidence of a differential response over time ($P < 0.001$), but no evidence of an interaction ($P = 0.994$) or of differences between the test varieties ($P = 0.797$). After 24 hours, significantly fewer aphids (Solstice - test = 3.07, SE 0.71, $P < 0.001$) settled on *Ae. speltoides* than on the hexaploid control (figure 1). Similarly, when *Ae. sharonensis* was tested against Solstice significantly more aphids settled on the control variety (Solstice - test = 2.882, SE 0.60, $P < 0.001$). No differences were found at 2 or 5 hours, and no difference was found between the test varieties in aphid settling behaviour.

Aphid fecundity tests on wheat plants

In each of three aspects of fecundity measured the B genome diploids proved more challenging to *R. padi* than the control hexaploid Solstice (figure 2). *R. padi* nymphs feeding on the diploid varieties took 8-12 days to start to reproduce, while nymphs feeding on the hexaploid variety took 7-9 days from birth ($P < 0.001$). The average daily nymph production was higher for Solstice, (5.2 nymphs/day; SE 0.44), than on the diploid varieties, (3.3 nymphs/day; SE 0.24) ($P < 0.001$). On the *Aegilops* species the average total nymph production was only 39 while on Solstice it was 63 during the test period ($P < 0.001$) (figure 2). This difference also showed in the size of the adult aphids, since aphids

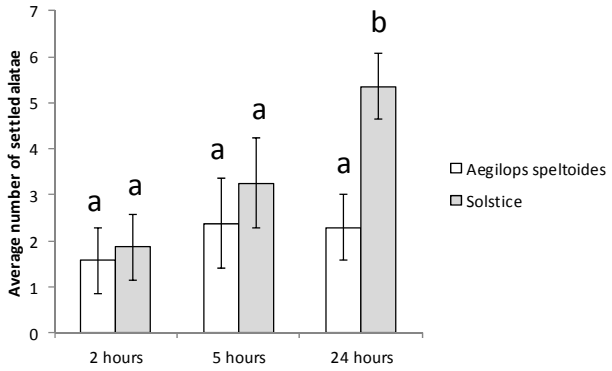

Figure 1. *R. padi* alate settlement on *Ae. speltoides* and the hexaploid control (Solstice) after 2, 5 and 24 hours. No differences were found in the number of settled aphids after 2 and 5 hours, but significantly more aphids settled on Solstice after 24 hours ($P < 0.001$). Different letters above the columns indicate significant difference at $P = 0.05$.

feeding on the diploid varieties were noticeably much smaller than those on Solstice. There was some variation between the diploid varieties ($P < 0.001$, figure 2a), with the number of nymphs produced per day on *Ae. sharonenesis* and *Ae. searsii* not significantly different to Solstice.

The lowest nymph production was noted on *Ae. speltoides* where aphids took on average 3 days longer to produce the first nymph. The difference in intrinsic rate of population increase between the varieties was highly significant ($P < 0.001$) the lowest r_m was calculated on *Ae. speltoides* 0.218 (SE 0.009), which was 33% lower than the hexaploid control Solstice 0.323 (SE 0.009) (figure 2b).

Hydroxamic acid in different plant tissues

When the coleoptiles and the roots of *Ae. sharonensis*, *Ae. longissima*, *Ae. bicornis* and *Ae. searsii* were analysed only low levels of HAs were detected compared to levels found previously in *Ae. speltoides* by Gordon-Weeks *et al*., (2010) (table 1).

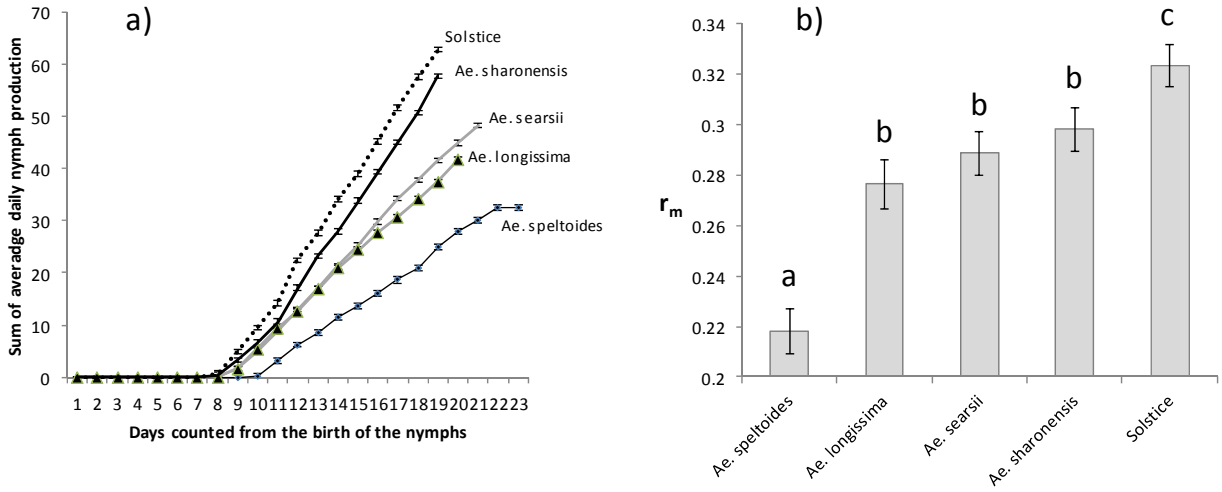

Figure 2. a) Sum of the average daily nymph production by *R. padi* on the B genome *Ae. speltoides*, *Ae. longissima*, *Ae. searsii*, *Ae. sharonenesis* and on the hexaploid Solstice. **b)** The intrinsic rate of population increase (r_m) of *R. padi* on *Aegilops* sp. and the hexaploid control Solstice ($P < 0.001$). Different letters above the columns indicate significant difference at $P = 0.05$.

Table 1. Average levels of hydroxamic acids (mmol/kg fresh weight) in the root, coleoptile and the leaf tissues of different B genome species at 6 (GS 11), 9 (GS 11-12) and 12 days (GS 12-13). The levels of HAs (mmol/kg fresh weight) in *Ae. speltoides* as measured by Gordon-Weeks *et al.* (2010) are presented in bold.

Sampled area	Species	6 days				9 days				12 days			
		DIMBOA-glucoside		DIMBOA (+HDMBOA-glu)		DIMBOA-glucoside		DIMBOA (+HDMBOA-glu)		DIMBOA-glucoside		DIMBOA (+HDMBOA-glu)	
		mmol/kg fresh weight											
Root	*Ae. sharonensis*	0.82	SE 0.08	0.28	SE 0.09	0.81	SE 0.08	0.43	SE 0.09	0.69	SE 0.08	0.44	SE 0.09
	Ae. longissima	0.9		0.25		0.6		0.28		0.53		0.47	
	Ae. searsii	1.35		0.48		0.91		0.33		0.53		0.28	
	Ae. bicornis	0.76		0.16		0.58		0.2		0.51		0.12	
	Ae. speltoides	**5**	**SE 0.37**	**14.5**	**SE 3.95**	**4.5**	**SE 0.28**	**10**	**SE 0.97**	**4.5**	**SE 1.25**	**5**	**SE 0.19**
Coleoptile	*Ae. sharonensis*	1.58	SE 0.22	1.74	SE 0.31	0.97	SE 0.22	1.41	SE 0.31	0.37	SE 0.22	1.27	SE 0.31
	Ae. longissima	1.3		0.78		0.85		0.86		0.47		0.8	
	Ae. searsii	2.97		3.22		0.61		1.5		0.44		1.16	
	Ae. bicornis	1.3		0.78		0.69		0.85		0.41		0.33	
	Ae. speltoides	**26.5**	**SE 1.82**	**25.2**	**SE 0.63**	**9**	**SE 0.75**	**29.7**	**SE 1.55**	**8**	**SE 0.59**	**18.9**	**SE 3.24**
Leaf	*Ae. sharonensis*	null		null		null		null		null		null	
	Ae. longissima	null		null		null		null		null		null	
	Ae. searsii	4.29 E-05		null		3.14 E		1.92 E-04		3.46 E-05		2.91 E-05	
	Ae. bicornis	null		null		null		null		null		null	
	Ae. speltoides	**27**	**SE 4.71**	**22.2**	**SE 3.30**	**18.5**	**SE 6.14**	**25**	**SE 4.35**	**17.5**	**SE 4.71**	**10**	**SE 1.21**

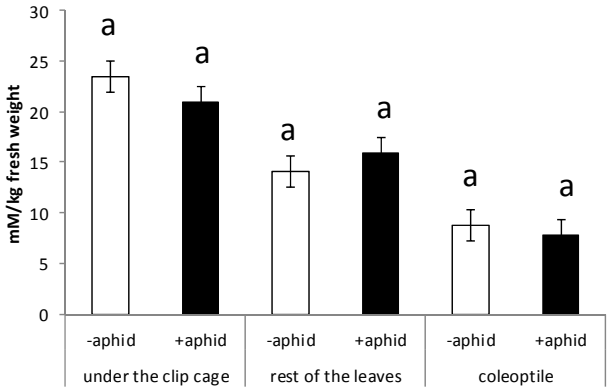

Figure 3. Changes in DIMBOA-glucoside level in the leaf tissue of *Ae. speltoides* after 48 hours of *R. padi* feeding. None of the parts of the plant tested showed any significant difference in the DIMBOA-glucoside level after aphid feeding. The above ground parts of the plant were analyzed separately; under the clip cage (the middle part of the first leaf where 25 aphids were feeding), the coleoptile and the rest of the leaves not including the tip of the first leaf.

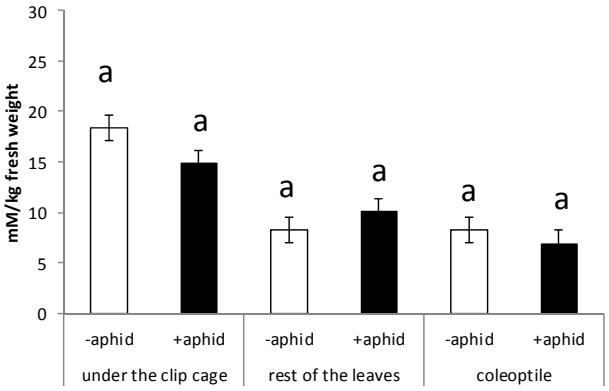

Figure 4. Changes in DIMBOA (+ HDMBOA-glucoside) level in the leaf tissue of *Ae*. *speltoides* after 48 hours of *R. padi* feeding. None of the parts of the plant tested showed any significant difference in the DIMBOA (+ HDMBOA-glucoside) level after aphid feeding. The above ground parts of the plant were analyzed separately; under the clip cage (the middle part of the first leaf where 25 aphids were feeding), the coleoptile and the rest of the leaves not including the tip of the first leaf.

In the roots DIMBOA-glucoside levels were recorded between the diploid species ($P = 0.021$) and there was evidence of significant changes over time ($P < 0.001$) due to a decrease with plant age. In the 6 day old plants the average DIMBOA-glucoside level was between 0.76 and 1.35 mmol/kg FW, which decreased by day 12 to 0.51-0.69 mmol/kg FW. The DIMBOA (+HDMBOA-glucoside) level varied between species ($P = 0.027$) but there was no evidence of differences over time. The average DIMBOA (+HDMBOA-glucoside) level was lower at between 0.16 and 0.48 mmol/kg FW in 6 day old plants and 0.12-0.47 mmol/kg FW in 12 day old plants.

Higher levels of HAs were measured in the coleoptiles and again there was evidence of differences with plant age ($P < 0.001$) and between the diploid species ($P = 0.02$) for DIMBOA-glucoside. In the coleoptiles of the 6 day old seedlings the DIMBOA-glucoside ranged across species from 1.3 to 2.97 mmol/kg FW, which decreased by day 12 to 0.37-0.47 mmol/kg FW ($P < 0.001$). The DIMBOA (+HDMBOA-glucoside) level showed some evidence of change over time ($P = 0.045$) due to a general decrease with plant age, to differences between the diploid species ($P = 0.002$) and to an interaction between variety and time point ($P = 0.042$), as one variety showed an increase rather than a decrease with plant age. The DIMBOA (+HDMBOA-glucoside) level was between 0.78 and 3.22 mmol/kg FW in 6 day old plants and between 0.33 and 1.27 mmol/kg FW by day 12.

HPLC of the 6 day old (GS 11) *Ae. speltoides* leaf tissue extracts detected high levels of DIMBOA-glucoside (27 mmol/kg FW) and DIMBOA (+HDMBOA-glucoside) (22.2 mmol/kg FW), but none of the known HAs could be detected in the leaf tissue of the other B genome species *Ae. sharonensis*, *Ae. longissima* and *Ae. bicornis* (table 1). In the leaf tissue extract of *Ae. searsii*, there were two peaks, which coincided with DIMBOA-glucoside and DIMBOA (+HDMBOA-glucoside). The peak areas for both retention times were too small to be able to confirm their identities and further analysis is required.

The leaf tissue of all the B genome species contain an unidentified compound which eluted on the HPLC at 13.6-13.7 minutes and this may be the same compound seen to elute at 13.6-13.8 minutes in *Ae. speltoides* and also in *T. aestivum* extracts (unpublished data).

Effect of aphid feeding on the HA levels of seedlings

Across the experiment *Ae. speltoides*, showed non-significant changes in HA levels in the leaf tissue sampled from immediately under the clip cage in comparison with control plants.

For the DIMBOA-glucoside in *Ae. speltoides*, there was evidence of a difference between parts of the plant ($P < 0.001$), with lower levels in coleoptiles compared to the leaves, as found by Gordon-Weeks *et al*., (2010) in 9 day old plants (table 1), but no other significant differences. After 24 hours of aphid feeding, the DIMBOA-glucoside level was similar in the presence and absence of aphids, in the leaf area where the aphids were feeding we recorded 20.9 mmol/kg FW and 23.5 mmol/kg FW in the control. In the coleoptile we measured 8.7 mmol/kg FW in the absence of aphids and 7.7 mmol/kg FW after aphid feeding (figure 3). After 48 hours we were still not able to record statistical difference in the plants in the presence and absence of the aphids.

Through the experiment for DIMBOA (+HDMBOA-glucoside) in *Ae. speltoides*, there was evidence of a difference between parts of the plant ($P < 0.001$), with increased levels in the middle of the leaf, compared to the coleoptiles and the rest of the leaves. There was also evidence of a three-way interaction ($P = 0.026$), due to an increase in levels between 24 and 48 hours in the middle

of the leaf compared to a slight decrease in the control. After 24 hours of aphid feeding, the DIMBOA (+HDMBOA-glucoside) levels were similar in the test and the control plants. In the leaf area where the aphids were feeding we recorded 14.8 mmol/kg FW and in the control 18.4 mmol/kg FW (figure 4), and the difference was still not significant after 48 hours of aphid feeding.

Ae. sharonensis was also tested in this aphid feeding assay, but neither DIMBOA-glucoside nor DIMBOA (+HDMBOA-glucoside) were detected by HPLC in the control leaf extract, with no aphid feeding, or in the leaf in the immediate region of the feeding aphids.

Discussion and conclusions

These studies pull together a series of observations which provide more evidence about the importance of wild relatives of polyploid wheat. The wild diploid species possess a wide range of adaptive diversity to diseases, pests and ecological stress for this reason these species could be a potential germplasm source for further crop improvement. Preliminary studies of the hexaploid (AABBDD, 2n = 42) and tetraploid (AABB, 2n = 28) varieties showed that the hexaploid wheat does produce hydroxamic acids, but in moderate concentrations of 1.2 to 7.8 mmol/kg FW (Elek *et al*., 2013). In the tetraploid *T. durum* there was a higher level of DIMBOA-glucoside leading to higher overall levels of 11.3 to 14.3 mmol/kg FW of total HA. This reduction in the level of HA from the 4× to 6× species was also reported by Niemeyer and Jerez (1997). When the A genome species *Triticum boeoticum* and its cultivated form *Triticum monococcum* (2n = 14) was investigated we could not detect any hydroxamic acid in the leaf tissue but we noticed negative effect on aphid settlement and nymph production on certain accessions (Elek *et al*., 2012). In our current studies we found similar effect during testing a group of B genome species including the closest ancestral of the B genome donor (*Aegilops speltoides*) of the hexaploid wheat. Most of the species tested have only very low or non-detectable levels of the known HAs in the leaves of seedlings. However, the accession of *Ae. speltoides* (214008) that was tested contained over 20 mmol/kg FW DIMBOA in the 6 day old seedling leaves, which is a considerably higher level of HAs than reported previously in either the 6× or 4× species. Niemeyer published similar observations in 1988, he also measured high level of hydroxamic acids in the *Ae. speltoides* species, 14.3 mmol/kg FW, although he was testing 10 day old seedlings, lower level of HAs was recorded in the tetraploid varieties (4.2 mmol/kg FW) and only 1.4 mmol/kg FW was detected in the hexaploid varieties. *Ae. sharonensis,* the other representative of the B genome, is a null hydroxamic acid expressor in the leaf but it can produce DIMBOA-glucoside and DIMBOA (+HDMBOA-glucoside) in the root and coleoptile, therefore the metabolic pathways are intact and there is a difference in the regulatory control of expression compared to *Ae. speltoides*. It would be unwise for us to generalise from these limited results on single accessions from each species, but it is clear that considerable scope exists among the potential diploid progenitors for variation in the levels of HA. It is very possible that the hybridisation event and the development of the polyploid species have limited the full range of allelic variation that could be available in the gene pool for breeding modern wheat cultivars.

Gianoli and Niemeyer (1997) studied the HAs level changes in a hexaploid wheat variety Paleta due to *R. padi* aphid feeding and he revealed an infestation of 25 aphids for 48 hours is required to elicit an induced response; the accumulation of HAs in the infested leafs. Studying the effect of *R. padi* feeding, we established that DIMBOA (+HDMBOA-glucoside) in the hexaploid and tetraploid varieties was higher after 24 hours of aphid feeding and the peak was maintained after 48 h, while the DIMBOA-glucoside levels was lower compared to plants with no feeding damage. We were able to record significant difference in the DIMBOA level between the control and the infested plants (hexaploid variety - Solstice) even after 24 hours of aphid feeding with a further increase after 48 hours. These effects within the host plant were localised and did not spread to the base of the leaf or the rest of the plant (Elek *et al*., 2013). The diploid *Ae. speltoides* behaved differently to the other species, in that there was only a small non-significant change in the HA level after aphid damage. Gordon-Weeks *et al*. (2010) made similar observations, and did not record significant changes in the levels of HAs produced by *Ae. speltoides* after aphid feeding. In *Ae. sharonensis* there were no HAs in the leaf tissue after aphid feeding. Since the HPLC method used could not separate DIMBOA and HDMBOA-glucoside, the possibility that HDMBOA-glucoside increases in *Ae. speltoides* in response to aphid feeding, as has been reported for maize (Ahmad *et al*., 2011), will be investigated in future work.

The settling test is a choice test based on physical and chemical differences between the test plant and the control plant (the hexaploid wheat variety Solstice). For the test, alate aphids were chosen because their olfactory receptors are more developed than in apterous aphids and alatae are the primary colonising morph. Both B genome varieties tested, whether they were HA producers or non-producers, were less attractive to *R. padi* alatae, and significantly fewer aphids settled on these plants compared to the control. However, when the diploid species were tested against each other there was no difference in aphid preference between them. The negative effect on aphids in the non-hydroxamic acid producing species could be caused by the unidentified compound, which was detected by HPLC and/or the chemical and physical characteristics of the plants, this will be investigated further.

Nymph production on hexaploid and tetraploid varieties was not significantly different in previous studies (Elek *et al*., 2013), but settlement and nymph production were significantly lower on the A and B genome diploid species compared to the hexaploid control (Elek *et al*., 2009; 2012).

The extreme levels of HAs in *Ae. speltoides* clearly had a negative effect on *R. padi* host plant selection and nymph production, which may indicate that this species

could be important in further crop improvement, by traditional breeding, to use as a resistance source and, combined with other defence mechanisms, to develop an effective aphid resistant variety.

The higher levels of HAs observed in *Ae. speltoides* correlates with the significantly lower intrinsic rate of population increase of *R. padi* compared to that on the hexaploid control. Certainly the development and production of the aphids on this diploid species was substantially reduced to a level which, if replicated in the field, would greatly facilitate protection of the crop from aphid damage. The small scale study reported here is unable to conclude that the HAs are definitely responsible for the observed effects upon aphid behaviour and growth. Indeed, the representatives of the species tested are extremely diverse and must vary in many aspects of their defences against pests. Nevertheless, the results are suggestive and the manipulation of HAs in cultivated wheat remains a candidate strategy towards the goal of enhancing host plant resistance to aphid damage. Furthermore, the identification of superior aphid resistance combined with higher HA levels in *Ae. speltoides* suggests that this accession (2140008) should form part of any follow up work designed to introgress novel resistance into modern cultivated wheats.

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