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The role of the benzoxazinone pathway in aphid resistance in wheat

By

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1.ABSRACT

The hydroxamic acids or benzoxazinones (BXs) are natural cereal defence compounds, biosynthesised early in development, that deter insects, pathogens and weeds. The most active are DIMBOA and DIBOA, which are stored as their inactive glucosides in the vacuole and released upon pathogen attack or wounding.

The aim of the project was to test whether BX production can be exploited to breed wheat varieties with sufficient resistance, mainly to aphids but to certain pathogens as well, to benefit UK agriculture. The wheat *BX* genes (*TaBX*s) have been sequenced, and the molecular genetic basis for BX accumulation to be analysed. We investigated the BX content and its variation in UK wheats by screening cultivars for *TaBX* expression and BX accumulation in different parts of the plant (leaf, coleoptile and root). We compared these measurements with the performance of two cereal aphids, the bird cherry oat aphid, *Rhopalosiphum padi,* and the grain aphid*, Sitobion avenae,* in bioassays to test settling preference, growth rate and fecundity on the different wheat lines. Additionally, we aimed to establish whether the pathway could be induced in the plant. This would be very advantageous as inducible defence systems are less costly to the plant. In hexaploid wheats we did not find significant variation in regulation of the pathway but *TaBX* gene expression was lowest and declined most dramatically in leaf tissue. We found that the enzymes that activate the glucoside were induced by aphid feeding in the leaf and that, consequently, DIMBOA levels increased. There was variation in the degree of this response but it did not correlate clearly with resistance, possibly because overall levels of DIMBOA were too low. When we broadened our screen to include wheats with different ploidy levels we found that a B genome wheat that contains 8 times the hexaploid levels of DIMBOA was the least favoured by aphids of all the wheats that we tested. This plant showed sustained levels of *TaBX* expression in the leaf, suggesting that regulation of the genes in the pathway may be important. DIMBOA was accumulated to particularly high levels in the leaf extracellular spaces through which the aphid must pass its stylet during the initial stages of feeding. However, other diploid wheats (A and B genome) that do not contain BXs in the leaves were also more resistant than the hexaploids. These findings demonstrate that ancestral wheats display aphid resistance traits, including both BX dependent and BX independent traits, that are absent from cultivated hexaploid wheat populations. Our findings can now be used for direct breeding strategies, or genetic modification, to produce wheat plants with enhanced resistance, but the former

would require wide crosses, combining different traits using germplasm from wheat with different ploidy levels.

2.SUMMARY

The hydroxamic acids or benzoxazinones (BXs) are a family of plant defence compounds produced in cereals, including wheat, maize and rye. The two most biologically active of the BXs, DIBOA, 2,4-dihydroxy -1,4-benzoxazin-3-one and, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) have been shown to be directly toxic to aphids when applied to artificial diets and to inhibit microbe and plant root growth. Hence, the broad ranging biocidal activity of these compounds makes them good candidates for developing both pest and disease resistance in crop plants. In maize and wheat, the concentrations of the BXs are highest in the young seedling and decrease as the plant matures. This has the advantage that the compounds, potentially unpalatable to humans, do not reach the human food chain.

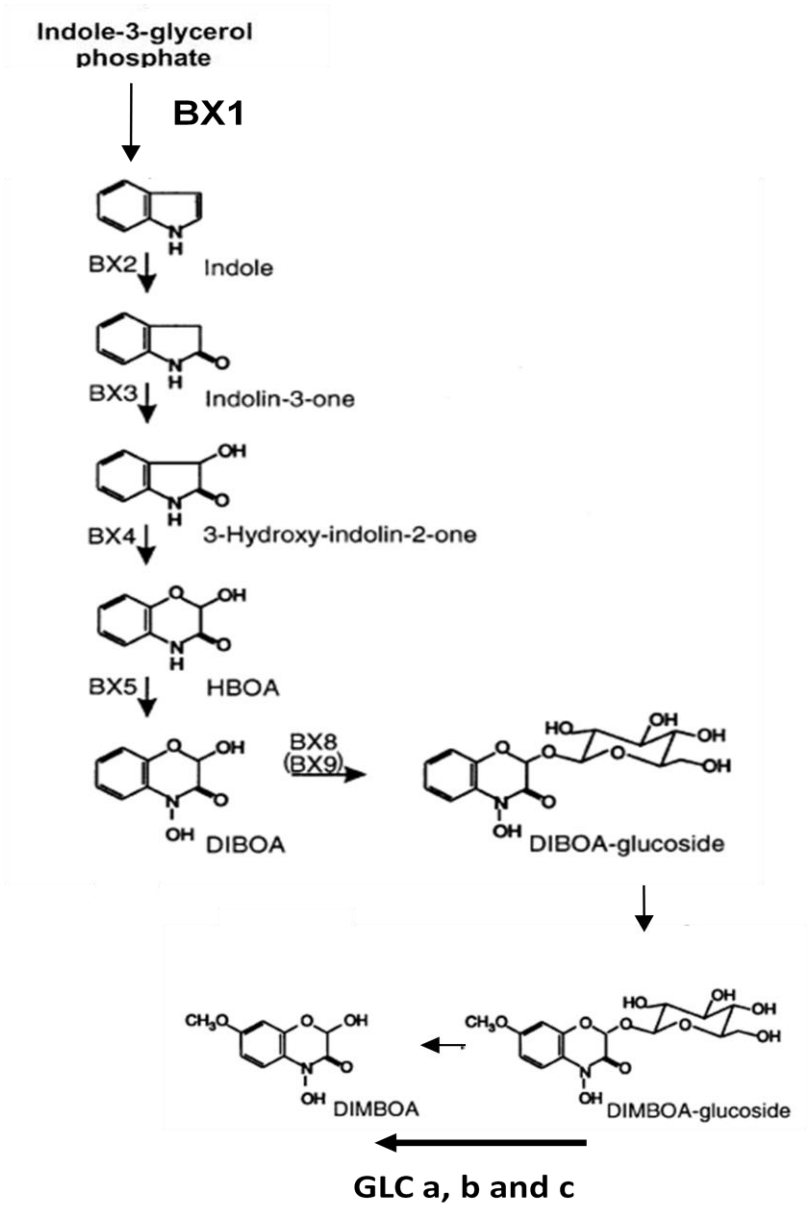


Figure 1. The BX pathway in maize

At the molecular level, the BX biosynthetic pathway has been fully characterised in maize but some genes remain to be cloned in wheat (see Figure 1). The pathway commences by the conversion of all cytosolic indole into DIBOA, catalysed by four Cytochrome P450 enzymes, BXs 2 – 5, which have been cloned and functionally

characterised in both wheat and maize. The final steps in the pathway have been identified in maize and this has revealed that as soon as DIBOA is formed it is glucosylated by two glucosyl transferases, BX8 and 9 and the glucoside (DIBOA-glu) is transported into the vacuole for storage. The activity of the pathway may also be regulated by release of unglucosylated DIMBOA or DIBOA (the aglucones) from the stored, inactive, glucoside by glucosidases. Three genes encoding BX specific glucosidases, *TaGlu a, b and c* have been cloned, sequenced and functionally characterised in hexaploid wheat.

2.1 Aims of the Project

The aim of this work has been to establish whether there is a relationship between BX concentration in wheat and aphid resistance. This has involved a detailed analysis of the localisation and regulation of the pathway in different parts of the plant during development in a range of hexaploid wheats and wheats of different ploidy levels have been studied. These studies were followed by a series of aphid bioassays to determine whether there was a correlation between BX levels and aphid resistance.

2.2 Plant materials

Analyses were performed on five commonly grown UK hexaploid winter wheat varieties, Claire, Malacca, Solstice and the orange wheat blossom midge resistant varieties Robigus and Welford, supplied by RAGT, LIMAGRAIN and KWS (British wheat breeders). A Chilean variety, Taluen, which has been shown to contain low levels of BXs immediately after germination compared to other local varieties (supplied by Professor Hermann Niemeyer), and an Australian variety, Tasman, (from the Australian Wheat Board) were included to provide a wider comparison. Tasman releases high levels of BXs from the roots, has high allelopathic activity and has been used as a mapping population parent. As an example of a tetraploid, Alifen, which is a durum wheat variety from Chile (supplied by Professor Hermann Niemeyer), shown to contain high BX concentrations immediately after germination was tested. The following diploid wheats were analysed: *Triticum monococcum* and *Triticum boeoticum,* provided by Kim Hammond-Kosack (Rothamsted Research)*,Triticum tauschii and Aegilops speltoides, longissima, sersii , bicornis* and *sharonensis* obtained from Mr. Stephen Reader at the John Innes Centre, Norwich, UK. *T. monococcum* and *T. boeoticum* were chosen as examples of A genome wheats. Although *T. monococcum* it is not believed to be the actual originator of the A genome

of hexaploid wheat it is the only diploid species to have been subjected to cultivation and has recently been developed as a reference species for wheat genetics and genomics . *Ae. speltoides* is possibly related to an ancestor of the S-genome progenitor of the B genome of hexaploid wheat. *T. tauschii* has been shown to be the D genome donor of the hexaploid by the creation of synthetic wheat with the tetraploid *Triticum dicoccoides* that has similar properties to *T. aestivum*.

2.3 Constitutive levels of BX content and gene expression in hexaploids

In order to establish a reliable method to quantify the BXs in wheat plant tissues we used a non aqueous extraction procedure, followed by high pressure liquid chromatography (HPLC), which we have adapted from earlier published methods (See Figure 2).

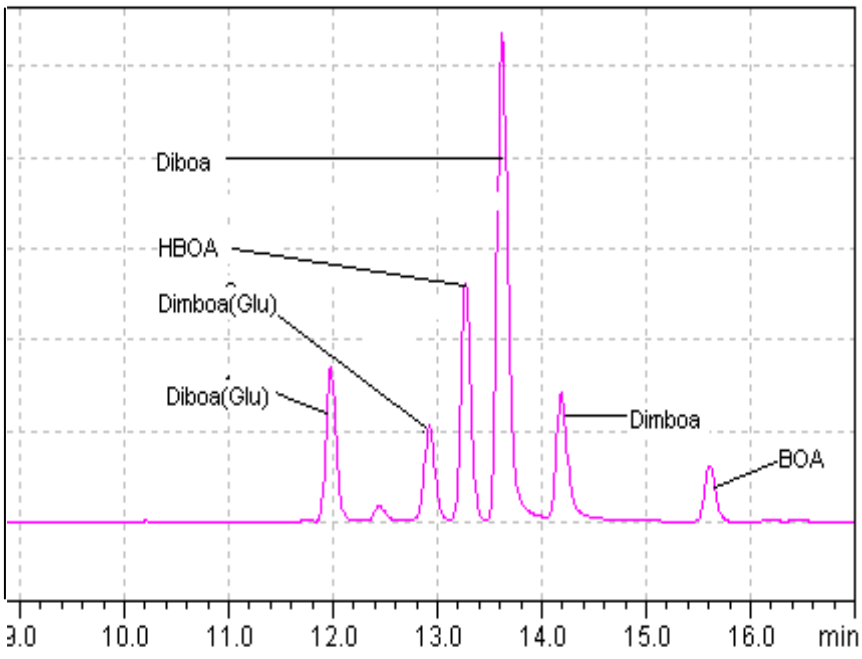


Figure 2 HPLC separation of BXs

Chemical analysis was performed over the first two weeks post sowing in the hexaploid wheat varieties. In these plants approximately similar amounts of both DIMBOA and DIMBOA-glu were present in the root and leaf tissues but there was more variation in the relative accumulation of the compounds in the coleoptile. The concentration of both compounds was less at the final sampling point than at 7 days, although in some plants, particularly Tasman root tissue, there was an increase of both compounds at 9 days. The highest concentration of both compounds was found in the coleoptile and the concentration of DIMBOA was in all cases slightly lower in the roots than in the rest of the plant. This may be because the compound is being released into the rhizosphere.

We also studied expression of the wheat BX genes (*TaBXs*) in the different plant tissues, using relative quantitative polymerised chain reaction (QPCR) analysis during

the early stages of development in the leaf, coleoptile and root tissue of the variety, Welford. This variety was selected because analysis had shown that the levels of the compounds in the different tissues were typically representative of the varieties sampled. The expression of all genes tested decreased over the time period, but the kinetics of the response was different in the different plant parts. Gene expression was generally lowest in the leaf and highest in the coleoptile. Also expression of all genes dropped precipitously to barely detectable levels in the leaf after 10 days whereas in the coleoptile and root the decrease was more gradual. The *Glus* also decreased in expression levels over the time period, although this was less noticeable in the coleoptile. *TaGlu b* was most highly expressed and relative expression of *TaGlu c* was higher in the root than in the other plant parts.

TaBX and *TaGlu* gene expression was also measured in the tissues of Solstice, Tasman and Taluen, but no significant differences could be detected between varieties.

2.4 Constitutive levels of BX content and gene expression in tetraploid and diploid wheats

Little variation in levels of BXs or in *TaBX* gene expression was detected within the hexaploid plants tested and this was particularly the case within the UK varieties. We therefore measured compound content and gene expression in a tetraploid wheat, (Alifen) and in diploid wheats containing the A, B or D genome. We wished to ascertain whether in these plants there was more variation and whether we could identify species that expressed BX pathway more highly. We found that there was much more variation in the levels of the compounds within the diploid and tetraploid wheats than had been observed within the hexaploid varieties. Also, the plants differed in the forms of the BXs that they contained. Alifen and *Ae. speltoides* contained both DIBOA and DIMBOA (and the corresponding glucosides), although DIMBOA was the predominant form. Only DIMBOA and its glucoside were present in *T. tauschii* but *T. monococcum* only contained DIBOA and DIBOA-glu.

The tissue concentration of both forms of the BXs generally decreased over the time period in all plants, but the absolute levels differed significantly. *Ae. speltoides* contained the highest concentrations, particularly in the leaf and coleoptile where both glucoside and aglucone levels were between five and eight fold higher than in Alifen, *T. tauschii* and Welford. However, the compounds were absent from the leaf tissue of the other B genome wheats, *Ae*. *longissima, sersii, bicornis* and *sharonensis* and the coleoptile contained only small amounts at the earliest time point. There was no

aglucone in leaf or coleoptile of *T. monococcum* or *T. boeoticum* and only trace amounts of glucoside in the coleoptile at the first time point.

In the root tissue, *Ae. speltoides* consistently contained slightly higher levels of glucoside although the difference compared to other wheats was much less than in the foliar tissue. Levels of DIMBOA were up to five times higher than in the other varieties over the first 9 days post sowing, but decreased rapidly. In roots of durum wheat, levels of the BXs, were slightly higher than in the hexaploid, or the D genome wheats, particularly at the later time points, but less than in *Ae. speltoides.* In roots of *T. monococcum and T. boeoticum*, BX levels were low, and DIBOA was undetectable after 6 days.

The expression of the *TaBX* genes was measured in the leaf, coleoptile and root of *T. monococcum, Ae. speltoides, T. tauschii* and Alifen. In the leaf tissue *TaBX2* was not expressed in any of the plants, *TaBX4* was highly expressed in *Ae. speltoides* and Alifen, but not in the other plants, and *TaBX5* was only expressed in *Ae. speltoides.* This pattern of expression was repeated in the coleoptile, apart from the presence of high expression of *TaBX2* in all plants except *T. tauschii.* The relatively high expression of *TaBX4* and *5* in the leaves and coleoptile of the B genome wheat correlates with the high accumulation of DIMBOA and DIMBOA-glu in these tissues, whereas expression of *TaBX2* shows the greatest variation in root tissue, with again the highest levels in *Ae. speltoides* and Alifen. The expression of *TaBX3* was much less variable between the plants and was expressed at similar levels in all tissues apart from *T. Tauschii* leaves, where it was not expressed.

2.5 Chromosome localisation of glucosidases

In the hexaploid, all of the *TaGlu* genes were expressed, but in the diploid wheats this was not the case. In *T. monococcum*, with exception of low expression of *TaGlu c* in the root tissue, no expression of any of the genes was detectable. In *T. tauschii,* only *TaGlu c* was expressed and this most strongly in the root. In Alifen and *Ae. speltoides,* all three genes were expressed in the root, but despite the relatively high levels of aglucone in the leaf and coleoptile of *Ae. speltoides*, only *TaGlu a* was expressed in the foliar tissue.

The *TaGlu* gene expression data described here raises the possibility that *TaGlu a, b* and *c* may be homoeologs, present on different chromosomes. To investigate this further, chromosome-mapping of the *BX* pathway-specific glucosidase genes in

wheat was undertaken. Using gene-specific primers RT-PCR successfully amplified products of approximately expected size from all these three genes confirming that these three genes are expressed in young shoot tissues of the wheat variety Chinese Spring. Five aneuploid wheat lines in the Chinese Spring background were then used for chromosomal mapping of these three genes. The results from these experiments indicated that *TaGlu a* and *b* are located on the long arm of chromosome 2B and *TaGlu c* is located on chromosome 2D. Therefore, *TaGlu c* could be classified as a homoeolog, but two forms of the gene are present on the B chromosome. The further significance of the failure to detect any *TaGlu* genes on the A chromosome will be discussed below.

2.6 Aphid bioassays

A series of bioassays were conducted to investigate the natural variation in antixenotic and antibiotic effects of wheat lines against two cereal aphid species, the grain aphid, *Sitobion avenae*, and the bird-cherry oat aphid, *Rhopalosiphum padi*. The different wheat lines that had been studied for BX production, and some additional hexaploid varieties and *T. monococcum* lines were used in the tests. Initially, settling tests were used, but more detailed developmental assays were performed, for a small number of hexaploid varieties that had elicited more extreme responses from the aphids, and for the tetraploid and diploid wheat species.

2.6.1 Settling tests

Settling tests were performed for both aphid species by giving replicated groups of 10 alate aphids the choice between two wheat seedlings at the first leaf growth stage, one of a standard variety, Solstice and the other of the test variety/species. The number of alates settled on each seedling was recorded at 2, 5 and 24 h and the number of nymphs produced on each seedling was recorded at 24 h. These data were then compared in a paired Students t test and the number of nymphs produced was expressed as a proportion of the nymphs produced on Solstice in the same assay, thus providing a "preference index" for both aphid species. Assays were repeated for varieties at extreme ends of the preference indices to confirm their position.

Although there were apparent differences in preference between the hexaploid wheat varieties for both aphid species, due to variation in nymph production there were none that were significantly different to Solstice for *S. avenae* and only one, Tasman, for *R. padi*. This response by *R. padi* to Tasman, was surprising and further investigated in

development trials and by HPLC (see effect on BX pathway by aphid feeding). The preference index for *R. padi* is less extensive than for *S. avenae*, but there are clear differences in preference between the species. However, since there were no clear differences in BX levels between the UK hexaploid varieties, which also contained lower amounts, few of these lines were further investigated. Settlement assays conducted for both aphid species on Alifen, *Ae. speltoides,* and *T. monococcum* lines MDR037 and 049 showed differences in preference between the two aphid species, with MDR049 preferred to MDR037 and Alifen being the most favoured. All of these wheat species were further investigated in development trials.

2.6.2 Development tests

Development trials were carried out by weighing neonate nymphs of *S. avenae* or *R. padi* and then transferring them to the first leaf of 7-8 day old seedlings of Solstice, as the standard variety, or of test plants. The nymphs were set up on each plant line enclosed in a clip cage and left to feed until they were re-weighed 6 to 7 days later. As opposed to settlement trials, these trials offer no choice and are more likely to unearth antibiotic or nutritional rather than antixenotic or morphological differences between plants.

After 6 or 7 days the Mean Relative Growth Rate (MRGR) was calculated as:

Mean Relative Growth Rate = (ln 6/7day weight – ln birth weight) / number of days

When MRGR between Tasman and Alifen were compared to Solstice, this revealed that, despite the slightly lower BX content in Solstice compared to both the other plants, *R. padi* developed better on both Tasman and Alifen than on Solstice and that *S. avenae* developed better on Alifen, but less well on Tasman than on Solstice. However, the performance of the aphids on *Ae. speltoides*, the wheat with the highest amount on BXs in the leaf tissue, showed that both aphids developed far less well than on Solstice.

The growth rate of both aphids on two *T. monococcum* lines, MDR 037 and MDR 049 was measured and this showed that MDR 037 sustained similar growth as Solstice, but that MDR 049 sustained significantly less. Since neither plant contains BXs in the leaf tissue, there must be other factors responsible for the poor growth rate of aphids on MDR 049. Also, the acceptability of the two *T. monococcum* lines as hosts in this assay was the reverse of the acceptability in settlement tests, where as discussed, morphological features may carry more weight than the nutritional status of the plant.

2.7 Population increase

For the intrinsic rate of population increase (r_m) study, neonate nymphs were caged and left to develop on Solstice or test plant seedlings. The time taken to moult to adult and produce their first nymph was recorded after which the number of nymphs produced per day was noted, the nymphs being removed to prevent overcrowding. The r_m was calculated using the time taken from birth to produce the first nymph (D) and the number of nymphs produced over a period equivalent to time D (FD) starting at the production of the first nymph. A constant obtained from the mean pre-reproductive times for numerous aphid species was used in the calculations using the equation:

$$r_m = 0.74 (\ln (FD) / D)$$

The r_m of *R. padi* and *S. avenae* on Tasman, Alifen and Solstice was compared and showed that the potential aphid population increase was greatest on Alifen, and on Tasman it was greater than on Solstice.

When the number of nymphs produced over the first 8 days was measured on a selection of diploid wheats (A and B genome) it was clear that the diploid wheats were less acceptable than the hexaploids and that *Ae. speltiodes*, the only diploid that contained BXs, was the least favoured as a host.

2.8 Effect of aphids on the BX pathway

The results of the three different types of bioassay suggest that BXs may play an important role in resistance in wheat, but that other factors are also affecting the preference and performance of aphids on the plants. The possibility that the varieties differed in their ability to respond to aphid attack by up regulating the pathway was considered. To test this, 25 nymphs were placed in clip cages and attached individually to the first leaf of wheat seedlings from a selection of the varieties. Half of the seedlings were harvested after 24 h and the rest at 48 h for assessment of gene regulation and BX levels. The area of leaf under the clip cage was cut out and analysed for compound accumulation and gene expression. The effect on these parameters in the rest of the plant was also measured.

This revealed that, in the hexaploid variety Welford, there was no effect on the expression of the *TaBX* genes anywhere in the plant. However, there was a rapid, transient increase in the expression of *TaGlu a* and *b* in the region covered by the clip

cage. It was highest at 24 hours and had started to decrease by 48 hours. The increase was also greater when the plants were fed upon by *R. padi* than *S. avenae*. When the rest of the foliar tissue was examined it was found that gene expression in the coleoptile was not affected, but expression of *TaGlu a* and *TaGlu b* was increased in the leaf tissue in the parts adjacent to the clip cage. This effect was delayed and was greater at 48 than at 24 hours. When the effect on the accumulation of the compounds was measured in the part of the leaf covered by the clip cage of Welford by HPLC, it was found the there was a conversion of DIMBOA-glu to DIMBOA after feeding by *R. padi* (the effect of *S. avenae* was not studied).

In order to establish whether there was any variation between different wheat varieties in the magnitude or duration of the respose, the effect of feeding by *R. padi* on the amounts of DIMBOA in the clip cage region were compared in *Ae. speltoides*, Alifen, Solstice, Welford and Tasman after 24 and 48 hours. The results indicated that there was considerable difference in both aspects of the response. No effect was observed in *Ae. speltoides* but, of the other three plants tested, all showed a response, with the least effect (not significant) being in Solstice and the greatest in Tasman. In Tasman there was a more sustained effect with the DIMBOA levels at 48 hours being greater than at 24 hours. This suggests that the induced response may be secondary, only occuring when the primary defence is weak, as it is greatest in the most susceptible plants.

2.9 BX content of honey dew

Aphids feed predominantly from the phloem, and only DIMBOA-glu has been found in phloem sap collected from wheat by aphid stylectomy. We were interested to establish whether resistance correlated with the presence of free DIMBOA in the phloem. Honey dew was collected by leaving clip cages, collected from aphid feeding trials on different wheat varieties for up to 24 h, in 100% humidity to allow the honeydew to take up water. It was then collected, using a micropipette, into a small Eppendorf tube for analysis by HPLC.

This showed that only DIMBOA-glu was present in the honey dew collected from *R. padi* and *S. avenae* fed on *Ae. speltoides* and *R. padi* fed on Tasman, Solstice and Alifen. However, honey dew from aphids fed on *T. monococcum* did not contain BXs, indicating that the DIMBOA-glu in the honey dew originated from the phloem. Honey dew from *R. padi* fed on artificial diets containing DIMBOA also contained no BXs, thus demonstrating that the aphid is not able to detoxify DIMBOA by glucosylation. The

DIMBOA-glu present in honey dew must have passed through the insect's gut but the DIMBOA must remain inside it.

2.10 BX content of interstitial spaces

In order to reach the phloem, the aphid stylet must pass through the extra cellular spaces surrounding the cells of the epidermal and mesophyll tissues. There is evidence that the insect ingests samples from the surrounding medium during the early stages of probing. Therefore, we were interested to establish whether any correlation between BX concentration in the extra cellular spaces and resistance could be determined. We prepared interstitial fluid extracts from foliar tissue from the hexaploid varieties, Welford and Tasman and from *Ae. speltoides.* Extracts were prepared by immersing leaf and coleoptile in distilled water in a petri dish and keeping them submerged by means of a metal grid. The petri dish was placed in a desiccator and subjected to a vacuum. After release of the vacuum, the tissue was removed, blotted dry with filter paper, rolled up and placed in an Eppendorf tube containing ball bearings and a small volume of extraction buffer and centrifuged. The plant material was removed and the liquid, comprising extraction buffer and interstitial fliud, was collected from beneath the ball bearings with a pasteur pipette and subjected to HPLC analysis.

The subsequent analysis showed that at 7 days post sowing the interstitial fluid from all varieties contained predominantly free DIMBOA and a smaller amount of DIMBOA-glu. This is in contrast to the ratio of the two compounds in whole leaf tissue extracts at the same developmental stage. In addition, *A. speltoides* contained over 5 times more DIMBOA than Tasman and 7 times more than Welford. This difference could contribute to the reduced susceptibility of *Ae. speltoides* to aphids compared to the hexaploid varieties.

3.TECHNICAL DETAIL

3.1 Introduction

The hydroxamic acids or benzoxazinones (BXs) are a family of plant defence compounds produced in cereals, including wheat, maize and rye (Kluge *et al* 1997; Niemeyer 1988; Sicker *et al* 2000; Wu *et al* 2001). The two most biologically active of the BXs, 2,4-dihydroxy -1,4-benzoxazin-3-one (DIBOA), and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) have been shown to be directly toxic to aphids when applied to artificial diets (Argandona *et al* 1980; Escobar *et a*l 1999) and to inhibit microbe and plant root growth (Bravo *et al* 1997; Corcuera *et al* 1978; Freibe *et al* 1998; González and Rojas 1999; Belz and Hurle 2005; Burgos and Talbert 2000; Wilkes *et al* 1999; Rice *et al* 2005). Hence, the broad ranging biocidal activity of these compounds makes them good candidates for developing both pest and disease resistance in crop plants.

Bxs have not been detected in brassicas although they have been found in some eudicotyledenous plant species (Baumeler *et al* 2000), where the biosynthetic pathway appears to have evolved independently (Schullehner *et al* 2008). Surprisingly, they are not present in cultivated barley although they have been found in some wild species (Barria *et al* 1992).

At the molecular level, the pathway has been fully characterised in maize but some of the wheat homologues (*TaBX*s) remain to be cloned (see Figure 1). It has, however, been demonstrated that in both plants the pathway proceeds by the conversion of all cytosolic indole into DIBOA (Nomura *et al* 2002; Nomura *et al* 2003; Frey *et al* 1997; Frey *et al* 2003). This conversion is catalysed by four Cytochrome P450 enzymes, BXs 2 – 5, all members of the family Cyp71C, which have been cloned and functionally characterised in both wheat and maize. Homoeologs of each of these genes have been identified in wheat and assigned to the different genomes (Nomura *et al* 2005). This has established that the B genome contributes the most to the BX biosynthesis in hexaploid wheat. The final steps in the pathway have been identified in maize and this has revealed that as soon as DIBOA is formed it is glucosylated by two glucosyl transferases, BX8 and 9 and the glucoside (DIBOA-glu) is transported into the vacuole for storage. DIBOA-glu is the substrate for the final two steps to produce DIMBOA-glu, BX6 and 7 (Jonczyk *et al* 2008). The last steps of the pathway in wheat are not known, but in rye only DIBOA-glu is present, suggesting that the enzymes responsible

for final steps may be absent in this plant. In maize and wheat, the concentrations of the BXs are highest in the young seedling and decrease as the plant matures (Thackray *et al* 1990) and some of the *BX* biosynthetic genes have been shown to be more highly expressed in young plants (Nomura *et at* 2005; Jonczyk *et al* 2008; von Rad *et al* 2001; Frey *et al* 2003). This has the advantage that the compounds, potentially unpalatable to humans, do not reach the human food chain.

The activity of the pathway may also be regulated by release of unglucosylated DIMBOA or DIBOA (the aglucones) from the stored, inactive, glucoside. The characterisation of glucosidase genes (*Glus*), responsible for the release of other plant secondary metabolites that are stored in this way, has been the focus of much recent scientific interest (Oba *et al* 1981; Gruhnert *et al* 1994). It appears that in monocots these enzymes are stored in the plastid or chloroplast whereas in dicots they are glycosylated, exported to the apoplast and may be bound to the cell wall or stored in protein bodies (Morant *et al* 2008). Three BX specific *Glu* genes have been cloned, sequenced and functionally characterised in hexaploid wheat (Sue *et al* 2006) and these genes are also under developmental regulation in the shoot tissue . It is not clear if they are homoeologs of the same gene.

Since their first discovery (Koyama and Yamato 1955), many chemical analytical investigations have attempted to establish a role for the BX pathway in plant defence, against aphids in particular, by establishing a positive correlation between tissue concentrations of the compounds and resistance (Thackray *et al* 1990: Bravo and Copaja 2002; Nicol *et al* 1992; Zheng *et al* 2005). Frequently, the objective of such studies was to establish whether these compounds could form the basis of a breeding program to generate resistant wheat varieties that contained elevated concentrations of the compounds (Escobar and Niemeyer 1993). Early methods did not always distinguish between different forms of the compounds (Thackray *et al* 1990; Baker and Smith 1977; Melanson *et al* 1997) and some included a hydrolysis step to convert glucoside to aglucone, to obtain a total combined value for active and inactive forms of the BXs (Gianoli and Niemeyer 1997; Nicol *et al* 1992; Reberg-Horton *et al* 2005; Larsen and Christensen 2000). Furthermore, accurate quantification of the BXs is problematic as they are highly unstable in aqueous media (Fomsgaard *et al* 2004). The glucoside is rapidly hydrolysed in disrupted tissue and this has lead to dispute over whether more than trace amounts of aglucone are ever present in plant tissue (Cambier *et al* 1999; Virtanen and Hietala 1960). Also, the aglucones themselves are rapidly converted to lactams and other products (Cambier *et al* 1999; Woodward *et al*

1978; Atkinson *et al* 1991; Villagrassa 2009). The spontaneous breakdown of the aglucones may reflect the fact that their degradation products play an important role in signalling within the rhizosphere (Macias *et al* 2005) but it has also been reported that pathogens are able to convert the most active BXs into less toxic products (Fomsgaard *et al* 2004; Friebe *et al* 1998; Weibull and Niemeyer 1995).

The aim of this work has been to establish whether there is a relationship between BX concentration in wheat and aphid resistance. This has involved a detailed analysis of the localisation and regulation of the pathway in different parts of the plant during development in a range of hexaploid wheatsand some wheats of different ploidy levels.

3.2 Materials and Methods

3.2.1 Compound extraction and quantification

In order to establish a reliable method to quantify the BXs in wheat plant tissues we used a non aqueous extraction procedure, followed by high pressure liquid chromatography (HPLC), which we have adapted from earlier published methods (Baumeler *et al* 2000; Nakagawa *et al* 1995). Tissue wascollected from plants at the time points indicated and individual plant parts (leaf, root and coleoptile) were harvested separately. Two methods of preparation were used. Where limited amounts of tissue were available, a sample was weighed accurately into an Eppendorf tube containing two ball bearings and frozen in liquid nitrogen before milling in a ball mill for 1 minute. Where larger amounts of tissue were available, the material was ground under liquid nitrogen with a pestle and mortar and transferred to a chilled Eppendorf tube. In both cases, the ground tissue was either processed immediately or stored at -80^{o}C before further analysis.

Extraction buffer (methanol/acetic acid, 98%/2%) (1 ml) was added to ball milled plant tissue samples without allowing the material to thaw. For the larger samples, an aliquot of ground tissue (approximately 25 mg) was accurately weighed into a tared Eppendorf tube containing 1 ml of extraction buffer. This process was also performed rapidly to prevent thawing of the tissue. In both cases, the mixture was sonicated for 10 minutes and centrifuged at 12,600 rpm for 10 minutes. The supernatant was transferred to an HPLC vial for further analysis. HPLC analysis was performed using a

Betasil C18 column, (purchased from Thermo Scientific, Hichrom Ltd, Theale, Reading Berkshire) with a particle size of 5 µ and dimensions of 250 by 4.6 mm. The column was eluted with a flow rate of 1 ml/min and detection was carried out at 254 nm. Samples (20 µl) were injected onto the column and eluted with a gradient profile of 90% solvent A (HPLC grade water), 10% solvent B (Methanol/Isopropanol (95/5) + 0.025% Acetic acid) for 0 – 2 min followed by an increase of solvent B from 10 to 50% from 2 – 11 min, constant conditions for 11 – 16 min and a final step (16 – 17 min) to return to starting conditions.

The elution positions of the different BXs (see Figure 2) were established by comparison with synthetic standards (kind gift from Prof. Dieter Sicker, University of Leipzig). The identity of the peaks present in tissue extracts was confirmed by Electro spray ionisation. Standard curves were constructed to establish a linear relationship between peak area and concentration and these were used to quantify the tissue content (mM/Kg fresh weight) of each compound.

3.2.2 Gene Expression analysis

We also studied gene expression in the different tissues, using relative quantitative polymerised chain reaction analysis (QPCR). RNA was extracted from approximately 200 mg of frozen powdered plant tissue in an Eppendorf tube (either ball milled material or from larger samples that had been ground with a pestle and mortar) with Trizol (Sigma Aldrich) (1.5 ml). The mixture was vortexed and incubated at room temperature for 5 minutes before centrifugation at 12,000 rpm for 10 minutes at 4 ^{o}C. The supernatant was transferred to a clean tube, one volume of chloroform was added and the tube contents were vortexed for 15 seconds. After centrifugation (12,000 rpm for 15 minutes at 4 ^{o}C) the aqueous upper phase was removed, transferred to a fresh tube and a further volume of chloroform/isoamyl alcohol was added. After vortexing and centrifugation, the upper phase was again removed and a 1/10 volume of sodium acetate (3.0 M) and 0.6 volume of isopropyl alcohol were added. The solution was incubated for 10 minutes at room temperature and centrifuged for 10 minutes at 12,000 rpm at 4 ^{o}C to pellet the RNA. The pellet was washed twice with 70 % ethanol and resuspended in 50 µl of sterile water. The RNA was quantified using a nanodrop and its integrity confirmed by electrophoresis on a 1% TAE agarose gel.

Single stranded cDNA was prepared from the RNA using SuperScript ™ lll RT (Invitrogen). Total RNA (1 µg) was incubated for 30 min at 37 °C in a nuclease free microfuge tube with 1 µl RNAse free DNAse and 1 µl 10 x DNAse buffer (PROMEGA) made up to a total volume of 10 µl with DEPC water. Oligo $(dT)_{12}$ (1 µl, 50 µM) and DNTP mix(1 µl of 10 mM of each dATP, dGTP, dCTP and dTTP) was added and the mixture was heated to 65 °C for 5 minutes. First strand buffer (4 µl, X5), dithiotheitol (1 µl, 0.1 M) and Superscript III (1 µl, 200 units/ µl) were added and the reaction mixture was incubated at 55 °C for 60 minutes. The reaction was terminated by heating to 70 °C for 15 minutes.

Table 1. Primers used to amplify *TaBX* and *TaGlu* genes in wheat

Gene	Forward	Reverse
TaBX2	CCCCGTGACCGCATG	ATATATGGGCGCACCAAATACC
TaBX3	TTCGCCGAAAGGAGAAGCT	GTGCTCATTCGAAATAAACTTACACATAG
TaBX4	TTGATATGACGGACCAGTTTGG	TGTAAATTTTGGGAACAAGGTGAA
TaBX5	CCCACCAGGGCCTTTACTC	TTTTGGCGGGTATGTCGTATC
TaGlu a	CGGCGGCTTCTTAAATAGGAA	TCACCCTGTCGCCGAAGT
TaGlu b	TCCCAAAACCCTTGCTTAAGAC	CACTTCGTTCTCCATCATACAGAAA
TaGlu c	TCGCCAAGGTGTGCTTTGA	AATATGTATGTGGCTCGTTAAA
Cyclofilin	CACCGTCCCCTGCAATTG	AGCCCACCTTCTCGATGTTCT
TaGlu a sps	CGATGTAGACGAGGCCGTAT	AGAAACTAAAGGGAGTGACGG
TaGlu b sps	GGCCTAACAGACCTCCTTCT	GAACCATCCCATGTTGTAGTCA
TaGluC c sps	TGGAGAAGGGGTAGTCATCC	ATACATATTGTTGCTTCTCCTATGG

Gene expression analysis was performed in a 7500 Thermolcycler (Applied Biosystems). Reactions were carried out in 96 well plates and contained X2 SYBR green mix, (5 µl) (Sigma Aldrich) internal reference (ROX, 0.1 µl) forward and reverse primers (200 nM final concentration) and cDNA from single strand preparation (1 µl), made up to a total volume of 10 µl with nuclease free water. Relative quantification of the expression levels of the target genes was performed using the comparative Ct method. Endogenous genes were selected that had the most consistent Ct values in the range of samples to be analysed (different tissues, plant ages or treatments). Target gene and endogenous control gene primers (shown in Table 1) were designed using Primer Express. The relative efficiencies of the primers were checked by plotting ΔCt target gene – ΔCt endogenous control gene, determined manually, against the log value of total cDNA volume over a range of 3 – 0.25 µl. Where the slope of the line was greater the 0.4 the primers were redesigned. The PCR reactions were carried out using an initial denaturing step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Three technical replicates were performed for

each target and endogenous gene measurement. Each experiment was performed on three separate occasions and the results shown are a typical result, as indicated in the text. The data was analysed with the Sequence Detection Systems (SDS) soft ware. For ΔCt analyses the Ct values of the target genes for each technical replicate were compared to the Ct values of each of the endogenous gene replicates to provide a mean ΔCt. Having demonstrated that amplification efficiencies of target and reverence primers were both approximately equal and close to unity, the amount of target cDNA present in each sample was calculated by the relationship:

$$\text{amount of target cDNA} = 2^{-\Delta Ct}$$

3.2.3 Plant material

These analyses were performed on wheat seeds obtained from different sources, which were sown in vermiculite and subjected to a light (16 hour) / dark (8 hour) regime at a constant temperature of 22 ^{o}C. Hexaploid wheat lines were supplied by RAGT, LIMAGRAIN and KWS (British wheat breeders) apart from Taluen and the tetraploid Alifen, which were supplied by Professor Hermann Niemeyer (University of Chile) and Tasman, which was obtained from the Australian Wheat Board. *Triticum monococcum* and *T. boeoticum* were provided by Kim Hammond-Kosack (Rothamsted Research). *Triticum tauschii, and Aegilops speltoides, longissima, sersii, bicornis* and *sharonensis* were obtained from Mr. Stephen Reader at the John Innes Centre, Norwich, UK. *T. monococcum* and *T. boeoticum* were chosen as examples of A genome wheats. Although *T. monococcum* is not believed to be the actual originator of the A genome of hexaploid wheat (Dubcovsky *et al* 1995) it is the only diploid species to have been subjected to cultivation and has recently been developed as a reference species for wheat genetics and genomics (Jing *et al* 2007). *Ae. speltoides* is possibly related to an ancestor of the S-genome progenitor of the B genome of hexaploid wheat. *T. tauschii* has been shown to be the D genome donor of the hexaploid by the creation of synthetic wheat with the tetraploid *Triticum dicoccoides* that has similar properties to *T. aestivum*. Alifen is a tetraploid Chilean durum wheat variety shown to contain high BX concentrations immediately after germination. Five commonly grown UK winter wheat varieties, Claire, Malacca, Solstice and the orange wheat blossom midge resistant varieties, Robigus and Welford, were used in the comparison. Also included to provide a wider comparison were the Chilean variety, Taluen, which has been shown to contain low levels of BXs immediately after

germination compared to other local varieties, and an Australian variety, Tasman, which releases high levels of BXs from the roots, has been shown to have high allelopathic activity and has been used as a mapping population parent, (Wu *et al* 2003).

3.2.4 Chromosomal localization of the *TaGlu* genes

Wheat seeds of Chinese Spring and its deletion lines CSDT2BL, CSN2DT2A, CSN2DT2B, CSN2BT2D, and CSN2BT2A were germinated on Whatman filter paper soaked in water at room temperature. Shoots of approximately 5-cm in length were collected in liquid nitrogen and stored in -80 ^{0}C freezer. RNA extraction and cDNA preparation were performed as described above.

To determine the presence of transcripts of each of the three glucosidase genes, SNP-based gene-specific (sps) primers were designed using the WebSNAPER programme (Drenkard, E. *et al* 2000). PCR was conducted in a total volume of 20 µl reaction, containing 10 µl ReddyMix PCR master mix (Thermo Scientific), 1 µl cDNA and 0.5 µM each of the primers. The gene-specific primers were used (see Table 1, *TaGlu a,b,c* sps) PCR reactions were performed using the same parameters for the expression of all the three genes. These were: initial denaturing at 94^{0}C for 1 min, followed by 30 cycles of 94^{0}C 45 sec, 57^{0}C 45 sec, and 72^{0}C 1 min. PCR products were analysed on agarose gel electrophoresis.

3.2.5 Interstitial fluid preparation

Interstitial fluid (IF) extracts were prepared from foliar tissue (leaf and coleoptile). The weighed plant material was immersed in distilled water containing proteinase K (14 µg/ml) in a petri dish and kept submerged by placing a metal grid on top of it. The petri dish was placed in a desiccator and subjected to a vacuum (-60 kPa) for 5 min. The tissue was removed, blotted dry with filter paper, rolled up and placed in an Eppendorf tube containing five ball bearings (3 mM diameter) and extraction buffer (0.5 ml) containing proteinase K (140 µg/ml) before centrifugation for 5 min at 10 x g. The plant material was removed and the liquid, comprising extraction buffer and IF, was collected from beneath the ball bearings with a Pasteur pipette and subjected to HPLC analysis.

3.2.6 Aphid Bioassays

A series of bioassays were conducted to investigate the natural variation in antixenotic and antibiotic effects of wheat lines against two cereal aphid species, the grain aphid, *Sitobion avenae*, and the bird-cherry oat aphid, *Rhopalosiphum padi*.
The aphids used in the laboratory experiments were obtained from cultures started from single field collected apterous virginopara maintained on wheat (cv. Tybalt) under controlled conditions (16:8 h L:D, 22 °C). Tybalt was chosen due to its comparative resistance to mildew disease, which can be a problem in laboratory maintained aphid cultures.

3.2.7 Settlement choice assay

For both aphid species, replicated groups of 10 alate aphids were given the choice between two wheat seedlings, at the first leaf growth stage, one of a standard variety, Solstice and the other of the test variety/species. Plants were grown in standard compost (75 % medium grade peat, 12 % screened sterilised loam, 3 % medium grade vermiculite and 10 % 5mm grit) in 2 x 2 cm pots and enclosed in a cylindrical glass test chamber (approximately 10 cm dia. X 17 cm high). The position of standard and test seedlings was alternated for each replicate to account for any influence of the lighting regime on aphid settlement. The number of alates settled on each seedling was recorded at 2, 5 and 24 h and the number of nymphs produced on each seedling was recorded at 24 h. These data were then compared in a paired Students t test and the number of nymphs produced was expressed as a proportion of the nymphs produced on Solstice in the same assay, thus providing a "preference index" for both aphid species. Assays were repeated for varieties at extremes ends of the preference indices to confirm their position.

3.2.8 No choice development assays

Five adult alate *S. avenae* or *R. padi* were placed in each of 30 clip cages, attached to leaves of wheat seedlings, cv. Tybalt, and left overnight to larviposit. For most trials, neonate nymphs, collected the following morning, were weighed in batches of five in a 0.2 ml Eppendorf tube on a microbalance (Cahn C33, Scientific and Medical Products Ltd, Manchester, UK) and then transferred to the first leaf of 7-8 day old seedlings of cv Solstice, as the standard variety, or of test plants. At least 12 batches of nymphs were set up on each plant line and each batch was enclosed in a clip cage. The seedlings were kept in a controlled temperature room (20 +/- 2 °C, 16:8 h L:D, 40 %

RH). Surviving nymphs were re-weighed in their batches after 6 or 7 days and the Mean Relative Growth Rate (MRGR) was calculated as:

MRGR = (ln 6/7day weight – ln birth weight) / number of days

In trials with *T. monococcum*, the adult aphids were caged directly on Solstice or on test plant seedlings and removed after 24 h. Neonate nymphs produced were counted and reduced to a maximum of 10 per cage, but were not weighed since survival of weighed neonates was poor on these plants. The surviving nymphs were weighed in their batches after 6 or 7 days and the weights compared.

All data were subjected to ANOVA to determine any significant differences.

For the intrinsic rate of population increase (r_m) study, individual neonate nymphs, produced as described above, were caged and left to develop on Solstice or test plant seedlings. The time taken to moult to adult and produce their first nymph was recorded after which the number of nymphs produced per day was noted, the nymphs being removed to prevent overcrowding. The r_m was calculated using the time taken from birth to produce the first nymph (D) and the number of nymphs produced over a period equivalent to time D (FD) starting at the production of the first nymph. A constant obtained from the mean pre-reproductive times for numerous aphid species was used in the calculations using the equation:

$r_m = 0.74 (\ln (FD) / D)$ (Wyatt and White, 1977)

Raw and log (ln) transformed data for mean time to production of first nymph (D), mean number of offspring produced over time D (FD) and the r_m were compared by ANOVA.

3.2.9 Aphid feeding assays

Wheat seedlings were grown in pure vermiculite in 3.5 cm pots. For both aphid species 13 days after sowing, at least 30 batches of 25 aphid nymphs (2^{nd} to 3^{rd} instar) were placed in clip cages and attached individually to the first leaf of each seedling per wheat line. The plants were then kept in a CE cabinet (20 +/- 2 °C, 16:8 h L:D, 40% RH). Half of the seedlings were harvested after 24 h and the rest at 48 h for assessment of gene regulation and BX levels. The area of leaf under the clip cage was cut out and the aphids carefully brushed off and discarded. The cut areas from 3-4 plants were pooled and wrapped in aluminium foil and placed in liquid nitrogen as

were the areas of leaf above and below the clip cage. The frozen samples were stored at -80 °C. For analysis of BX levels in the honeydew produced by the aphids, the clip cages were left for up to 24 h in 100 % RH to allow the honeydew to take up water after which it could be collected, using a micropipette, into a small Eppendorf tube for analysis by HPLC.

A feeding test using a simple artificial diet was set up to test the toxicity of DIMBOA against *R. padi*. Replicated plastic rings (0.5 cm high and 3 cm diameter) were used to set up the experiment. One side of each ring was covered tightly with two layers of parafilm, and 0.5 ml of 30 % sucrose solution containing 0.5, 1 or 2 mmol DIMBOA was injected between the parafilm layers, which were then sealed. A 30% sucrose solution was used as a control. Twenty mixed age aphids were put onto each ring and the numbers of surviving and feeding aphids were counted after 24, 48 and 72 hours.

3.3 Results

3.3.1 Regulation of the BX pathway in different tissues of hexaploid wheat during early development

The concentration of the BXs in different plant parts in the seven hexaploid wheat varieties was compared using HPLC as described in materials and methods. The major forms of the BXs in all the lines tested were DIMBOA and DIMBOA-glu, and the concentrations of these in the different varieties are shown in Figure 3. However, at the earliest time point in the root tissue of Tasman, Robigus and to a lesser extent, Claire, there were also significant amounts of DIBOA and DIBOA-glu. In all varieties approximately similar amounts of both DIMBOA and DIMBOA-glu were present in the root and leaf tissues but there was more variation in the relative accumulation of the compounds in the coleoptile. The concentration of both compounds was lower at the final sampling point than at 7 days, although in some plants, particularly Tasman root tissue, there was an increase of both compounds at 9 days. The highest concentration of both compounds was found in the coleoptile and the concentration of DIMBOA was in all cases lowest in the roots.

Gene expression analysis was measured during the early stages of development in the leaf, coleoptile and root tissue of the variety, Welford. This variety was selected because analysis (Figure 3) had shown that the levels of the compounds in the different tissues were typically representative of the varieties sampled. The expression

of all genes tested (4 *TaBX* genes and 3 *TaGlu* genes) decreased over the time period (6 to 18 days post sowing) but the kinetics of the response was different in the different plant parts. For the *TaBX* genes, with the exception of *TaBX3*, expression levels were lowest in the leaf and highest in the coleoptile (Figure 4 illustrates this for *TaBX4*). Also, expression of all genes dropped precipitously to barely detectable levels in the leaf after 10 days, whereas in the coleoptile and root the decrease was more gradual. Expression of *TaBX3*, on the other hand, was expressed at similar levels in leaf tissue over the time period. The *TaGlus* also decreased in expression levels over the time period, although this was less noticeable in the coleoptile. *TaGlu b* was most highly expressed (minimum Ct value, 15) and relative expression of *TaGlu c* was higher in the root than in the other plant parts.

TaBX and *TaGlu* gene expression was also measured in the tissues of Solstice, Tasman and Taluen, but no significant differences could be detected between varieties (data not shown).

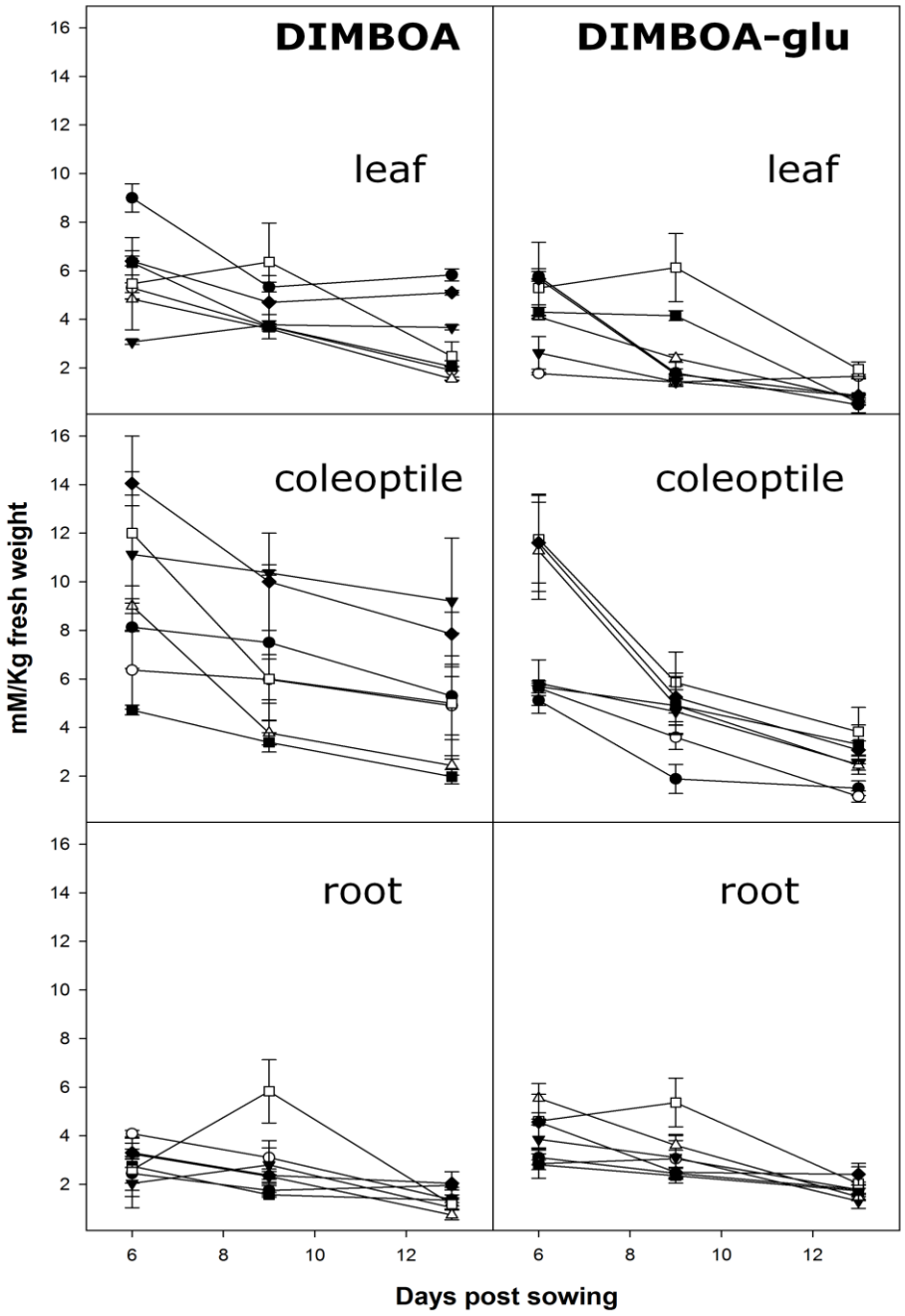
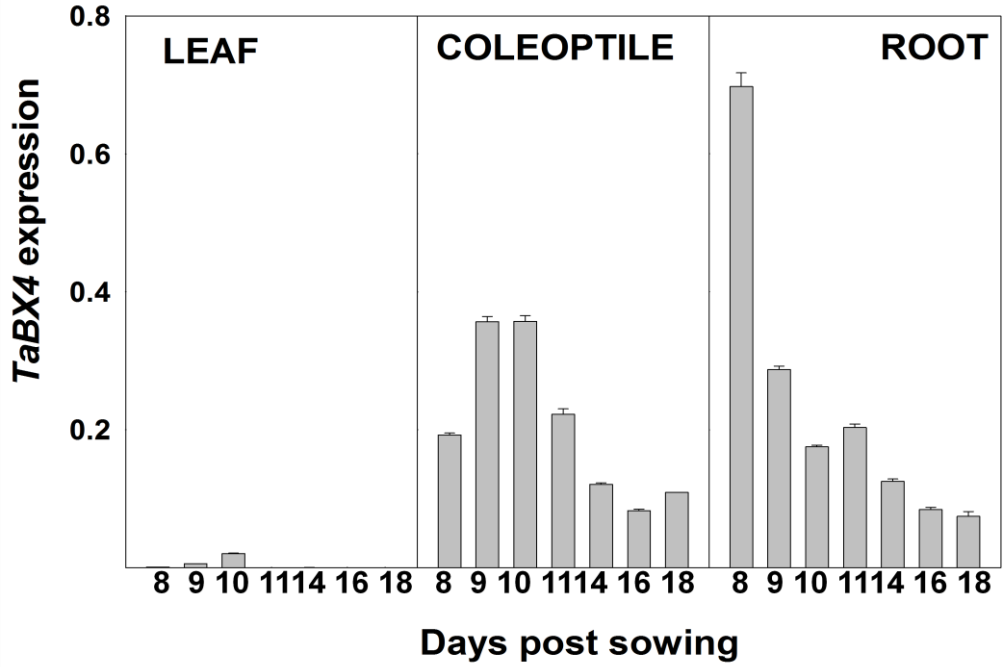


Figure 3 Concentration of BXs (DIMBOA and DIMBOA-glu see Figure 1) in different tissues of hexaploid wheats. Mallaca(▼), Welford (◆), Tasman (□), Claire (■), Robigus (○), Solstice (△), Taluen (●). Mean of three experiments.



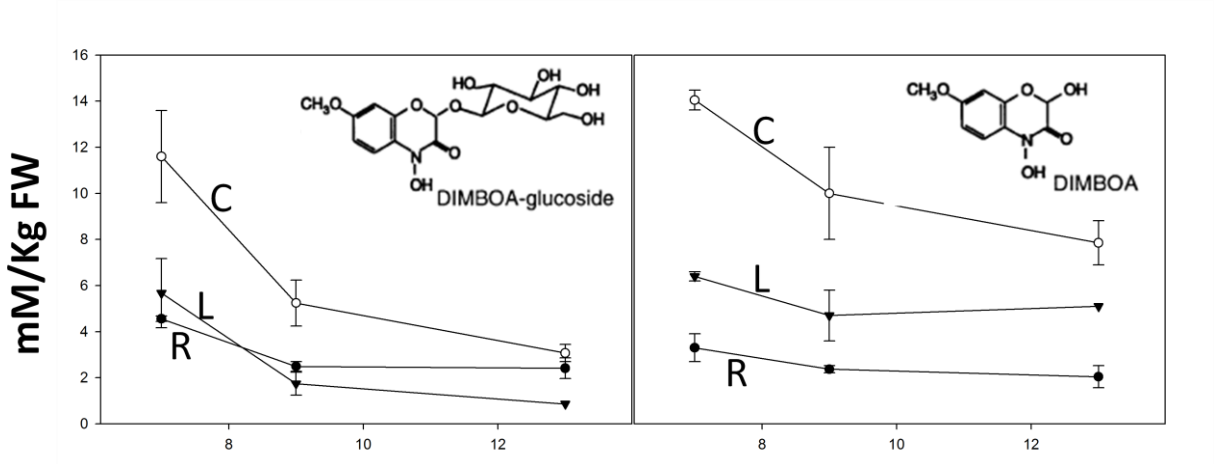


Figure 4 Expression of *BX4* in different tissues of the hexaploid wheat, Welford over the first 18 days post sowing (upper panel). Compound accumulation in coleoptile (C), leaf (L) and root (R) of Welford (lower panel). Results of gene expression analysis shown are the mean of three technical replicates. The experiment was performed three times and this is a typical result. Results of chemical analysis shown are the mean of three experiments.

3.3.2 Regulation of the BX pathway in tetraploid and diploid wheats

Since little variation was detected in BX levels or gene expression within the hexaploid plants tested, diploid wheats containing the A, B or D genome, representing relatives of the ancestors of cultivated wheat, and the tetraploid wheat, Alifen, were analysed.

Figure 5 shows that there was much more variation in the levels of the compounds within the diploid and tetraploid wheats than had been observed within the hexaploid varieties. Also, the plants differed in the forms of the BXs that they contained. Alifen and *Ae. speltoides* contained both DIBOA and DIMBOA (and the corresponding glucosides), although DIMBOA was the predominant form. Only DIMBOA and its glucoside were present in *T. tauschii,* but *T. monococcum* only contained DIBOA and DIBOA-glu.

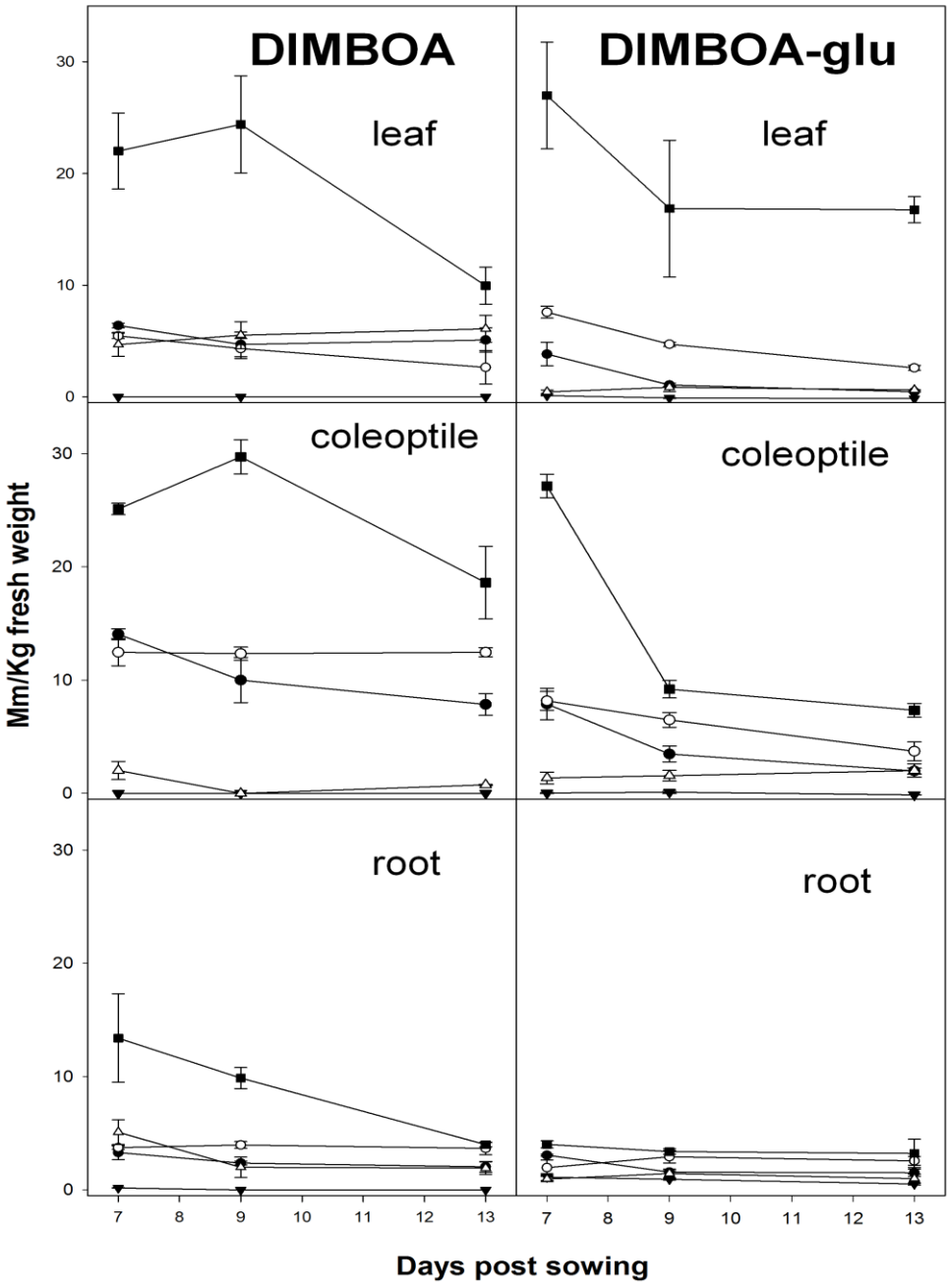


Figure 5. Concentration of BXs in different tissues of diploid, tetraploid and hexaploid wheats. *T. Monococcum* (▼), *Ae. speltoides* (■), Alifen (○), *T. tauschii* (△), Welford (●). Results show mean of three experiments.

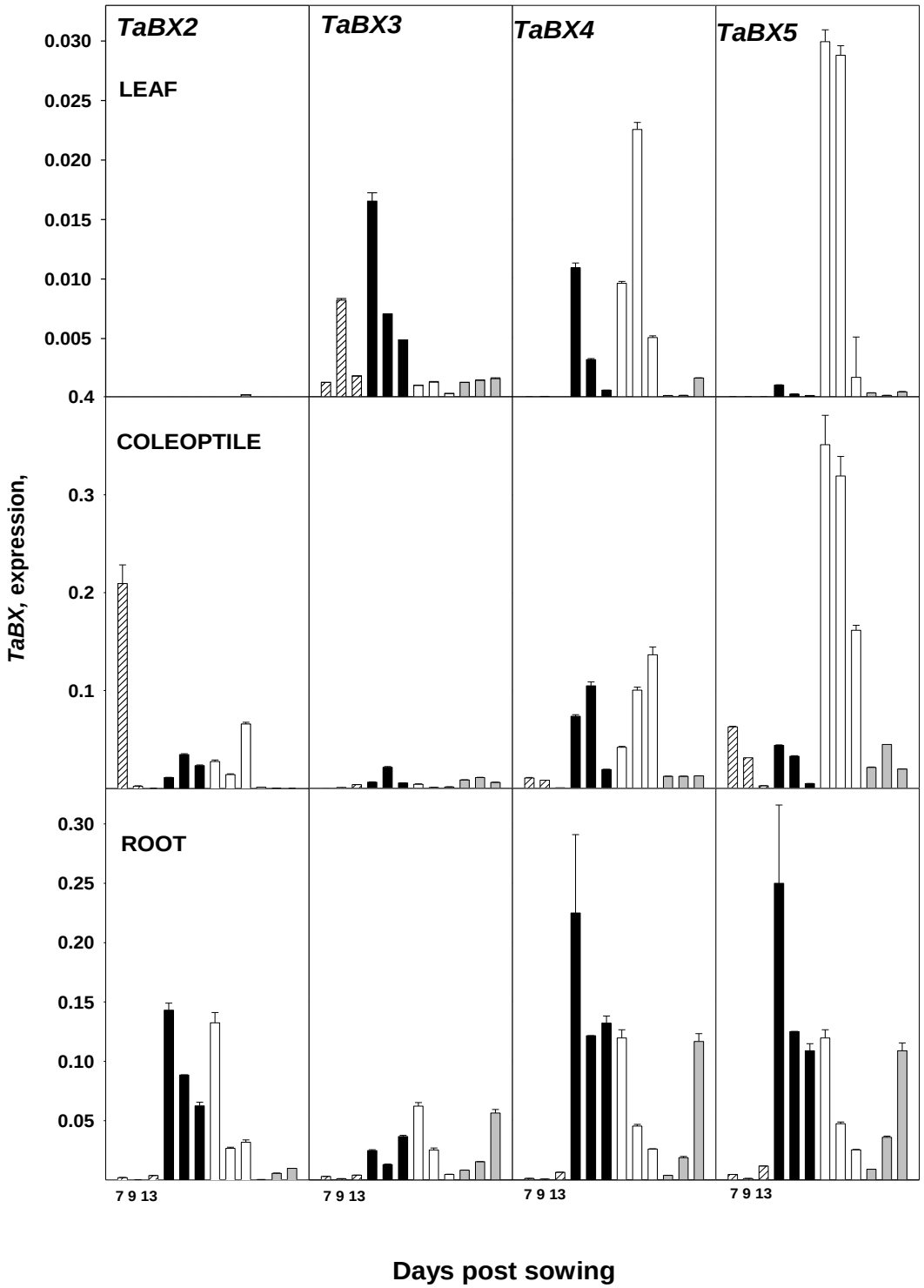


Figure 6. Q PCR of the expression of the 4 TaBX genes (BXs 2-5) in different plant tissues of T. monococcum (striped bars), Alifen (solid bars), Ae. speltoides, (open bars) and T.tauschii, (grey bars). Three measurements were made for each tissue at 6, 9 and 13 days post sowing. The experiment was done three times and the figure shows a typical experiment with the error bars indicating the mean SE of three technical replicates.

The tissue concentration of both forms of the BXs generally decreased over the time period in all plants, but the absolute levels differed significantly. *Ae. speltoides* contained the highest concentrations, particularly in the leaf and coleoptile where both glucoside and aglucone levels were between five and eight fold higher than in Alifen, *T. tauschii* and Welford (included for comparison). However, the compounds were absent from the leaf tissue of the other B genome wheats, *Ae*. *longissima, sersii , bicornis* and *sharonensis* (data not shown) and the coleoptile contained only small amounts at the earliest time point. There was no aglucone in leaf or coleoptile of *T. monococcum* or *T. boeoticum* and only trace amounts of glucoside in the coleoptile at the first time point.

In the root tissue, Ae*. speltoides* consistently contained slightly higher levels of glucoside although the difference compared to other wheats was much less than in the foliar tissue. Levels of DIMBOA were up to five times higher than in the other varieties over the first 9 days post sowing but decreased rapidly. In roots of durum wheat, levels of the BXs, were slightly higher than in the hexaploid, or the D genome wheats, particularly at the later time points, but less than in *Ae. speltoides.* In roots of *T. monococcum and T. boeoticum*, BX levels were low, and DIBOA was undetectable after 6 days.

The expression of the *TaBX* genes was measured in the leaf, coleoptile and root of *T. monococcum, Ae. speltoides, T. tauschii* and Alifen (Figure 6). In the leaf tissue *TaBX2* was not expressed in any of the plants, *TaBX4* was only expressed in *Ae. speltoides* and Alifen and *TaBX5* only in *Ae. speltoides.* This pattern of expression was repeated in the coleoptile, apart from the presence of detectable levels of expression of *TaBX2* in all plants except *T. tauschii.* The relatively high levels of expression of *TaBX4* and *5* in the leaves and coleoptile of the B genome wheat correlates with the high accumulation of DIMBOA and DIMBOA-glu in these tissues, whereas expression of *TaBX2* also showed significant variation in root tissue with again the highest levels in *Ae. speltoides* and Alifen. In particular, the pathway was upregulated in Alifen roots. The expression of *TaBX3* was less variable between the plants and was expressed at similar levels in all tissues.

In the hexaploids, all of the *TaGlu* genes were expressed, but in the diploid wheats this was not the case. In *T. monococcum*, with the exception of low expression of *TaGlu c* in the root tissue, no expression of any of the genes was detectable. In *T. tauschii,* only *TaGlu c* was expressed and this most strongly in the root. In Alifen and *A. speltoides,* all three genes were expressed in the root, but despite the relatively

high levels of aglucone in the leaf and coleoptile of *Ae. speltoides*, neither *TaGlu b or TaGlu c* were expressed in the foliar tissue.

3.3.3 Chromosomal localisation of the *TaGlu* genes in hexaploid wheat

The *TaGlu* gene expression data described here raises the possibility that *TaGlu a, b* and *c* genes may be homoeologs, present on different chromosomes. To investigate this further, chromosome-mapping of the BX pathway-specific glucosidase genes in wheat was undertaken. Using gene-specific primers (*TaGlu a,b,c* sps, see Table 1), RT-PCR successfully amplified products of approximately expected size from all these three genes (*TaGlu a*, 347 bps; *TaGlu b*, 344 bps; *TaGlu c*, 330 bps), confirming that these three genes are expressed in young shoot tissues of the wheat variety Chinese Spring. Five aneuploid wheat lines in the Chinese Spring background were then used for chromosomal mapping of these three genes (Figure 7).

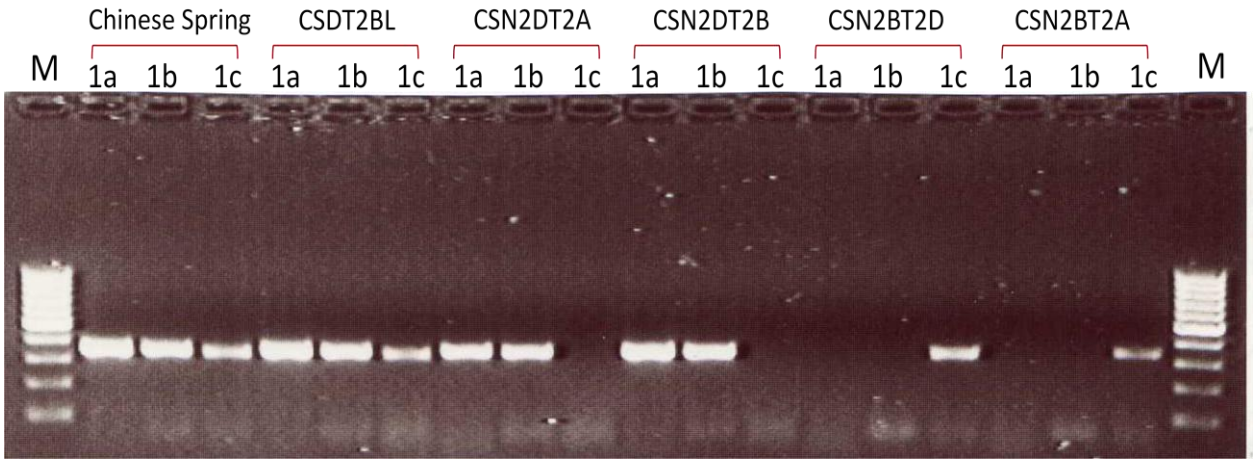


Figure 7. RT-PCR analysis of *TaGlu a, b*, and *c* in aneuploid lines CSDT2BL, CSN2DT2A, CSN2DT2B, CSN2BT2D, and CSN2BT2A. The presence and the absence of gene-specific PCR products of *Glu a* (1a), *Glu b* (1b), and *Glu c* (1c) were shown on the agarose electrophoretic gel. M, DNA molecular marker.

The expression of all the three genes was detected in the ditelosomic line CSDT2BL. This indicated that all these three genes were not located on the short arm of chromosome 2B. The expression of *TaGlu a* and *b* was detected in nullisomic 2D-tetrasomic 2A line (CSN2DT2A) but not for *TaGlu c*. This indicated that *TaGlu c* could only be located on chromosome 2D, but not possibly on chromosome 2A or anywhere

else in the wheat genome. The same result on the analysis of another wheat aneuploid line with nullisomic 2D-tetrasomic 2B (CSN2DT2B) further supported the mapping of *TaGlu c* on chromosome 2D. Interestingly, the expression of both *TaGlu a* and *TaGlu b* was not detected on either aneuploid line CSN2BT2D (nullisomic 2B-tetrasomic 2D) or CSN2BT2A (nullisomic 2B-tetrasomic 2A) while it was detected for *TaGlu c* in both lines. This clearly indicated that *TaGlu a* and *b* were located on chromosome 2B; they were not present on chromosome 2A, 2D, or anywhere else in the wheat genome. Considering the result that these two genes were not on the short arm of chromosome 2B, we conclude that *TaGlu a* and *b* are located on the long arm of chromosome 2B.

3.3.4 Aphid bioassays to compare susceptibility of different wheats

Initially, the differences in colonisation and short-term nymph production by alate aphids were determined on a series of elite UK hexaploid wheat varieties, chosen by the BWB. More detailed developmental assays were performed for a small number of varieties eliciting more extreme responses from the aphids. The study was then extend ed to include some hexaploid varieties from outside the UK, including some with resistance to other aphid species, and a limited number of tetraploid and diploid wheat species.

Settlement trials Although there were apparent differences in preference between the hexaploid wheat varieties for both aphid species (see preference indices in Figs 8 and 9, upper panels), due to variation in nymph production there were none that were significantly different to Solstice for *S. avenae* and only one, Tasman, for *R. padi*. This response by *R. padi* to Tasman was surprising and was further investigated in development trials and by HPLC (see effect on BX pathway by aphid feeding). The preference index for *R. padi* is less extensive than for *S. avenae*, but there are clear differences in preference between the species. However, since there were relatively low levels and no clear differences in BX levels between the hexaploid varieties that we had tested (see Figure 3), few of these lines were further investigated. Settlement assays conducted for both aphid species on Alifen, *Ae. speltoides,* and *T. monococcum* lines MDR037 and 049 (Figs 8 and 9, lower panels) also showed differences in preference between the two aphid species, with MDR049 preferred to MDR037 and Alifen being the most favoured. All of these wheat species were further investigated in development trials.

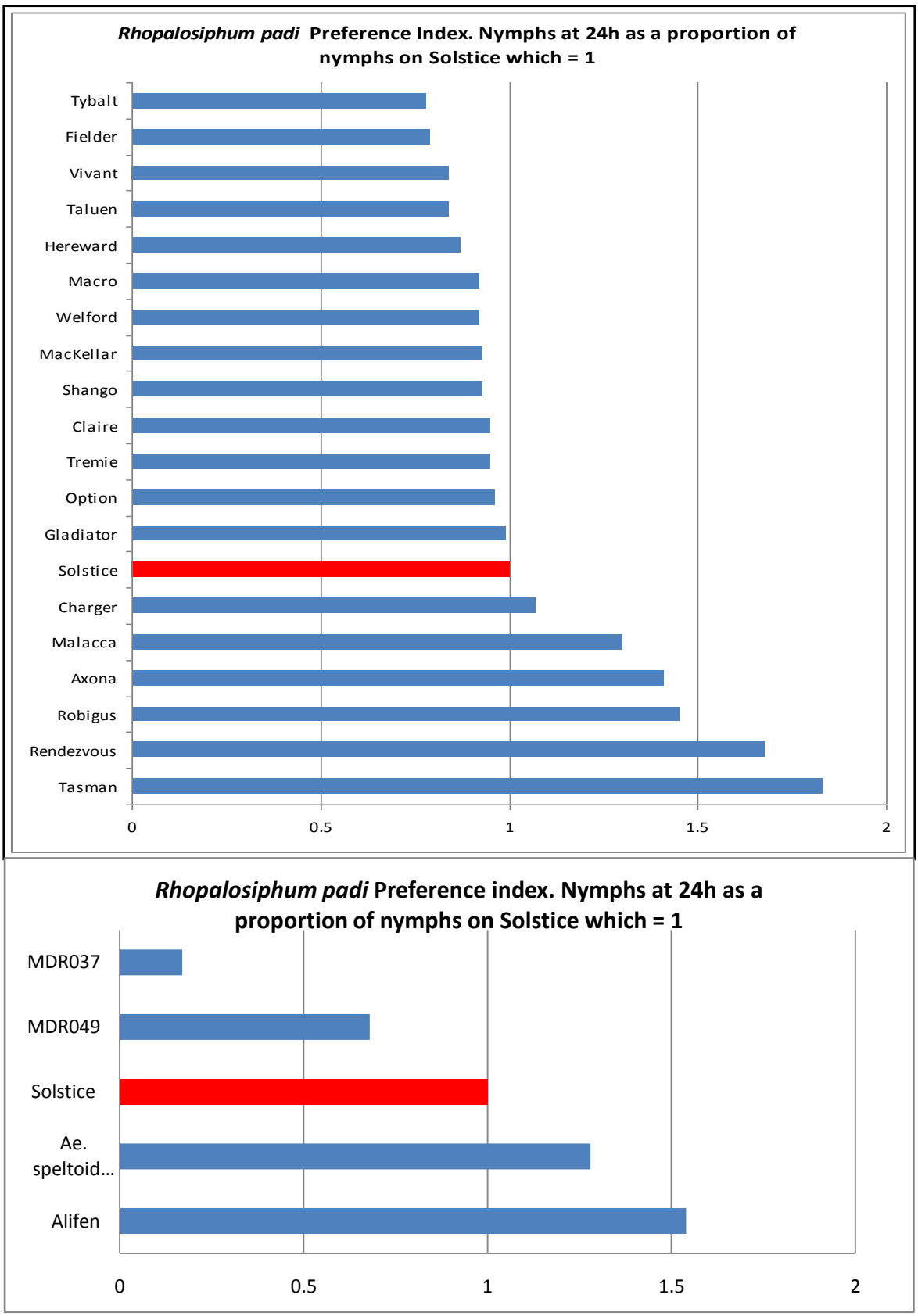


Figure 8 Preference index for *R. padi* on hexaploid wheats (upper panel) and on other wheat species (lower panel).

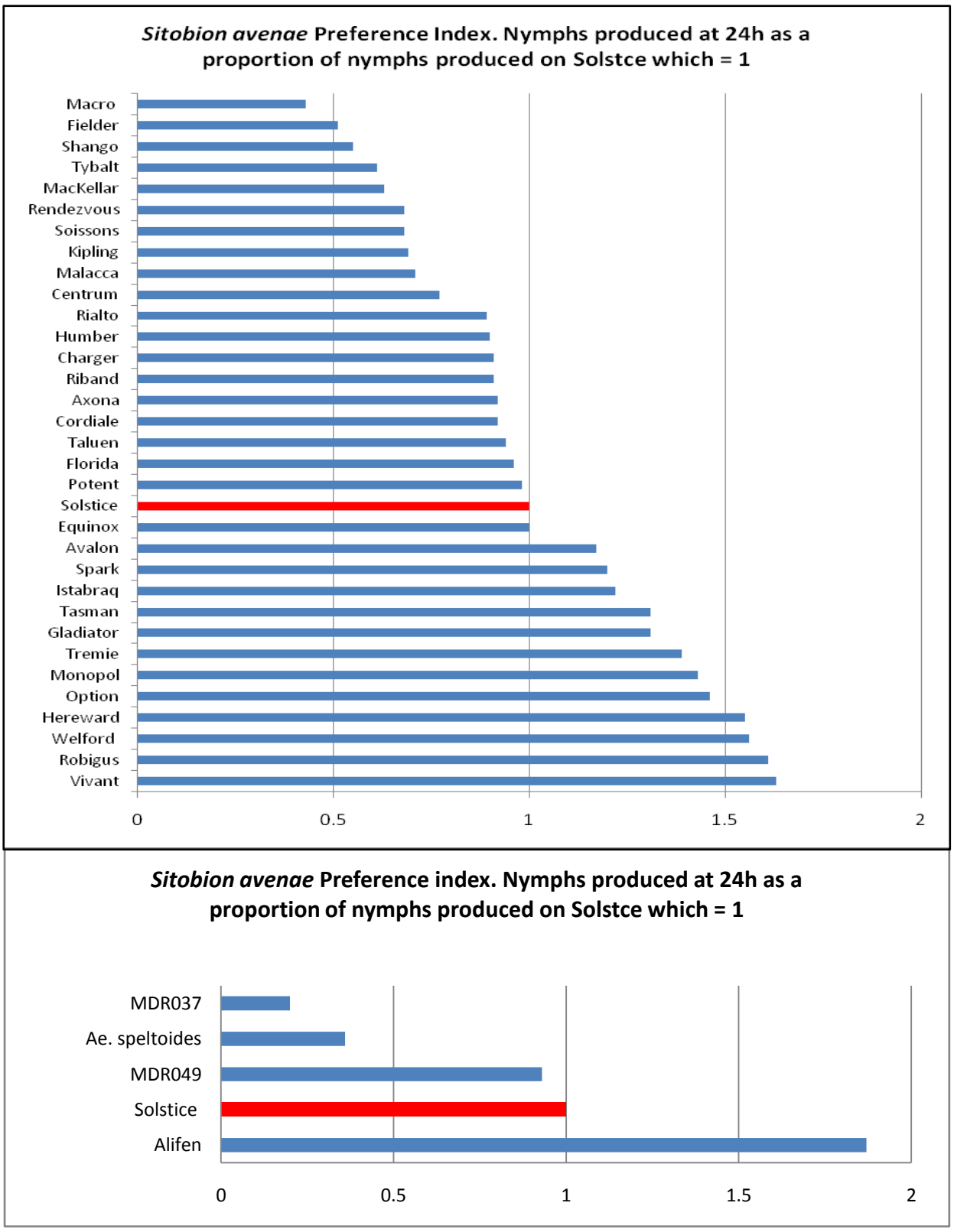


Figure 9 Preference index for *S. avenae* on hexaploid wheats (upper panel) and on other wheat species (lower panel).

Development trials Mean Relative Growth Rates between Tasman and Alifen were compared to the reference hexaploid, Solstice. This revealed that despite the slightly lower BX content in Solstice than both the other plants, *R. padi* developed better on both Tasman and Alifen than on Solstice and *S. avenae* developed better on Alifen, but less well on Tasman than on Solstice (Figures 10 and 11).

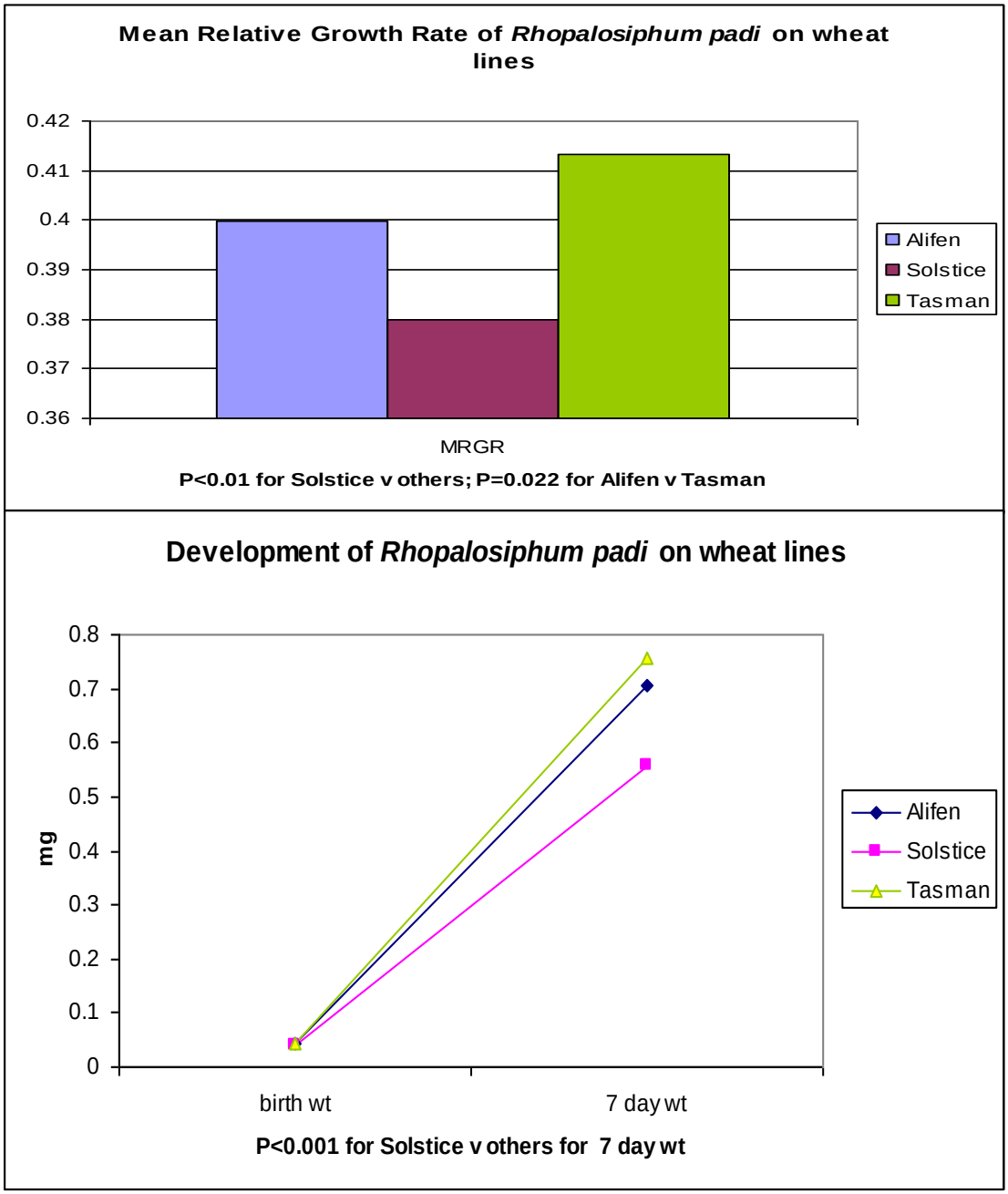


Figure 10 Growth and development of *R. padi*

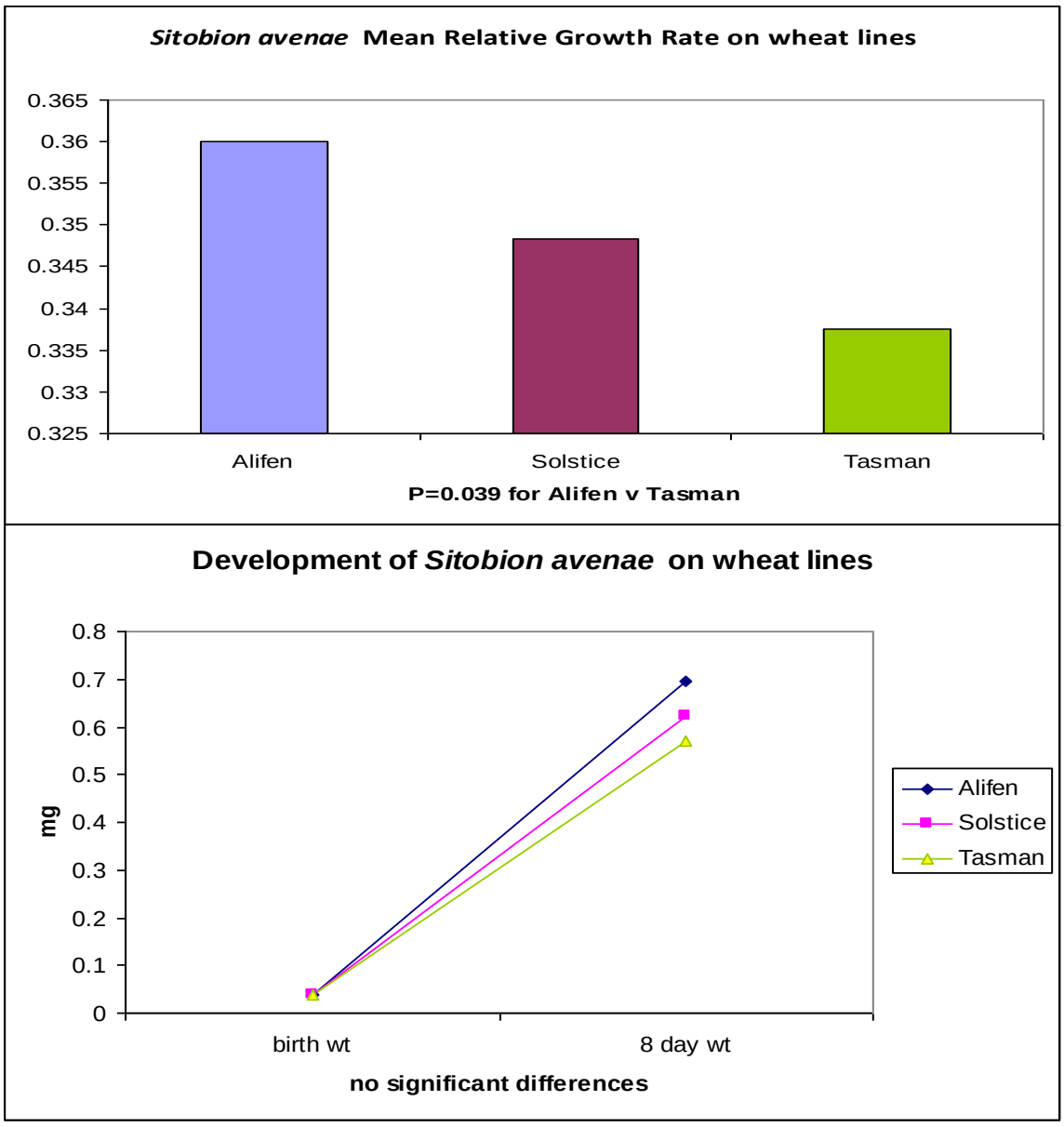


Figure 11 Growth and development of *S. avenae*

However, in the case of *Ae. speltoides*, the wheat with the highest amount on BXs in the leaf tissue, both aphids developed less well than on Solstice (Figure 12).The growth rate of both aphids on two *T. monococcum* lines, MDR 037 and MDR 049 was measured (Figure 13) and this showed that MDR 037 sustained similar growth as Solstice, but that MDR 049 was a poor host. Since neither plant contains BXs in the leaf tissue, there must be other factors responsible for the poor growth rate of aphids on MDR 049.

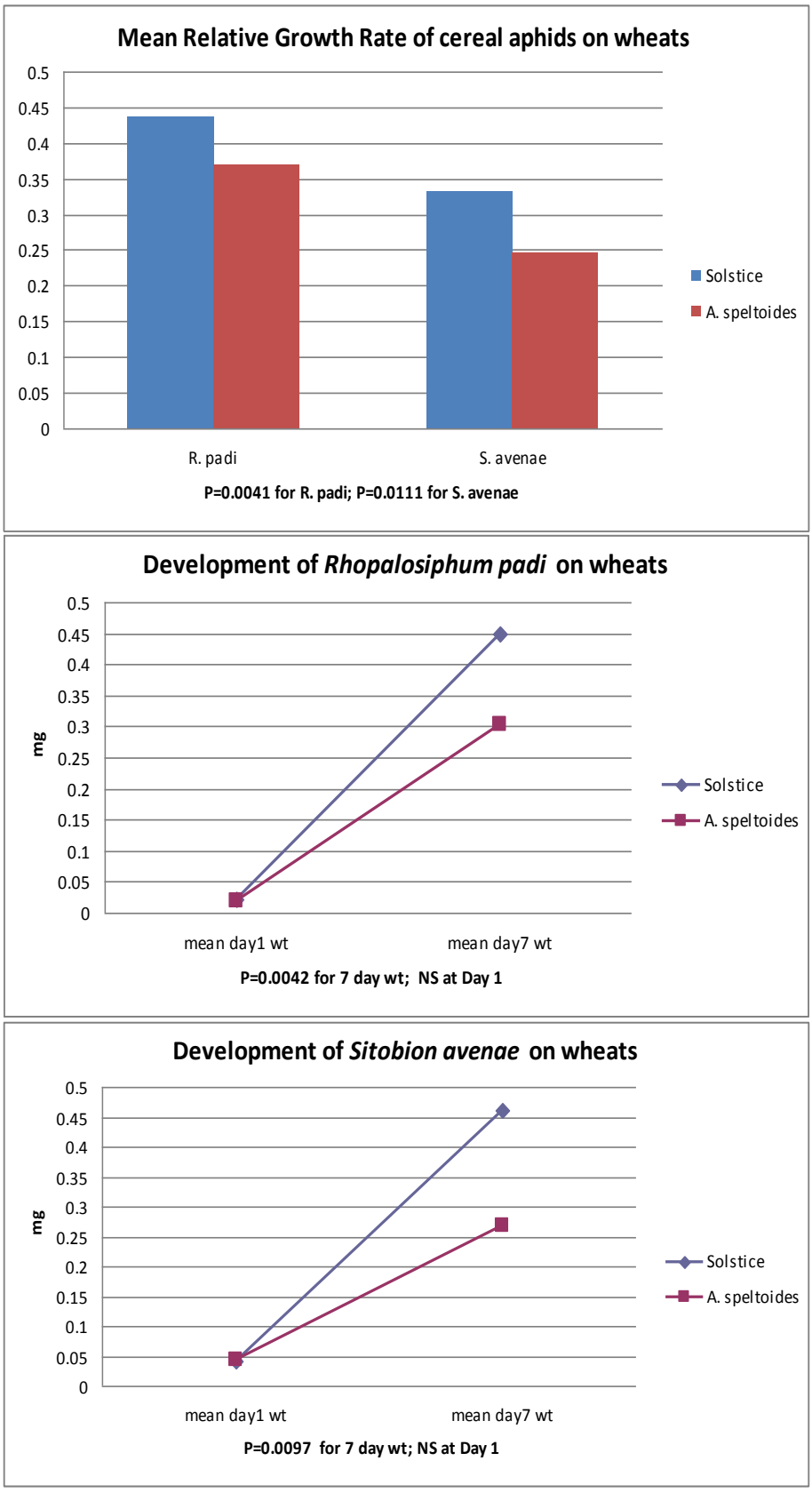


Figure 12 Growth and development of *R. padi* and *S. avenae* on Solstice and *Ae. speltoides*

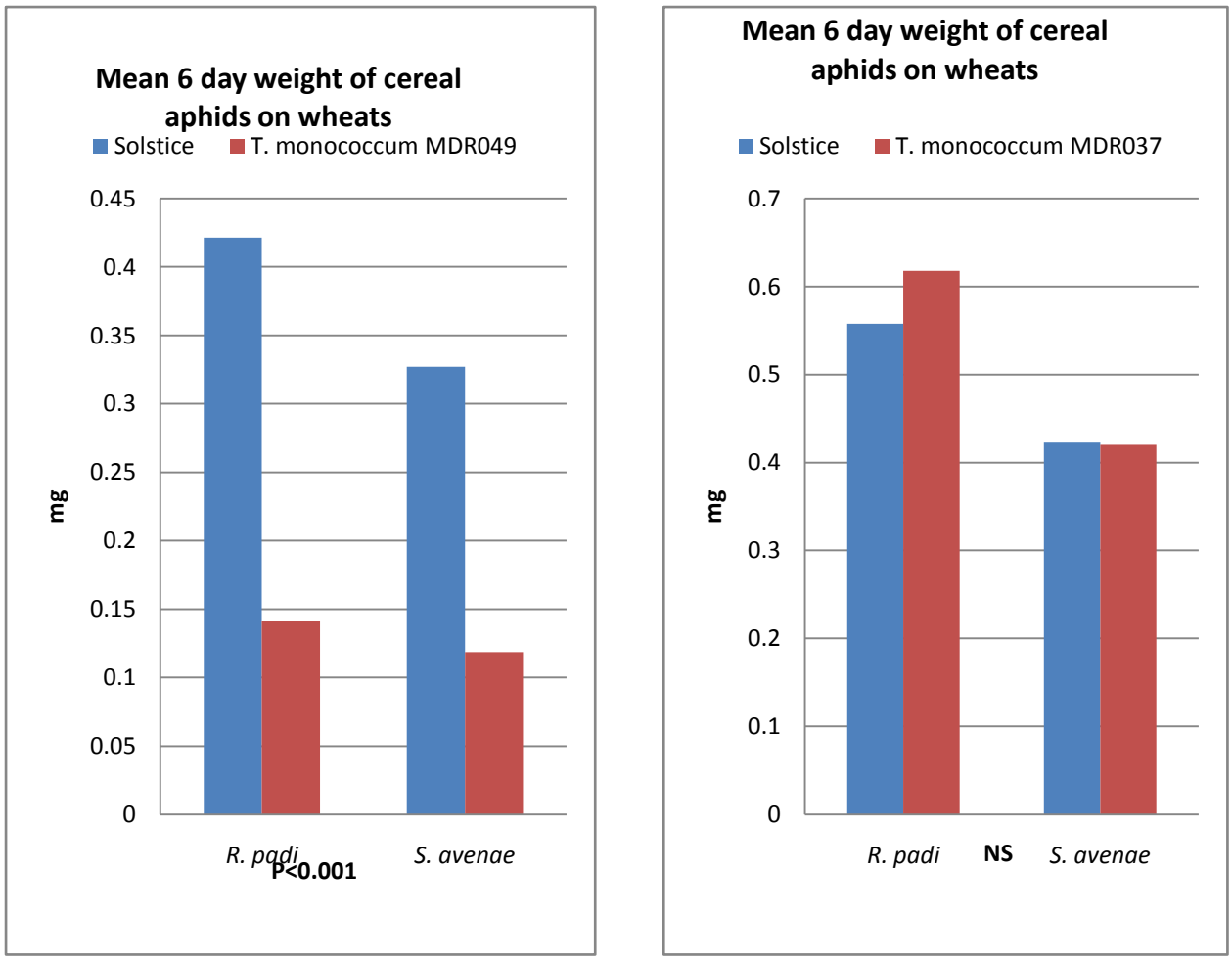


Figure 13 Development of *R. padi* and *S. avenae* on Solstice and two *T. monococcum* lines.

3.3.5 Intrinsic rate of population increase

The intrinsic rate of population increase (r_m) of *R. padi* and *S. avenae* on Tasman, Alifen and Solstice was compared (Figure 14). This showed that the aphid population increase was greatest on Alifen and on Tasman it was greater than on Solstice.

When the number of nymphs produced over the first 8 days was measured on a selection of diploid wheats, (A and B genome) it was clear that the diploid wheats were less acceptable than the hexaploids and that *Ae. speltiodes*, the only diploid that contained BXs, was the least favoured as a host (Figure 15).

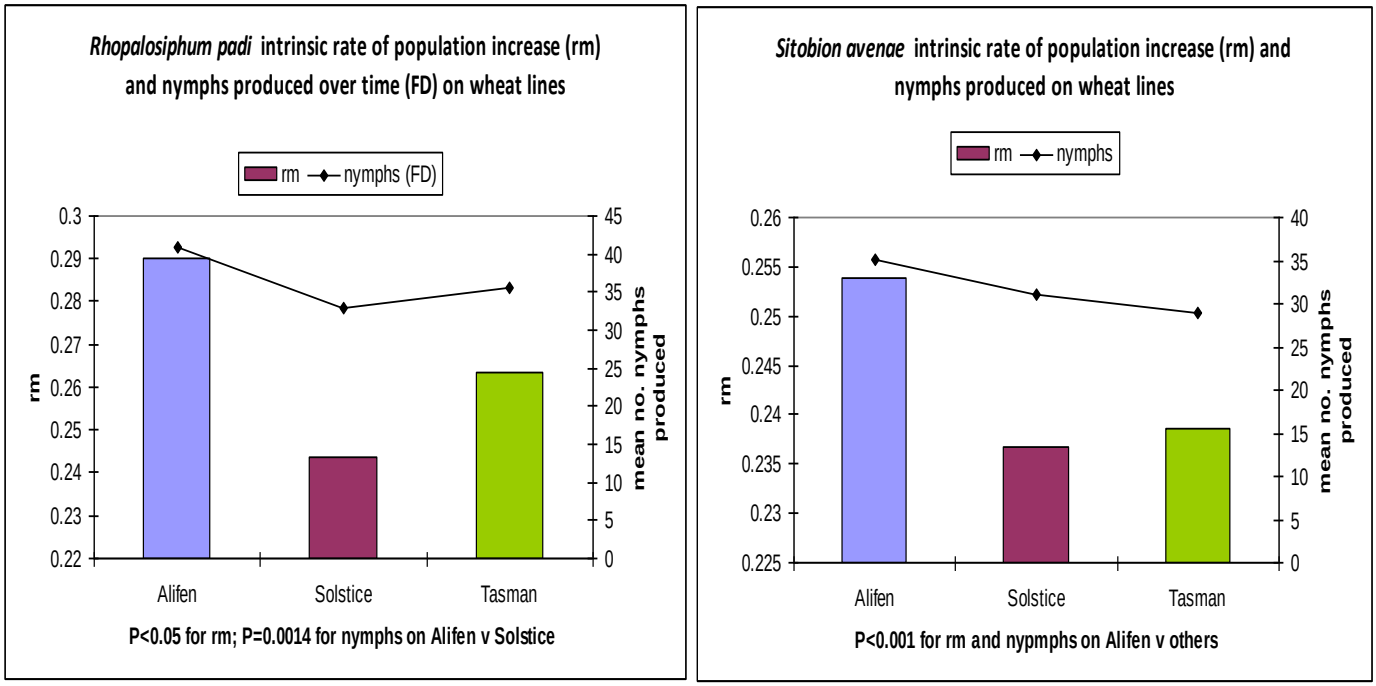


Figure 14 Intrinsic rate of population increase of *R. padi* and *S. avenae* on Solstice, Tasman and Alifen

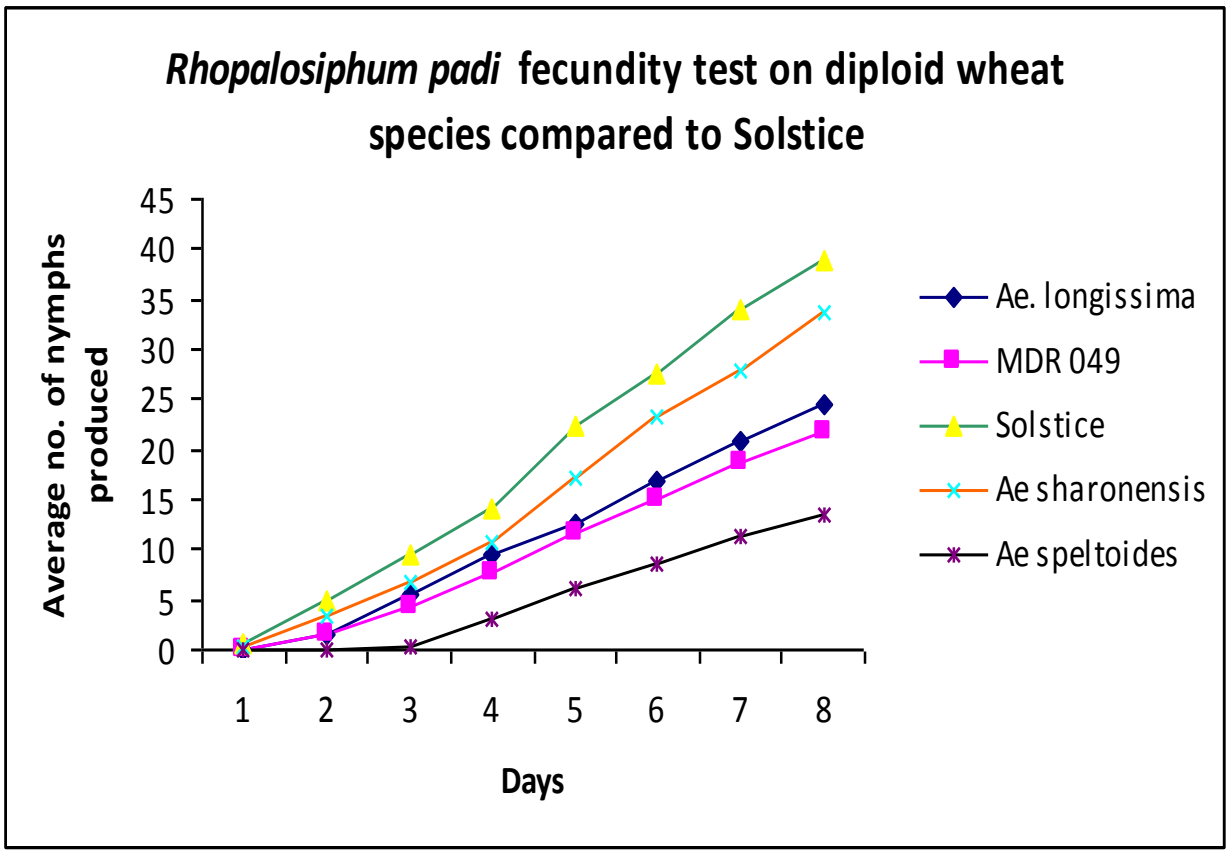


Figure 15. Average number of nymphs produced on diploid and hexaploid wheats.

3.3.6 The effect of aphid feeding on the BX pathway

The expression of the *TaBX* and *TaGlu* genes and the concentration of DIMBOA and DIMBOA-glu was measured in the hexaploid variety, Welford. The area under the clip cage, the rest of the plant and the coleoptile were measured separately. This showed that there was no effect on the expression of the *TaBX* genes anywhere in the plant (not shown). However, there was a rapid, transient increase in the expression of *TaGlu a* and *b* in the region covered by the clip cage (Figure 16). It was highest at 24 hours and had started to decrease by 48 hours. The increase was also greater when the plants were fed upon by *R. padi* than *S. avenae*.

When the rest of the foliar tissue was analysed it was found that there was no change in expression of the *TaGlus* in the coleoptile but in the part of the leaf adjacent to the clip cage there was an increase in both genes but it was weaker and greater at 48 than at 24 hours (not shown).

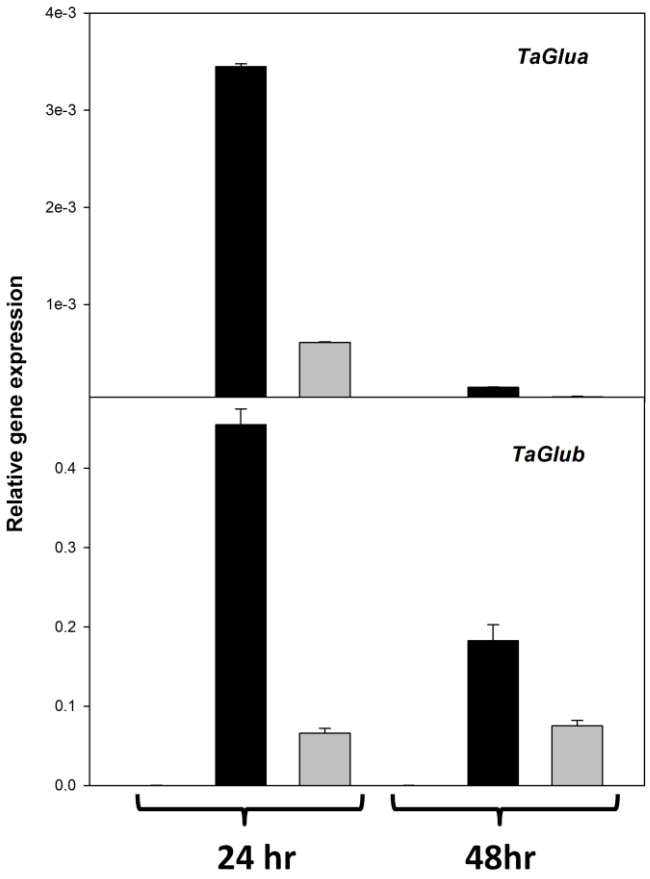


Figure 16. The effect of aphid feeding on *TaGlu* gene expression in Welford.

In Welford, when the effect on the accumulation of the compounds was measured in the part of the leaf covered by the clip cage by HPLC, it was found the there was a conversion of DIMBOA-glu to DIMBOA after feeding by *R. padi* (the effect of *S. avenae* was not studied) (Figure 17).

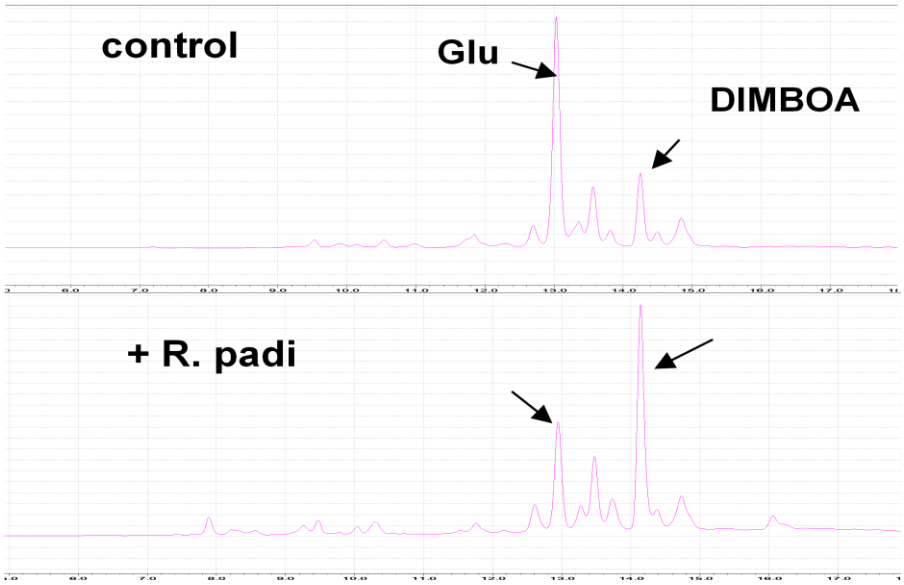


Figure 17. The HPLC trace of extract of Welford leaf tissue covered by clip cage to show the effect of aphid feeding (for 24 hours)on the ratio of DIMBOA-glu to DIMBOA.

In order to establish whether there was any variation between different wheat varieties in the magnitude or duration of the response, the effect of feeding by *R. padi* on the amounts of DIMBOA and DIMBOA-glu in the clip cage region were compared in *Ae.speltoides*, Alifen, Solstice, Welford and Tasman after 24 and 48 hours.

The results are shown in Figure 18 and indicate that there was considerable difference in both aspects of the response. Suprisingly, there was a slight decrease in the concentrations of both compounds in *Ae. speltoides.* However, in this plant no increase in *TaGlu* expression was found (not shown). This decrease in compound accumulation was not only in the clip cage region, but also occurred in the coleoptile (not shown). There was no change in the rest of the leaf tissue. The other three plants tested all showed a response, with the least effect (not significant) being in Solstice and the greatest in Tasman. In Tasman there was a more sustained effect with the DIMBOA levels at 48 hours being greater than at 24 hours.

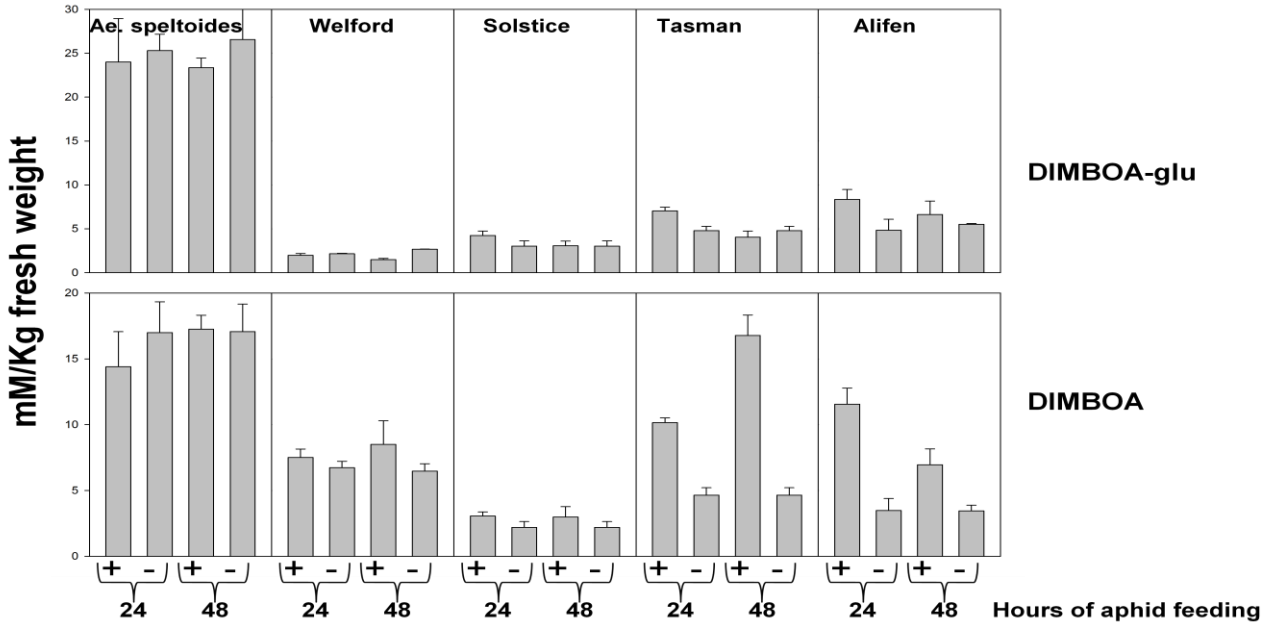
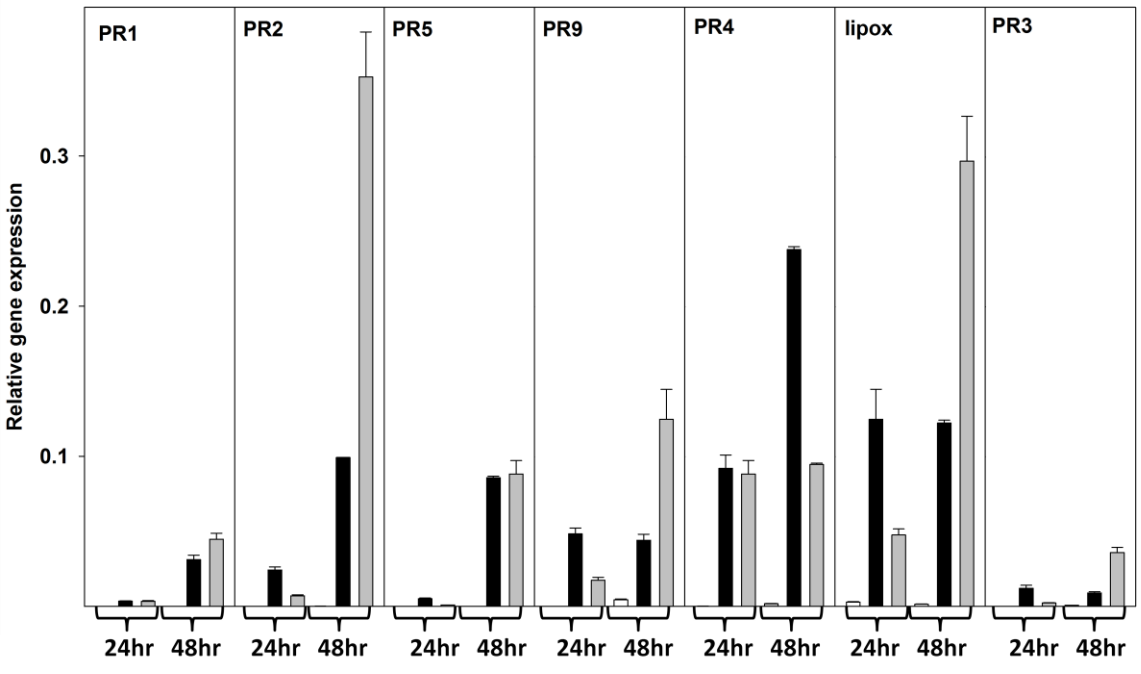


Figure 18 The amounts of DIMBOA-glu / DIMBOA measured by HPLC in the clip cage region of different wheat varieties after feeding (+) or the absence of feeding by *R. padi*



The

Figure 19. The effect of aphid feeding on defence related gene expression in Welford. PR1 -9 , are pathogenesis related proteins, lipoxygenases are involved in jasmonic acid mediated defence (Wasternack 1997) . Control=red, *R. padi*=green, *S. avenae*=blue.

effect of aphid feeding on other genes associated with defence was also measured in Welford (Figure 19) and this showed that mainly genes associated with the salicylic acid pathway, but not the jasmonate pathway were up regulated. This increase in expression occurred predominantly at the later time point (48 hours). Although the effects of *R. padi* on those defence genes that were upregulated was also localised to the clip cage region, *S. avenae* also induced expression in the region below the clip cage (data not shown).

3.3.7 The presence of compounds in aphid honey dew from different wheat varieties

Aphids feed predominantly from the phloem, and only DIMBOA-glu has been found in phloem sap collected from wheat by aphid stylectomy (Givovich *et al* 1994).

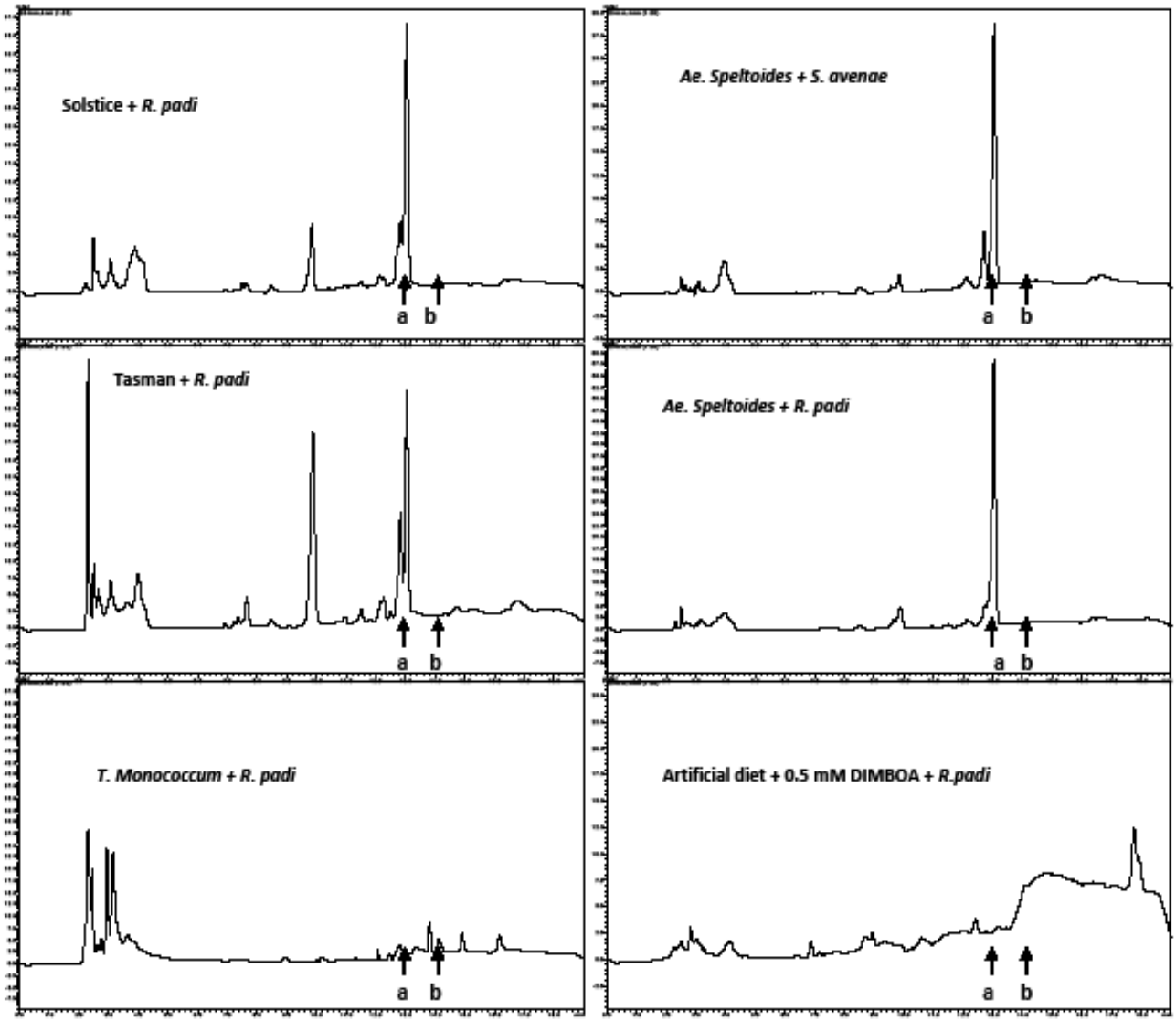


Figure 20. HPLC of honey dew collected from aphids (R. padi or S. avenae as indicated) feeding on different wheat varieties and artificial diet containing DIMBOA. Arrows indicate elution positions of a, DIMBOA-glu and b, DIMBOA.

Consistent with this observation, we were only able to detect the glucoside in aphid honey dew. We compared the constituents of honey dew collected from aphids fed on different wheat varieties and on artificial diets containing DIMBOA. We were able to confirm that only DIMBOA-glu was present in the honey dew collected from *R. padi* and *S. avenae* fed on *Ae. speltoides* and *R. padi* fed on Tasman and Solstice (Figure 20) *.* This was also the case for honey dew from aphids fed on other hexaploids (for example Welford) and the tetraploid Alifen. However, honey dew from aphids fed on *T. monococcum* did not contain BXs in the leaves, indicating that the DIMBOA-glu in the honey dew originated from the plant on which the aphid was feeding*.* Honey dew from *R. padi* fed on artificial diets containing DIMBOA also contained no BXs. There was an additional peak present in Solstice and Tasman that co eluted with the secondary metabolite dhurrin, produced in sorghum.

3.3.8 The presence of compounds in interstitial fluid (IF) extracts from different wheat varieties

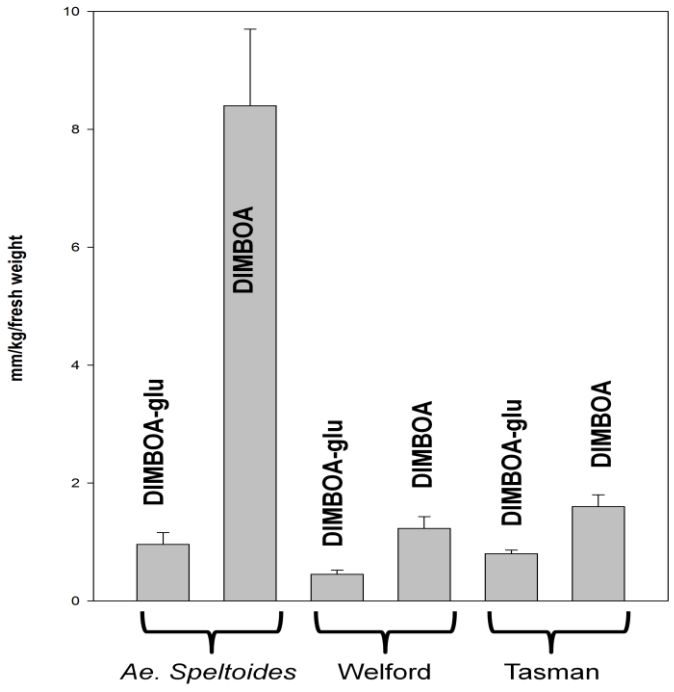


Figure 21. BX levels of in interstitial fluid extracts from *Ae. Speltoides*, Welford and Tasman.

In order to reach the phloem the aphid stylet must pass through the extra cellular spaces surrounding the cells of the epidermal and mesophyll tissues. There is evidence that the insect ingests samples from the surrounding medium during the early stages of probing. Therefore, we were interested to establish whether any correlation between BX concentration in the IF and resistance could be determined. We prepared IF extracts from leaf tissue from the hexaploid varieties, Welford and Tasman and *Ae. speltoides.* HPLC analysis showed that at 7 days post sowing the IF from all varieties contained predominantly free DIMBOA and a smaller amount of DIMBOA-glu (Figure 21). This is in contrast to the ratio of the two compounds in whole leaf tissue extracts at the same

developmental stage. In addition, *Ae. speltoides* contained over 5 times more DIMBOA than Tasman and 7 times more than Welford.

3.4 Discussion

Although the BX pathway has been the subject of scientific interest for several decades and its toxicity in artificial diets has been demonstrated, unequivocal proof that the compounds play a significant role in reducing susceptibility of wheat plants to aphids has remained lacking. None the less, the question that still requires to be answered is whether, if the BXs are present at high enough concentrations and tissue localisation, the compounds could confer aphid resistance in wheat. The presence of three genomes in hexaploid wheat renders reverse genetic approaches to confirm a relationship between BX content and resistance challenging as each *TaBX* homoeolog has been shown to be functional (Nomura *et at* 2005). In addition, it is not envisaged that the BXs are solely responsible for resistance. The effects of the compounds may be masked by other factors that contribute to resistance or susceptibility, including colour, nutritional status of the plant, cuticle structure or hairiness of the leaves. These factors will have a differential impact on the different types of bioassays that are used to assess the acceptability of a particular plant as a host for the aphid. In this work we have undertaken a detailed analysis, both of the regulation of the pathway and of the localisation of its products, in different types of wheat. We have then tried to establish a correlation between these parameters and the development and feeding of aphids on the different wheats. This has enabled us to establish that although BXs may contribute to resistance in some types of wheat, (Gordon-Weeks *et al* 2010) and this observation is supported by our recent findings in maize (Gordon-Weeks, Smart and Ton, personal communication), others possess mechanisms that are clearly independent of the products of the pathway.

One of the objectives of this study was to establish whether there was sufficient variation in the levels of BX accumulation that correlated with aphid resistance within the UK hexaploid population to form the basis of a breeding program. However, study of the levels of BX accumulation in different plant tissues from a range of hexaploid wheat varieties showed only a limited degree of variation between the varieties tested even though within the sample we had included the variety Tasman, the exudates from the roots of which have been shown to have high allelopathic activity (Wu *et al* 2003).

When we compared the compound accumulation and the expression of the genes responsible for the regulation of the BX pathway in different parts of a standard UK hexaploid variety (Welford) over the first 18 days post sowing, we found that, as anticipated, the levels of both gene expression and compound concentration decreased over the time period in all tissue types. It has already been well documented that the BX pathway is naturally active predominantly in the young plant (Thackaray *et al* 1990) but as it matures other defence mechanisms start to take over the protective role and BX levels decline. A beneficial aspect of this is that the compounds are not present in the seed (Niemeyer 1988), and will therefore not contaminate the human food chain, although it could be envisaged that it would be advantageous for enhanced disease protection if the pathway were expressed for a longer period of the plant's development. Sustained expression of the BX pathway has been observed in some dicot species that produce the compounds (Schullehner *et al* 2008). Predominantly the compounds are present in the root tissues of these plants, concentrated at the root tip (Baumeler *et al* 2000). However, no correlation has been made between their presence in roots of dicots and prolonged resistance to soil borne pathogens.

We found that the expression of the *TaBX* genes declined most dramatically in leaf tissue of Welford, apart from *TaBX3,* which maintained an almost constant level of expression over the time period. The decrease in compound concentration was more gradual, indicating that the enzymes, or their products, turn over at a relatively slow rate. Although there was a similar amount of gene expression in the root and coleoptile and much less in the leaf (except for *TaBX3*, which was expressed more evenly throughout the plant) there was an equal amount of DIMBOA/DIMBOA-glu in the root and the leaf. This suggests that the compounds may be transported from the root and coleoptile to the leaf. This would incur benefits for protection against attack from sucking and chewing insects and air borne bacteria. There may also be release of the BXs from the root into the rhizosphere, where they, or their metabolites, reduce colonisation by soil borne pathogens, insect larvae or nematodes.

The explanation for the higher and more sustained expression of *TaBX3* in the leaf is unclear, but it could indicate that the enzyme, or indeed its product, performs an alternative role. Comparison of the promoter sequences of *TaBX3* and *TaBX4* revealed no sequence homology between them (Nomura *et al* 2008) although there were some common *cis*-regulatory elements. The promoter regions of *TaBX2,* and *5,* which show almost identical expression patterns to *TaBX4,* have yet to be sequenced. Nomura et

al (2005) carried out a detailed analysis of the expression of each of the three homoeologs of the genes *TaTABX1 - TaTaBX5* and accumulation of the BXs in the hexaploid wheat, Chinese Spring. These authors found that the expression of *BX5* was higher than the other genes, which we were not able to demonstrate, but they analysed earlier time points than those that we studied (36 hours and 5 days). Nomura *et al* (2005) also found that the expression of all the genes was lower in the root than in the shoot, but they did not separate leaf from coleoptile and their results cannot be directly compared to ours.

A further surprising finding was that free aglucone levels were similar to those of the glucoside. Some conversion of glucoside to aglucone may occur during extraction, but exogenous addition of glucoside to the extraction mixture indicates that this is less than 2% and would not account for the amounts of DIMBOA measured in the tissues. It was initially believed that final steps in the BX pathway in maize involved conversion of DIBOA to DIMBOA and that both aglucones were glucosylated and transported into the vacuole. More recent data now indicate that the substrate for the methyl transferase is DIBOA-glu and that its conversion to DIMBOA-glu occurs in the cytoplasm. It is not known if this is also the case in wheat, but if it were, it would mean that the free DIMBOA must have been released from DIMBOA-glu, presumably by the action of a TaGlu enzyme and is not a biosynthetic precursor *en route* to production of the stored compound.

Consistent with the findings of Sue *et al* (2000;2006), we found that the expression of the three *TaGlu* genes in Welford declined more or less in parallel with the time dependent decline in *TaBX* expression and compound accumulation. The highest fold decrease in *TaGlu* expression was also in the leaf tissue. *GLU a* and *GLU b* showed similar spatial and temporal expression patterns to *TaBX 2, 4* and *5,* although *GLU b* was much more highly expressed throughout the plant. *GLU c* was expressed significantly more in the root than elsewhere in the plant. In contrast, Sue *et al* (2006) found that expression of *GLU a* accounted for 85% of the total glucosidase gene activity in whole plant seedlings of Chinese Spring.

Gene expression and compound accumulation in wheats with different ploidy levels, revealed a much greater degree of variation between these plants than we were able to detect in the hexaploid population. Cultivated wheat, bred for high yield under conditions where exposure to biotic or abiotic stress is minimised, may have lost traits associated with resistance to environmental threat. Wild relatives of ancestral wheats

that are adapted to a wide range of conditions will contain more diversity than cultivated species that have been developed in a more uniform, protected environment.

In *T. monococcum* (A genome) the only BX compound clearly detectable after the first time point was DIBOA-glu. This was only in root tissue and no BX compounds accumulated in the foliar tissue. It appears that *T. monococcum* is unable to convert DIBOA-glu to DIMBOA-glu, suggesting that the pathway may be incomplete. The pattern of compound accumulation was identical in *T. boeoticum*, an A genome relative of *T. monococcum* (data not shown) and this wheat is subject to natural mutations of the pathway, leading to accessions that do not contain BXs. Although some of the hexaploids, the tetraploid Alifen and *Ae. speltoides* contained DIBOA and DIBOA-glu in young tissues, the predominant form was DIMBOA/DIMBOA-glu in all the plants apart from the A genome wheat. This is in contrast to the findings of Nomura *et al* (2005) who only detected DIBOA-glu in the foliar tissue of *Ae. speltoides*.

Ae. speltoides contained more BXs in all plant parts, but the difference was most striking in above ground tissue. The expression of *TaBX4 and TaBX5* was significantly enhanced in the foliar tissue of this plant, but *TaBX2* was only up regulated in the roots. Surprisingly, the expression of *TaBX3* is suppressed in *Ae. speltoides* leaves compared to *T. monococcum* and Alifen.These findings are open to a number of interpretations, but would suggest that transcriptional control of *TaBX4 and TaBX5* are responsible for the higher accumulation of the compounds in the leaf tissue where the levels of expression of *TaBX2* and *TaBX3* are sufficient to provide the required amount of substrate (hydroxyl indole). In the root, the limiting step appears to be the conversion of indole to indole-3-one by *TaBX2*.

It has previously been established that the Kms of TaBX4 and TaBX5 B genome homoeologs are lower than those of the A genome counterparts, although the A genome *TaBX3* homoeolog is more active than the B genome enzyme (Nomura *et al* 2005). This would suggest that the differences in gene expression are magnified by the enzymic activity of their products. Within the B genome wheats the high BX accumulation in the leaf tissue is unique to this particular plant and leaves of *Ae. longissima, sersii , bicornis* and *sharonensis* did not contain even detectable levels of BXs. This observation lends support to the suggestion that *Ae. speltoides* is the closest relative to the true ancestor of hexaploid wheat (Golovnina *et al*. 2007) as

both BX levels and *TaBX4 and TaBX5* expression in the tetraploid leaf are intermediate between those found in *Ae. speltoides* and A genome wheats. A comparison between the levels of expression of these genes in leaf tissue of the other B genome wheats is now required as translocation of the compounds from the coleoptile may also be more efficient in *Ae. speltoides* or differences in post translational regulation of the TaBXs may take place.

Comparative analysis of the expression patterns of the *TaGlu a* and *b* in the different wheat species showed that no expression could be detected in the A or D genome wheats, but that both were expressed in the B genome and tetraploid wheats. *GLU c* appeared to be expressed at low levels in the tetraploid and B genome root tissue and throughout the D genome plant at higher levels. It has been speculated that the three forms of the glucosidase may be homoeologs, each existing on separate chromosomes, but our expression data did not fully support this. We found some expression of *TaGlu c* in the roots of *Ae. speltoides* and *T. tauschii* and *TaGlu a* and *b* were both expressed in *Ae. speltoides*. There may be sequence differences between the genes present in the different wheat species, such that the genes may be expressed, but not amplified by the primers. For example the primer used to amplify *TaBX 3* in hexaploid wheat did not produce a product in *T. monococcum,* and a specific primer was designed for this species, but this would not explain our findings. Chromosome mapping of the different genes using the aneuploid lines of Chinese Spring enabled us to establish that *TaGlu a* and *b* are present on the chromosome 2B and *c* on 2D. This location is particularly significant as screening of a double haploid population derived from the hexaploid wheats, Sunco (weakly weed suppressing) and Tasman (strongly weed suppressing) allowed a QTL for allelopathic activity to be identified on chromosome 2B (Wu *et al* 2003).

Many plant defence pathways are induced either by plant signalling compounds or in response to insect attack. In the latter case the defence eliciting stimulus is a combination of wounding and inducing compounds released from the attacker, that are recognised by the host and that cause an augmentation in basal defence (Heil and Ton. 2008; Ahmad *et al* 2010). Induced defence is more economical for the plant in terms of metabolic resources and less likely to lead to resistance in pest populations. We were therefore interested to establish whether the BX pathway could be induced by aphid feeding. Our results clearly demonstrate that this is the case as there was a rapid, localised and transient increase in expression of the *TaGlu a* and *TaGlu b* in hexaploid and tetraploid wheats. Other defence genes, mainly associated with salicylic

acid defence, were also affected, but this response was slightly delayed. Coupled with the change in gene expression, there was an associated conversion of DIMBOA-glu to DIMBOA, but there was either no effect on the biosynthetic genes or there was a slight down regulation. This could be a result of feedback inhibition of the pathway caused by accumulation of the toxic product.

Some damage to plant cells may also occur while the aphid stylet is probing through the plant tissue, but such damage would be minimal compared to that which would occur during attack by chewing insect pests. Presumably, factors present in the aphid saliva are responsible for causing the changes in the regulation of the BX pathway. There have been several reports in the recent literature of the identification of plant defence elicitors, predominantly proteins, in aphid saliva (Miles, 1999). Also, it has been established that the aphid secretes a different type of saliva during the initial stages of colonisation, while the stylet is moving through the leaf tissue, to the saliva released when it is feeding from the phloem (Miles, 1999). Different active substances could be present in both types of saliva that would exert their effects either in the initial stages of colonisation or once the aphid has started to feed from the phloem. The effect was first detected after 24 hours of feeding, but it must be taken into account that the onset of feeding is delayed and it takes some time before a significant number of aphids start to probe. The fact that the response of the BX pathway is localised and decreases rapidly, however, indicates that it is likely to be elicited during probing through the epidermal and mesophyll tissue rather than during phloem feeding. It appears that once sustained feeding has commenced the saliva containing the elicitor is no longer released. Also, elicitors released into the phloem would be transported throughout the plant, particularly to the region below the clip cage and would be expected to have a systemic effect. We did not observe such plant-wide induction of the BX pathway, although induction of PR proteins in response to feeding by *S. avenae* was less restricted. The PR protein genes are mainly salicylic acid responsive genes and previous evidence has suggested that aphid feeding induces the salicylic acid dependent defence pathway, rather than the jasmonic acid pathway (Moran and Thompson 2001).

When we compared the degree of conversion of DIMBOA-glu to DIMBOA in different types of wheats, we found that there was a significant degree of variation. Alifen and Tasman had the highest response, but no induction, either of gene expression or change in compound levels could be detected in *Ae. speltoides*. This could be interpreted to indicate that the magnitude of the response is indirectly proportional to

the concentration of DIMBOA in healthy tissue. This is not supported by the observation that in both Alifen and Tasman DIMBOA levels are higher than in Solstice, where a very weak induced response occurred. We conclude that the excessively high levels of DIMBOA in *Ae. speltoides* may represent a threshold amount capable of inhibiting the TaGlus that has not been reached in Alifen or Tasman.

When we then examined the performance of the two cereal aphid species, *R. padi* and *S. avenae* on different varieties and compared results of the different bioassays to constitutive and induced levels of BXs in the leaf tissue we did not find a consistent correlation within the hexaploid plants that we analysed where the overall levels tended to be low. Tasman, which had the highest constitutive levels, displayed a strong induced response and yet was preferred as a host by *R. padi* to Solstice in terms of preference index, growth rate and population increase. The results were similar for *S. avenae*, for all but the growth rate bioassay where Tasman supported a reduced growth rate compared to Solstice. Overall the difference between the bioassay results was less marked between varieties for this aphid. Alifen also contained higher constitutive BX levels and responded strongly, but it was also favoured by both aphids in all bioassays. With a few, exceptions diploid wheat species were less acceptable as hosts in all assays, and because, with the exceptions of *Ae spletoides*, they did not contain BXs, other resistance mechanisms are implied.

Careful interpretation of the results of these bioassays is required before any clear conclusion can be drawn with respect to the role in aphid defence played by the BX pathway in plants currently available. Choice tests are more likely to show up subtle differences in preference between varieties than no choice tests where the aphids have to feed or die. However, comparison of the preference indices for alate aphid settlement in choice tests with subsequent nymph development trials in a no choice situation, show that adult preference and early nymph production is not always influenced by possible antibiotic factors within the plant. In fact, *R. padi* would seem to prefer plants with higher levels of BXs and this could be due to its feeding habit, preferring the stem and younger leaves compared to *S. avenae,* which prefers to feed on upper leaves and developing ears . This aphid may have developed ways to overcome the toxicity of DIMBOA, as is the case with certain brassica specialist insect feeders that seek out hosts with higher glucosinolate levels to avoid competition with more generalist feeders (Hopkins *et al* 2008). As already alluded to, adult preference may also be influenced by other factors such as leaf surface chemistry or morphology.

Therefore both approaches are necessary for a full assessment of the potential of plant lines to "resist" aphid colonisation in the field.

The observation that *Ae. speltoides* was the plant least susceptible to both aphids in all respects would suggest that here the high BX levels may have an effect, but that this is in combination with other defence traits. It is clear from our results, obtained from studying aphid behaviour on the diploid wheat species, that BXs are not the only defence chemistry influencing aphid development. This is particularly true for *T. monococcum* where the observation that there are accessions that are resistant to aphids has already been reported (Caillaud *et a*. 1995, Migui *et al* 2004). No BXs are found in the leaves of this plant, but two of the accessions that we studied, MDR 037 and 049, differ significantly in their acceptability as hosts for both aphids. Whereas aphids feeding on MDR 037 displayed a similar growth rate to those feeding on Solstice the growth rate of those feeding on MDR 049 was reduced by 75%. However, our latest results would suggest (not shown) that MDR 049 is favoured in settling tests and MDR 037 appears to have morphological features that initially deter aphids, despite the plant being a better host in terms of its nutritional value to the aphid compared to MDR 049. These trials were conducted with young seedlings since the emphasis of the project was on BX levels, which have been shown to decline as the plant ages. However, to determine, if other chemical defences are involved, trials with plants of different ages would be necessary.

Here we have considered the ability of aphids to exploit advantageously the BX pathway and breakdown the correlation between resistance and BX levels and the implication of other defence factors that may over ride the importance of the nutritional status of the plant. However, another reason why higher BX levels may not have a clearly demonstrable antibiotic effect on aphid development at the levels present in currently available wheats is that the compounds are not present in the right area of the plant. The major food source for the aphid is the phloem sap, which is under high pressure and is forced through the aphid's alimentary canal. Hence, the content of the aphid honey dew reflects the contents of the phloem to a large extent and both contain a high proportion of sugar related compounds. It has previously been reported that only DIMBOA-glu, and not DIMBOA is present in aphid honey dew (Givovich *et al* 1993) and likewise, it has also been demonstrated that the intact glucosinolate is present in the phloem, as opposed to the toxic catabolite, isothiocyanate (Chen *et al* 2001). We were also only able to detect DIMBOA-glu in the honey dew of both *R. padi* and *S. avenae*, and we demonstrated that the compound

originated from the plant from which the aphid was feeding, as it was not present in honey dew from aphids that had fed on *T. monococcum.* However, we considered that it was possible that the aphid may be capable of ingesting DIMBOA and converting it into the non toxic glucoside by the action of its own intestinal glucosyl transferases. We found that aphids fed on DIMBOA alone did not secrete DIMBOA or DIMBOA-glu, suggesting that when the aphid ingests DIMBOA it remains, or more likely is toxically metabolised in the insect's body and does not pass into the honey dew. This would mean that the absence of DIMBOA in honey dew is not definitive proof that the compound is not in the phloem although relevance of honey dew collected from artificially fed insects must be viewed with some caution as, unlike the phloem sap, the feeding source is not under pressure.

Phloem extracts obtained by aphid stylectomy also only contain glucoside (Givovich *et al* 1994) which would appear to provide further confirmation that no DIMBOA is present in the sap. However, sap collected in this way has also been exposed to aphid saliva, which may contain substances capable of inhibiting the activity of plant Glus. This would lead to an absence, or to significantly reduced levels of DIMBOA in stylectomy extracted phloem but DIMBOA could still be present in the sap from an uninfested plant.

It is known that aphid saliva contains calcium binding proteins that are capable of preventing the plant from responding to sieve element wounding (Will *et al* 2007). Forisomes, or protein bodies in the sieve elements, respond to elevated calcium concentration by changing their conformation so that they obstruct phloem transport. When the calcium concentration drops, the forisomes contract and free phloem movement is restored. Aphids appear to be able to manipulate this response to their advantage by lowering calcium concentrations to maintain phloem movement, which is essential for their ability to feed. Whether aphid saliva also contains proteins or other substances that interfere with Glu activity is not known.

Although the insects feed from the phloem, there is considerable evidence that gustatory cues detected during stylet penetration of peripheral plant tissues affect aphid feeding behaviour (Miles, 1999). These cues may be in the form of defence related proteins (PR proteins) or secondary metabolites that are present in the extra cellular spaces of the epidermal and mesophyll layers of the leaf that the aphid tastes as its stylet passes between the cells. For this reason we also undertook an analysis of the interstitial fluid (IF) from the leaves of different types of wheat plants. This

revealed that predominantly DIMBOA, and relatively low amounts of DIMBOA-glu were present in the IF and the ratio between the two compounds was very different from that found in whole leaf tissue extracts. Although it has to be considered that breakdown of DIMBOA-glu could occur during IF extraction, our evidence from preparation of IF from maize, where the predominant form is DIMBOA-glu would refute this. Also, our experiments were performed under highly controlled conditions and when DIMBOA-glu was added to the extraction it did not degrade. Furthermore, the presence of such large amounts of DIMBOA in the IF provides a convincing explanation for how the plant is able to tolerate the surprisingly high concentrations of the compound that were detected in whole tissue extracts (see above).

Our observation that *Ae. speltoides* contains the highest amounts of DIMBOA in the IF on a fresh weight basis could explain why this plant is relatively resistant. In the hexaploid and tetraploid plants there was no correlation between DIMBOA levels in the IF and resistance; in Tasman the concentration was higher than in Solstice or Welford, but the variety tended to be more susceptible. We would therefore hypothesise that a threshold level of the compound is required in the extracellular compartment to provide defence. This could be mediated either by acting directly as an antixenotic, or indirectly by stimulating callose deposition. There is good evidence that glucosinolates are involved in this latter defence response in Arabidopsis thaliana (Clay *et al* 2009) and, although we have not yet compared callose levels in the leaves of different wheat plants, we are currently exploring the role of DIMBOA in callose deposition in maize (Ton, and Veryrat, personal communication).

Having established that significant amounts of aglucone are present in the extra cellular spaces of wheat leaves, in particularly high amounts in the least susceptible variety, it is now important to confirm that this property is indeed associated with resistance. This could be achieved by constructing mapping populations between *Ae. speltoides* and a B genome wheat that does not contain DIMBOA in the leaf tissue, which could then be screened for IF DIMBOA concentrations and aphid resistance. This strategy is included in our planned future research.

It would also be valuable to determine the mechanism involved in the accumulation of the compound outside the cell, because a better understanding of the process may indicate means by which it can be manipulated to enhance resistance. Of the possibilities to be considered, the most obvious would involve the direct release of DIMBOA from the cytoplasm, mediated via a plasma membrane transporter system.

However, unless the final steps in the BX pathway in wheat differ from maize, DIMBOA-glu is derived from DIBOA-glu, through a process that occurs in the cytoplasm and the glucosides are sequestered into the vacuole (Jonczyk *et al* 2008). This would indicate that all cytoplasmic DIMBOA must have been be generated from hydrolysis of glucoside, which has either not been successfully transported across the tonoplast membrane or has been released from the vacuole. This seems unlikely as the conversion of DIBOA to DIBOA-glu is believed to be instantaneous, and as it is presumed to occur as a measure to protect the plant cell from the toxicity of the aglucone (Jonczyk *et al* 2008) it is not clear why the step would be reversed. It would expose the cytoplasm unnecessarily to the compound. Furthermore, hydrolysis of the glucoside is only believed to take place after pathogen attack or wounding (Sicker *et al* 2000), but we found a significant amount of DIMBOA in the IF of healthy plants.

The scenario described above relies on the assumption that the TaGlus that catalysed the hydrolysis of DIMBOA-glu are either located in the cytoplasm or are stored within the plastids (Sue *et al* 2006). However, the subcellular localisation of the Glus has not been directly established and the available evidence is based on protein structure. In monocotyledons, the enzyme has an N-terminal transit peptide that is believed to direct the protein into the plastid (Gusmayer *et al* 1994). In eucotyledenous plants, the protein possesses a signal peptide, which has been shown to be associated with secretion through the endoplasmic reticulum and export from the cell (Ahn *et al*. 2007) and it has been suggested that the enzyme is either located in the apoplast or in intracellular protein bodies (Poulton and Li 1994). Some evidence that a proportion of the Glu exists outside the cell is obtained from the work of Nikus *et al* (2001). These authors have shown that in wheat, but to a much lesser extent in maize, Glu immuno labelling can be detected in the cell wall as well as in the cytoplasm and plastids. If TaGlus were, in fact, present in the extracellular spaces of wheat plants, it could be envisages that the DIMBOA in the IF could originate from DIMBOA-glu that has been exported from the cell and hydrolysed by extracellular enzymes. DIMBOA-glu could accumulate in the extracellular space as a result of a diversion in the transport of some of the newly biosynthesised compound across the plasma membrane, as opposed to through the tonoplast, to the vacuole. However, the compound could have been stored in the vacuole initially, but then packaged into secretory vesicles, which are released into the cytoplasm, and moved to the plasma membrane where the contents are released into the extracellular space (Echeverría 2000).

Whatever the mechanism involved, the presence of DIMBOA in the extracellular spaces would provide a deterrent both to aphids and to pathogens that colonise the apoplast. A question that still remains to be answered however, is whether the observed increase in DIMBOA concentration, caused by aphid feeding, involves an increase in the concentration of the extracellular located compound. If the hypothesis described above, involving secretion of DIMBOA-glu into the extracellular space, is correct, both release of DIMBOA-glu and TaGlu from the cell must be considered. It is clear that the response is also associated with a sharp increase in *TaGlu* expression in some wheat plants, but that no increase in *TaBX* expression could be detected. This implies that both enzyme and substrate would be released because there is only a low concentration of DIMBOA-glu in the apoplast in the healthy plant.

These considerations remain speculative as we have not investigated the effect of aphid feeding on apoplastic DIMBOA concentrations, and we have only made measurements at a whole tissue level. However, we have observed that plants that mount the strongest response in terms of hydrolysis of DIMBOA-glu (Tasman for example) are often relatively susceptible. Although Tasman contains relatively high levels of BXs compared to other hexaploids, the levels are significantly lower than in *Ae. speltoides* in the IF. This leads us to conclude that it may represent a secondary response that is only activated if the primary defence mechanism is inadequate. The primary defence could, in the case of *Ae. speltoides*, be high apoplastic DIMBOA, but there are also clearly other mechanisms that have to be considered. A further conclusion is that the induced response is not entirely effective, presumably because it is not sufficiently rapid or that it does not indeed deliver the compound to the required location, which we would speculate to be either the apoplast or the phloem.

3.5 Conclusions

In this study we have established a reliable method to quantify the BXs in wheat plant tissues using a non aqueous extraction procedure, followed by HPLC. We have compared the levels of the compounds in different plant parts during early development in hexaploid wheats and in wheats with different ploidy levels and we have measured the expression of *TaBX* and *TaGlu* genes in the same tissues. We have then examined the mean performance of two cereal aphid species, *R. padi* and *S. avenae* on varieties that differ significantly in levels of BXs in their leaf tissue. This has revealed that a B genome wheat that contains the highest amounts of the compounds is the least favoured by aphids, and that other diploid wheats that do not contain BXs

in the leaves have resistance based on other mechanisms . Although we have been able to demonstrate that the pathway is induced by aphid feeding, this response does not correlate with resistance. This would suggest that it is a secondary defence strategy. An important factor may be the concentration of the active BX aglucone in the extracellular spaces of leaves.

3.6 References

Ahmad S., Gordon-Weeks R., Pickett J., Ton J. 2010. Natural variation in priming of basal resistance: from evolutionary origin to agricultural exploitation. *Molecular Plant Pathology, (in press).*

Ahn Y.O., Saino H., Mizutani M., Shimizu B., Sakata K. 2007. Vicianin hydrolase is a novel cyanogenic b-glycosidase specific to b-vicianoside (6-O-a-Larabinopyranosyl-b-D-glucopyranoside) in seeds of Vicia angustifolia. *Plant Cell Physiol*., **48**, 938–947.

Argandona V.H., Luza L.C., Niemeyer H.M., Corcuera L.J. 1980. Role of hydroxamic acids in the resistance of cereals to aphids. *Phytochemistry,* **19:** 1665-1668

Atkinson J., Morand P., Arnason J., Niemeyer H., Bravo H. 1991. Analogues of the cyclic hydroxamic acid 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one: decomposition of benzoxazolinones and reaction with beta-mercaptoethanol. *J. Org. Chem.,* **56:** 1788-1818

Baker E.A., Smith I.M. 1977. "Antifungal compounds in winter wheat resistant and susceptible to *Septoria nodorum*" *Ann. Appl. Biol.,* **87:** 67-73

Barria B.N., Copaja S.V., Niemeyer H.M. 1992. Occurrence of DIBOA in wild*Hordeum* species and its relation to aphid resistance. *Phytochemistry,* **31:** 89-91

Baumeler A., Hesse M., Werner C. 2000. Benzoxazinoids-cyclic hydroxamic acids, lactams and their corresponding glucosides in the genus *Aphelandra* (Acanthaceae). *Phytochemistry,* **53:** 213-222

Belz R.G., and Hurle K. 2005. Differential exudation of two benzoxazinoids – one of the determining factors for seedling allelopathy of Triticeae species. *J. Agri. Food Chem.,* **53:** 250–261.

Bravo H.R., Copaja SV. 2002. Contents and morphological distribution of 2,4-dihydroxy-1,4-benzoxazin-3-one and 2-benzoxazolinone in Acanthus mollis in relation to protection from larvae of *Pseudaletia impuncta. Annal App. Bol*., **140:** 129-132

Bravo H. R., Copaja S.V., Lazo W. 1997. Antimicrobial activity of natural 2-benzoxazolinones and related derivatives. *J. Agric. Food Chem*., **45:** 3255-3257

Burgos NR, Talbert RE. 2000. Differential activity of allelochemicals from *Secale cereale* in seedling bioassays. *Weed Sci*., **48:**302-310

Caillaud C.M., Pierre J.S., Chaubet B., Di Pietro J.P. 1995. Analysis of wheat resistance to the cereal aphid Sitobion avenae using electrical penetration graphs and flow charts combined with correspondence analysis. *Ent. Exp. App*., **75:** 9-18

Cambier V., Hance T., de-Hoffmann E. 1999. Non-injured maize contains several 1,4-benzoxazin-3-one related compounds but only as glucoconjugates. *Phytochem. Anal.,* **10**,119-126

Chen S., Petersen B.L., Olsen C.E., Schulz A., Halkier B.A. 2001. Long-Distance Phloem Transport of Glucosinolates in Arabidopsis. *Plant Phys.,* **127:** 194-201

Clay N.K., Adio A.M., Carine Denoux C., Jander G., Ausubel F.M. 2009. Glucosinolate Metabolites Required for an *Arabidopsis* Innate Immune Response. *Science,* **323:** 95 - 101

Corcuera L.J., Woodward M.D., Helgeson J.P., Kelman A., Upper C.D. 1978. 2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one, an inhibitor from *Zea mays*Zea mays with differential activity against soft rotting *Erwinia* Erwinia species. *Plant Physiol*., **61:** 791-795

Drenkard E., Richter B. G., Rozen S., Stutius L. M., Angell N. A., Mindrinos M., Cho R .J., Oefner P. J., Davis R. W., Ausubel F. M. 2000. A simple procedure for the analysis of single nucleotide polymorphisms facilitates map-based cloning in Arabidopsis. *Plant Physiol*., **124:** 1483-92

Dubcovsky J., Luo M.C., Dvora**k J. 1995.** Differentiation between homoeologous chromosomes 1A of wheat and $1A^m$ of *Triticum monococcum* and its recognition by the wheat *Ph1* locus. *Proc Natl Acad Sci USA.,* **92:** 6645-6649.

Echeverría E. 2000. Vesicle-Mediated Solute Transport between the Vacuole and the Plasma Membrane . *Plant Pysiol*., **123:** 1217-1226.

Escobar C.A., Niemeyer H.M. 1993. Potential of hydroxamic acids in breeding for aphid resistance in wheat. Acta Agriculturae Scandinavica section B-soil and plant science, **43:** 163-167.

Escobar C.A., Sicker D., Niemeyer H.M. 1999. Evaluation of Dimboa Analogs as Antifeedants and Antibiotics Towards the Aphid *Sitobion avenae* in Artificial Diets. *J. Chem. Ecol*., **25:** 1543-1554.

Fomsgaard I.S., Mortensen A.G., and Carlsen S.C.K. 2004. Microbial transformation products of benzoxazolinone and benzoxazinone allelochemicals-a review *Chemosphere*., **54:** 1025-1038.

Friebe A., Vilich V., Hennig L., Kluge M., Sicker D.1998. Detoxification of benzoxazolinone allelochemicals from wheat by *Gaeumannomyces graminis* var. *tritici*, *G. graminis* var. *graminis*, *G. graminis* var. *avenae*, and *Fusarium culmorum*. *Appl. Environ. Microb*., **64:** 2386-2391.

Frey M, Chomet P, Glawischnig E, Stettner C, Grun S, Winklmair A, Eisenreich W, Bacher A, Meeley RB, Briggs SP, Simcox K, Gierl A. 1997. Analysis of a chemical plant defense mechanism in grasses. Science , **277:** 696-699

Frey M., Huber K., Park W.J., Sicker D., Lindberg P., Meeley R.B., Simmons C.R., Yalpani N., Gierl A.2003. A 2-oxoglutarate-dependent dioxygenase is integrated in DIMBOA-biosynthesis. *Phytochemistry,* **62:** 371-376.

Gianoli E., Niemeyer H.M.1997. Environmental effects on the accumulation of hydroxamic acids in wheat seedlings: the importance of plant growth rate. *J. Chem. Ecol.,* **23:** 543-551.

Givovich A., Morse S., Cerda H., Niemeyer H.M., Wratten S.D. Edwards P.J. 1993. Hydroxamic acid glucosides in honeydew of aphids feeding on wheat. *J. Chem. Ecol.,* **18:** 841-846.

Givovich A., Sandström J., Niemeyer H.M., Pettersson J. 1994. Presence of a hydroxamic acid glucoside in wheat phloem sap, and its consequences for performance of *Rhopalosiphum padi* (L.) (Homoptera: Aphididae). *J. Cem. Ecol.,* **20:** 1923-1930.

Golovnina K.A., Glushkov S.A., Blinov A.G., Mayorov V.I., Adkison L.R., Goncharov N.P.2007. Molecular phylogeny of the genus *Triticum* L. *Plant Systematics and evolution*, **264**: 195-216.

González L.F., Rojas M.C. 1999. Role of wall peroxidases in oat growth inhibition by DIMBOA. *Phytochemistry*, **50:** 931-937.

Gordon-Weeks R., Smart L., Ahmad S., Elek H., Zhang Y., Martin J., Pickett J. 2010. The regulation of the a natural plant defence pathway in wheat and its role in aphid resistance. *MPMI Proceedings of the 14th International Congress on Molecular Plant-Microbe Interactions*.

Gruhnert C., Biehl B., Selmar D. 1994. Compartmentation of cyanogenic glucosides and their degrading enzymes *Planta*, **195:**36-42.

Gusmayer S., Brunner H., Schneiderpoetsch H.A.W., Rudiger W. 1994. Avenacosidase from oat – purification, sequence analysis and biochemical characterization of a new member of the bga family of beta-glucosidases. *Plant Mol. Biol.,* **26:** 909–921.

Heil M., Ton J. 2008. Long-distance signalling in plant defence. *Trends in Plant Science,* **13:** 264-272.

Hopkins RJ, van Dam NM, van Loon JJA 2008. Role of Glucosinolates in Insect-Plant Relationships and Multitrophic Interactions *Ann. Rev. Ent*., **54:** 57-83.

Jing H-C., Kornyukhin D., Kanyuka K., Orford S., Zlatska A., Mitrofanova O.P., Koebner R., Hammond-Kosack K. 2007. Identification of variation in adaptively important traits and genome-wide analysis of trait–marker associations in *Triticum monococcum. J. Ex. Bot*., **58:** 3749–3764.

Jonczyk R., Schmidt H., Osterrieder A., Fiesselmann A., Schullehner K., Haslbeck M., Sicker D., Hofmann D., Yalpani N., Simmons C., Frey M., Gierl A. 2008. Elucidation of the Final Reactions of DIMBOA-Glucoside Biosynthesis in Maize: Characterization of TaBX6 and TaBX7. *Plant Physiol*., **146:** 1053-1063.

Kluge M., Grambow H.J., Sicker D. 1997. (2R)-2-beta-D-Glucopyranosyloxy-4,7-dimethoxy-2H-1,4-benzoxazin-3(4H)-one from *Triticum aestivum*. *Phytochemistry*, **44:** 639-641.

Koyama T., Yamato M. 1955. Constituents of the root of *Coix lachryma-jobi*. J. *Pharm Soc. Japan*., **75:** 699-701.

Larsen E., Christensen L,P,. 2000. Simple Method for Large Scale Isolation of the Cyclic Arylhydroxamic Acid DIMBOA from Maize (*Zea mays* L*.) J. Agri. Food Chem*., **48:** 2556-2558.

Macías F.A., Marín D., Oliveros-Bastidas A., Castellano D., Simonet A.M., Molinillo J.M.G. 2005. Structure−Activity Relationships (SAR) Studies of Benzoxazinones, Their Degradation Products and Analogues. Phytotoxicity on Standard Target Species (STS). *J. Agri. Food Chem.,* **53:** 554-561

Melanson D., Chilton M-D., Masters-Moore D., Scott Chilton W.1997. A deletion in an indole synthase gene is responsible for the DIMBOA-deficient phenotype of TaBXTaBX maize*. Proc. Natal .Acad. Sci*., **94:** 13345-13350.

Migui S.M., Lamb R.J. 2004. Seedling and adult plant resistance to Sitobion avenae (Hemiptera: Aphididae) in Triticum monococcum (Poaceae), an ancestor of wheat. *Bulletin Ent. Res*., **94:** 35-46.

Miles P.W. 1999. Apid saliva. *Biol. Rev.,* **74:** 41-85.

Moran P.L., Thompson G.A. 2001. Molecular Responses to Aphid Feeding in Arabidopsis in Relation to Plant Defense Pathways. *Plant Physiol*., **125:** 1074-1085.

Morant A.V., Jørgensen K., Jørgensen C., Paquette S.M., Sánchez-Pérez R., Møller B.L., Bak S. 2008. β-Glucosidases as detonators of plant chemical defense. *Phytochemistry,* **69:** 1795-1813.

Nakagawa E., Amano T., Hirai N., Iwamura H. 1995. Non-induced cyclic hydroxamic acids in wheat during juvenile stage of growth. *Phytochemistry*, **38:** 1349-1354.

Nicol D., Copaja S.V.., Wratten S.D., Niemeyer H.M. 1992. A screen of worldwide wheat cultivars for hydroxamic acid levels and aphid antixenosis. *Ann.App.Biol.,* **121:** 11-18.

Niemeyer H.M. 1988. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones) defence chemicals in the Gramineae. *Phytochemistry,* **27:** 3349**-**3358.

Nomura T., Ishihara A., Imaishi H., Endo T.R., Ohkawa H., Iwamura H. 2002. Molecular characterization and chromosomal localization of cytochrome P450 genes involved in the biosynthesis of cyclic hydroxamic acids in hexaploid wheat. *Mol. Genet. Genom.,* **267:** 210-217.

Nomura T., Ishihara A., Imaishi H., Ohkawa H., Endo T.R., Iwamura H. 2003. Rearrangement of the genes for the biosynthesis of benzoxazinones in the evolution of Triticeae species. *Planta,* **217:** 776-782.

Nomura T., Ishihara A., Yanagita R.C., Endo T.R., Iwamura H.2005. Three genomes differentially contribute to the biosynthesis of benzoxazinones in hexaploid wheat. *Proc. Natal. Acad. Sci.,* **45:** 16490-16495.

Nomura T., Nasuda S., Kawaura K., Ogihara Y., Kato N., Sato F., Kojima T., Toyoda A., Iwamura H., Endo T.R. 2008. Structures of the three homoeologous loci of wheat benzoxazinone biosynthetic genes TaTaBX3 and TaTaBX4 and characterization of their promoter sequences. *Theor. Appl. Genet*., **116:** 373–381.

Oba K., Conn E.E., Canut H., Boudet A.M. 1981. Sub-cellular localization of 2-(beta-d-glucosyloxy)-cinnamic acids and the related beta-glucosidase in leaves of *Melilotus alba* Desr. *Plant Physiol.,* **68:** 1359-1363.

Poulton J.E., Li C.P. 1994. Tissue level compartmentation of (R)-amygdalin and amygdalin hydrolase prevents large scale cyanogenesis in undamaged prunus seeds. *Plant Physiol*., **104:** 29–35.

Reberg-Horton S.C., Burton J.D., Danehower D.A., Ma G., Monks D.W., Murphy J.P., Ranells N.N., Wiliamson J.D., Creamer N.G. 2005. "Changes over time in the allelochemical content of ten cultivars of rye (*Secale cereale* L.)" *J. Chem. Ecol*., **31:** 179-193.

Rice C.P., Park Y.B., Adam F., Abdul-Baki A.A., Teasdale J.A.2005. Hydroxamic Acid Content and Toxicity of Rye at Selected Growth Stages. *J. Chem. Ecol*., **31:** 1887-1905.

Schullehner K., Dick R., Vitzthum F., Schwab W., Brandt W., Frey M., Gierl A.2008. Benzoxazinoid biosynthesis in dicot plants. *Phytochemistry,* **69:** 2668-2677.

Sicker D., Frey M., Schulz M., Gierl A. 2000. Role of natural benzoxazinones in the survival strategy of plants. *Int. Rev. Cyt*., **198:** 319-346.

Sue M., Yamazaki K., Yajima S., Nomura T., Matsukawa T., Iwamura H., Miyamoto T. 2006. Molecular and Structural Characterization of Hexameric β-D-Glucosidases in Wheat and Rye. *Plant Physiol*., **141:** 1237-1247.

Thackaray D.J., Wratten S.D., Edwards P.J., Niemeyer H.M.1990. Resistance to the aphids *Sitobion avenae* and *Rhopalosiphum padi* in Gramineae in relation to hydroxamic acid levels. *Ann. Appl. Biol.,* **116:** 573-582.

Villagrasa M., Eljarrat E., Barcelo Villagrassa D.2009. Analysis of benzoxazinone derivatives in plant tissues and their degradation products in agricultural soils. *Trends Anal. Chem.,* **28:** 1103-1114.

Virtanen A., Hietala P.1960. Precursors of benzoxazolinone in rye plants. I. Precursor II, the aglycone*. Acta Chem. Scand,.* **14:** 499-502.

von Rad U., Hüttl R., Lottspeich F., Gierl A., Frey M. 2001. Two glucosyltransferases are involved in detoxification of benzoxazinoids in maize. *Plant J*., **28:** 633-642.

Weibull J., Niemeyer H.M. 1995. Changes in ihydroxymethoxybenzoxazinone glycoside content in wheat plants infected by three plant pathogenic fungi. *Physiol. Mol. Plant Pathol.,* **47:** 201–212.

Wilkes M., Marshall D.R., Copeland L. 1999. Hydroxamic acids in cereal roots inhibit the growth of take-all. *Soil Biol.Biochem*., **31:** 1831-1836.

Will T., Tjallingii F.J., Tho¨ nnessen A., van Bel A.J.E. 2007. Molecular sabotage of plant defense by aphid saliva. *Proc. Nat Acad. Sci*., **104:** 10536-10541.

Woodward M., Corcuera L., Helgeson J. 1978. Decomposition of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one. *Plant Physiol.,* **61:** 796-802.

Wu H., Haig T., Pratley J., Lemerle D. , An M. 2001. Allelochemicals in wheat (Triticum aestivum L.): Production and exudation of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one . *J. Chem. Ecol.* , **27:** 1691-1700.

Wu H., Pratley J., Ma W., Haig T. 2003. Quantitative trait loci and molecular markers associated with wheat allelopathy. *Theor. Appl. Genet* ., **107:** 1477–1481.

Wyatt IJ & White PF 1977. Simple estimation of intrinsic increase rates for aphids and tetranychid mites. *Journal of Applied Ecology*, **14:** 757-766

Zheng Y.Q., Zhao Y., Dong F.S., Yao J.R., Hurle K .2005. relationship of DIMBOA content in wheat seedlings and its resistance to plant pathogens. *Allelopathy Journal,* **15:** 137-144 .