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Structural Damage in the Vascular Tissues of Resistant and Non-Resistant Barley (*Hordeum Vulgare* L.) by Two South African Biotypes of the Russian Wheat Aphid

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Abstract : *The effects of the structural damage caused by the two South African biotypes of the Russian wheat aphid (RWA, Diuraphis noxia Kurdjumov), RWASA1 and RWASA2, through formation and distribution of wound callose in the phloem tissues of non-resistant and resistant barley (Hordeum vulgare L.) cultivars were investigated. Our results revealed that RWASA2 breeds faster than RWASA1 and both cause the deposition of feeding induce wound callose in the phloem tissue within 24h of infestation on both resistant and non-resistant barley lines, but the deposition was higher in the former than the latter. This wound callose deposition became more extensive and pronounced with sustained feeding exposure of 3, 7 and 14 days, yet, it is more extensive in the non-resistant than in the resistant plants. The reduction in the amount of wound callose found in the veins of the resistant plants indicates that the resistance gene in them may have mitigated the effects of feeding by the two RWA biotypes. It is suggested that the resistant lines should be studied further to unravel the mechanism behind the seemingly differences in their responses to infestation by the two biotypes, in the endeavour to develop RWA-resistant barley lines.*

Keywords: Aphid biotypes; aphid feeding damage; barley; resistance; Russian wheat aphid; wound callose quantification.

Introduction

As a result of feeding on the phloem, aphids remove essential food materials and growth substances from their host plants (1; 2; 3). This results in stunted growth and low crop yield (4). The feeding also caused extensive damage to the cells of the host plants resulting in cascades of wound responses among which is the formation and deposition of wound callose (5; 6; 7). Callose is a β-1, 3-glucan, a carbohydrate compound that is reported to be rapidly deposited by plants in sieve pores and plasmodesmata between sieve tube-companion cell complex in response to wounding of their cells (8; 7). This deposition of callose is considered to be a defence response which plants employ to effectively seal sieve plates, pore plasmodesmal areas and sieve area pores against the feeding aphid thereby reducing assimilate loss through them (9).

Recent studies have demonstrated that in response to feeding damage caused by Russian wheat aphids (RWA, *Diuraphis noxia* Kurdjumov), wound callose is deposited along sieve plates and pore-plasmodesmal units in the companion cell-sieve tube complex of susceptible wheat and barley cultivars (5; 6; 10; 7; 11). However, this wound response is reduced in resistant wheat cultivars especially the cultivars carrying *Dn1* gene (6; 10). Engineered wheat cultivars carrying *Dn1* gene are known to act by eliciting antibiosis effect against RWASA1, suppressing its population growth, fecundity and biomass (12; 13; 14; 15). A more virulent second biotype of RWA (RWASA2) reported from South Africa, appears not to be affected by *Dn1* gene in wheat (16).

Qualitative data have been previously presented on damage inflicted by feeding aphids through wound callose deposition in host plants (5; 6; 7). In addition, it was reported (11) that when the two RWA biotypes were allowed to feed for 10 days, there was no reduction in cellular damage as visualised by callose deposition in resistant cultivars in comparison to the non-resistant counterpart. The paper also revealed that there was no detectable difference in the amount of callose distribution between both RWASA1 and RWASA2 biotypes. This informed the current study which is set to further examine the level of structural damage caused by the two RWA biotypes in the vascular tissues of resistant and non-resistant barley hosts, after short- and long-term feeding exposures. The focus is on differences between RWASA1 and RWASA2 feeding effect through examination of the formation, deposition and distribution of wound callose in the phloem of non-resistant and resistant barley cultivars. In the study, morphometric analysis of wound callose deposition was carried out and hereby presented. We used this analysis to quantify the level of damage caused by RWASA1 and RWASA2 feeding. Our approaches were to determine whether there are variations in callose formation, deposition and distribution in resistant and non-resistant barley hosts during short- and long-term aphid infestations.

Materials and Methods

Aphid colonies and maintenance

The two South African biotypes of the Russian wheat aphid (RWASA1 and RWASA2) were obtained from the Agricultural Research Council (ARC), Small Grain Institute, Bethlehem, South Africa. The colonies were maintained on young barley (cv. Clipper) cultivar reported to be susceptible to RWASA1 (2). They were kept in insect cages in separate controlled environment cabinets (Conviron S10H Controlled Environment Ltd., Winnipeg, Manitoba, Canada) maintained at day time conditions of 24°C and 66% relative humidity (RH), night-time conditions of 22°C, 60% RH and a 14-h photoperiod. The light source was a combination of fluorescent tubes (F48T12.CW/VHO 1500, Sylvania, Danvers, MA) and frosted incandescent 60W bulbs (Phillips, Eindhoven, The Netherlands), with a photosynthetic active radiation (PAR) level of 250 $\mu mol^{-2} s^{-1}$ 30cm below the light source. The protocol for the maintenance is as previously published (2; 11).

Barley lines

Four barley lines were used in the study, namely STARS-0502B (PI 47541), STARS-9301B (PI 573080) and STARS-9577B (PI 591617) and PUMA. The STARS lines were developed by the United States Department of Agriculture, Agricultural Research Station (USDA-ARS), Aberdeen/Stillwater, Oklahoma and demonstrated to be resistant to some biotypes of RWA in the USA (17; 18; 19; 20; 21; 22). PUMA, a widely cultivated barley cultivar grown commercially in South Africa, was used as a non-resistant control line. Seeds of the four lines were obtained from the ARC, Bethlehem, South Africa. Seeds were raised and maintained as previously described (23).

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Experimental design and wound callose studies

The four barley lines were tested against the two RWA biotypes using clip cages (7), these clip cages were used to enclose a 5cm-long segment of either the second or the third leaf above the coleoptile of each experimental plant. Control plants were also fitted with the clip cages but were not infested with aphids. Ten replicates of each treatment combination (2 aphid types × 4 plant types × 4 assays) were set up and 10 control (uninfested) plants were included (330 plants in total). Plants were exposed to 24h, 72h (short-term), 7d and 14d (long-term) aphid feeding periods which constituted the assays (treatments) mentioned above.

For the purpose of studying wound callose formation, ten random samples were used. The control in this case was the uninfested samples. The clip cages were carefully removed and the caged region of the leaves were marked with a soft tip marker, whole leaf excised at the base and then transferred immediately to Ca^{2+}-free buffer (10 mM 2- [morpholino] ethanesulfonic acid (MES), 0.5mM $MgCl_2$, 0.5 mM KCl and 125 mM mannitol, adjusted to pH 7.2). The stock solution of aniline blue fluorochrome (4'4-[carbonyl bis (benzene 4, 1- diyl) bis (imino)] bis benzensulphonic acid), Biosupplies Australia Pty Ltd, used was prepared as previously described (7). Procedures for the preparations of samples are as previously described (7). The procedures were repeated twice and sections were examined for callose fluorescence using an Olympus BX61 wide-field fluorescence Digital Imaging Microscope (Olympus, Tokyo, Japan supplied by Wirsam Scientific, Johannesburg, South Africa), fitted with aniline blue specific filter cube (excitation of 425-444nm; emission of 475nm). High-resolution images were saved in a database using the programme analySIS (Soft Imaging System GmbH, Münster, Germany), and imported as bitmaps to CorelDraw version 12 for presentation.

Morphometric analysis of wound callose distribution

Ten high-resolution images of the same magnification were randomly selected as samples for each treatment (24h, 72h, 7d and 14d) as well as the uninfested control. Quantitative morphometric measurement of wound callose was carried out using phase analysis (analySIS program, Soft Imaging System GmbH). The programme automatically measures the area covered by the feeding-related wound callose deposited in sieve tubes of infested plants. Data collected for the area of callose in images selected for each feeding exposure treatment and its control were entered into a spreadsheet and subjected to further statistical analysis using Statistica version 9. Three-way ANOVA was used to examine the differences in the area of wound callose per image which now forms the dependent variable.

Results

Formation of wound callose in leaf tissues

Figure 1(A) shows a segment of leaf from control (uninfested) tissues. As expected, very little callose was found in the control (uninfested) leave tissues of all lines. Development of wound callose due to scraping of the leaves was minimized utilizing Ca^{2+}-free MES buffer at pH 7.2. The callose-associated fluorescence observed were those associated with sieve plates (SP) and lateral sieve area pores in longitudinal and cross (XV) veins.

Formation of wound callose appeared in infested tissues within 24h of feeding by RWASA1 and RWASA2, in both resistant and non-resistant barley lines (Fig. 1B|). Aphid stylets (ST) were seen inserted into the veins and wound callose associated with stylets or stylet tracks was visible. Majority of the wound callose was however associated with the sieve plates and pore-plasmodesmata units in the common walls between the sieve tube members and their associated parenchyma elements.

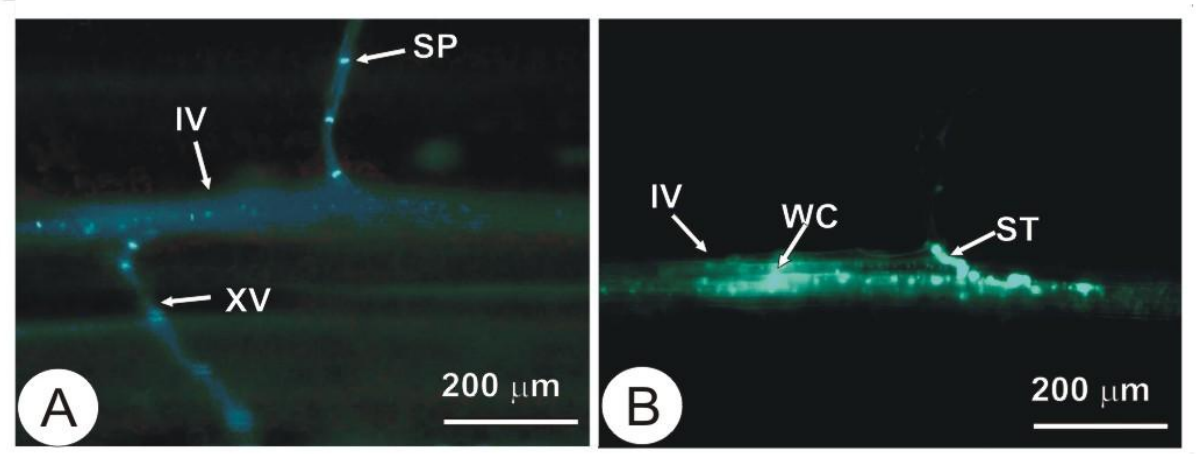

Fig. 1: Callose formation in uninfested (control, A) and infested (B) leaf tissues of barley.
IV = intermediate vein; XV = cross vein; SP = Sieve plate; WC = wound callose.

Distribution of wound callose in leaf tissues

The morphometric analysis of the area of wound callose distribution in control as well as infested leaf tissues are presented as mean values in Figs. 2A and B. Figure 2A illustrate deposition during short-term and Fig. 2B shows the long-term feeding periods. Each aphid infestation caused a progressive increase in the area of wound callose with increasing days of infestation. RWASA2 had higher mean deposition values on each barley line than RWASA1.

The results of the three-way ANOVA at various levels of interactions for the area of callose measured morphometrically in randomly selected images for each feeding treatment is shown in Table 1. It is indicated that individual effects of the two aphid biotypes, the four barley lines and days of feeding exposure are significantly different ($p<0.01$) with respect to area of wound callose measured on images. It also shows that the effect of the interaction between the barley lines and days of infestation is significantly different ($p<0.01$). However, interactions between the aphids and the barley lines, aphids and Day After Infestation as well as the three-way interaction effects of the aphids, barley lines and days of infestation are all not significantly different at $p<0.01$.

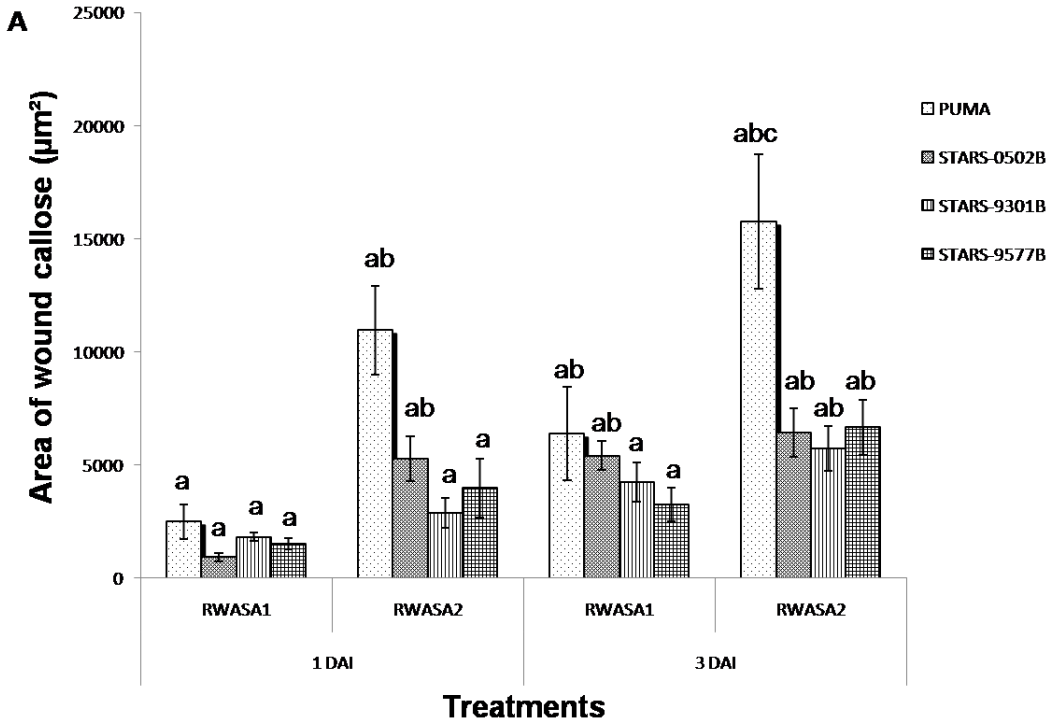

Fig. 2A: Distribution of wound callose in barley leaves during short-term feeding by RWASA1 and RWASA2. Vertical bars are standard error; N=10). Similar letters on tops of corresponding bars indicate statistically non-significant differences at p<0.05 for each barley line.

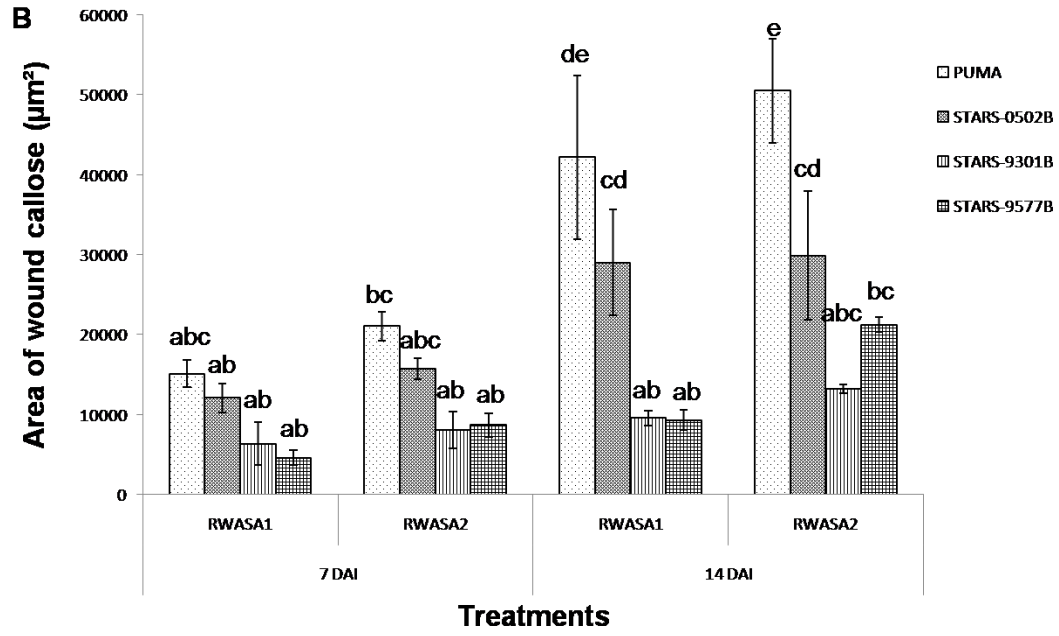

Fig. 2B: Distribution of wound callose in barley leaves during long-term feeding by RWASA1 and RWASA2. Vertical bars are standard error; N=10). Similar letters on tops of corresponding bars indicate statistically non-significant differences at p<0.05 for each barley line.

Table 1: Comparison of levels of interactions on the areas of wound callose recorded at 1, 3, 7 and 14 days of feeding

Interactions	Area of wound callose
Aphids	$F_{1,288}$ = 16.46 *
Barley lines	$F_{3,288}$ = 34.30 *
Days of infestation	$F_{3,288}$ = 77.03*
Aphids X Barley lines	$F_{3,288}$ = 1.63 n.s
Aphids X Days of infestation	$F_{3,288}$ = 0.27 n.s.
Barley lines X Days of infestation	$F_{3,288}$ = 8.61 *
Aphids X Barley lines X Days of infestation	$F_{3,288}$ = 0.35 n.s.

Levels of significance are indicated as: n.s. (not significant) as p> 0.01 and * (significant) at p< 0.01.

Discussion

Observation from this study showed that both aphid biotypes bred faster on the non-resistant PUMA cultivar compared to any of the three resistant barley lines (data not shown). This is in agreement with results of similar studies on aphid population on non-resistant and resistant barley cultivars (24; 25). Populations of aphids feeding on leaves of host plants usually result in structural damage to vascular tissues which are accompanied with a number of responses from infested plants (2; 3; 7). These responses include the formation and deposition of wound induced callose in the veins of the leaves of infested plants (5; 7).

The results of this study show that RWASA1 and RWASA2 feeding caused formation of wound callose upon infestation of resistant and non-resistant barley lines. Though restricted to sieve plates and pore-plasmodesmal units, wound callose is visible within the sieve elements when compared to control aphid-free plants. This is in agreement with earlier studies (6; 10) using the aphid *Sitobion yakini* on non-resistant Betta and resistant Betta-*Dn* wheat cultivars which showed that wound callose was absent or greatly reduced in the resistant wheat cultivars bearing *Dn* resistance genes. Interestingly, this is in stark contrast to the results reported here where wound callose is present in both resistant and non-resistant barley lines. This supports the idea that plant response to feeding is aphid species-specific (26; 7). It is possible that the two aphid biotypes produced signals which elicited varying responses in the barley and wheat hosts.

Morphometric analysis of wound callose distribution in leaf tissues carried out in this study shows variation in the formation of wound callose observed in resistant and non-resistant lines fed upon by either RWASA1 or RWASA2. This is because wound callose deposition, measured on an area basis, increases in each of the four barley lines as days of feeding exposure advances. Irrespective of aphid biotype, area of wound callose is higher in PUMA than in any of the three resistant lines. This supports previous data (10) where it was demonstrated that callose deposition is higher in the non-resistant Tugela than in its near-isogenic resistant Tugela-*Dn* counterpart. However, PUMA fed upon by RWASA2 showed more evidence of callose than those fed upon by RWASA1. From this study, the trend of callose distribution among resistant lines was STARS-0502B > STARS-9577B > STARS-9301B. The relative reduction in the levels of wound callose in the resistant lines recorded in this study when compared to the non-resistant PUMA suggests that the action of resistance may reside in the relative potency of the resistant lines to ensue breakdown of callose as soon as it is formed.

The ANOVA result from this study shows that the individual effects of interaction between the two aphid biotypes, four barley lines and days of aphid feeding behave differently ($p<0.01$) with respect to formation of wound callose. These clearly reinforces earlier position that plants response to aphid infestation is species specific (26; 7) and even between biotypes (10). This data equally suggests that the duration of infestation is critical in determining the level of response of plants to infestation by aphids through wound callose formation. However, the synergistic effects of the interactions between the aphids and the barley lines on one hand, the aphids and DAI on another as well as the three-way interaction which are not significantly different ($p<0.01$) may have affected callose formation. The reason for this may reside in the complexities behind the mechanism of interaction between the aphid and the host plant which are yet to be completely unravelled. Our view here has been previously canvassed by a number of authors studying the interactions between aphid pests and their host plants (27; 28).

In conclusion, our results showed that wound callose formation occurred in both resistant and non-resistant barley lines, though with a higher intensity in non-resistant PUMA and also that this intensity is increased with infestation time. The study further showed that RWASA2 feeding induced greater wound callose than RWASA1 in both resistant and non-resistant barley lines. This might be due to variations in the salivary components of each of the two biotypes. This should be studied further at molecular and biochemical levels in order to understand the seemingly differences in the feeding mechanism and behaviour of the two biotypes.

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