EFFECTS OF SUSTAINED RUSSIAN WHEAT APHID (*Diuraphis noxia* Mordvilko) FEEDING ON LEAF BLADES OF WHEAT (*Triticum aestivum* L. cv Adamtas)

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ABSTRACT

Penetration of sink as well as source leaves of wheat plants by the Russian wheat aphid, *Diuraphis noxia* (Mordvilko) was investigated using light, fluorescence and transmission electron techniques, to determine the feeding strategies adopted by the aphid in penetrating and successfully feeding from the phloem, and to assess the structural effects of the probing and feeding behaviour of *D. noxia* on the feeding sites.

Examination of aphid-infested sink, as well as source leaf tissue, showed that *D. noxia* probed in cells of the vascular bundle more frequently than mesophyll cells. Within the vascular bundle, thin-walled sieve tubes were visited (probed) more than the other cells. In sink leaf material, 68 of 82 (83%) stylets and stylet tracks encountered during the examination of 1000 serial sections (from 5 different plants) terminated in thin-walled sieve tubes and only 14 (17%) in thick-walled sieve tubes. Thin-walled sieve tubes were visited more significantly than thick-walled sieve tubes.

However, examination of the aphid-infested sink leaf on a per centimetre basis, from the tip of the leaf, revealed that thick-walled sieve tubes in the area closest to the tip (0-2cm from the tip) were as attractive to the aphid as were thin-walled sieve tubes, with no significant difference in the number of times thick- and thin-walled sieve tubes were probed in this area. Some 2-4cm from the tip however, thin-walled sieve tubes were significantly more probed and therefore more attractive than thick-walled sieve tubes.

Examination of 2000 serial sections using aphid-infested source leaf tissue, showed that the thin-walled sieve tubes were significantly more probed than thick-walled sieve tubes, along the whole leaf, expressed as a total of all leaves, as well as on a per centimetre basis along the length of the leaf, with 212 (95%) of 222 terminations within the thin-walled sieve tubes and only 10 (5%) in thick-walled sieve tubes.

The aphid probed the small vascular bundles (loading bundles) many more times than intermediate or large transport vascular bundles, in sink as well as source leaf. Of a total of 82 stylets and stylet tracks encountered in sink leaf tissue, 31 terminated in small vascular bundles and the remaining 28 and 16 were located within large and intermediate vascular bundles respectively. In source leaf tissue 121 of 222 stylets and stylet tracks encountered were associated with small vascular bundles and only 58 tracks and 43 tracks with intermediate and large vascular bundles, respectively.

The effect of sustained RWA feeding on the transport capacity was examined after the application of 5,6 carboxyfluoresceine diacetate (5,6-CFDA) in control (sink and source leaf tissue) and aphid-infested (source) wheat leaves, using fluorescence microscopy. After 3h acropetal longitudinal transport of 5,6-CF had occurred in sink leaves in longitudinal veins, as well as a lateral transfer via cross veins and subsequent unloading into mesophyll cells close to the tip of the leaf was observed.

In control leaf tissue, the fluorescence front was detected up to about 5cm from the point of application and was only associated with the phloem and not unloaded. In contrast, aphid-infested leaf tissue showed very little 5,6-CF transport, being limited to 2cm or less from the point of application.

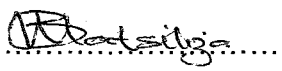
Structural damage to the phloem in general and to the sieve tubes in particular within of control and infested wheat leaves was investigated using transmission electron microscopy (TEM). In addition, leaf strips were mounted in aniline blue to visualise callose deposition using the fluorescence microscopy. At the TEM level. infested leaf tissue showed various abnormalities, which included destruction of cell contents, membrane damage and subsequent loss of cell contents. TEM studies suggest severe osmotic shock resulted from the aphid's probing. Examination of leaf tissue using fluorescence microscopy showed that there was very little characteristic aniline blue-stained callose visible in control leaf tissue, other than the thin diffuse patches along the sieve plates and punctate spots associated with pore plasmodesmatal areas and plasmodesmatal aggregates. In contrast, the aphid-infested leaf tissue was heavily callosed, with callose deposited not only within the phloem tissue but also in neighbouring vascular parenchyma cells as well.

The data collectively suggest that *D. noxia* feeds preferentially within thin-walled sieve tubes, within the small longitudinal vascular bundles in sink , as well source leaf tissue. Based upon the data presented here the thin-walled sieve tubes in the wheat leaf appear to be more attractive to the aphid and that they are probably more functional in terms of transport system and unloading in sink leaves. Aniline

blue stained leaf material that had previously hosted large aphid colonies showed evidence of extensive callose deposits 24 to 36h after the aphids were removed, suggesting that the aphids caused severe mechanical damage to the vascular tissue and mesohyll cells as well. Damage (transient or more permanent) and the subsequent deposition of wound callose, disrupted phloem transport and hence the export of photoassimilate from the leaves.

DECLARATION OF ORIGINALITY

I, Babalwa Matsiliza, hereby declare that this thesis represents my own original work, except where the technical assistance was offered or asked, or publications of others have been duly acknowledged.



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CHAPTER 1: GENERAL INTRODUCTION

1.1 Life history and distribution of the Russian Wheat aphid (*D. NOXIA*)

The Russian wheat aphid, *Diuraphis noxia* (Mordwilko) is a small aphid of about 1.4-2.3mm in length with a spindle shaped, lime green to grey body. It can be easily distinguished from other small grain aphids (eg. Bird-cherry oat aphid, *Rhopalosiphum padi* (L) and the greenbug, *Schizaphis graminum* (Rondani) by its extremely short antennae, greatly reduced cornicles ("tailpipes") and a projection above the last abdominal segment which gives *D. noxia* a forked tail or double tail appearance when viewed from the side.

As with other aphids which attack small grains in South Africa, two forms of the adult *D. noxia* occur, namely winged (alate) and wingless (apterous) females occur. The wingless females spend most of their time on the plant, feeding and reproducing. Adult females give birth to instars which mature and start to reproduce within two weeks under favourable conditions (Walters et al 1980). Individuals live for 60 to 80 days and produce about 80 offspring (Peairs (1998). The winged females develop under specific conditions for example, when the host plants are under stress, or when the growth stage of the plant is such that it no longer provides a favourable habitat for the pest (Walters et al 1980, Kriel et al 1984). They then spend less time on the plant and are responsible for both local and long distance distribution to areas where host plants are in a more favourable growth stage. They therefore have less reproductive and damage potential under these

conditions.

D. noxia is indigenous to Russia, hence the name Russian wheat aphid. It was already regarded as pest of wheat and barley in Russia from as early as 1912 and has since been recorded as a minor pest of small grains in various countries of the Middle East and the Mediterranean littoral of North Africa and Europe (Walters *et al.* 1980). It was first recorded as a pest in South Africa during 1978 where it was confined to the eastern Orange Free State (Walters *et al.* 1980). It has remained a persistently serious pest and has since spread to other countries in Asia, the rest of Africa and recently north and south America (Walters 1982, Stoetzel 1987). Although widely distributed, *D. noxia* aphid is a major pest of small grains primarily in South Africa, the United States and Canada (Walters 1982, Hein et al 1990).

D. noxia has been found to be more cold tolerant than greenbug, which helps explain its successful survival, particularly in the northern states (Peairs 1998, CAPS Program 1994). Individual aphids can survive temperatures as low as -25^0C, although temperatures below -10^0C reduces reproduction and life span. Also critical to its survival is ability to survive the oversummering period between small grain harvest and fall planting. Even where the aphid is not able to oversummer or overwinter, it may still be a major pest. Its ability for rapid dispersal has been seen in its spread throughout the western United States states (Peairs 1998). This ability to disperse rapidly and high reproduction make possible the re-infestation of any areas where it has been eliminated by a severe winter, lack of oversummering hosts or any other adverse environmental factors.

1.2 *D. noxia* feeding damage

1.2.1 Host plants

As mentioned, *D. noxia* has been a pest of small grain in areas of Russia since 1912. Wheat, barley and *Triticale* are the preferred hosts of *D. noxia* and it can breed rapidly on these crops. It also infests a number of cool-season grasses, particularly wheat grasses. These grasses serve as alternate hosts for *D. noxia* during the period between grain harvest and the appearance of new wheat. The availability of host plants plays a decisive role in the seasonal history of *D. noxia*. A limited plant range means that large numbers can only build up during periods of the year when wheat and barley are in the fields.

1.2.2 Symptoms of D. NOXIA infestation

The *D. noxia* feeds on younger leaves of wheat and barley and as result prevents normal unrolling of leaves. Damaged leaves remain tightly rolled (Hewitt et al 1984) and *D. noxia* colonies are found within the tubes formed by these tightly curled leaves. This not only makes it difficult to achieve good insecticide coverage but also interferes with the ability of predaceous and parasitic insects to reach and attack the aphids.

Damage is the greatest when crops start to ripen concurrently with peak aphid numbers. At this stage, the colonies are found on the green parts of the plant, the plant inflorescence, and even as low as the first node of the stem. The aphid not only withdraws plant sap from the phloem but is also known to inject a toxin (Hewitt et al 1984, Fouche' 1983, Fouche' *et al.* 1984) which leads to several characteristic symptoms. Such symptoms include longitudinal white or yellowish streaks and less

frequently areas of purplish discolouration. In the later stages the aphid often infests the flag leaf. After flowering, some heads are twisted or distorted and have a bleached appearance and may contain poorly formed or blank heads. Heads often have a "fish hook" shape caused by awns trapped in the tightly curled flag leaf an indication that the yield will be poor.

Yield loses are variable. When the pest outbreaks occur, yield of winter wheat can be reduced by as much as 60% (Du Toit and Watlters 1984, Archer and Bynum 1992). The cumulative economic loss attributed to *D. noxia* in the western United States since its introduction in 1986 exceeds USD 320 million dollars, with more than 70 million spent on control and 250 million in lost production (Hein *et al.* 1990, Peairs 1998).

1.2.3 Ultrustructural damage

Limited research has been done to investigate the cellular destruction to wheat leaves resulting from *D. noxia* infestation. Laboratory experiments conducted by Fouche' (1983) and Fouche' *et al.* (1984) showed that changes occurred in the cell organisation of wheat tissue treated with aphid extracts. After 72h the plasmalemma was situated further from the cell wall than usual and had a convoluted appearance. Membranes of some chloroplasts also showed these changes. Initially the orderly peripheral arrangement of the chloroplasts was disrupted, subsequently, the chloroplast membranes disintegrated such that no chloroplast was visible after a period of 240h.

1.2.4 Physiological damage

Kruger and Hewitt (1984) investigated the effects of *D. noxia* extracts (macerated aphids in NaCl) on the primary processes of photosynthesis of isolated chloroplasts in order to explain *D. noxia* feeding damage on a physiological basis. Their results showed a pronounced reduction of chlorophyll content (up to 80%) which they suggested was due to the disruption of the thyllakoid membranes (as observed in cytological studies by Fouche' (1983) and Fouche' *et al.* (1984) setting free membrane-bound chlorophyll molecules. The authors also reported an increase of up to 50% of the rate of oxygen evolution which they suggested was due to uncoupling of photophosphorylation by the aphid extract which stimulates uncontrolled electron flow (Avron and Neuman, 1968) as they partially destroy the membrane structure (Jagendorf 1967).

D. noxia has been shown to induce a stress condition for both resistant and susceptible wheat. Metabolically, resistant plants have a better ability to survive the stress condition imposed by sustained infestation. Studies conducted by Van der Westhuizen and Pretorius (1995) showed a reduction of total soluble protein content of wheat leaves caused by *D. noxia* feeding in both susceptible and resistant wheat, but demonstrated that the resistant wheat maintained a higher protein content throughout the infestation. Greenhouse experiments conducted by Girma, Wilde and Harvey (1993) showed a significant reduction of weight and number of spikes as a result of *D. noxia* feeding. In addition, yield per plant was significantly reduced throughout the heading stage. Other studies have shown that *D. noxia* feeding damage can be expressed as interference with cold hardening and predeposition to winterkill (Thomas and Butts 1990) and disruption of

osmoregulatory process and the occurrence of drought stress symptoms in leaves of infested plants (Riedell 1989).

Clearly *D. noxia* poses a serious threat to the wheat industry. Its successful control depends on a sound background knowledge of the aphid's ecology and biology, which include *D. noxia* feeding & damage on the phloem, which is the main feeding site of this pest.

1.3 The phloem in wheat: Source of nutrition for the aphid

1.3.1 Cellular composition of wheat leaf blade vascular bundles

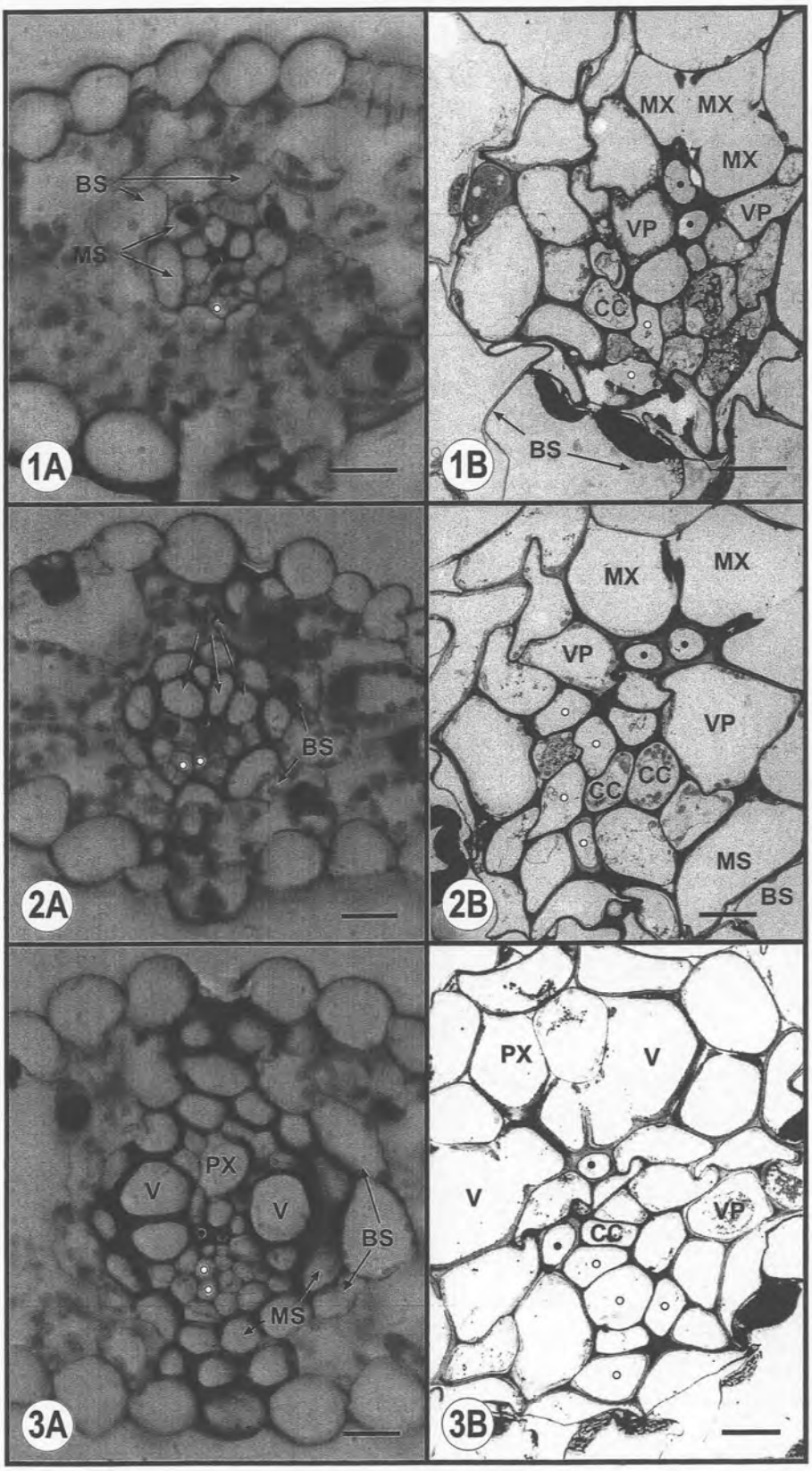
The leaf blade of wheat has typical Pooid grass anatomy, with a system of longitudinal vascular strands interconnected by numerous transverse veins (Blackman 1971) and vascular bundles widely separated by a loosely-arranged mesophyll. Like most other grasses, three orders of longitudinal vascular bundles are present namely, large (first order, Fig.1.3a & b), intermediate (second order, Fig. 1.2 a & b) and small (third order, Fig. 1.1 a & b). As in other grasses, large and intermediate bundles are associated with girders or strands of hypodermal sclerenchyma (Botha *et al.* 1982b, Botha and Cross 1997, Evert, Russin and Botha 1996, Dannenhoffer, Ebert and Evert 1990). The vascular bundles are surrounded by two sheaths, an inner mestome sheath, and an outer parenchymatous bundle sheath.

When viewed in transection, mature large bundles are characterised by the presence of a protoxylem lacuna (PX) and two large metaxylem vessels (V), one on either side of the lacuna. The intermediate bundles are associated with girders or

strands of hypodermal sclerenchyma which are often reduced and the strands are not always in contact with the vascular bundles. They lack a protoxylem lacuna as well as the large metaxylem vessels which are characteristic of the large bundles only. Small bundles are generally smaller than the intermediate bundles. They are similar in structure to the intermediate bundles and have often been classified as such merely by the absence of spatial association with hypodermal sclerenchyma (Botha *et al.* 1982b, Dannenhoffer, Ebert and Evert 1990).

The protophloem sieve tubes in large bundles border the mestome sheath abaxially and are usually collapsed or obliterated in such bundles, leaving only functional metaphloem. In addition, the large bundles contain vascular parenchyma cells, some of which occur on the xylem side of the bundles, spatially associated with the vessels. On the phloem side of the bundle, some parenchyma cells abut both vessels and sieve tubes, whilst others abut only sieve tubes and their associated companion cells. In intermediate bundles the protophloem may be present, but in mature bundles these are mostly collapsed or obliterated. Small bundles contain vascular tissue composed of metaxylem and metaphloem.

Like most other members of the Poaceae (e.g. barley, sugarcane, maize and southern African grasses such as *Themeda triandra),* wheat (Fig. 1A-3B) contains both thin- (open circles) and thick-walled sieve tubes (solid dots), at least in the intermediate and small leaf-blade bundles (see Dannenhoffer, Ebert and Evert 1990; Kuo and O'Brien 1974; Colbert and Evert 1982; Walsh 1974; Botha *et al.* 1982b and references cited therein). Within monocots studied to date, these are classified as first-formed and late-formed metaphloem.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Figs. 1.1A&B – 1.3A&B show light (left) and transmission electron micrographs (left) of transverse sections of a small, intermediate and large longitudinal vascular bundles of wheat.

Fig. 1.1 A light, Fig. 1.1B transmission electron micrograph (TEM) showing a small vascular bundle in transection. Two thick-walled sieve tube (solid dot) occuring adjacent to the metaxylem (MX) and two thin-walled sieve tubes (open circles) are present. A parenchymatous bundle sheath (BS) surrounds a mestome sheath (MS).

Fig. 1.2A light, Fig. 1.2B transmission electron micrograph showing an intermediate vascular bundle in transection. Note that these bundles lack large metaxylem vessels (MX) and protoxylem. Both thick- (solid dot) and thin-walled sieve tubes (open circles) are present.

Fig. 1.3A light, Fig. 1.3B transmission electron micrograph showing a large vascular bundle in transection. Such bundles are characterised by the presence of large metaxylem vessels (V), one on either side of the protoxylem (PX) (Fig. 1.3A). Thick-walled sieve tubes (solid dot) occur next to the xylem and thin-walled sieve tubes (open circles) below.

Bar=10μm (A), 2μm (B)

The first-formed metaphloem consists of sieve tubes and associated companion cells. These have cellulosic walls and are called thin-walled sieve tubes, because their walls are thin compared to the walls of the last late-formed sieve tubes which form towards the end of the differentiation cycle. These last-formed metaphloem elements differentiate next to the metaxylem vessels. According to available literature, these late-formed sieve tubes lack companion cells (see Evert, Eschrich and Heyser 1978, Evert, Russin and Botha 1996, Botha and Cross 1997 and literature cited). The thick-walled sieve tubes in the wheat leaf were said to be lignified (Kuo and O'Brien 1974) but those in the leaves of sugarcane, maize and *Themeda triandra* apparently are not.

The thick walled sieve tubes are much fewer in number compared to the thin-walled sieve tubes, depending on the size of the vascular bundles. Based upon micrographs shown here, as well as on other data, the intermediate and small vascular bundles all contained at least one, but not more than two thick-walled sieve tubes, and between 3 and 5 thin-walled sieve tubes (also see Kuo and O'Brien 1974). On average the ratio of thick to thin-walled sieve tubes is 1:2. The vascular parenchyma cells occupy most of the interface between the sieve tube-companion cell complexes and the mestome sheath and commonly separates the sieve tube-companion cell complexes from thick-walled sieve tubes.

Studies undertaken of the small and intermediate bundles of the barley source leaf by Evert, Russin and Botha (1996) and Botha and Cross (1997) showed that the thick-walled sieve tubes have very few plasmodesmata in their common walls with other cell types. These authors found that plasmodesmatal connections between

sieve tube-companion cell complexes and other cell types of the leaf, including vascular parenchyma cells and thick-walled sieve tubes, were also rare. The authors therefore concluded that both the sieve tube-companion cell complexes and the thick-walled sieve tubes may based on plasmodesmatal frequencies be virtually symplastically isolated from the rest of the leaf. This contrasts sharply with that in maize and sugarcane bundles, where only the sieve tube-companion cell complexes are symplastically isolated (Evert, Eschrich and Heyser 1978, Robinson-Beers & Evert, 1991).

Haupt *et al.* (2001) reported that intermediate and large bundles of barley sink leaves contained relatively high frequencies of plasmodesmata between cells in the vascular bundles. These authors further reported that unlike source leaves, connections between thick-walled sieve tubes and adjoining cells were abundant in sink leaves. They also stated that thick-walled sieve tubes were connected to the companion cells by pore plasmodesmata and these companion cells were connected to vascular parenchyma by conspicuous fields containing simple plasmodesmata. In addition, they also stated that thin-walled sieve tubes were also connected to companion cells by pore plasmodesmatal units and showed numerous plasmodesmatal connections with surrounding cells.

Several authors have suggested that the reliance upon plasmodesmatal frequency alone as a determinant of cell–to-cell transport is difficult to support (see Botha and van Bel 1992, Evert, Russin and Botha 1996 and Botha and Cross 1997) and should be treated with caution when interpreting results. Botha and van Bel (1992) argued that plasmodesmatal frequency data merely gave an indication of the

maximum potential pathway of symplastic transport and does not take the transport capacity of the plasmodesmata into account. The use of fluorescent dyes such as lucifer yellow and 5,6-carboxyfluorescein diacetate (5,6-CFDA) which are able to move symplasmically, has provided more information on symplastic continuity. Where plasmodesmatal frequency studies are coupled with microinjection and microiontophoretic studies, they provide more meaningful information about cell-to-cell transport in the phloem.

Lucifer yellow (LYCH) was used by Farrar *et al.* (1992) to demonstrate symplastic continuity in mature leaves of barley. These investigators concluded that LYCH injected into parenchymatous bundle sheath and mestome sheath cells, was transferred symplastically to the sieve tubes of intermediate bundles. In a subsequent study, Botha and Cross (1997) specifically focused on plasmodesmatal frequency in relation to short distance transport and phloem loading in barley. Plasmodesmatal frequency data, together with electrophysiological and intercellular microiontophoretic injection of LYCH suggested a two-domain phloem loading strategy in barley, confirming the near or possibly complete isolation of the sieve tube-companion cell complex from the vascular parenchyma. In addition, plasmodesmatal frequency studies together with carboxyfluorescein (CF) transport studies in sink leaves of barley suggested a symplastic sieve element unloading pathway involving thick- and thin-walled sieve tubes.

1.3.2 Functionality of the thin- and thick-walled sieve tubes

The presence of two types of sieve tubes in grass leaves has resulted in speculation about their possible role in phloem loading and transport. Kuo and

O'Brien (1974) first suggested that the thick-walled sieve tubes in *Triticum aestivum* (wheat) may be specialised for either long distance transport, or serve as temporary storage reservoirs for sugar in excess of what can be transported by the thin-walled types of sieve tubes. However, results from a subsequent microautoradiographic study of the transport of ^{14}C-photosynthates transport do not support a functional role for the thick-walled sieve tubes, which in this species is involved neither in storage nor directly in the transport of photosynthates (Cartwright, Lush and Canny. 1977).

Evert, Eschrich and Heyser (1978) suggested that the thick-walled sieve tubes in *Zea mays* may play a role in the retrieval of solutes entering the leaf apoplast from the transpiration stream. This suggestion was based on the observed close spatial association between the thick-walled sieve tubes and the metaxylem vessels, as well as on their possession of plasmalemma tubules, which apparently greatly increase the apoplast-symplast interface (Evert *et al.* 1977). Microautoradiographic studies of phloem loading and transport in the leaf of maize (Fritz, Evert and Heyser 1983) showed that the thin-walled sieve tubes accumulated sucrose and photosynthate from the apoplast, without the companion cells serving as an intermediary stage in this process. In addition, this study showed that the thin-walled sieve tubes are primarily involved with the uptake of photosynthate. From this study, it is apparent that the thick-walled sieve tubes received photosynthate and that it was transferred to the thick-walled sieve tubes after accumulation by contiguous vascular parenchyma cells. The authors also suggested that the thick-walled sieve tubes could accumulate assimilates through retrieval from the xylem, into which solutes could have leaked from the phloem free space. The

microautoradiographic study by Fritz, Evert and Heyser (1983) further suggested assimilates could be transferred from the thick-walled sieve tubes to the thin-walled sieve tubes and were thus unlikely to be involved in long distance transport. However, a cautionary note may need to be sounded here as the experiments were conducted on detached leaves. Later, Evert and Mierzwa (1989) suggested that in barley, the presence of a pectin-rich wall layer permeated with microvilli-like evaginations of the plasmalemma could greatly facilitate the movement and uptake of water and solutes across the cell wall-plasmalemma interface.

Free-space marker studies on the leaves of *Themeda triandra, Zea mays, Saccharum officinarum* and *Bromus uniloides* (Botha *et al.* 1982a, Evert, Botha and Mierzwa 1985, Botha and Evert 1986) showed the accumulation of Prussian blue crystals in the cell walls of both thin- and thick-walled sieve tubes, demonstrating the potential ease with which water (implied from crystal deposition) moves from the lumina of the vessels to the walls in both thin- and thick-walled sieve tubes and their associated apoplasts. The uptake of Prussian blue suggests that the cell walls of the thick- and the thin-walled sieve tubes are freely permeable to water and solute, and thus do not or should not impede solute transfer across the cell wall – interface up to the plasmamembrane.

Even though there is evidence that both thin- and thick-walled sieve tubes are capable of assimilate uptake and transfer (Fritz, Evert and Heyser 1983) and again that the cell walls of thin- and thick-walled sieve tubes are apparently freely permeable to solutes (Botha and Evert 1986 and literature cited), convincing evidence for a long-term function for the thick-walled sieve tubes in source leaves

of grasses is still lacking. Clearly, alternative methods need to be explored to determine the role of the thick-walled sieve tubes in grasses such as wheat.

1.4 Rationale of using *Triticum aestivum* L. cv Adamtas and *Diuraphis noxia*

Wheat has been ranked as the most important crop in the world (Rogers and Dahlman (1993), highlighting its high economic significance. Since the discovery and subsequent recognition of the Russian wheat aphid as the most destructive pest of wheat, the two species have attracted the attention of a number of researchers. However research on *D. noxia* remains focused on developing efficient methods to control this pest. In South Africa, control is achieved by spraying expensive mixtures of systemic contact insecticides, supplemented by the eradication of volunteer wheat which serves as a host between seasons (Du Toit 1989). The availability of resistant cultivars offers a positive alternative to the application of expensive insecticides. Fast development of new resistant cultivars has therefore become a priority. Whilst all these methods have proved to be more or less successful, one cannot exclude the possibility that new *D. noxia* biotypes may evolve with the ability to overcome existing resistance in wheat. Plant breeders therefore require more effective and less time-consuming selection procedures.

In searching for more resistant wheat varieties, a more profound knowledge of the phloem, the main feeding site of this pest is essential as little is known of the long-term effects of aphid feeding on the phloem.

Previous studies on phloem transport in grasses conducted by Matsiliza and Botha

(2002) showed that the aphid *Sitobion yakini* fed preferentially in the thin-walled sieve tubes in barley. Whilst a great deal of information was obtained from this study, the aphid species used was not a known pest of barley. In order to confirm results obtained from the previous study it was decided that a follow up study should be undertaken, using the Russian wheat aphid whose preferred host plant is wheat.

1.5 Research Objectives

Previous studies of *D. noxia* have shown it to be a sheath feeder (Miles and Taylor, 1994). The aphid frequently probes in the phloem tissue (Girma, Wilde and Reese 1992). Beyond this, there is very little information available on feeding strategies adopted by *D. noxia* in feeding from the phloem. Issues such as the preferred feeding site and effects of *D. noxia* feeding on the phloem are still not clearly understood. Whilst the study by Matsiliza and Botha (2002) showed preference for thin-walled sieve tubes over thick-walled sieve tubes in barley leaves, there is still no clear understanding of the role of the thick-walled sieve tubes. These authors assumed that the thin-walled sieve tubes function as the principal conduit for the long-distance transport of photoassimilates from sites of synthesis (source) to sites of storage or use (sink). Clearly feeding from the functional phloem by the aphid must have an effect on the capacity to transport. In addition, long-term feeding could have deleterious effects on the functional transport capacity of the sieve tubes.

It is clear that a detailed study of the effects of long-term feeding are essential, if we

are to understand the effect the aphid has on the functional state of the phloem. An examination of damage effected by the aphid is thus essential. Several varieties of wheat have been bred to be aphid-resistant. The Western Cape cultivar Adamtas was chosen for a baseline study of the long-term effects of phloem-feeding by the aphid, *Diuraphis noxia,* as this variety is known to be highly susceptible to aphid feeding. Future studies could then focus on the less susceptible varieties such as Tugela DN.

The objectives of this study therefore were:

1. To obtain detailed information on the selectivity of feeding patterns of the Russian wheat aphid in source and sink leaves of wheat, in order to determine if a preferential feeding pattern existed. This information would also be useful in determining the significance of the thin- and thick-walled sieve tubes.
2. To examine the effects of sustained *D. noxia* feeding on the transport capacity in maturing wheat leaves using fluorescence microscope techniques.
3. To examine the extent of damage caused by sustained *D. noxia* feeding on functional phloem tissue, using fluorescence microscopy and electron microscope techniques.

1.6 Hypotheses

The hypotheses upon which this research is based are:

1. The thin-walled sieve tubes were the principal solute transport conduit in

source and sink leaves.

2. Transport phloem (thin-walled sieve tubes) will, after sustained feeding show evidence of damage, and non-functional phloem will not show signs of damage.

CHAPTER 2: MATERIALS AND METHODS

2.1 Aphid colony maintenance and infestation

The aphid colony used in the experiments was donated by the Agricultural Research Council (ARC, Bethlehem, South Africa). The colony was maintained on young wheat plants and kept in insect cages in the greenhouse. Greenhouse settings were 25^{0}C, 14h sunlight/day, with high relative humidity. Wheat plants were infested with *D. noxia* at the 2-leaf stage. Each plant was infested with 10-12 adult aphids per plant.

2.2 Plant host material

Wheat seeds (*Triticum aestivum* L. *cv.* Adamtas) were pre-germinated in petri dishes and thereafter sown in potting soil in plastic pots (diameter ≒ 17cm). Plants were watered on alternate days with full strength Long-Ashton nutrient solution (Hewitt 1966). When the plants were two weeks old, colonies of the aphid, *Diuraphis noxia* (Mordvilko) were transferred to them and the aphid-containing plants were kept in insect cages either under controlled conditions (Conviron (S10H), Controlled Environments Limited, Winnipeg, Manitoba, Canada) at 28.8^{0}C and RH 50 or in the greenhouse (between 20 & 30^{0}C). Aphid colonies were transferred to new young wheat plants every two weeks to ensure succulent hosts. Plants with suitably-established aphid colonies were selected for further study.

2.3 Light microscopy

Aphids were killed *in situ* by rapidly exposing them to an atmosphere of 100% acrolein vapour (Merk Chemicals, Germany). Leaf segments containing attached aphids selected from both source and sink leaves (5-7cm in length), were fixed in FAA for 24 hours. The leaf segments were then gently cut into smaller, more manageable pieces and dehydrated through an alcohol and tertiary butyl alcohol series. The material was then infiltrated with a number of changes of paraplast wax over three days, in an embedding oven at 60^0C. Blocks were mounted and trimmed and serial transverse sections were cut at 15μm using a Minot rotary microtome (Leitz Wetzlar, Germany). Sections were stained in Safranin and Fast green. The stained sections were mounted on slides using Canada Balsam and dried in an oven at 37^0C for three weeks. Serial sections were carefully examined for evidence of aphid feeding, by careful examination for stylet tracks or sheaths which are easily seen after staining the sections. Data was collected concerning point of origin (ad or abaxial leaf surface), successful or unsuccessful probes, probes of thin- or thick-walled sieve tubes, successful evidence for feeding in thin- or thick-walled sieve tubes.

2.4 Electron microscopy

2.4.1 Transmission electron microscopy (TEM)

Some leaf blade segments of uninfested plants (control) and those with dead attached aphids were carefully cut into smaller pieces and fixed in 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer for 24 hours. The segments were

washed in two 30 min. changes of 0.1M cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 90 min. After post-fixation, pieces were rinsed in cold cacodylate buffer and dehydrated in a cold, graded ethanol series, followed by two 30 min. changes of propylene oxide. Embedment was in a Araldite Taab 812 resin mixture. Monitor sections of the leaf were cut with glass knives and mounted in water, on microslides and dried down at 60°C. Sections were stained in toluidine blue for 1min., rinsed in distilled water, and then mounted under coverslips. Ultrathin sections (silver to gold) were cut using a diamond knife (Drukker, Netherlands) and were collected on 300 mesh copper grids (SPI suppliers, Philadelphia, USA). They were then stained in uranyl acetate and lead citrate. Sections were viewed using a JEOL JEM 1210 transmission electron microscope (JEOL, Tokyo, Japan).

2.4.2 Scanning electron microscopy

Leaf blade segments of to which aphids were still attached were carefully cut into smaller pieces and fixed in 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer for 24 hours. The segments were washed in two 30 min. changes of 0.1M cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 90 min. After post-fixation, pieces were rinsed in cold cacodylate buffer and dehydrated in a cold, graded ethanol series (30%, 50%, 70%, 80%, 90% & 2x100%). The final ethanol was replaced with 75:25 ETOH: amyl acetate, and allowed to infiltrate for 20 minutes. This was repeated with 50:50 and 25:75 ethanol : amyl acetate and 100% amyl acetate. The samples were placed in CPD baskets under 100% amyl acetate in a CPD boat and critical point dried in a Polaron E3000-049 Critical Point Drier (Polaron Equipment, Inc., Hatfield, Pa., USA). The samples

were then attached to brass stubs with graphite tape (SPI Suppliers, Structure Probe, Inc., West Chester, USA) and gold coated in an E5100 SEM Coating Unit (BioRad, Polaron Division: Polaron Equipment, Inc., Hatfield, Pa., USA). The sections were examined in a JEOL JSM 840 Scanning Electron Microscope (JEOL, Tokyo. Japan) and areas of interest were photographed on Agfapan ISO 100/21 film (Agfa-Gevart AG, Germany).

2.5 Fluorescence microscopy

2.5.1 Aniline blue treatment

Leaf strips (approximately 3cm long) of both infested and control wheat were cut out from whole leaves. The abaxial leaf surface was gently scraped in places, under MES (morpholinoethanesulfonic acid, pH 7.2) with a sharp single-edged razor blade. This was done to remove the cuticle and the underlying epidermal tissue in order to expose "windows" into the underlying mesophyll and vascular tissues. Aniline blue (0.05%, w/v) was applied drop-wise to the exposed area. After 5-15 minutes, leaf strips were examined for callose fluorescence using a Zeiss Standard Junior 18 microscope fitted with a 50W HBO UV lamp (Zeiss, Johannesburg, South Africa). Callose was visualised using a Chroma (#1103) violet filter set (BP 425-440, LP 475, FT 460nm, Chroma technology, Battlebro, USA). Callose related fluorescence was photographed using an Olympus DP10 camera (Wirsam Scientific, Johannesburg, South Africa) and downloaded as tagged information format images at 144 pixels per inch resolution. The digital images were imported to Microsoft Photo Editor and adjusted if necessary.

2.5.2 Preparation of plant tissue for 5,6-carboxyfluorescein diacetate (5,6-CFDA) treatment

5,6-CFDA was made up by adding 100mg of the compound to 1ml DMSO, after which 1-2µl of stock solution was diluted to 1.0ml with distilled water. Until used, the use 5,6-CFDA working solution was foil-wrapped and kept frozen at -4°C in small polypropylene centrifuge tubes, and thawed out just prior to use. Unless specifically mentioned, leaf strips from the second visible leaf (counting from the top of the plant) of both infested and control wheat were used in all transport experiments. They were gently scraped on the adaxial surface under MES with a sharp single-edged razor in order to expose small 'windows' into the mesophyll. 5,6-CFDA was introduced drop-wise to these exposed windows, covered with a cover-slip, and left to be taken up into the mesophyll. 5,6-CFDA is membrane-permeable and is therefore readily taken up from damaged to undamaged cells. The diacetate is cleaved by whole cells and resultant 5,6-CF is taken up by the phloem and is passively transported via the sieve tubes. After 2-3 hours, 5,6-CF related transport was viewed using a Zeiss fluorescence microscope fitted with a standard FITC filter set (Zeiss, BP 450-490, LP 520, FT510). 5,6-CF related fluorescence was photographed using an Olympus DP10 camera (Wirsam Scientific, Johannesburg, South Africa) and downloaded as tagged information format images at 144 pixels per inch resolution. The digital images were imported to Microsoft Photo Editor and adjusted if necessary.

2.6 Data analysis

In order to determine aphid feeding preferences, two hundred serial sections cut from 5 replicates, and 400 serial sections cut from 5 replicates were examined in sink and source leaves of wheat, respectively. Data collected were analysed using Sigma Stat (1994, Jandel Scientific Sigma Suite, San Rafael, CA). The significant differences between the number of times the aphid probes a sieve tube (thin- or thick-walled sieve tube) and/or vascular bundle (small, intermediate or large) were calculated using a One-way Analysis of Variance (ANOVA). The significant difference between aphid stylet length and phloem depth in three kinds of vascular bundles was calculated using a Two-way Analysis of Variance (ANOVA).

CHAPTER 3: PREFERENTIAL FEEDING OF D. NOXIA ON WHEAT LEAF BLADES

3.1 Introduction

3.1.1. Morphology of aphid's mouthparts and feeding strategies

Aphids are small insects which feed by inserting their stylets into the plant tissue, where they feed on cell sap. Several studies of stylet anatomy carried out at electron microscope level (see Mittler 1957, Evert *et al.* 1968, Botha *et al.* 1972) demonstrated their remarkable adaptation for puncturing and penetrating plant tissues. The aphid stylet bundle consists of an outer pair of mandibular stylets and an inner pair of maxillary stylets (Dixon 1978). The mandibular stylets possess hook-like projections on their surfaces and the maxillary stylets possess a series of lateral tooth-like projections. These features are presumed to play a part in the penetration of the stylet group into the plant tissue.

As an aphid inserts its stylets into plant tissue, it secretes a stylet sheath around the stylets. Having punctured a functional sieve element (which is assumed to be under full turgor) the aphid is virtually force-fed and is believed to remove chiefly amino acids and sugars for its own dietary needs, excreting the non-essential liquid as well as any undigested sap as honeydew. When the aphid withdraws its stylets the stylet sheath remains in the plant tissues, and indicates the exact passage taken by the stylets during feeding. The point where the sheath terminates indicates the tissue on which the aphid has fed and this termination usually proves to be sieve tubes (see Evert *et al.* 1968; Bornman and Botha 1973, Botha, Evert and Walmsley

1975a,b; Botha and Mabindisa 1977; Botha and Evert 1978; Prado and Tjallingii 1994; Caillaud and Niemeyer 1996 and literature cited).

Aphids mostly aggregate on parts of a plant where food is of high quality (Kennedy and Booth 1951). In other words, they select their feeding sites according to the quantity and quality of the food that should be yielded at that site. It has been shown, using membrane-feeding systems that the probing behaviour of some aphid species and feeding can be influenced by plant-specific chemicals which are found in parenchyma cells (for further details see Montgomery and Arn 1974). High levels of sucrose, amino acids and inorganic ions, (which are characteristic constituents of phloem sap) may provide the stimuli by which phloem feeding aphids "recognise" sieve elements (Mittler and Dadd 1965). The suggestion is that phloem feeders therefore feed at the site of high sucrose loading, that is, on the functional phloem. Changes in the distribution of an aphid with time could therefore indicate changes in concentration gradients within the phloem sap itself.

Clearly aphid feeding involves not only the phloem sap, but also other plant tissues overlying and associated with the vascular tissues, which may interact with the insect mouth parts and therefore play an important role in the final acceptance of plants as suitable hosts.

Recently-developed methods for studying the feeding behaviour of aphids include electronic feeding monitors which give information about the activities of aphids while their stylets are inside plant tissue. Studies carried out using the above method demonstrate that aphids are capable of two activities during sieve element

puncture (Prado and Tjallingii 1994): 1) salivary excretion into the sieve element where the fluid filled canal does not allow saliva to be ingested, 2) passive ingestion of sieve element sap which is mixed with the concurrently secreted saliva. The secreted saliva in the latter case does not reach the plant (sieve element) but is mixed with the sap. The first example is suggested in this thesis to refer to a non-functional feeding environment and the second example referring to a functional feeding environment.

Many aphids show a non-random distribution over the surfaces of leaves on which they feed. Their location is related to differences in the size of the leaf veins from which the aphids feed (Kidd 1976). Larger aphids tend to settle on larger veins, while the smaller aphids settle on the smaller veins. In some species (see Gibson 1972, Elliot and Hodgson 1996) this pattern of distribution is simply the result of the stylets of small aphids being too short to reach the deeper phloem of the larger veins, while the larger aphids with their longer stylets are able to accomplish this.

3.1.2 Investigation of phloem sieve tube physiology, using feeding aphids and aphid stylectomy techniques.

From the earliest days of the study of phloem transport, investigators have attempted to probe the mysteries of the functional sieve-tube system by obtaining samples of the nutrient stream which moves through it. The dependence of aphids on phloem sap as a source of food was exploited over the years in a number of laboratories, in the attempt to solve the as yet, unknown issues related to phloem transport and sieve tube content. A popular technique which has been used over many years, is to sever the stylets of feeding aphids and collect the sap that

exudes from the cut ends of the stylet bundle left embedded in the host plant. Kennedy and Mittler (1953) used this technique to determine the composition of the food digested by aphids. Weatherley, Peel and Hill (1958) used the technique to study sieve tube physiology. Recently, Pritchard (1996) has used this method to measure the osmotic pressure of functional sieve tubes. Clearly, this technique has been used extensively in an attempt to rationalise some of the many problems and major controversies associated with phloem translocation.

Whole aphids have frequently been used to collect sieve tube sap in the form of excreted honeydew (Ho and Peel 1969). Studies undertaken by several authors (Esau, Namba and Rasa 1961, Evert *et al.* 1968, Bornman and Botha 1973, Botha *et al.* 1975a, b, Botha and Evert, 1978,) demonstrated that aphids are useful tools in the study of functional phloem tissue. The use of live, feeding aphids was necessary in these experiments as this allowed the aphids to settle and feed undisturbed. As a result thus more information on stylet position as well as about the effects of prolonged feeding by the aphids could be obtained.

Feeding aphid colonies present opportunities to examine potential functionality of specific phloem groups as was the case in studies by Botha, Evert and Walmsley (1975); Botha and Mabindisa (1977); Botha, Malcom and Evert (1977); Botha and Evert (1978), where preferential feeding habits were revealed.

Given that grasses have thick- and thin-walled sieve tubes and given the controversy surrounding their potential roles in retrieval, loading and transport it became clear that some of the as yet, unanswered questions relating to function

could possibly be answered, by examining preferential feeding sites of aphids in source and sink leaves blade bundles in a grass such as wheat. In addition, evidence of selectivity (if any) in either thin- or thick walled sieve tubes could be useful in developing wheat varieties resistant to *D. noxia*.

3.1.3 Objectives

Fouche' 1983 and Fouche' *et al.* (1984) used light and transmission electron miscroscope techniques to investigate the feeding habit of *D. noxia*. The main focus of their study was to determine if *D. noxia* had any preferential penetration and feeding site. Their results showed that no preferences occurred. Their study suggest that penetration did not follow any pattern and appeared to occur at random. Recent studies by Ni and Quisenberry (1997) compared the distribution of *D. noxia* salivary sheath in susceptible (Arapahoe) and resistant (Halt) wheat leaves. Their results showed that *D. noxia* produced both single and multiple-branched salivary sheaths on both cultivars, with more salivary sheaths terminating in vascular bundles than in mesophyll tissue. Whilst a great deal of useful information was gained from these studies, the authors did not indicate which (thin- or thick-walled) sieve tubes were penetrated.

A recent study using barley source leaves conducted by Matsiliza and Botha (2002) showed preference of thin-walled sieve tubes by the aphid *Sitobion yakini,* suggesting that the thin-walled sieve tubes were a more attractive feeding source than the thick-walled sieve tubes. The authors further suggested that the thin-walled sieve tubes were more functional in phloem loading and transport than the thick-walled sieve tubes.

In this chapter I am reporting on my investigation of the preferences by *D. noxia*, (as described by Matsiliza and Botha, 2002) in an attempt to determine the feeding pattern of *D. noxia* in source and sink leaves of wheat and to determine the role of the thin- and thick-walled sieve tubes in wheat.

This chapter therefore focuses attention on the point of initiation, the probe pathway and point of termination of stylets and stylet tracks of *D. noxia.*

3.2: Results

3.2.1 Relationship between phloem depth/penetration depth and stylet length

In total 30 transverse sections (10 sections of each of the 3 types of vascular bundles) showing evidence of stylet penetration were examined. These sections were selected in different areas along the length of the leaf in sink as well as source leaf. Table 3.1 shows the mean distance measured from the adaxial and abaxial epidermis to the point of stylet penetration in the phloem of small, intermediate and large vascular bundle, and the mean length of stylets terminating in these vascular bundles. In all three kinds of vascular bundles and in both sink and source leaf tissue the distance from the adaxial leaf surface to the point of penetration in phloem was longer than the one from the abaxial leaf surface to the same position in the phloem (Table 3.1). In addition, phloem depth increased with the increase in size of the vascular bundles. The phloem in large vascular bundles was situated deeper within the leaf than in the small and intermediate vascular bundles.

The length of stylets inserted during feeding was significantly longer than phloem penetration depth in small, intermediate and large vascular bundles and in the mean total of all three types of vascular bundle (Table 3.1). In addition, the length of stylet required for feeding increased with the increase in vein size, with the shortest stylets associated with small vascular bundles and the longest with the large vascular bundle.

Table3.1 Mean distance (μm) from the adaxial and abaxial epidermis to the point of stylet penetration in the phloem (phloem/penetration depth) and mean stylet and track lengths (μm) of the Russian wheat aphid feeding in different vascular bundles of the sink and source leaf of wheat. Means with different letters **(a or b), ((x or y), (c or d), (e or f)** within a row were significantly different ($P<0.05$) according to a two way ANOVA.

Type of vascular bundle	Penetration depth/Phloem depth (μ)				Stylet length (μ)	
	Sink		*Source*		*Sink*	*Source*
	Adaxial	Abaxial	Adaxial	Abaxial		
Small	60	48	71	51	77	88
	±9	±6	±16	±10	±6	±17
Inter-	65	50	78	55	82	99
mediate	±7	±5	±9	±12	±10	±16
Large	79	52	105	73	96	124
	±10	±15	±8	±8	±6	±11
Mean	68^{a}	50^{x}	85^{c}	60^{e}	85^{by}	104^{df}
SD	±10	±2	±10	±18	±12	±18

3.2.2 Distribution of stylets and salivary sheaths/stylet tracks on sink leaf blade.

Two hundred serial sections were cut from leaf segments of sink leaves (4-5cm in length) and were examined microscopically for evidence of stylet penetration. Stylet tracks were examined for the point of origin/initiation (ad- or abaxial leaf surface) to the point of termination (at the thin- or thick-walled sieve tubes. The principal data obtained are presented in Table 3.2 and Figs. 3.1-3.6.

In total, 100 stylets and stylet tracks were observed in 5 different sink leaves (82 phloem sieve tubes and the rest in other tissues). Both single and multiple branched salivary sheaths were observed during this investigation with single branched salivary sheaths terminating only in one type of leaf tissue whilst the multiple-branched terminated in either of these tissues. Fig.3.1 shows distribution of probes in different types of tissues of the leaf blade. As observed by Ni and Quisenberry (1997) a majority of salivary sheaths and stylets observed terminated in cells of the vascular bundle (asterix) (i.e. bundle sheath, mestome sheath, xylem, phloem and vascular parenchyma cells) and very few terminated in mesophyll tissue. Amongst cells of the vascular tissue, most probes terminated in thin-walled sieve tubes of the phloem, followed by thick-walled sieve tubes. The least number of probes was in the vascular parenchyma and bundle sheath cells. In most cases these cells were penetrated by stylets passing through to the sieve tubes of the phloem (see Figs. 3.3 – 3.6)

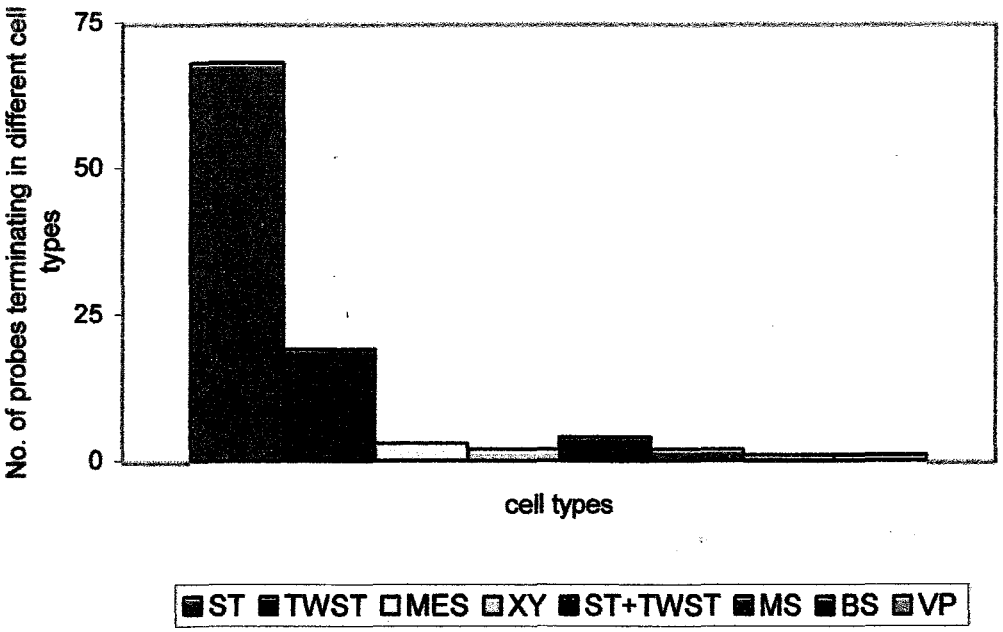


Fig. 3.1 shows distribution of probes in sink leaf tissue. Note 1: Determined from stylets (in thin- (ST) and thick-walled sieve tubes (TWST) and stylet tracks/salivary sheaths (other cell types)

The total number of probes terminating in the phloem tissue was lowest close to the leaf tip and increased away from the leaf tip becoming highest close to the base of the leaf (Fig. 3.2). In addition the number of probes terminating in thin-walled sieve tubes also apparently increased towards the base of the leaf. In contrast, probes terminating in thick-walled sieve tubes were highest close to the tip and decreased away from the tip becoming lowest close to the base of the leaf (Fig. 3.2).

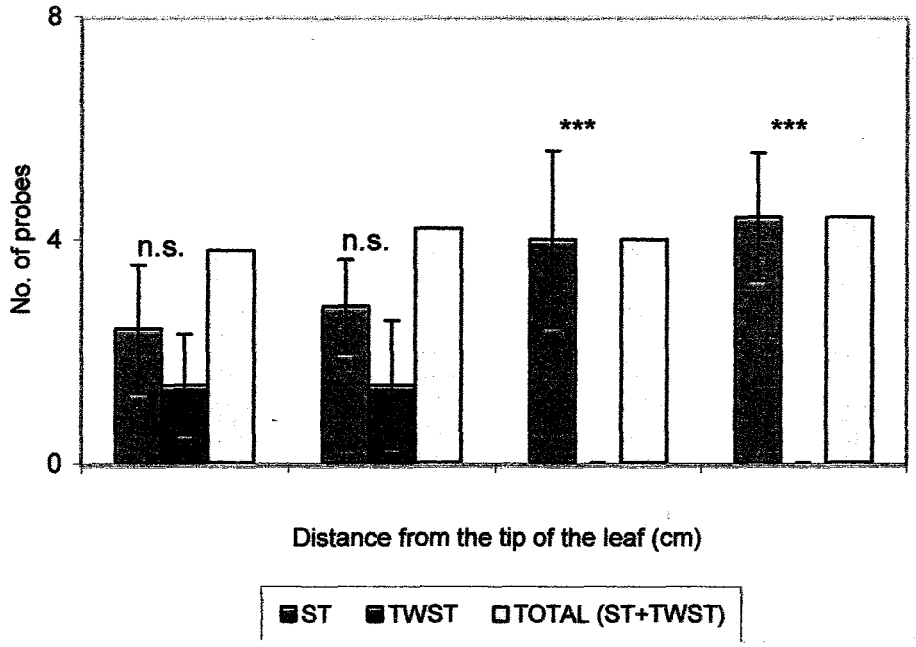


Fig. 3.2 shows the mean number of probes terminating in thin- (ST) and thick-walled sieve tubes (TWST) and the total number of probes observed along the length of a sink leaf. (n = 5, sink leaf = 5cm long). Level of significance, n.s.= not significant, *** $P<0.05$.

Sixty-eight (78%) of eighty two stylets and stylet tracks observed in the phloem terminated in thin-walled sieve tubes and the remaining 14 (22%) in thick-walled sieve tubes. Statistical analysis of results of the total of all plants show a highly significant difference between thin- and thick-walled sieve tube penetration (Table 3.2). The thin-walled sieve tubes (ST, 13.6, ±3.6) are significantly more attractive to the aphids than the thick walled sieve tubes (TWST, 2.8, ±1.3). However, statistical analysis of the data per cm from the tip of the leaf, show that there is no significant difference of the number of times thin- and thick-walled sieve tubes are penetrated

between 0-1cm (ST, 2.4, ±1.1 and TWST, 1.4, ±0.9) and 1-2cm (ST, 2.8, ±0.8 and TWST, 1.4, ±1.1). On the other hand, thin walled sieve tubes between 2-3 cm (ST, 4.0, ±1.6 and TWST, 0.0, ±0.0) and 3-4 cm (ST, 4.4, ±1.1 and TWST, 0.0, ±0.0) from the tip were significantly more attractive to the aphid than thick-walled sieve tubes (Table 3.2).

Of a total of 82 stylets and stylet tracks encountered during this study 31 were associated with small vascular bundles and the remaining 28 and 16 with large and intermediated vascular bundles, respectively. The large vascular bunles were selected nearly as many times as the small vascular bundles. Statistical analysis of results showed a significant difference in the number of times each of the longitudinal vascular bundles was penetrated. The small vascular bundle was significantly more penetrated followed by the large and then the intermediate vascular bundle.

Most stylets and stylet tracks encountered during this study were initiated from the adaxial /upper surface of the leaf (Table 3.3). Very few were observed emanating from the abaxial /lower surface. This observation was uniform throughout the entire leaf and for all leaves examined.

Table 3.2-Results of a one-way ANOVA for the variation between the number of times a sieve element (thin-walled sieve tube (ST) or thick-walled sieve tube (TWST) are visited along the length of a wheat sink leaf. Level of significance: n.s.- not significant, ***P<0.05.

Distance from the leaf tip (cm)	**0-1**		**1 – 2**		**2 - 3**		**3 - 4**		**Total no. of probes for whole leaf**	
Replicate	ST	TWST	ST	TWST	ST	TWST	ST	TWST	ST	TWST
1	3	1	4	1	4	0	6	0	17	2
2	2	2	2	3	3	0	5	0	12	5
3	2	2	3	1	6	0	4	0	15	3
4	4	2	3	0	5	0	4	0	16	7
5	1	0	2	2	2	0	3	0	8	3
Total	**12**	**7**	**14**	**7**	**20**	**0**	**22**	**0**	**68**	**14**
Mean &	2.4	1.4	2.8	1.4	4.0	0.0	4.4	0.0	13.6	2.8
S.D.	±1.1	±0.9	±0.8	±1.1	±1.6	±0.0	±1.1	±0.0	±3.7	±1.3
Significance in difference		**(n.s.)**	**(n.s.)**			***		***		***

Table 3.3 Origin and distribution of stylets and stylet tracks of *D. noxia* feeding in longitudinal bundles of the sink leaf blades of wheat. Different letters (**x** or **y**) within a column, and (**a** or **b** or **c**) within a row indicate significant difference according to One-Way ANOVA test ($P<0.05$).

Source	Termination	Small vascular bundle	Intermediate vascular bundle	Large vascular bundle	Total
Abaxial epidermis	ST	2	1	2	5
	TWST	0	0	0	0
Total					**5^{x}**
Adaxial epidermis	ST	29	11	21	63
	TWST	2	5	7	14
Total					**77^{y}**
Total		**31^{a}**	**16^{b}**	**28^{c}**	

Figs. 3.3-3.6. serve to illustrate various aspects of stylet penetration of the phloem in sink leaves of wheat. Figs. 3.3 and 3.5 show penetration of large vascular bundles by stylets of *D. noxia*. In Fig. 3.1 penetration of the mesophyll, bundle sheath and xylem was largely intercellular, apparently without attempting to feed or sample the contents of these cells. During this penetration, the aphid first probed two thick-walled sieve tubes before terminating in a thin-walled sieve tube. In Fig. 3.5 the tips of the styles (unlabelled arrow) are visible inside a thin-walled sieve tube and are free of any mucilaginous deposits, or salivary material. This is an indication that the aphid was feeding at the time it was killed (Evert *et al.* 1968, Botha, Evert and Walmsley 1975a,b, Matsiliza and Botha 2002). Branched stylet tracks were relatively frequent and occurred mostly near or inside the vascular bundles. Fig. 3.4 shows penetration of a small vascular bundle in which a thick-walled was penetrated. Examination of several serial sections showed that the aphid first probed several thin-walled sieve tubes, as evidenced by salivary sheath left behind, (unlabelled arrowhead), then redirected its stylets and penetrated a thick-walled sieve tube. Fig 3.6 shows penetration of a large vascular bundle. Examination of serial sections revealed that the aphid which formed this sheath first penetrated a vascular parenchyma cell and a thin-walled sieve tube. Partly withdrawing the stylets, the aphid then penetrated a thick walled sieve tube and several vascular parenchyma cells before terminating in a thin-walled sieve tube.

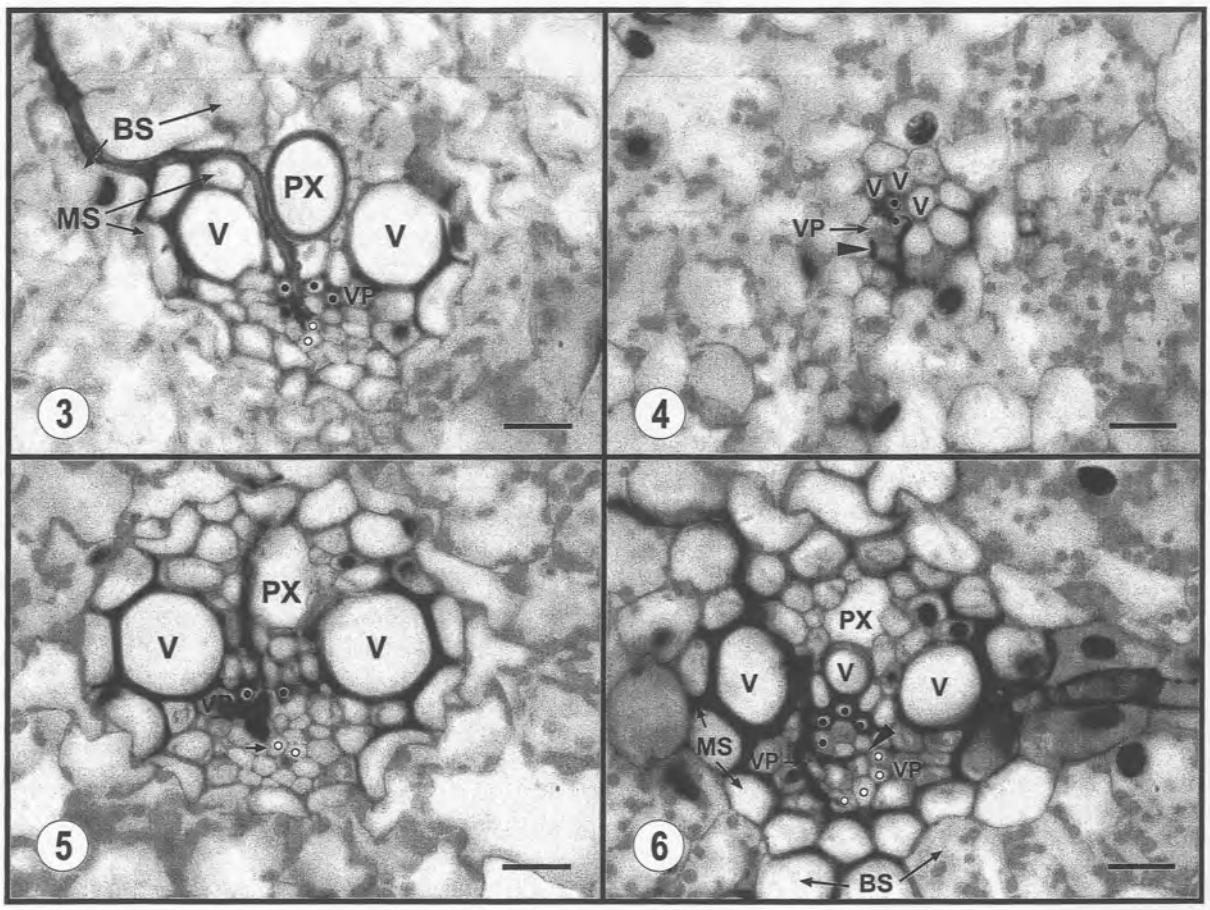

BS
MS
PX
V
V
VP
3
V
V
V
VP
4
PX
V
V
V
5
PX
V
V
V
MS
VP
VP
6
BS

Fig. 3.3-6 show penetration of large (Figs 3.3, 5-6) and small (Fig. 3.4) vascular bundles in sink leaf tissue.

Fig. 3.3 Shows penetration of large vascular bundle from the adaxial surface of the leaf. The aphid probed the bundle sheath (BS), mestome sheath (MS) and xylem intercellularly, then penetrated a thick-walled sieve tube (solid dot) before terminating in a thin-walled sieve tube.

Fig. 3.4 and 3.6 show examples of branched stylet tracks. Fig 3.4 shows penetration of a small vascular bundle. The aphid penetrated this vascular bundle from the side, re-curved its stylet and penetrated a vascular parenchyma cell (VP). The aphid apparently withdrew its stylets and successfully penetrated a thick-walled sieve tube (solid dot). In Fig 3.6 the aphid penetrated a large vascular bundle from the adaxial surface of the leaf. The pathway of stylets is partly intercellular and in part intracellular. The aphid first penetrated a vascular parenchyma cell, then withdrew its stylets and continued further down terminating successfully in a thin-walled sieve tube (open circle).

Fig. 3.5. Shows penetration of a large vascular bundle. The aphid penetrated the xylem (V) both intercellularly and intracellularly, then penetrated two thick-walled sieve tubes and a vascular parenchyma cell before terminating successfully in a thin-walled sieve tube. Stylet tips (unlabelled arrow) are free of salivary material, suggesting that the aphid fed from this sieve tube.

Scale bars (Figs. 3.3-3.6) = 10μm

3.2.3 Penetration of the source leaf blade of wheat by stylets of D. noxia

As with sink leaf tissue, information on the manner of penetration of the leaf blade by the aphid was obtained by observing the path taken by the aphid towards the phloem, by observing the stylet tracks or sheaths in serial transverse sections of the host tissue. Stylet tracks were examined for point of origin (i.e., from the ad- or abaxial leaf surface) and to the point of termination (i.e., in the thin- or thick-walled sieve tubes). The principal data obtained during this study are presented in Table 3.4 & 5 and Figs. 3.7-12.

Fig. 3.7 shows the distribution of stylets (only in thin-walled sieve tubes) and salivary sheaths in tissues of the source leaf. Of a total of 264 stylets and stylet tracks/salivary sheaths encountered in source leaves during this study, 260 were associated with cells of the vascular bundle (i.e. bundle sheath (BS), mestome sheath (MS), xylem (XY), vascular parenchyma (VP) and sieve tubes (ST & TWST) and the remaining 4 with mesophyll cells (MES). Within cells of the vascular bundle, the highest number of probes terminated in thin-walled sieve tubes. Examination of serial sections revealed that most probes terminating in cells other than sieve tubes were simply penetrations caused by stylets passing through on the way to the phloem.

In contrast to the pattern of distribution of probes observed along the length of sink leaves, the aphids in source leaf appeared to feed anywhere on the leaf (Fig. 3.8). Although the number of probes observed in the middle section of the leaf seems to be higher than in other areas, the difference becomes very little if one takes into account the standard deviation.

Results of a One-way ANOVA for the variation between the number of times the two kinds of sieve tubes are penetrated show a significant difference between thin- and thick-walled sieve tubes for the whole leaf, total of all leaves examined and per centimetre along the length of the leaf. The thin-walled sieve tubes were probed significantly more times than the thick-walled sieve tube (Table 3.4 & 5). Of a total of 222 stylets and stylet tracks encountered during this study, 212 (95%) terminated in thin-walled sieve tubes and the remaining 10 (5%) in thick-walled sieve tubes (Table 3.5).

One hundred and twenty one stylets and stylet track encountered terminated in the small leaf blade bundles and the remaining 58 and 43 in intermediate and large vascular bundles, respectively (Table 3.5). Statistical analysis of results show that the small vascular bundles were significantly more visited that the other two vascular bundles.

D. noxia stylets penetrated wheat source leaves almost exclusively from the adaxial surface, with very few instances observed of stylets penetrating from the abaxial surface (Table 3.5).

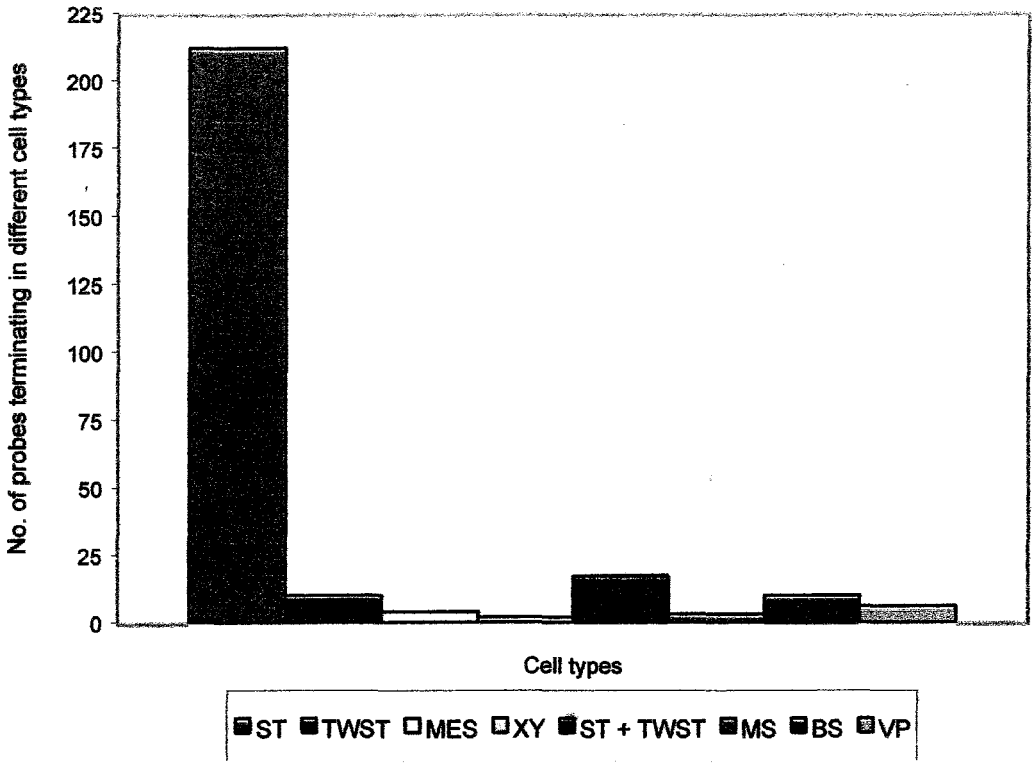


Fig. 3.7 Distribution of stylets (only in thin-walled sieve tubes (ST) and stylet tracks/salivary sheath in source leaves of wheat.

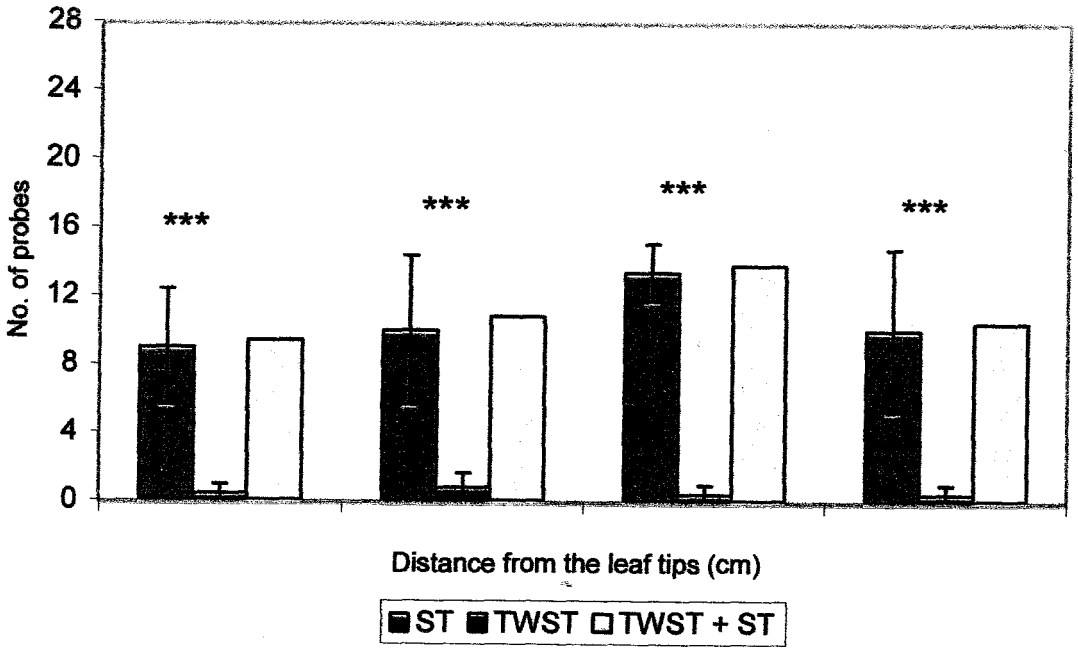


Fig. 3.8 Mean and SD, and total number of stylets and stylet tracks terminating in thin- (ST) or thick walled (TWST) sieve tubes along the length of a source leaf (n=5, the source leaf is 12-14cm long). Level of significance *** $p<0.05$.

Table 3.4-Results of a one-way ANOVA for the variation between the number of times a sieve element (thin-walled sieve tube (ST) or thick-walled sieve tube (TWST) are visited along the length of a wheat source leaf. Level of significance: n.s.- not significant, ***P<0.05.

Distance from the leaf tip (cm)	**1 - 2**		**3 – 4**		**5 - 6**		**7 - 8**		**Total no. of probes for whole leaf**	
Replicate	ST	TWST	ST	TWST	ST	TWST	ST	TWST	ST	TWST
1	7	0	10	1	11	0	9	0	37	1
2	10	1	12	0	13	0	3	0	38	1
3	9	0	18	1	15	0	12	1	54	2
4	14	0	7	2	12	1	16	1	49	4
5	5	1	8	0	11	1	10	0	34	2
Total	**45**	**2**	**55**	**4**	**62**	**2**	**50**	**2**	**212**	**10**
Mean & S.D.	9 ±3.4	0.4 ±0.6	11 ±4.4	0.8 ±0.8	12.4 ±1.7	0.4 ±0.6	10 ±4.7	0.4 ±0.6	42.4 ±8.6	2 ±1.2
Significance in difference		***		***		***		***		***

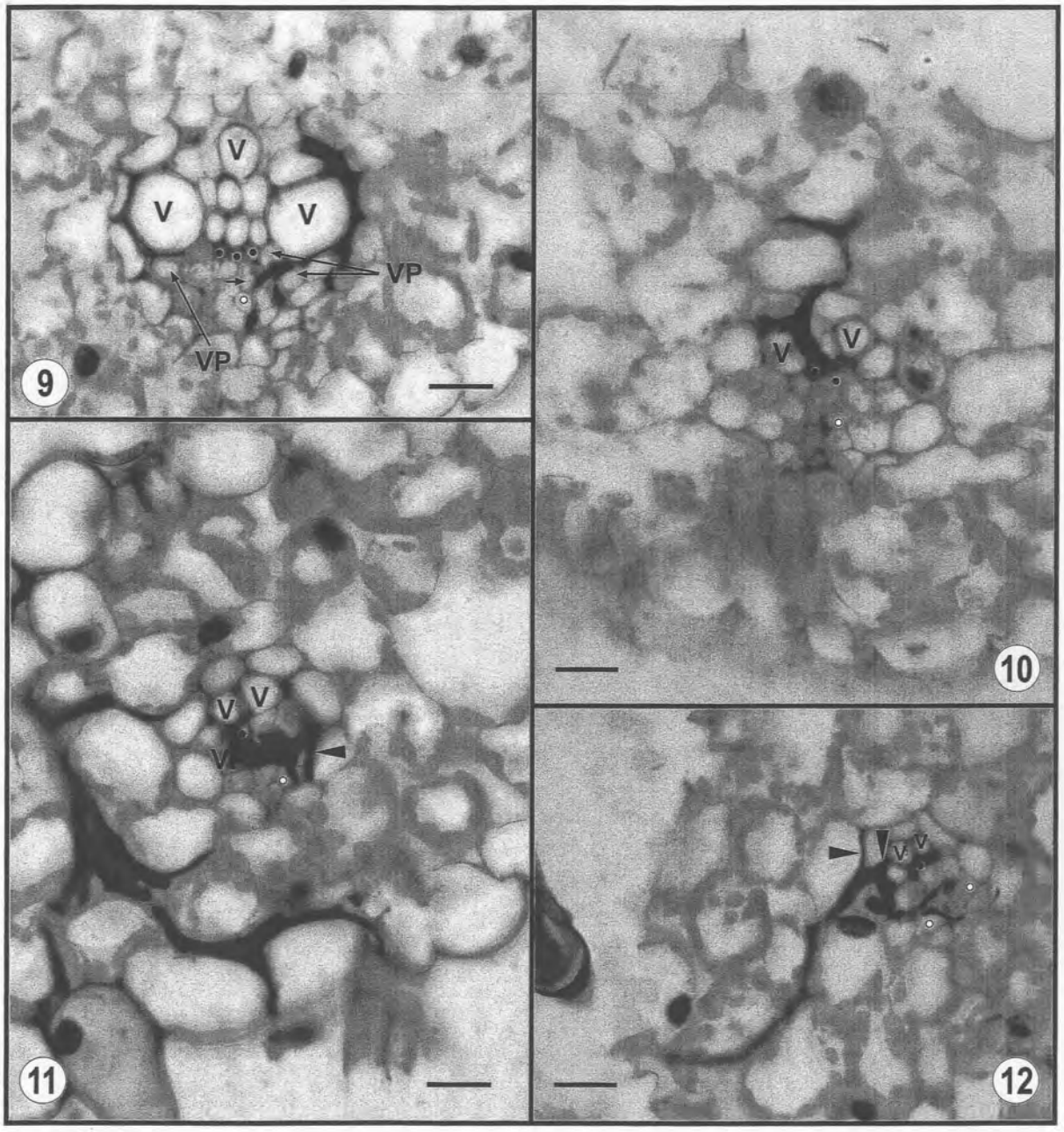
Table 3.5 Statistical analysis of the origin of probes at the adaxial and abaxial epidermis, the distribution of stylets and stylet tracks within the leaf and the termination of stylet tips and tracks in either thin- or thick-walled sieve tubes, together with the number of times the thin (ST) and thick (TWST) are visited, with the mean standard deviation and variation between and within different vascular bundles. Different letters **a, b** and **x, y** indicate significant difference. Level of significance according to One-Way ANOVA, *** $p < 0.05$.

Termination of aphid stylets and stylet sheaths in different sieve tube types

Probes as indicated by Stylets and stylet sheaths	ST	TWST	Total
Adaxial origin	197	10	207^{x}
Abaxial origin	15	0	15^{y}
Total probes	212 (95%)	10 (5%)	222
Mean and SD	$42.40^{a} \pm 8.62$	$2.00^{b} \pm 1.22$	
Distribution of probes in different bundles			
Small vascular bundle	120	1	121
Intermediate vascular bundle	55	3	58
Large vascular bundle	37	6	43
Significant difference between bundles	***	***	***

As with most other relatively small aphids (Evert *et al.* 1968, Botha, Evert and Walmsely 1975a, b, Matsiliza and Botha 2002), penetration of the source leaf was largely intercellular (Figs. 3.9-12). The aphid probed the leaf between epidermal cells or through the stomata and proceeded in a more or less zig-zag pathway along the surfaces of the mesophyll cell walls to the vascular tissues. In all successful probes of the phloem tissue only the tips of the maxillary stylets were inserted into the sieve tubes (fig. 3.9). Fig. 3.9 shows penetration of a large vascular bundle by stylets of *D. noxia* . Penetration of stylets between xylem cells, mestome sheath cells was intercellular, the aphid the penetrated two vascular parenchyma cells before terminating in a thin-walled sieve tube. This micrograph shows that only the tips of the maxillary stylets (arrow) entered the thin-walled sieve tube. They are free of salivary material, suggesting active feeding before the aphid was killed. Unlike in Fig. 3.9 where stylet tips are visible, in Figs 3.10-12 the stylet tips are masked by salivary sheath material. In these micrographs, it is likely that the aphid was disturbed and withdrew its stylets, during manipulation of the leaf, leaving behind the salivary sheath. Fig. 3.10 shows one of the few instances where a thick-walled sieve tube was penetrated. The aphid first penetrated a bundle sheath and vascular parenchyma cell in this intermediate vascular bundle before terminating in a thick-walled sieve tube. Fig. 3.11 shows penetration of several thin-walled sieve tubes of a small vein. Massive saliva deposited after penetration or feeding in vascular parenchyma cells and thin walled sieve tubes is evident in this vascular bundle. Branched stylet tracks were seldom encountered in the mesophyll cells. Fig. 3.12 shows penetration of a small vascular bundle by stylets initiated from the adaxial surface of the leaf. The pathway of stylets in the mesophyll is largely intercellular, apparently becoming intracellular once inside the vascular

bundle. The stylet track in this micrograph branched 4 times, with two unsuccessful probes towards the xylem and bundle sheath cells and the remaining two terminating in thin-walled sieve tubes.


V
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V
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12

Figs. 3.9-3.12 show penetration of vascular tissue in a source leaf of wheat by stylets of *D. noxia.*

Fig. 3.9 Shows penetration by stylets of a thin-walled sieve tube in a large vascular bundle. The aphid's stylet tips (unlabelled arrow) are visible just beneath a vascular parenchyma cell. Lack of saliva associated with stylet tips suggest that the aphid was feeding from a functional sieve tube.

Fig. 3.10 Shows a successful probe which terminated at a thick-walled sieve tube. In this instance, the aphid first probed the xylem intracellularly before proceeding towards the thick-walled sieve tube.

Fig. 3.11 Shows penetration of a small vascular bundle from the abaxial surface of the leaf. After penetrating a vascular parenchyma several successive thin-walled sieve tubes were probed, and massive salivary sheath were deposited in the process (arrowhead).

Fig. 3.12 Shows salivary sheath which originated at the adaxial surface of the leaf, branching and unsuccessfully probing the bundle sheath and xylem (arrowheads). Two probes terminated in thin-walled sieve tubes.

Scale bars (Figs. 3.9-3.12)= 10μm

3.4 Discussion

The data presented in Tables 3.1-5, Figures 3.1-12 present point of initiation of styets and stylet tracks of *D.noxia,* their distribution and point of termination within source and sink leaves of wheat plants.

These results show that *D.nox*ia, like *S. yakini* (Matsiliza and Botha 2002), feeds preferentially on the thin-walled sieve tubes of wheat plants. Most stylets and stylet tracks encountered in sink (for the whole leaf) as well as source leaves terminated in thin-walled sieve tubes. Of a total of 222 stylets and stylet tracks encountered in source leaves of wheat, 212 terminated in thin-walled sieve tubes (see table 3.5). Furthermore, 68 of 82 stylets and stylet tracks encountered in sink leaves terminated in thin-walled sieve tubes (see table 3.3). However, there was no significant difference in the number of times thin- and thick-walled sieve tubes are visited within the first two centimetres from the tip of a sink leaf. Thick-walled sieve tubes in this area of the leaf appear to be just as important and attractive as a source of food to the aphid as the thin-walled sieve tubes. On the other hand, thin-walled sieve tubes in the area of the sink leaf between 2-4cm from the tip were significantly more visited than the thick-walled sieve tubes. However analysis of the sink leaf data for the whole leaf clearly showed that the thin-walled sieve tubes were the preferred feeding site.

This data support the hypothesis that the thin-walled sieve tubes are more attractive to *D. noxia* as a feeding site than thick-walled sieve tubes in both sink and source leaves of wheat.

There was a significant difference in the number of times each of the longitudinal vascular bundles were selected in sink as well as source leaf. Significantly, the majority of tracks and stylets encountered were associated with the small longitudinal vascular bundles in sink as well as in source leaf.

The preference for smaller veins in source leaves is likely to be related to both the quality and quantity of the food (for example the total available soluble sugars, carbohydrates and proteins). Altus and Canny (1982) and Evert, Russin and Botha (1996) suggested that these veins were involved in assimilate loading process, and that the large bundles are primarily involved in longitudinal transport. The preference of smaller veins in sink leaves suggest that these veins might be involved in the unloading process. If the small bundles are implicated in loading and unloading then clearly the closer the stylets are to the site of vein loading and unloading the higher both the quality and quantity of the food available to them should be. The preponderance of stylets and tips associated with the thin-walled sieve tubes of small longitudinal bundles suggests potentially higher osmotic potentials within these veins.

Gibson (1972), Kidd (1976) and Elliot and Hodgson (1996) have suggested that vein-selection by aphids can be affected by several factors. First, the fact that the phloem of major veins is situated deeper within the leaf than is the case in minor veins, means that the aphid will expend more energy and time to reach its objective in sieve tubes. Feeding on minor veins means a shorter pathway to the functional sieve tubes. Secondly, in many plants the larger veins are associated with a thick mestome or sclerenchyma sheath surrounding the vascular bundles, while smaller

veins are not. Feeding on minor veins should therefore be easier for the aphid. Sclerenchyma girders are associated with large vascular bundles of wheat leaves, and are found on the adaxial and abaxial side of the bundle. *D. noxia* has been found to approach the bundle obliquely and not directly from above it seems quite possible for the aphid to avoid this structural obstacle and the position of sclerenchyma should not therefore impede the aphid's probings. By feeding on the minor, rather than major veins, the aphid has the advantage of a 'shorter' pathway to sieve tubes, less sclerenchyma to impede the passage of its stylets, and also a food supply which arguably is richer in both sugars and proteins.

In addition, when phloem depth was compared with the length of stylet used to reach the phloem, it was clear that stylet lengths were significantly longer than the distance to the phloem for each of the vascular bundles and for the mean total of all vascular bundles (Table 3.1). This in part may be due to the fact that the stylets were winding in search of a suitable feeding site and in part because in most instances the aphid approached the vascular bundles obliquely and directly from above. There was very little difference between the mean stylet length in small vascular bundles and mean phloem depth in large vascular bundles. In addition, the mean stylet length in intermediate bundles was longer than the phloem depth in large bundles. This clearly shows that the aphid's stylets were long enough to reach most of the phloem in all three kinds of vascular bundles. In addition, feeding aphids never seemed to insert the full length of their stylets (Gibson, 1972) and so the effective length of stylets for reaching the phloem would seem to be somewhat less than their actual length. In light of this, the preference of small vascular bundles by the *D. noxia* observed in this study could not have been affected by the

length of stylets.

The surprising selection of larger vascular bundles by the aphid in sink leaves might be because of their early development (Patrick 1972, Trivett and Evert 1998) compared to the smaller veins and their direct involvement in importing of assimilates to sink leaves (therefore contained a high enough proportion of some desirable assimilates). The latter would provide the aphid with a continuous supply of assimilates before they are unloaded to the rest of the sink leaf. Selection of smaller veins on the other hand could simply be because they are easily accessible to the aphid.

Of interest too was the fact that the aphids probed preferentially from the adaxial surface of the leaf in both sink and source leaves. The choice of penetration location exhibited by *D. noxia* could simply be due to the fact that the adaxial surface together with the observed (also see Hewitt *et al.* 1984) upward curling of infested leaves offered more protection for the aphid than the now exposed abaxial surface would have done. Thus, the need for protection from potential predators and possibly to avoid being dislodged, could therefore account for the distribution of *D.noxia* on the adaxial surface.

Several explanations have been put for the predominance of intercellular penetration of tissues by aphid stylets (see Evert *et al.* 1968 and literature cited therin). Firstly, that the intercellular course offers the line of least resistance. Secondly, that the cell wall offers support to the stylets. Thirdly, that smaller aphids are unable to penetrate the tissue by pressure alone and that their saliva is not potent enough to enable them to take a straight course to their objective. From the

present investigation, the stylets of *D. noxia* (which is a relatively small aphid species) follow an apparently intercellular (from epidermis through the mesophyll) and then intracellular (near and inside the bundle) pathways.

While some workers have suggested that the aphids and other suctorial insects they have studied find their objectives by trial and error (Evert *et al.* 1968 and literature cited therein, Botha *et al.* 1975a,b), others have reported evidence which suggests that the aphid's stylets do not enter the tissues haphazardly, but are directed to their objective with marked precision (Matsiliza and Botha 2002). Based on my observations, *D. noxia* appears at times to locate the phloem by chance (Fig. 3.4,6 & 8-10) and at other times with some degree of precision (Fig. 3.3 & 11). However, it would seem that precise location may be more infrequent, as branching of tracks, suggestive of trial and error, was relatively frequent. It is not possible to rule out the probability that penetration releases pressure within the sieve tubes, and as such leads to decreased flow rate, at which point the aphid could withdraw its stylets, to look for a new feeding site. Quite possibly, multiple probes could indicate a long-term feeding pattern.

Prado and Tjallingii (1994) reported that during ingestion of phloem sap, the stylet tips of aphids projected beyond the salivary sheath which terminates at the cell wall, as the salivary sheath does not reach the sieve element. Several other authors also demonstrated that the maxillary stylet tips (of other aphids) projected beyond the salivary sheath during penetration (see Evert *et al.* 1968; Botha, Evert and Walmsely 1975a,b; Botha, Malcom and Evert 1977; Botha and Mabindisa 1977; Botha and Evert 1978, Matsiliza and Botha 2002). In this study, it has been

demonstrated that the maxillary stylet tips of *D. noxia* project beyond the salivary sheath in some cases and are open within the sieve tube being tapped. It would seem then, that the presence of open stylet tips and lack of associated salivary sheath material within the sieve elements, still constitutes sufficient evidence for such sieve elements to be identified as functional.

The fact that *D. noxia* feeds almost exclusively on the thin-walled sieve tubes may be due to the following reasons: Firstly, the structure or composition of the walls of the thick-walled sieve tubes makes it more difficult for the aphid to penetrate than that of the thin-walled sieve tubes. However, it must be born in mind that aphids have been found to penetrate or probe the bark of trees (see Evert *et al.* 1968) and penetrate long distances both intercellularly and intracellularly through xylem (Botha, Evert and Walmsely 1975a) in search of internal phloem in stems of *Gomphocarpus physocapus*.

However, it is more plausible that the thin-walled sieve tubes contain some substance or substances which are either lacking in the thick-walled sieve tubes, or present in lesser amounts and that such substances are highly desirable to *D. noxia* within the sieve tubes of wheat. Alternatively, perhaps greater quantities of assimilates are transported in the thin-walled sieve tubes than in the thick-walled sieve tubes and the aphids are subsequently drawn to higher sucrose concentration in these sieve tubes. In addition, given that the thick-walled sieve tubes are situated closer to the adaxial surface than the thin-walled sieve tubes, it would be expected that the aphid would feed in the thick-walled sieve tubes. However, results obtained in this clearly show that the aphid's stylets probed all the

way bypassing or passing through thick-walled sieve tubes in search of a suitable feeding site i.e. thin-walled sieve tubes. If the phloem composition in these sieve tubes was identical or had the same concentration of nutrients, it would be expected that the aphid would feed equally in both kinds of sieve tubes. The earlier formation and quantitative dominance of the thin-walled sieve tubes over thick-walled sieve tubes lends support to the idea that more assimilates are translocated in thin- than in the thick-walled sieve tubes.

Another consideration is that there could be resistance factors, which could possibly render the thick-walled sieve tubes unsuitable, or normally unpalatable to the aphid. However, if this was indeed the case, then one would have expected to see many more unsuccessful probes in the vicinity of the thick-walled sieve tubes in all bundle sizes than reported here. As this was not observed, the only conclusion one can come up with is that the thick walled sieve tubes do not possess these specific resistance factors.

Careful attention needs to be devoted to possible resistance factors. Two reasons are suggested in the literature which result in unsuitability of certain plants. These are chemical deterrence mechanism and mechanical interference (Caillaud and Niemeyer 1996). Possible chemical deterrence mechanisms in the phloem could include the occurrence of a feeding deterrent or the absence of a feeding stimulant, or a cardiac glycoside as was reported to exist in Asclepiadaceae and Apocynaceae which render the aphid, *Aphis nerii* immune to attack by predators (see Botha, Malcom and Evert 1977). These plant-derived toxic steroids (cardiac glycosides) are synthesised by *Apocynales* as an effective defence strategy against

herbivores. The protective efficacy of a single cardiac glycoside in *Aphis nerii* fed on *Gomphocarpus physiocarpus* was indicated by the death of a dwarf chamaeleon which was fed about 200 *Aphis nerii* (Malcom 1976). In another study, Montgomery and Arn (1974) showed that phlorizin, a flavonoid in *Malus* sp, promotes probing in *Aphis pomi* and inhibits ingestion. This chemical also inhibited both probing and ingestion of the non-apple feeding species, *Amphorophora agathonica*. A mechanical mechanism could involve the activation of the phloem sealing system of the plant which would result in the blocking of sieve elements at the sieve plates by callose or alternatively, in the plugging of the aphid's stylets, thus reducing sap availability for ingestion. In addition, resistance factors have been shown to be located in the sieve elements in wheat which are able to inhibit the ingestion of sap by the aphid *Sitobion avenae* (Caillaud *et al*. 1995).

Haupt *et al*. (2001) presented evidence showing that thick-walled sieve tubes in sink leaves are involved in the unloading process. In their study, thick-walled sieve tubes, like thin-walled sieve tubes, were found to be symplastically connected to the mesophyll cells and to be able to translocate 5,6-CF. Results of the present study show that there is no significant difference in the number of times thin- and thick-walled sieve tubes are penetrated in the upper area (0-2cm from the tip) of the sink leaf. Thick-walled sieve tubes in this area appear to be as attractive as a feeding source as thin-walled sieve tubes. This suggests that thick-walled sieve tubes in this area of the sink leaf are functional in phloem translocation supporting the findings of Haupt *et al*. (2001).

3.5 Concluding remarks

Results of this study suggest that aphids may select their feeding sites according to the quality and quantity of the food they yield, so feeding pattern may well be dictated by localisation of the phloem or of the sieve tubes within which the aphid will have access to both the quality and quantity of food required (soluble sugars, carbohydrates and proteins) where they also obtain other substances desirable or other essential food substances for them. Similar studies on phloem using aphids have also demonstrated a similar feeding strategy (Botha, Evert and Walmsely 1975a, Botha and Evert 1978, Matsiliza and Botha 2002).

Other studies (see Altus and Canny 1982, Evert , Russin and Botha 1996) suggest that the small veins in leaf blades of wheat are involved in assimilate uptake and thus must have high solute concentrations which in this study, is confirmed as *D. noxia's* preferential feeding site in source leaves, and then that thin-walled sieve tubes are preferred over the thick-walled sieve tubes in sink (whole leaf) as well as in source leaves.

CHAPTER 4: EFFECTS OF SUSTAINED *D. Noxia* FEEDING ON TRANSPORT CAPACITY OF WHEAT LEAVES.

The phloem is an essential tissue in all higher plants, functioning primarily in the long distance transport of photoassimilates from sites of synthesis (source) to sites of storage or use (sink). Given that *D. noxia* has been shown to feed preferentially from the conducting phloem, it is reasoned that the probing and feeding behaviour of *D. noxia* on this tissue must essentially affect changes to the normal functioning of the phloem. The purpose of the investigation was thus to answer several questions which are critical to our understanding of the long-term effects of sustained aphid feeding. Principal questions for which answers were sought include: Can the damage sustained by the phloem as a result of aphid feeding be visualised? Could the functional state of the transport system be assessed using fluorochrome probes? How does aphid feeding affect the physiological capacity of the translocating phloem? What are the effects of sustained feeding of *D. noxia* on the phloem? and finally what, if any, long-term effects are directly attributable to sustainable aphid feeding?

4.1.1 Visualisation of the assimilate transport pathway

Fluorescent tracer dyes such as 5,6-carboxyfluorescein (5,6-CFDA) have been used extensively to visually trace the symplastic continuity and implied by this, the movement of assimilates in leaves and stems (see Grignon, Touraine and Durand 1989, Oparka *et al.* 1994, Haupt *et al.* 2001). Applied in a diacetate form, 5,6-CF does not fluoresce, is non-polar and is readily taken up by plants cells, usually across cell walls and membranes of damaged cells. Once contained within

physiologically intact systems, the diacetate is cleaved and the polar free 5,6-CF fluoresces. It is reasonably membrane impermeant, but is known to accumulate in some cell vacuoles. Appearance in contiguous cells is thought to be confined to plasmodesmal trafficking. If a symplasmic route to the sieve tubes is available, then 5,5-CF should be transported by the phloem. Grignon, Touraine and Durand (1986) suggest 5,6-CFDA to be a useful tool for tracing phloem transport because it is stable, non-permeant, and probably innocuous molecule, which obeyed source-sink relationships and in their study remained confined to the phloem tissue for up to four days. From the literature it appears that 5,6-CF transport occurs from source to sink and mimics the distribution of fluorescence and ^{14}C radioactivity distributions (Grignon , Touraine and Durand 1989, Cook and Oparka 1983, Haupt *et al*. 2001).

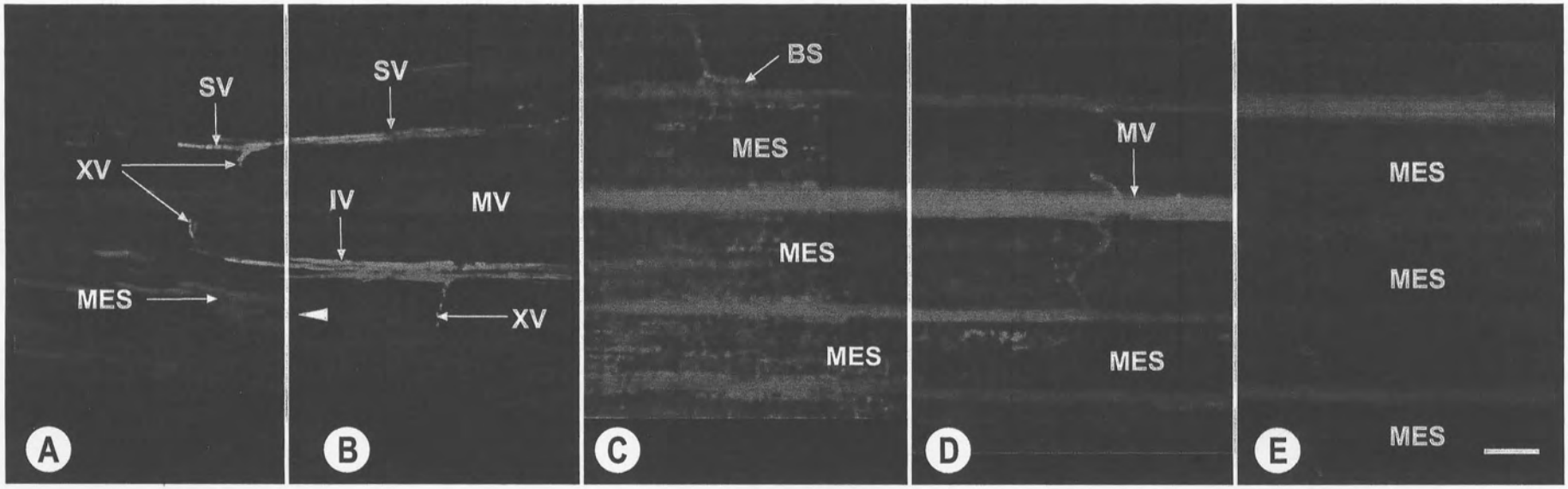
It was therefore decided to make use of 5,6-CF to illuminate the phloem loading and unloading pathway in *D. noxia* -infested and control leaf material. It was hoped tha 5,6-CF transport would allow investigation of the transport pathway, and the effects of *D. noxia* feeding on transport capacity of these leaves.

4.2 Results

4.2.1 Transport of 5,6-CF in control sink leaves of wheat

Sink leaves used in this investigation were between 4-5 cm. 5,6-CFDA was applied at about 2.5 cm from the tip of attached leaf. Two to three 3 hours after application of 5,6-CFDA, leaf segments were examined for evidence of 5,6-CF transport.

Figs 4.1A -E show the distribution of 5,6-CF in a sink leaf. Fig. 4.1C was taken at the point of application. The fluorochrome is taken up in the mesophyll, bundle sheath and is visible in the small longitudinal veins. Fig 4.1A and 4.1B show movement of 5,6-CF away the point of application (Fig. 4.1B) and the beginning of the unloading process at the tip of the leaf (Fig. 4.1A). In Fig. 4.1B the fluorochrome is only visible in the two longitudinal veins and in one transverse vein to the left of the intermediate vein (1-2cm from the tip). In Fig. 4.1A (0-1cm from the tip) 5,6-CF is evident in the intermediate and small longitudinal veins and the transverse veins connecting the longitudinal veins to the midvein but not in the midvein. Some evidence of the unloading process is visible in this micrograph as 5,6-CF associated fluorescence is visible in the mesophyll cells (to the left of the intermediate vein). The distribution of the dye was continuous along the length of these longitudinal veins. Fig 4.1D shows very little 5,6-CF associated fluorescence in some mesophyll cells and in the midvein. No fluorescence was visible beyond this area (Fig. 4.1E).


SV
XV
MES
A
SV
IV
MV
XV
B
BS
MES
MES
MES
C
MV
MES
D
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MES
MES
E

Figs. 4.1 A-E show an acropetal movement and unloading of 5,6-CF in wheat sink leaf, 2-3hrs after application of 5,6-CFDA. Figs. 4.1A&B micrographs were taken from above the point of application and Fig. 4.1D&E were taken below the point of application.The sink leaf used was 5-6cm in length.

Fig. 4.1A shows 5,6-CF unloading at the tip of the leaf. The fluorochrome is still visible in the small (SV), intermediate (IV) and cross vein (XV). Note that the mesophyll cells (MES) to the left of the intermediate vein also labelled with 5,6-CF.

Fig. 4.1B shows progression of 5,6-CF between 1-2cm from the point of application. There is evidence of longitudinal transport in the intermediate, small veins and lateral transfer in the cross vein to the left of the intermediate vein. Note that there is no 5,6-CF associated fluorescence visible in the mesophyll.

Fig. 4.1C shows the region of 5,6-CFDA application, 3hrs after application of 5,6-CFDA. The fluorochrome is distributed within the flap-fed mesophyll (MES) and there is loading into the bundle sheath (BS) cells.

Fig. 4.1 D&E were taken 1-2 cm below the point of 5,6-CFDA application. Very little 5,6-CF associated fluorescence is visible in some mesophyll cells (Fig. 4.1D). There is no fluorochrome visible in Fig. 4.1E taken below Fig. 4.1D.

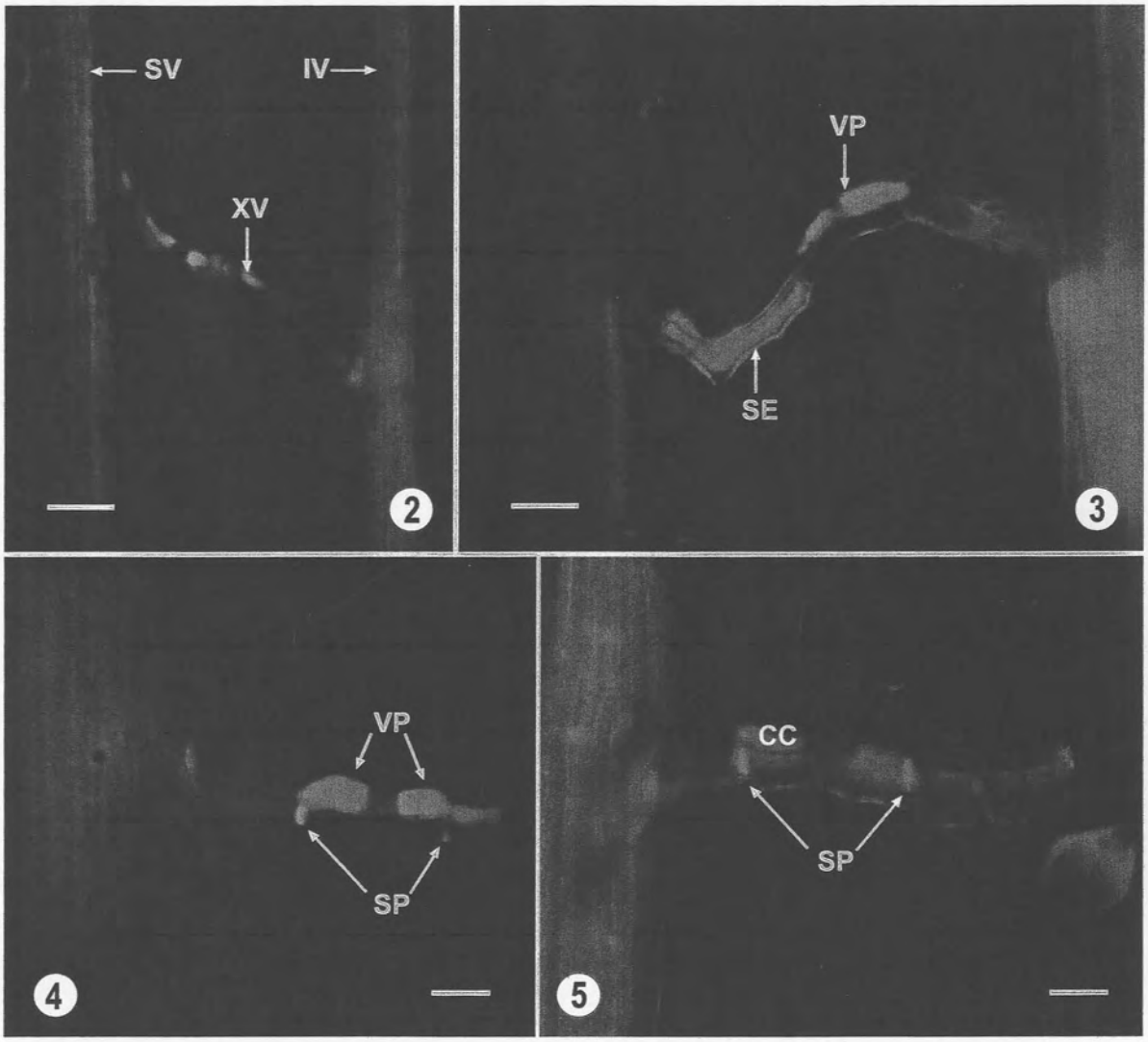
Scale bar (Figs. 4.1 A-E) = 20μm

4.2.2 Transport of 5,6-CFDA in control source leaf tissue

Three hours after application of 5,6-CF diacetate, the leaf tissue was examined for evidence 5,6-CF transport. 5,6-CF when viewed with the appropriate filter set, could be easily distinguished by its bright yellow-green fluorescence under the microscope. In total, thirty source leaves were examined, using the flap-feeding technique described elsewhere.

The dye front was observed up to 6cm from the point of CFDA application. The rate of transport measured as the distance (in cm) up to where the fluorescent dye is visible from the point of feeding over time (in hrs) was slow, at approximately 2cm/h (n=30).

The dye was observed in small as well as in the larger longitudinal veins. In most cases however, 5,6-Cf fluorescence was observed in the small veins, and it was more intense in the smaller than than in larger veins. In cases where fluorescence was observed in larger veins, transverse veins connecting the small to larger veins also contained 5,6-CF. Fig. 4.2 and 4.3 show a typical transport sequence of a fluorochrome in a small vein (to the left), in which the 5,6-CF has been transported via a transverse vein which is also connected to an intermediate vein. In Fig. 4.2 the 5,6 CF is associated with a single file of cells in the cross vein, while Fig. 4.3 shows the fluorochrome apparently associated with two files of cells in the cross vein. It is not possible to distinguish cell types in either case.


SV
IV
XV
2
VP
SE
3
VP
SP
4
CC
SP
5

Figs. 4.2 – 5 show transport of 5,6-CF in the phloem and phloem parenchyma of cross veins between small and larger veins.

Fig. 4.2 & 4.3. show a transverse vein interconnecting an intermediate (right) and small vein (left). Fig. 4.37 further shows association of the dye with two files of cells in the transverse vein. It was not always possible to determine if the dye was associated either with vascular parenchyma elements (VP), or with the single sieve tube visible in these sections. Bar = 20 µm (Fig. 4.2), Bar = 10 µm (Fig. 4.3)

Fig. 4.4 & 4.5 show distribution of 5,6-CF after co-staining with aniline blue.
Fig. 4.4 shows 5,6-CF fluorescence in vascular parenchyma cells adjacent to the thin-walled sieve tubes (characterised by the presence of sieve plates (SP).
Fig. 4.5 shows some evidence of 5,6-CF in the companion cell (CC) as well as in the sieve tube itself (three sieve plates visible in this transverse vein).
Bar = 10 µm (Fig.4.4 & 4.5).

Co-staining the tissue with aniline blue allowed for rapid easy identification of sieve plates, lateral sieve area pore plasmodesmata between sieve tubes and companion cells and plasmodesmata. Figs. 4.4 and 4.5 show leaf strips examined after application of 5,6-CFDA and subsequent co-staining with aniline blue. Fig.4.4. shows bright dye fluorescence in vascular parenchyma cells next to a sieve tube with bright fluorescing sieve plates labelled by aniline blue. The dye is not visible in the adjacent sieve tube. Fig.4.5. shows sharp discrimination of 5,6-CF in companion cells and sieve tube members of a transverse vein. In this instance the dye appears to be loaded into the sieve tube-companion cell complex of the transverse veins. Again, the sieve tube can be identified easily after staining with aniline blue, by the bright callose fluorescence associated with the sieve plates. According to the literature (Altus and Canny, 1982) and as evidenced in these micrographs, the transverse vein in wheat contains only one sieve tube (as in Figs. 4.4 & 4.5) and transport in this vein to adjacent longitudinal vein must therefore be via the metaphloem (thin-walled sieve tube).

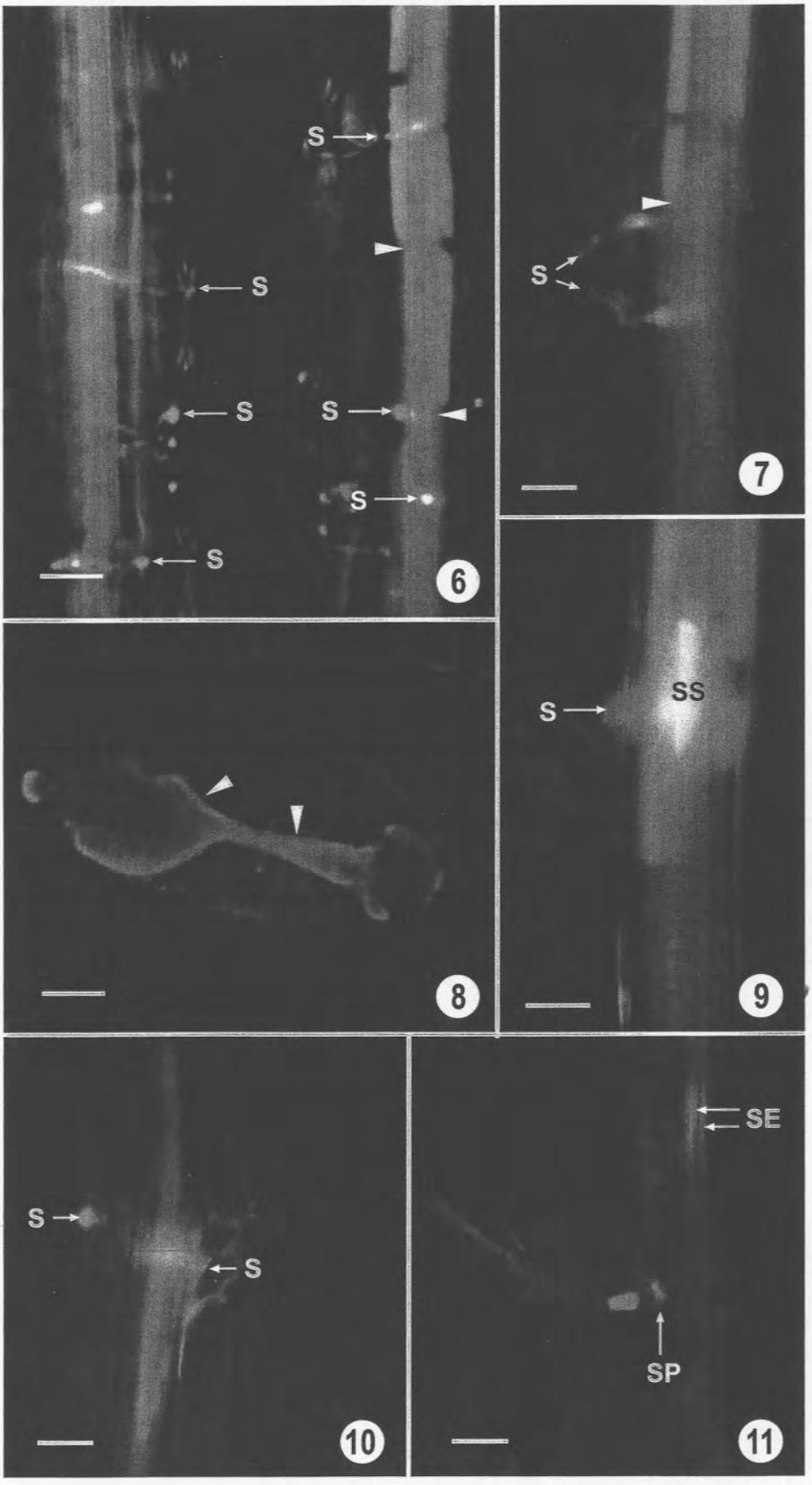
4.2.3 Transport of 5,6-CF in infested source leaf tissue

As in the control tissue, 5,6-CF was allowed to be taken up and transported for 3h after application of 5,6-CFDA via flap-feeding, before the material was examined. After this period, very little dye was seen to have moved in the leaf so uptake and transport time was increased to 4h and before leaf strips were examined. In contrast to the control leaf tissue, the dye front was observed to have moved only approximately 2cm from the point of application. The rate of appearence measured as the distance (in cm) moved by the fluorescent dye from the point of feeding over time (in hrs) was approximately 0.5cm/h (n=30). One centimetre from the point of

application of 5,6-CFDA, the distribution was already apparently discontinuous, or patchy along the length of longitudinal veins (Figs 4.6, 4.7 & 4.11).

As in control leaf tissue, most of the fluorescent dye observed was in smaller veins. Several stylets were observed lodged at different sites along the same longitudinal vein (Figs. 4.6 & 4.7). Fig. 4.6 shows an example in which stylets tracks from several aphid stylets in a small and intermediate longitudinal vein are visible. The aphid appears to have penetrated this leaf, in some instances, through the stomata as stylet tracks can be seen starting from the stomata. Fig. 4.7 further shows two *D. noxia* stylets still lodged in a small vein where the aphids had been feeding. In this, and other examples in which either aphid stylet tracks, or aphid stylets were visible, it was noted that the fluorochrome front only occurred only up to the point where the stylets were inserted and not beyond this point. There is no salivary sheath material visible which suggests that the aphid was still feeding at the time of manipulation. Fig. 4.8 shows an example of *D. noxia* that had been feeding undisturbed at approximately 4cm from the point of 5,6-CFDA application. The aphid ingested the 5,6-CF with assimilates as bright fluorescence associated with 5,6-CF is evident inside the aphid's gut. In contrast to Fig.4.6 & 4.7, Fig. 4.9 shows bright fluorescence of salivary sheath material which had been deposited in a small vein after feeding. The aphid in this instance must have been feeding and then disturbed during the course of this experiment, withdrawing its stylets, leaving behind a mixture of the salivary sheath mixed 5,6-CF-containing phloem sap which appears to have moved acropetally as well as basipetally. There is no evidence in this and other examples where disturbance occurred to suggest that 5,6-CF-associated sap passed the point of feeding.

Very few transverse veins showed fluorescence associated with 5,6-CF. Fig. 4.10 shows one of the few instances where *D. noxia* stylets penetrated a transverse vein. Of interest is the fact that the stylets are lodged at the junction between the transverse vein and a longitudinal vein and no fluorochrome is visible in this cross vein, beyond the point of stylet insertion, supporting our evidence that very little evidence of lateral assimilate transfer occurs after aphid feeding . Fig. 4.11 shows one of the few instances where photoassimilates were not ingested by the aphid, i.e. stylets and stylet sheaths were not observed in veins. The patchiness of the dye is evident in this micrograph. The fluorochrome can only be seen in part of two phloem strands in a longitudinal vein and appears to be confined to one sieve tube member of the sieve tube as there is no dye visible in other sieve tube in the rest of the sieve tube.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Figs. 4. 6 and 4.7, show several stylets and or tracks still lodged within longitudinal veins. Note that 5,6-CF occurs within the stylet tracks and stylet tracks within the phloem which is evidence that the aphids had been ingesting 5,6-CF-containing phloem sap, prior to examination. Fig. 4.7 shows tracks of two *D. noxia* aphids within in the small vein. S= stylet track; Dart = fluorochrome front. Bar = 20 µm (Fig.4.6), 10 µm (Fig. 7).

Fig. 4.8. Shows an example of an aphid which contains 5,6-CF within its gut. Bar = 250 µm.

Fig. 4.9. Shows bright fluorescence associated with salivary sheath material which had been deposited in a small vein after feeding. The aphid withdrew its stylets, leaving behind a mixture of the salivary sheath mixed with 5,6-CF-containing phloem sap, which is confined to the vascular tissues, but appears to have moved acropetally as well as basipetally. S = stylet sheath; SS = salivary sheath material. Bar = 10 µm.

Fig. 4.10. Shows a penetration near the junction with a transverse vein. The stylets are lodged at the junction between the transverse vein and a longitudinal vein. Note that no fluorochrome is visible in this cross vein, behind the stylet insertion region. S = stylet. Bar = 10 µm

Fig. 4.11. Shows bright fluorescence associated with a single sieve tube member in a transverse vein and also in two strands of the phloem tissue of a longitudinal vein. Bar = 10 µm

4.3 Discussion

Results of this study unequivocally demonstrate that after uptake by the mesophyll cells in source leaves of wheat, 5,6-CF arrives in the sieve tube-companion cell complex of both longitudinal and transverse veins. This suggests that there is symplastic loading component involved in the transport of 5,6-CF, which was apparently unrestricted at least in the control leaf tissue. Only acropetal movement of 5,6-CF was observed in sink leaves of wheat - basipetal transport was not observed in any of the sink leaves examined during the study. The small and intermediate longitudinal and transverse veins appear to support the loading and unloading process in source and sink leaves, respectively. These observations contrast with those of Haupt *et al.* (2001) in that in their study, transverse veins in sink leaves of barley apparently did not support CF unloading. The latter agrees with the suggestion made by Altus and Canny (1982) that transverse veins may function only during the loading phase in the lateral transfer of assimilates from small bundles to large bundles. However, the presence of the fluorochrome in transverse vein in the apical region of the sink leaf cannot be ignored as it indicates possible involvement of these veins in the unloading process. These transverse veins perhaps, as suggested to be the opposite situation in source leaves, transfer assimilates from larger veins to smaller veins during the unloading process in sink leaves.

In contrast to control leaf tissue where several transverse veins showed fluorescence, dye fluorescence was seen in very few transverse veins in leaf material on which aphids had been feeding. This observation suggests the damage

caused by the aphid during feeding on longitudinal veins prevented further movement of the fluorochrome into the connecting transverse veins from interconnected longitudinal veins. In addition, aphid feeding at or near the junction between a transverse vein and a longitudinal vein,resulted in redirection of the assimilates normally trafficked across these veins, into the aphid's gut. Clearly feeding aphids appear to receive a continuous supply of diverted nutrients which was evidenced in the 5,6-CF transport experiments, as very little 5,6-CF fluorescence was ever observed beyond the point of stylet penetration. The observation suggests that the established feeding aphids had themselves become secondary sinks, which was evidenced by the lack of fluorescence in cross veins, below aphid feeding sites. It was possible that the feeding pattern of the aphid (preferentially within thin-walled sieve tubes (Matsiliza and Botha 2002) affected the transport capacity of the phloem severely at this juncture. The assumption that lowering or altering the transport capacity through injury occurred is evidenced by the large areas of wound-related callose seen in all aphid infested material examined.

Support for this argument is found in the work of Nielson, Lamp and Stutte (1990) who reported that the upward movement of photosynthates in alfalfa was disrupted by feeding of the potato leafhopper. Their study showed that the rate of photosynthate transport to the tips of exposed stems was reduced by up to 62% of that calculated for control plants. Another study conducted by Cagampang (1974) showed that the rate of upward sap transport for rice plants infested by *Nilaparvata lugens* (Stal) was only 60% that of their healthy counterparts. The results of the present study showed a significant reduction (25% of the calculated value for

control plants) of the rate of 5,6-CF transport in wheat leaves infested by *D. noxia*. This observation can be attributed to several factors caused by *D. noxia* feeding.

The work presented by Hicks *et al.* (1984) showed that the quantity of tagged assimilates in leaf blades was 4 times more above petioles that had been girdled by two nymphs of the three-cornered alfalfa hopper and less than half the normal amount below girdles. Nielson, Lamp and Stutte (1990) reported over-accumulation (194%) of translocate at the exposed sites in leafhopper-injured stems which suggested phloem blockage and induction of a permanently diminished assimilate transport rate, as recovery was not observed even after 4days. Results of the present study however, showed very little evidence of accumulation of translocates, which were represented by the apparent confinement of the fluorescent dye observed in sieve tube members. Most assimilate appeared to be ingested immediately by feeding aphids along the length of the longitudinal vein.

Results presented in this study unequivocally confirm that feeding aphids form local sinks, once their stylets have penetrated functional phloem. Hill (1962) demonstrated that if the drain of assimilates is sufficiently strong and localised, the plant reacts to it in some respects as if it were a bud. The aphid not only ingests products of photosynthesis but also competes directly with other parts of the plants which are denied assimilate as result. Individual aphids have been shown to grow faster in communities than when isolated (Dixon and Wratten 1971) as they collectively establish a sink of sufficient magnitude thereby attracting significant amounts of assimilate. Increasing the number of aphids feeding on wheat plants would therefore increase the size of the sink. This argument finds support in the

work by Girma, Wilde and Harvey (1993) who presented evidence that plant height, shoot weight and number of spike were significantly reduced by *D. noxia* feeding. Results of the present investigation provide clear evidence of direct ingestion of assimilates by *D. noxia* which must greatly reduce the quantity of nutrients transported to roots or parts of the shoot resulting in symptoms such as those described by Girma, Wilde and Harvey (1993). Some observable damage of wheat plants by *D. noxia* feeding can be ascribed to the insects' extraordinary rate of multiplication which may result in a high demand for nutrients and in a heavy fluid and assimilate drain during feeding. If the drain exceeds the capacity of the plant for compensatory growth, it will result in an apparently systemic reduction of overall growth.

The use of 5-6, CFDA in this study made visualisation of the translocation pathway possible and therefore enabled assessment of the state of transport system before and after aphid feeding. The study clearly demonstrates that *D. noxia* feeding on phloem tissue does affect the functioning and therefore transport capacity of this system, thus effecting development adversely. This aspect of the research also demonstrates that during phloem feeding assimilate flow was redirected by *D. noxia*, causing massive, possibly long-term cellular damage in the process. Finally, this aspect of research clearly shows that ingestion of assimilates by *D. noxia* whilst they are still in smaller and larger longitudinal veins thus leads to a noticeable decline in the availability of assimilates either loaded and transported (in source leaves) or unloaded (in sinks).

CHAPTER 5: EXAMINATION OF DAMAGE CAUSED BY *D. noxia* FEEDING IN LEAF BLADES OF WHEAT

Fouche' (1983) and Fouche' *et al.* (1984) previously made use of light and electron microscope techniques to study the nature and extent of injury or damage caused as a result of *D. noxia* feeding. The authors observed disruption of cell membranes as well as chloroplasts. The disintegration of chloroplasts was caused by treatment with *D. noxia* extracts (macerated aphids in 0.154M NaCl). However, the study is questionable, in that the authors did not specify which tissues of the leaf the damaged chloroplasts were from. In addition, the study by Fouche' (1983) and Fouche' *et al.* (1984) did not pay attention to the phloem or its associated vascular parenchyma tissues. Given that the pathway of stylets of *D. noxia*, like most other aphids, has been shown to be mainly intercellular from the epidermis through the mesophyll and only intracellular once inside the vascular bundle, one would expect that damage would be more severe inside the vascular bundle, including the phloem, than in mesophyll cells. In addition, the authors only focused on salivary sheath (use of aphid extract) as a cause of damage to the plant and excluded mechanical damage caused by the actual penetration, movement and probing of sieve tubes by *D. noxia* 's stylets. Additionally, no work has been undertaken to localize callose in aphid-infested material either.

In this chapter the results of my investigation of cellular and sub-cellular damage caused by sustained *D. noxia* feeding on leaf blades of wheat is reported, with special emphasis on the vascular bundles.

5.1.1 Callose deposition as an indicator of the extent of damage resulting from D. noxia feeding

Callose, a β(1-3)-glucan, has been used in several studies to identify phloem elements active in sap transport (see Botha *et al.* 2000 and literature cited). Its synthesis is initiated when β-1,3-glucan synthetase is activated subsequent to the disruption of plasmamembrane integrity (Kauss 1985). It is deposited between the plasma membrane and the wall upon wounding of the plant and is electron lucent when viewed in electron micrographs. (Stone and Clarke 1992).

Other common sites of callose deposition are the connections that unite the protoplasts of contiguous cells, ranging from single plasmodesmata, through plasmodesmatal aggregates, pore-plasmodesmata to sieve plate pores. The presence of callose in almost all preparations of the phloem makes it a comparatively easy marker for microscopic investigation of sieve tubes. Many studies have shown that sieve tube callose, which can be regarded as a consistent component of sieve tubes, varies in quantity depending on several factors (Eschrich 1975, Fincher and Stone 1981, Kauss 1985, Galway and McCully 1986, Radford, Vesk and Overall 1997, Botha *et al.* 2000). These factors include mechanical, chemical, environmental or parasite-related stresses that plants may be subjected to. In event of wounding plants must be able to down regulate plasmodesmatal size exclusion limit or seal off the plasmodesmata of intact cells rapidly, preventing the loss of metabolites (Lucas et al. 1993). Callose formation is also known to occlude plasmodesmatal fields following wounding events involving loss of turgor pressure (Galway and McCully 1987, Lucas et al. 1993, Apitius and Lehmann 1995, Sjolund 1997). This wound response is essential in plants since the inability to seal sites of

mechanical damage is potentially catastrophic.

Examination of callose deposition sites was therefore considered to be is an ideal tool to investigate the extent of damage caused by *D. noxia* feeding on the phloem tissue.

5.2 Results

5.2.1 Localization of callose deposition in control leaf tissue

Twenty leaf strips were examined for evidence of callose deposition after a minimum of 5 min staining with analine blue. Of all cell types, sieve tubes showed the greatest density of callose. This occurred as thin diffuse patches along sieve plates and as sparsely distributed, punctate spots in pore-plasmodesmatal areas of lateral walls shared with companion cells, as well as between companion cells and vascular parenchyma.

Fig. 5.1. shows typical distribution of callose-associated fluorescence in a longitudinal vein and transverse vein in which bright fluorescence associated with sieve plates and pore-plasmodematal units (PPU's) is clearly visible in the transverse vein. Other than the sieve plates, very little callose fluorescence is evident along the longitudinal vein. Fig. 5.2. shows a detail of a transverse vein, in which callose fluorescence occurs in association with pore-plasmodesmatal units as well as with sieve plates. Callose appears as bright punctate spots in the walls of sieve tube members, possibly marking sites of junction with companion cells

and/or related vascular parenchyma cells.

5.2.2 Callose deposition in aphid infested leaf tissue

Twenty leaf strips were examined for evidence of callose deposition after at least 5 minutes after staining with analine blue.

Callose-associated fluorescence was most evident in longitudinal veins and less so in transverse veins. Figs. 5.3-6 show examples of the distribution of callose, after prolonged aphid feeding. Fig. 5.3 shows massive callose deposited along longitudinal vein. Callose is evident not only in the phloem strand but also in other cells neighbouring the phloem. Of interest is the fact that the aphid's stylet track can be seen from the mesophyll into the phloem of the longitudinal vein. Note that area around the stylet track is heavily callosed and that callose is not only visible next to the point of stylet penetration but also further down along the length of the longitudinal vein. It is clear that sustained aphid feeding and probing of the phloem has resulted in the damage visible in this micrograph. Fig.5.4. illustrates the distribution of wound callose in a transverse vein. Whilst extensive callose deposits are associated with the sieve tube members closest to the longitudinal vein to the right, the sieve tube members at the lower left show massive callose deposits, which is clear evidence of severe damage caused by the aphids feeding in the vicinity, presumably in a cross vein (not shown) to the left.

It was necessary to determine if the wounding effect (due to aphid feeding) was transient or not. To that end a number of leaf strips 24 and 36h after removal of feeding aphids were examined. Twenty-four hours after removal, there was still

evidence for massive damage to the phloem system. Fig.5.5 shows that there are large callose deposits associated with the phloem tissue. Both the longitudinal and transverse veins still show evidence of wound callose deposited in sieve plates. By 36h (Fig. 5.6) callose was still evident at least in the phloem tissue. Although other tissues appear to have recovered as they do not show wound callose, the phloem core in the longitudinal vein and sieve plates in the transverse vein still have areas showing large callose deposits.

The data shown here conclusively demonstrate that using aniline blue to localise callose deposition in control and aphid-infested plant tissues provides conclusive evidence of physical and associated physiological damage. When compared to control tissues, the massive callose deposits observed in infested leaf tissue are strong evidence of severe damage to the phloem and its associated parenchymatic elements.

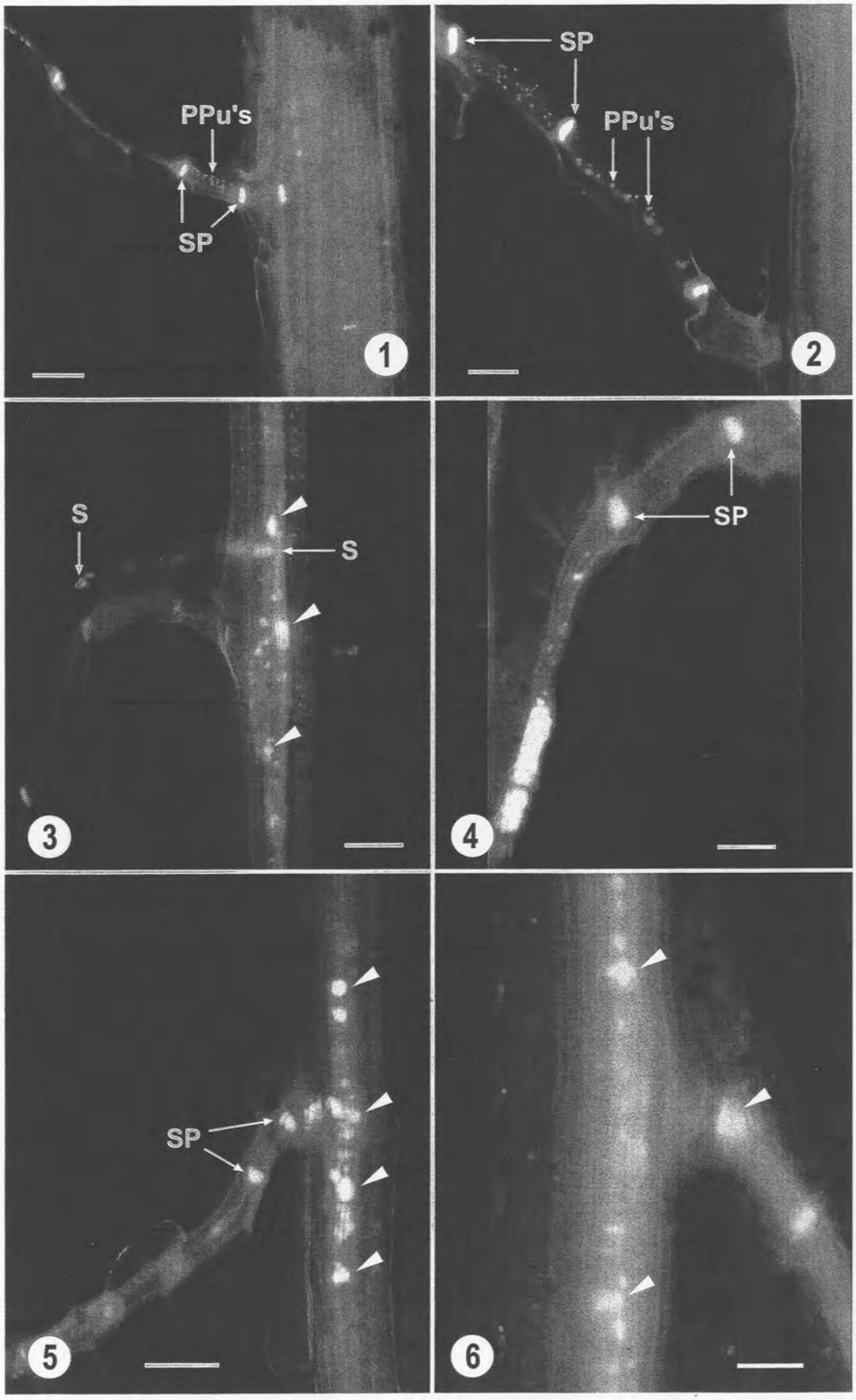
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Fig. 5.1. Shows the distribution of callose-associated fluorescence in a longitudinal vein and its associated transverse vein. Note bright fluorescence is associated with sieve plates and pore-plasmodematal units (PPU's) in the transverse vein sieve element. Other than the sieve plates, very little callose is evident along the longitudinal vein. SSP = sieve plate; PPU's = pore-plasmodesmal units. Bar = 20 µm.

Fig. 5.2. Shows a detail a transverse vein in which callose occurs in association with sieve plates and PPU's. Callose appears as bright punctate spots in the walls of the sieve tube members. SP = sieve plate; PPU = pore-plasmodesmal units. Bar = 10 µm.

Fig. 5.3. Shows a stylet track terminating just above a cross vein. This track is itself massively callosed near the phloem, as is the vascular strand within this intermediate vascular bundle. Darts point to large callose deposits; S = stylet track. Bar = 10 µm.

Fig. 5.4. Illustrates the distribution of wound callose in a transverse vein. The vein contains a single sieve tube. A massive wound callose deposit is visible in the lower-most sieve tube member. Note aggregates associated with the sieve plates as well as large areas associated with the PPU's. Bar = 10 µm.

Figs. 5.5 -6. Show wound callose deposits, 24 (Fig. 5.5) and 36 h (Fig. 5.6) after removal of feeding aphids. There is some evidence for a slow reversal of the wounding effect, as is evidenced in this, as well as other examples, by the decrease in fluorescence activity after 36h (Fig. 5.6). Darts = large wound callose formations in phloem and associated parenchyma tissue; SP = sieve plates. Bar = 20µm (Fig.5.5), 10 µm (Fig. 5.6).

5.2.3 Ultrastructure of penetrated vascular tissue

Leaf material was collected for examination at least 120h after aphids were introduced to source leaves. Despite several attempts I was unsuccessful in obtaining good sections of embedded plant tissues in which the aphid stylets remained attached. The aphids might have been pulled out by the mass of their dead bodies during fixing and embedding or else the resin used (Araldite Taab 812) was softer and less likely to hold up under the beam. Those stylets that did remain in the tissue did not infiltrate with the resin and as result tore out while sectioning, when mouthparts were sectioned transversely and especially when they were sectioned longitudinally. The salivary sheath material however, infiltrated better but not completely, as parts of it too, were lost along with the stylets. Consequently most of the study was undertaken on sections including salivary sheaths that lacked *in situ* stylets and consequently, as none of the micrographs of embedded sections shown here contain stylets, the study is based upon tissue containing salivary sheaths material.

Leaf tissue which had been subjected to aphid probing and feeding showed various structural abnormalities compared with control tissue. As described by Fouche *et al.* (1984), damage to the mesophyll cells involved destruction of the cell contents, some membrane damage and subsequent loss of cell contents. Figs. 5.7 shows evidence of salivary sheath material between adjacent mesophyll cells, where the aphid probed intercellularly. Note the loose fibrillar deposits, interspersed with more solid-looking, more electron-dense material in the salivary sheath (SS) and sheath associated material. Mesophyll cells (MES) and their contents are extensively disrupted, indicative of plasmolytic shock due to aphid feeding. Fig. 5.8 shows

massive damage caused by probing aphids to the cytoplasm as well as the disruption of the chloroplasts envelope and stroma lamellae. The plastid envelope (T) of the two chloroplasts (CL) is extremely convoluted and has formed vesicles surrounding a greater part of the area between chloroplast contents and the plastid envelope. Grana (G) in one chloroplast (to the right) are obviously swollen. Fig. 5.9. demonstrates extreme damage, typical of tissue which had been extremely probed by the aphids. Note that the plasma membrane (PL) is free of the cell wall (CW) (indicative of plasmolytic damage) and is vesiculate (Ve).

Three types of penetration were observed during the present transmission electron microscope study; intercellular (Fig. 5.12), intracellular (Fig. 5.11) and "intermediate" or in part inter- and intracellular (Fig. 5.10). Fig. 5.10 shows the aphid's salivary sheath (SS) deposited between the cell wall and the plasma membrane. The aphid's stylets (S) have unfortunately collapsed under the electron beam. The plasma membrane of one of the bundle sheath cells (BS) has been pushed away from the cell wall and is convoluted whilst the one on the cell below this one seems to be broken and therefore not visible on this micrograph. Fig. 5.11 shows an example of an intracellular penetration. The aphid's stylets have penetrated a cell wall of a sieve tube (SE) subsequently punctured the plasma membrane. The stylets have unfortunately either collapsed under the electron beam or had not been infiltrated by the embedding medium but salivary sheath deposited by the aphid is visible outside this sieve tube but not inside, suggesting that the aphid was feeding before it was killed. Note that the sieve tube next to the one penetrated by the aphid's stylets is severely plasmolysed. In Fig. 5.12 the salivary sheath bundle is deposited between two cells. It is likely that the aphid

withdrew its stylets after feeding or during manipulation of the leaf, leaving behind the salivary sheath. The cell walls of the two cells adjacent to the salivary sheath show evidence of damage.

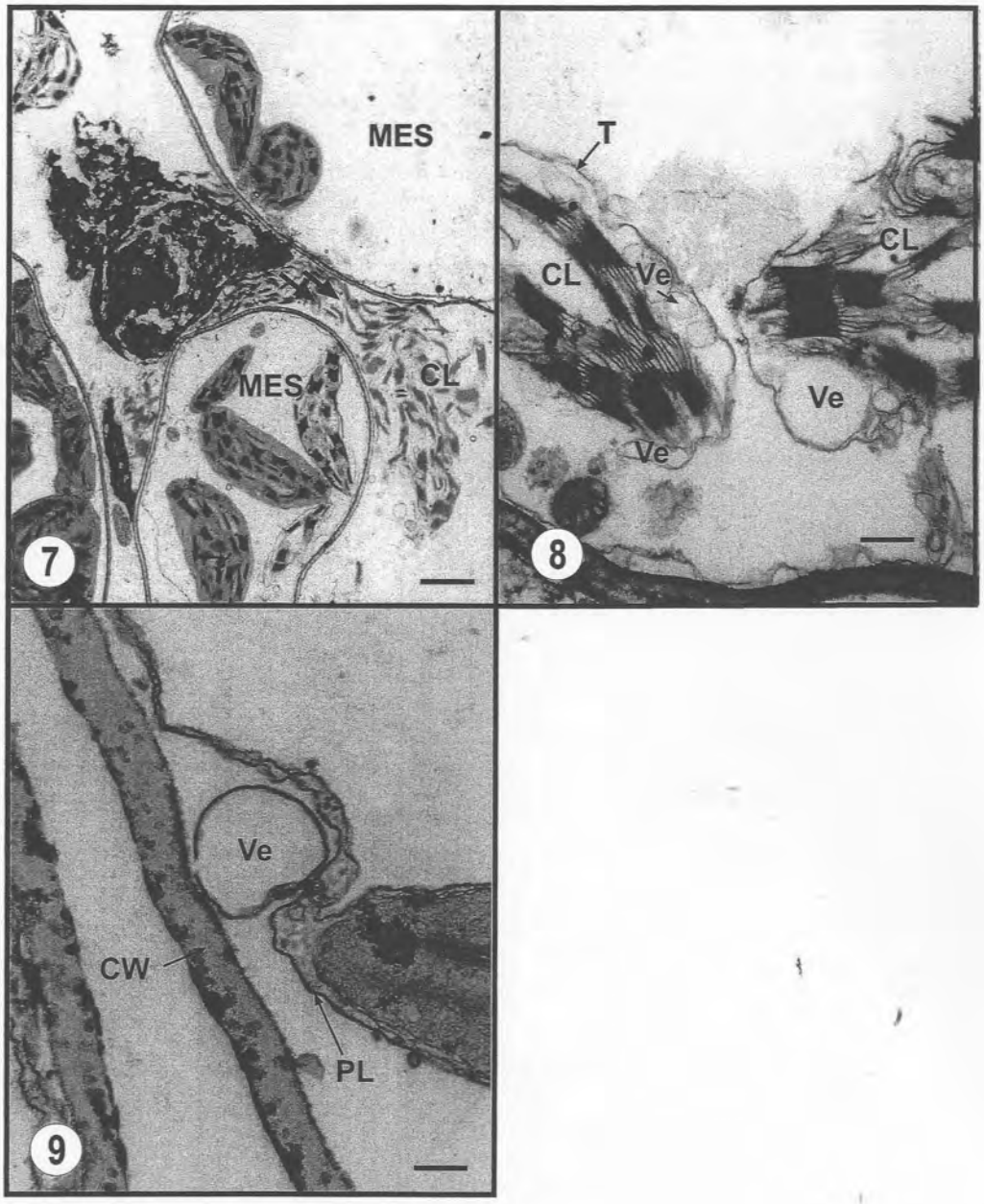
MES
MES
CL
7
T
CL
Ve
CL
Ve
Ve
8
Ve
CW
PL
9

Fig. 5.7 – 9 Transmission electron micrographs of wheat leaf tissue, showing damage caused by prolonged aphid feeding. Aphid probing has caused extensive damage to mesophyll cells, puncturing and rupturing of the cells appears to have caused massive turgor changes, leading to membrane and organelle disruption

Fig. 5.7. Shows aphid's salivary sheath (SS) deposited between adjacent mesophyll cells (MES). Cell contents are extensively disrupted and some have disintegrated, an indication of plasmolytic shock. Bar = 500μm

Fig. 5.8. Shows a convoluted plastid envelope (T) of the two chloroplasts (CL) forming vesicles (Ve). A few grana (G) are slightly swollen. Bar = 200nm

Fig. 5.9. Shows a convoluted plasma membrane (PL) forming vesicles (Ve).
Bar = 100nm

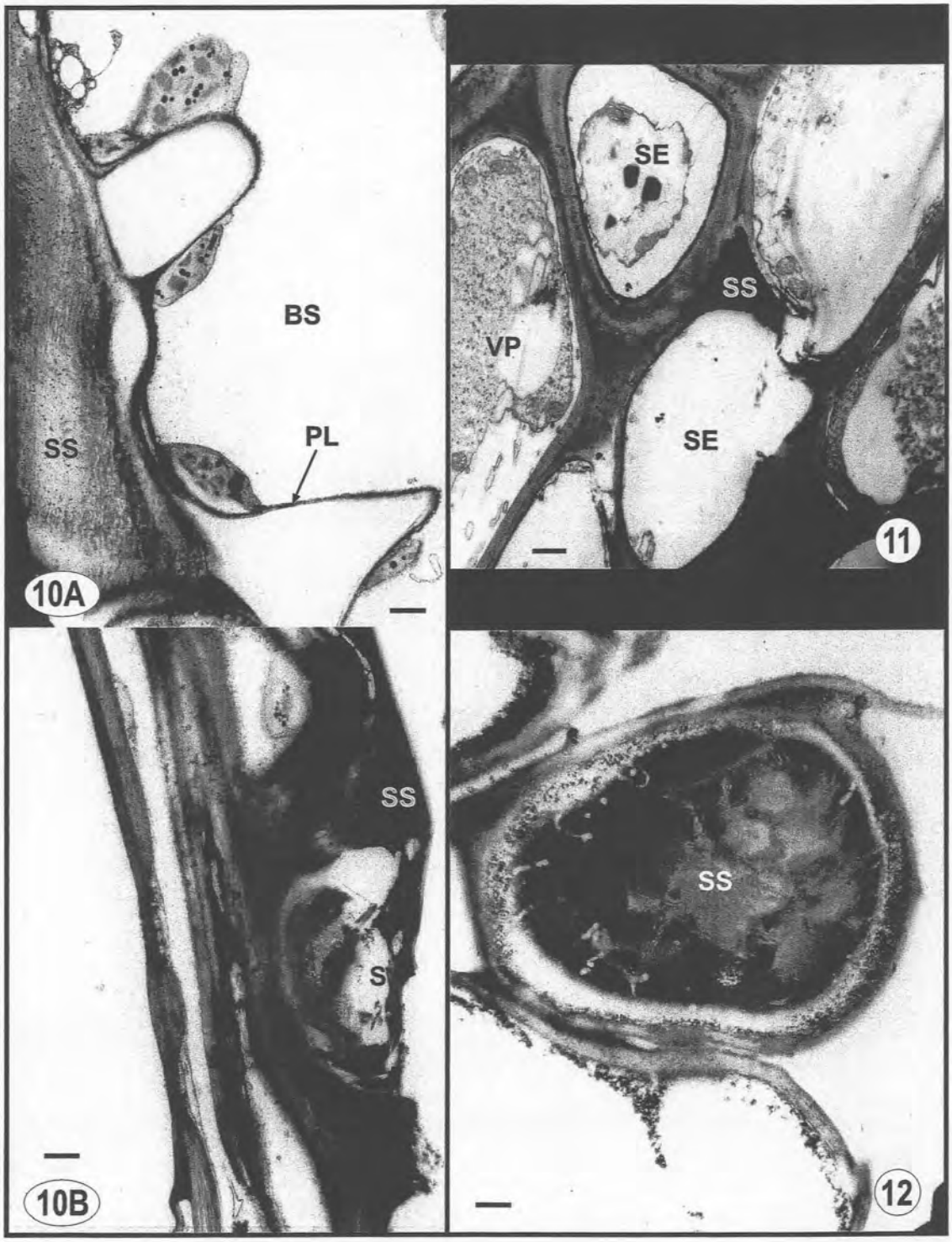
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Fig. 5.10 – 5.12 Transmission electron micrographs illustrating aspects of stylet penetration.

Fig. 5.10A & B Shows longitudinal section of aphid's stylets (s) (disintegrated) and a massive deposit of salivary sheath (SS) material between the cell wall (CW) and the plasma membrane (PL). The plasma membrane and the protoplast have been pushed away from the cell wall by the sheath material. Bar = 500nm.

Fig. 5.11 Shows intracellular penetration of a sieve element (SE) by aphid's stylets (disintegrated) with salivary sheath deposited outside this sieve element. The cell wall and plasma membrane of this and adjacent cell are disrupted by track or sheath material. The sieve tube next to the penetrated one is severely plasmolysed Bar = 500nm

Fig. 5.12. Shows the aphid's salivary sheath in near transection, in an intercellular space between two vascular parenchyma cells. Bar = 200nm

5.3 Discussion

Results presented in this chapter show clear evidence of mechanical damage caused by aphid stylet penetration, salivary sheath deposition and long-term feeding. The extent of the damage is inferred from the size and extent of the callose deposition as well as from the transmission electron micrograph study, which showed major disruption of the cytoplasm and its constituents – all evidence of rapid turgor loss during penetration

The effects of penetration and feeding of aphids and other suctorial insects upon host tissues have been reported by many investigators (see Evert *et al.* 1968; Evert *et al.* 1973; Fouche *et al.* 1984, Brzezina, Spiller and Llewellyn 1986, Ecale Zhou and Backus 1999). The effects range in severity from no apparent injury in some cases, to widespread disorganisation of cells in others as presented here. In this study damage resulting from *D.noxia* probing and feeding includes destruction of cell contents, severe membrane damage and subsequent loss of cell contents. The observed cellular disruption in areas not associated with salivary sheaths (Figs. 5.8 & 5.9) suggest that *D. noxia* feeding damage is more than just a mechanical wounding by the insect stylets and that some substance or substances in the insect's saliva is involved. In addition to the signs of osmotic shock, the dissolution of cell walls and disintegrated appearance of organelle membranes suggest that *D. noxia* saliva may contain hydrolytic enzymes capable of interfering with cellular metabolism.

It is generally assumed that as long as aphids are not transmitting phytopathogenic

viruses and injecting toxins, causes little or no observable damage to their host plants. Feeding of individual Aphidoidea have been reported to have very few, if any mechanical consequences (Pollard 1973, Gibson 1974), possibly no more than just the histologically detectable death of a few cells that suffered direct and irreparable damage. Results of the present study clearly show evidence of severe mechanical damage which included punctured cell walls during stylet penetration and resulting from this disorganised cell organelles and cell membranes caused by salivary sheath deposited inside or next to these cells. Stylets and salivary sheaths of *D. noxia* were observed in intercellular spaces, in damaged cells (intracellular) and between cell walls and protoplasts (intermediate). In the intermediate route, the stylet sheath was in direct contact with the plasma membrane, with no obvious signs of damage, suggesting that chemical constituents of the saliva do not degrade membrane constituents. However, hydrolytic enzymes released from cell vacuoles may contribute to organelle and cell damage (Boller and Kende, 1979). Most evidence of damage was associated with the intracellular route. The cell wall and cell membrane were punctured by penetrating stylets and salivary sheaths deposited resulted in major disorganisation of cell contents.

Miles (1987) suggested that an additional function of the salivary sheath for typical sheath-feeders is that it may minimize the amount of plant damage during feeding by absorbing and inactivating defensive compounds produced by the plant. However, in this study cells around salivary sheaths of *D. noxia* were extremely disrupted.

These results show clear evidence of formation of massive callose deposits in

infested leaf tissue. The pattern of wound callose deposition along veins penetrated by the aphid shows that deposition of callose occurred not only in phloem cells penetrated by the aphid, but also in other cells adjacent to the phloem. This may occur as a result of the presence extensive and rather special lateral connections between sieve elements and phloem (vascular) parenchyma (Esau and Thorsch 1985) which may rapidly transmit turgor loss laterally. Turgor loss by plasmolysis has been shown to induce deposition of callose at plasmodesmata of parenchyma cells (Drake, Carr and Anderson 1978)

It is well known that callose, together with P-protein, serve to seal the sieve plate pores of injured sieve tubes thus preventing the loss of assimilates (Eschrich 1975, Sjolund 1997). Plugging of sieve plate pores with P-protein has been reported to be an almost instantaneous reaction to pressure release in active sieve tube (Knoblauch and van Bel, 1998) whilst the rate of callose deposition has been reported to vary considerably from species to species (Galaway and McCully 1986, Apitius and Lehmann 1995, Botha *et al.* 2000). Evert *et al.* 1968 also presented evidence of blockage of sieve tubes by tylosoids in plants that had been punctured by aphids. The authors suggested that the loss of turgor through aphid feeding was responsible for formation of tylosoids in living sieve tubes. Results of the present investigation showed evidence of intense deposition of callose in response to wounding. This could lead to constriction of the symplasmic connections within the vascular bundle and also between sieve tube members and associated vascular bundle cells, slowing down the rate of diffusion as well as of the rate of active movement of solutes. In this study, the massive amount of callose deposited in the phloem and neighbouring cells along the length of longitudinal bundles should

affect the passage of assimilates in these veins. In addition, the apparent confinement and patchiness of the fluorescent dye observed in infested leaf tissue (Fig. 4.15) is attributed to the deposition of massive wound callose zones, which resulted in blockage of the sieve plate pores.

Results of this study clearly demonstrate that *D. noxia* causes severe damage to its host plants, by the combination of mechanical damage and salivary secretions during its active probing behaviour, both of which result in severe damage of the infested leaf. The large callose deposits observed in aphid-infested leaf tissue must affect the movement of assimilates in these leaves (as observed in Chapter 4). Whether mechanical wounding or *D. noxia* salivation alone can quantitatively produce the severe damage caused by *D. noxia* still needs to be investigated, but it is clear that mechanical damage (the disruption of the cell wall and rupture of the plasma membrane) must cause extensive and associated physiological damage.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

This study had from the outset, two principal components. First, to determine the feeding preference (thin- *vs* thick-walled sieve tubes), vein size and order preference (large, intermediate or small) as well as leaf positional preference (adaxial *vs* abaxial probe origin) of *D. noxia* on the wheat leaf blade. Secondly, the study was directed at examining the effects of long-term aphid feeding on the transport effectiveness of the phloem tissue.

The hypothesis was divided into two parts, with the second expected to lead logically from the first . In the first part of my hypothesis preferential feeding of *D. noxia* in either thick- or thin-walled sieve tubes in the phloem was considered to be an indication of functionality with respect to phloem loading and transport. The assumption was that the aphid would feed preferentially in thin-walled sieve tubes, indicating these are more functional than thick-walled sieve tubes. The second part involved consideration of the preferred feeding site (i.e. the transport phloem), for evidence of damage following sustained Russian wheat aphid feeding. Here it was assumed that damage caused by aphid feeding would be evident at the light and electron microscope level.

6.1 Preferential feeding

The presence of two kinds of sieve tubes i.e. thin- and thick-walled sieve tubes in grasses and the roles played by these sieve tubes in phloem loading, translocation

and unloading has been a subject of debate for the past two decades. Several studies have been conducted in an attempt to determine if there are different functional roles for these sieve tubes. Several studies have been undertaken using feeding aphid colonies to examine potential functionality of specific phloem groups (Botha and Mabindisa 1977, Botha, Malcom and Evert 1977, Botha and Evert 1978) where preferential feeding was revealed. Aphid feeding in these studies was equated to preference feeding implying that it is the functional phloem that is penetrated. Recently, Matsiliza and Botha (2002) showed that the aphid *Sitobion yakini* fed preferentially on thin-walled sieve tubes in barley. The authors therefore suggested that the thin-walled sieve tubes were more functional in phloem loading and transport than were the thick-walled sieve tubes.

The present study shows unequivocally that *D. noxia* feeds preferentially from thin-walled sieve tubes in sink as well as source leaves of wheat (Table 3.2 & 3.4). This observation together with those of Cartwright, Lush and Canny (1977), Fritz, Evert and Heyser (1983) and Matsiliza and Botha (2002) on translocation, phloem loading and transport in leaves of wheat, maize and barley, respectively, more strongly support an important role for the thin-walled sieve tubes in phloem loading and transport, than the thick-walled sieve tubes.

The relatively low percentage of stylets and stylet tracks seen terminating at the thick-walled sieve tubes, suggests that the thick-walled sieve tube is an unsuitable feeding site, or perhaps it does not contain enough attractive substance (whether it is sucrose or not remains to be determined). Alternatively, the thick-walled sieve tubes may not have a sufficiently high functional osmotic potential, to allow feeding

by *D.noxia* from them. Something is obviously missing from the ingredients required to make the thick-walled sieve tubes attractive to *S. yakini* with respect to providing an adequate food source.

However, the probing of thick-walled sieve tubes observed in tips of the sink leaf (Table 3.2) suggests maturity and some functionality in unloading/or loading phase- however care needs to be taken in interpreting the data, as we do not as yet have functional evidence based on transmission electron microscope studies, to prove this. Whilst the study conducted by Haupt *et al.* (2001) support the suggestion of functionality for the thick-walled sieve tubes, a cautionary note needs to be sounded here, as the authors did not specify which area of the sink leaf they examined.

This study also shows that *D.noxia* fed preferentially from the small longitudinal bundles in sink as well as source leaf blades. The preference of these veins is likely to be related to the quality and quantity of assimilates in them as these veins have been suggested to be involved in the assimilate loading in source leaves (Altus and Canny 1982, Evert, Russin and Botha 1996). The length of aphid's stylets did not appear to have any influence on vein selection as they were found to be long enough to penetrate any of the three longitudinal veins.

D. noxia fed preferentially from the adaxial surface of sink as well as source leaves. The fact that the distance from the adaxial surface to the feeding site (i.e. the phloem) was longer than that from the abaxial leaf surface did not affect the aphid's leaf positional preference. The choice of penetration location exhibited by *D. noxia*

could simply be because the leaves were curled upward and thus feeding on the adaxial leaf surface offered protection than the now exposed abaxial leaf surface.

6.2 Damage to the phloem

Results of the present study clearly show that the interaction of aphids and their host plants is much more dynamic than might be inferred from the absence of readily observable symptoms caused by viruses and salivary toxins. Mechanical damage observed in the present study causes extensive damage to the phloem and other cells than was previously thought. It is not clear however, whether mechanical damage alone could result in observed symptoms associated with Russian wheat aphid infestation, as these symptoms only become visible days after the aphids started feeding (i.e. probing and feeding resulting in mechanical injury) on the host plants. Possibly mechanical effects on their own depend on the phenology of the tissues affected and on the frequency of occurrence of the damage.

Results of this study also suggest that recovery of plant tissue after Russian wheat aphid infestation is highly unlikely. Examination of leaf material 24 and 36h after the aphids were removed still showed evidence of extensive wound callose deposits associated with the phloem. In addition, water imbalances expressed as loss of turgor have been found to be the primary response of wheat plants to Russian wheat aphid feeding (Burd and Burton 1992). Plants infested with Russian wheat aphid failed to osmoregulate (Burd and Burton 1992, Riedell 1989). Plasmolytic shock (expressed as retraction and convolution of the plasmalemma) observed in

this study (also see Fouche' *et al.* 1984) implies a loss of cellular turgidity as a result of aphid feeding.

Data presented in this study clearly show that there was no impediment to symplasmic loading/transport and unloading of 5,6-CF (therefore assimilates) in control source and sink leaves, respectively. Phloem blockage (by salivary sheath material deposited and wound callose) led to accumulation of assimilates together with the drain of assimilates, observed in infested leaf material should affect the functioning of the transport system. Phloem blockage could result in the inability of phloem translocated substances to either enter or exit leaves, which has been suggested to cause yellowing of leaves in alfalfa (Nielson, Lamp and Stutte 1990). The aphid thus presented itself as a localised assimilate sink thereby redirecting and draining assimilates to itself which were meant to be transported to other parts of the plant.

There are two possibilities which, based on the present study, as well as previous studies that can explain how *D. noxia* feeding damages wheat plants. The first is that a salivary toxin is injected during and after feeding (Fig. 3.3-6, 3.9-12, 4.9 and also Fouche' *et al.* (1984) resulting in destruction of cell contents. The second one is that phloem tissue and transport phloem is blocked and disrupted as a result, by mechanical damage by either stylets, salivary sheath deposited and callose deposited in response to wounding. Whatever the case or outcome, the aphid causes severe, possibly irreversible damage during feeding.

6.3 Future prospects based on thesis

Notwithstanding the shortcomings of this thesis, I hope that it will provide a better understanding of how the Russian wheat aphid penetrates the leaf blade of wheat, where it feeds in the phloem, and how its feeding affects the phloem of wheat plants.

The current study provides new information on preferred feeding site which may be useful to plant breeders in their attempt to develop more wheat cultivars that are more resistant to the Russian wheat aphid. Further studies could focus on identification of molecules present in the two kinds of sieve tubes that attract or repel the *D. noxia* to investigate the possibility of resistance in thick-walled sieve tubes. In addition, more research still needs to be done in order to determine whether the preference of thin-walled sieve tubes is only due to high concentration of solutes in these sieve tubes or other factors are involved.

In addition, the use of feeding aphids in this research project has provided highly significant information supporting the preferential feeding of the aphid on the thin-walled sieve tubes and adds to the small data pool previously available on the possible functions of thin- and thick-walled sieve tubes in grass leaves. It does not however, provide evidence for functionality of thick-walled sieve tubes in grasses, except for the assumed possible functionality at the tips of sink leaves, where aphids tended to probe both thick- and thin-walled sieve tubes. They remain as much of an enigma as they were when they were first reported 26 years ago by Kuo and O'Brien (1974). Much work remains to be done in order to determine the

possible role of the thick-walled sieve tubes in grasses. Future studies could focus on conducting experimental studies using fluorochromes to investigate further the possible functional role of thick-walled sieve tubes in source and sink leaves wheat. Based on this study, I would suggest that the tip region of sink leaves needs to be investigated carefully in an integrated phloem offloading study, which is coupled to a detailed investigation of the ontogeny and functionality of the thick-walled sieve tubes in this region. Fritz, Evert and Heyser (1983) suggested that thick-walled sieve tubes may be involved in the retrieval of photosynthates from the transpiration stream and/ or apoplast in grasses. Future studies may therefore investigate whether thick-walled sieve tubes were perhaps involved in the retrieval of solutes from the xylem, using tracers, such as those used here.

The use of suctorial insects such as aphids in this and previous studies (Botha 1974, Botha *et al.* 1972, Botha, Evert and Walmsley, 1975a, Botha, Malcom and Evert 1977, Botha and Evert 1978, Matsiliza and Botha 2002) clearly has provided useful foundation information in phloem structure/ function studies - without which we would be far less informed, and not able to assess the roles of thin- and thick-walled sieve tubes in nature.

Microscopy is a frustrating reality in this and future studies. The use of more suitable techniques (confocal microscopy) to visualise assimilate transport will provide a more detailed information and hopefully answer some of the yet unanswered questions on phloem translocation in grasses.

6.4 Conclusions

The original hypotheses have been examined in the preceding chapters. I believe that the two hypotheses for the research held true in part:

- *D.noxia* fed preferentially in thin-walled sieve tubes of the small longitudinal veins, in sink as well as source leaves of wheat.
- This suggests that these sieve tubes are more attractive to the aphid as a feeding source than thick-walled sieve tubes.
- Based on the evidence presented in this thesis it appears that the the thin-walled sieve tubes are more functional and important in phloem loading, transport and unloading than thick-walled sieve tubes, with the possible exception of the tips of sink leaves.

These results therefore fulfilled the expectations set out in the first hypothesis mentioned earlier.

The second part of the hypothesis did not work really so well due to the lack of suitable *in situ* localization systems (digital deconvolution or confocal facilities) to enable through-focus of whole leaf tissue and more clear differentiation between thin- and thick-walled sieve tubes. Fluorescence microscopy proved frustratingly difficult. The use of a more advanced camera system will help future studies.

The use of transmission electron coupled with fluorescence microscope techniques however, provided some very useful information on assimilate movement and subsequent aphid-related damage to the vascular tissue and phloem sieve tubes.

D. noxia feeding affected the transport system in two ways. Firstly, it induced occurence of plasmolytic shock during probing resulting in the destruction of cell wall, cell membranes and cells contents, and subsequent deposition of massive callose areas in the phloem and surrounding cells in response to wounding, which lead to blockage of the phloem per se. Secondly, by forming a local sink thereby redirecting assimilates, which now moved towards the aphids via their stylets. Given the limitations discussed, it was however not really possible to tell which sieve tube (thin- or thick) was damaged except in cross veins where only a thin-walled sieve tube is present.

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APPENDIX A

The full Long-Ashton nutrient solution used in this study as taken from Hewitt (1966).

SALT	Wt used (g)	V. stock Sol. (ml)	V. stock sol. dil. In 25 L (ml)	Conc. In final V. of 25 L (mM0
Macronutrient				
KNO_3	101	500	25	2
K_2SO_4	43	500	25	1
$Ca(NO_3)_2$	164	500	25	4
$CaCl_2$	111	500	25	4
$MgSO_47H_20$	92	500	25	1.5
$NaH_2PO_4.2H_2O$	104	500	25	4
Micronutrient				
$MnSO_4.4H_2O$	11.20	500	2.5	0.02
$CuSO_4.5H_2O$	1.25	500	2.5	0.002
$ZnSO_4.7H_2O$	1.45	500	2.5	0.002
H_3BO_3	15.50	500	2.5	0.05
$Na_2Mo_4.2H_2O$	0.605	500	2.5	0.0005
NaCl	29.30	500	2.5	0.1
Fe-Citrate (3H2O)	29.90	500	2.5	0.6

APPENDIX B

Table 1.: Alcohol dehydration series (modified from Sass, 1958 and from Botha, 1994)

	Step	Time in hours
1.	FAA	24h
2.	50% EtOH	12h
3.	70% EtOH	12h
4.	35% Butyl-alcohol	12h
5.	55% Butyl-alcohol	12h
6.	75% Butyl-alcohol	12h
7.	100% Butyl-alcohol	3 changes of 12 hours each
8.	100% n-Butanol (liqiud parrafin)	12h

Table 2: Composition of F.A.A.

Ethyl alcohol	50 cc
Glacial acetic acid	5 cc
Formaldehyde (37-40 %)	10 cc
Water	35 cc

Table 3.: Staining series (from Botha, 1994)

	Stain	Time
1.	Xylol	3 min
2.	Xylol	3 min
3.	Xylol	3 min
4.	Xylol : Alc. 50 : 50	3 min
5.	Abs. Alc.	2 min
6.	90% Alc.	2 min
7.	70% Alc.	2 min
8.	50% Alc.	2 min
9.	Safranin	24 hours
10.	70% Alc.	2 min
11.	90% Alc.	2 min
12.	95% Alc. + picric acid	dip drain
13.	95% Alc.= NH_3	dip drain
14.	Abs. Alc.	2 min
15.	Abs. Alc.	2 min
16.	Fast green	1 min
17.	clove oil	dip drain
18.	clearer	dip drain
19.	Xylol	2 min
20.	Xylol	2 min
21.	Xylol	max. 5-6 min

Table 5: T.B.A (Tertiary butyl alcohol)series.

T.B.A.	95% ethyl alcohol	Absolute ethyl alcohol	T.B.A.	Water	Paraffin oil
1	50	0	10	40	0
2	50	0	25	30	0
3	50	0	35	15	0
4	50	0	50	0	0
5	0	25	75	0	0
6	0	0	50	0	50
7	0	0	100	0	0